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Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis

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Summary. A glutamine synthetase (GS) cDNA isolated from an alfalfa cell culture cDNA library was found to represent a cytoplasmic GS. The full-length alfalfa GS_1 coding sequence, in both sense and antisense orientation and under the transcriptional control of the cauliflower mosaic virus 35S promoter, was introduced into tobacco. Leaves of tobacco plants transformed with the sense construct contained greatly elevated levels of GS transcript and GS polypeptide which assembled into active enzyme. Leaves of the plants transformed with the antisense GS_1 construct showed a significant decrease in the level of both GS_1 and GS_2 polypeptides and GS activity, but did not show any significant decrease in the level of endogenous GS mRNA. We have proposed that antisense inhibition using a heterologous antisense GS RNA occurs at the level of translation. Our results also suggest that the post-translational assembly of GS subunits into a holoenzyme requires an additional factor(s) and is under regulatory control.

Key words: Glutamine synthetase – Heterologous antisense RNA – Transgenic plants – Tobacco

Introduction

Glutamine synthetase (GS; EC 6.3.1.2) catalyzes the ATP-dependent formation of glutamine from glutamate and ammonia. GS catalyzes the reassimilation of ammonia released from a variety of metabolic pathways such as photorespiration, catabolism of amino acids and metabolism of phenylpropanoids. GS is also involved in nitrate (or nitrite) assimilation in leaves and roots and in dinitrogen fixation in root nodules of legumes (Miflin and Lea 1980). In plants, GS is an octamer and has a native molecular weight of approximately 320–380 kDa (Stewart et al. 1980).

In plants, GS is encoded by a small multigene family that show organ-specific patterns of expression, with separate genes encoding leaf cytoplasmic and chloroplastic GS isoforms (GS_1 and GS_2, respectively), root GS and nodule GS isoforms in legumes (Tingey et al. 1987; 1988; Gebhardt et al. 1986; Lightfoot et al. 1988; Forde and Cullimore 1989; Peterman and Goodman 1991). Based on the site of localization, the different GS isoforms assimilate ammonia derived from different sources. Analysis of photorespiratory mutants of barley has established that reassimilation of photorespiratory ammonia is the function of the chloroplast-localized GS_2 protein (Blackwell et al. 1987). Furthermore, analysis of transgenic plants containing GS_2 or GS_1 promoters, has shown that GS_1 genes are only expressed in the cells around the phloem, suggesting that the GS_1 isoform functions in generating glutamine for nitrogen transport (Brears et al. 1991).

The regulation of GS gene expression is not yet fully understood. The genes for GS_1 and GS_2 are differentially expressed during plant growth and development (Forde and Cullimore 1989; Edwards et al. 1990; Brears et al. 1991; Kamachi et al. 1991), reflecting the different roles and cellular compartmentalization of the two isoenzymes (Mann et al. 1980; Tingey et al. 1988; Edwards and Coruzzi 1989). In addition, it has been shown that the pea GS_2 promoter is light-induced (Tingey et al. 1988; Edwards et al. 1990) and that the accumulation of GS mRNA may in part be due to a phytochrome-mediated response (Sakamoto et al. 1990). Photorespiratory ammonia production also regulates pea GS_2 expression (Edwards and Coruzzi 1989). In root nodules, a nodule-specific GS isoform that appears to be primarily under developmental control has been reported for several species, including bean (Lara et al. 1983; Forde et al. 1989), alfalfa (Dunn et al. 1988), lupin (Konieczny et al. 1988) and soybean (Sengupta-Gopalan and Pitas 1986;

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Sengupta-Gopalan et al. 1991). However, soybean nodules also seem to have a cytoplasmic GS gene that is not nodule specific and appears to be regulated by ammonia or a related metabolite (Hirel et al. 1987). The promoter of the gene when used in β-glucuronidase (GUS) fusions, is ammonia-inducible in transgenic *Lotus corniculatus* but not in transgenic tobacco (Miao et al. 1991).

To increase our level of understanding of GS function and regulation in plants, we have attempted to alter the levels of GS expression, using both overexpression and downregulation, by genetic engineering (van der Krol et al. 1988b). Successful downregulation of plant genes using homologous antisense genes has been reported for a growing number of different systems: chalcone synthase (van der Krol et al. 1988a; 1990a, b), polygalacturonase (Smith et al. 1990), ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (Quick et al. 1991) and the 10 kDa photosystem II polypeptide (Stockhaus et al. 1990). However, few examples of successful down-regulation in a heterologous system have been reported (van der Krol et al. 1988b; Visser et al. 1990; Schuch et al. 1990).

In this study, we have used an alfalfa GS_1 antisense RNA to test if it can down-regulate both GS_1 and GS_2 gene expression in the heterologous system, tobacco. Our experiments, while clearly demonstrating the effectiveness of this approach, suggest that the mechanism of downregulation using a heterologous antisense RNA does not fit the established model of RNA:RNA (or RNA:DNA) duplex formation resulting in rapid degradation or impaired transcript processing (van der Krol et al. 1988b). We have also overexpressed the alfalfa GS_1 gene in tobacco and established that the encoded polypeptide can assemble into a functional enzyme. The results of preliminary biochemical and molecular analysis of some of these plants are presented.

Materials and methods

Recombinant DNA techniques. Standard procedures were used for recombinant DNA manipulations (Maniatis et al. 1982). Plasmid pGS100 containing an alfalfa GS cDNA isolated from a *Medicago sativa* cell culture line (DasSarma et al. 1986) was a gift from Dr. H.M. Goodman (Dept of Molecular Biology and Genetics, Harvard Medical School, Mass., USA). A 1.35 kb *Ssp*I fragment encoding the entire GS coding region and extensive 5′ and 3′ untranslated regions was subcloned in both orientations into the *Sma*I site of pSP73. The gene was recovered as a *Cla*I-*Sal*I fragment that was inserted into the *Cla*I-*Xho*I polylinker sites of pMON316 (Rogers et al. 1987) in both sense and antisense orientations relative to the 35S cauliflower mosaic virus (CaMV) promoter and nopaline synthase (NOS) 3′ transcription terminator. The resulting plasmids, pGS111 and pGS121, contained the alfalfa GS gene in sense and antisense orientations with respect to the 35S promoter (Figs. 2 and 3).

Plant transformation. Plasmids pGS111 and pGS121 were mobilized from *Escherichia coli* DH5α into the *Agrobacterium tumefaciens* receptor strain pTiT37ASE by triparental mating as described by Rogers et al. (1987). *Nicotiana tabacum* cv. Xanthi plants were transformed using the leaf disc transformation procedure (Horsch et al. 1985). Transformants were selected and regenerated on MS medium containing 100 μg of kanamycin per ml. Shoots appeared 4–6 weeks after inoculation. These shoots were rooted on the same medium containing kanamycin, but minus the hormones, and transfered to potting soil:perlite:vermiculite (3:1:1) for maintenance in a greenhouse.

Isolation of poly(A)$^+$ RNA and Northern blot analysis. Total RNA was isolated using the LiCl precipitation procedure described by De Vries et al. (1982). Poly(A)$^+$ RNA was isolated by subjecting total RNA to poly(U) Sepharose chromatography (Murray et al. 1981). Poly(A)$^+$ RNA was fractionated in 1% agarose/formaldehyde gels and blotted onto nitrocellulose. Hybridization was carried out in 50% formamide at 42° C using standard conditions (Maniatis et al. 1982). Probes were prepared by either labeling purified DNA fragments by random priming (Feinberg and Vogelstein 1983) or using strand-specific RNA probes. The 1.0 kb *Bgl*II-*Bam*HI fragment from pGS100 that included most of the alfalfa GS coding region was subcloned into the *Bam*HI site of pSP73 in the sense orientation relative to the SP6 RNA polymerase transcription initiation site to give pGS301. A ^{32}P-labeled single-stranded GS antisense RNA probe was synthesized in vitro using *Sma*I-linearized pGS301, T7 RNA polymerase and [^{32}P]CTP (Riboprobe System, Promega, Wis.). Following hybridization using a riboprobe, the filter was washed at low stringency, 3 × 15 min at 42° C in 2 × SSC, 0.1% SDS. The extensive nonspecific background binding was removed by rinsing the filter in 2 × SSC followed by incubation in 1 μg/ml RNAase A in 2 × SSC at room temperature for 30 min. Finally the filter was washed at higher stringency in 0.1 × SSC, 0.1% SDS (2 × 15 min at 42° C).

Hybrid select translation. This was carried out essentially as previously described (Sengupta-Gopalan and Pitas 1986). The insert DNA (*Ssp*I fragment of pGS100) was immobilized on nitrocellulose filter disks and hybridized with 10 μg poly(A)$^+$ RNA isolated from alfalfa leaf and root. The selected RNA was recovered using 10 μg of calf liver tRNA as a carrier. The RNA was translated in vitro using the wheat germ system (Promega, Wis.) using [^{35}S]methionine (NEN) as the tracer amino acid. The translation products were separated by SDS-PAGE and made visible by fluorography and autoradiography (Laskey and Mills 1975).

Polyacrylamide gel electrophoresis. Two different PAGE systems were employed, both using the BioRad Protean II system:

A. SDS-PAGE system according to Laemmli (1970) using 14% slab gels. Proteins were then blotted onto nitrocellulose electrophoretically in 25 mM TRIS, 192 mM glycine, 5% methanol, pH 8.2. The nitrocellulose was blocked overnight with 1% BSA in TRIS-buffered saline

containing 0.05% Tween 20 and probed with a suitable antibody. The antisera used was raised against: (1) pea seed GS (Langston-Unkefer et al. 1987), diluted 1:1000; (2) *Mesembryanthemum crystallinum* phosphoenolpyruvate carboxylase (PEPC), diluted 1:1000 (a gift from Dr. H.J. Bohnert); and (3) NADH-dependent hydroxypyruvate reductase (HPR), diluted 1:16000 (Titus et al. 1983). Cross-reacting bands were made visible using an alkaline phosphatase-linked second antibody employing the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, used according to the supplier's instructions (Promega, Wis.).

B. A native PAGE system using 7.0% slab gels run in 25 mM TRIS, 192 mM glycine overnight at 4° C and 25 mA constant current. The GS activity on native polyacrylamide gels was detected using the transferase assay (Shapiro and Stadtman 1970). Following staining, the activity gels were photographed with Tech Pan film (2415) using a blue filter.

Determination of glutamine synthetase activity. Tobacco leaf tissue was ground in a mortar with ice-cold 100 mM TRIS-HCl (pH 7.8) containing 100 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5 mM glutamate and 1% (w/v) isoluble polyvinylpyrrolidone. An extraction buffer to tissue ratio of 4:1 was found to maximize the stabilization of GS activity even in older tobacco leaf tissue. The homogenate was clarified by centrifugation (40000 *g* for 15 min at 4° C). The GS activity of the supernatant was measured using the ADP-dependent transferase assay of Shapiro and Stadtman (1970). One unit of GS activity is defined as 1 µmol of γ-glutamyl hydroxamate formed per min. Protein concentration was determined by the dye-binding method of Bradford (1976) with bovine serum albumin as a standard.

Results

The GS cDNA clone from alfalfa cell culture represents the GS_1 gene and does not hybridize to GS_2 mRNA

The full-length alfalfa GS cDNA clone obtained from Dr. Goodman was isolated from a cDNA library made from RNA from alfalfa suspension culture (DasSarma et al. 1986). To determine which form of GS the cDNA clone represents and establish its relative level of expression in different alfalfa tissues, we analyzed the RNA from alfalfa root, nodule and leaf by Northern blot hybridization. As seen in Fig. 1A and B, all three probes (the 5′ and 3′ untranslated regions of the cDNA and the coding region) hybridized strongly to the root and nodule mRNA and very weakly to leaf RNA. Of the three probes, the coding sequence exhibits the highest relative level of hybridization to the leaf RNA (probe 2, Fig. 1A). Alfalfa leaves contain higher levels of GS protein and activity than roots and the major fraction of GS protein in leaves can be attributed to the chloroplast form. The absence of any significant hybridization between the alfalfa GS cDNA and leaf RNA would suggest that the cDNA represents a cytoplasmic GS and

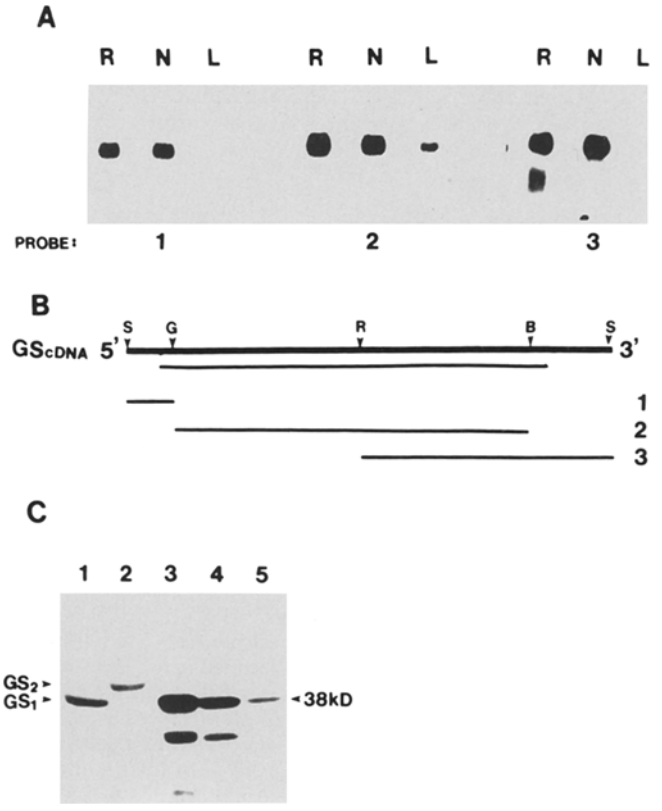

Fig. 1A–C. Characterization of the glutamine synthetase alfalfa (GS) cDNA clone (DasSarma et al. 1986). **A** Northern blot analysis of 3 µg poly(A)$^+$ RNA isolated from alfalfa root (R), nodule (N) and leaf (L). The blots were hybridized with ^{32}P-labeled DNA fragments 1, 2 and 3 isolated from the alfalfa GS cDNA (**B**). **B** Positions of the restriction sites used in the preparation of the probes: S, *Ssp*I; G, *Bgl*II; R, *Eco*RI; B, *Bam*HI. **C** Comparison of Western blot analysis of alfalfa root (lane 1) and leaf (lane 2) total soluble protein extracts (100 µg/lane) separated by SDS-PAGE and probed with pea seed GS antibody compared with the in vitro translation products obtained following hybrid select translation of alfalfa nodule (lane 3), root (lane 4) and leaf (lane 5) poly(A) RNA using the plasmid pGS100

that the cloned cDNA does not hybridize significantly to GS_2 mRNA.

The GS_2 and GS_1 polypeptides can be distinguished by their molecular weights. Thus, the primary translation products of mRNA hybrid-selected with the GS cDNA from the total mRNA population of leaf and root, should reflect the extent of sequence homology between the GS cDNA and GS_1/GS_2 mRNAs. While the root contains only GS_1 mRNA, the leaf tissue contains predominantly GS_2 mRNA and only a small fraction of GS_1 mRNA (Tingey et al. 1987; Cock et al. 1990; Peterman and Goodman 1991). The GS cDNA was used to hybrid select GS mRNA from root, leaf and nodule poly(A)$^+$ RNA populations. The RNA was translated in vitro in the wheat germ system and the translation products subjected to SDS-PAGE in parallel with total soluble proteins from root and leaf. Following electroblotting onto nitrocellulose, the strip containing the translation products was subjected to autoradiography while the strip with the total soluble protein was sub-

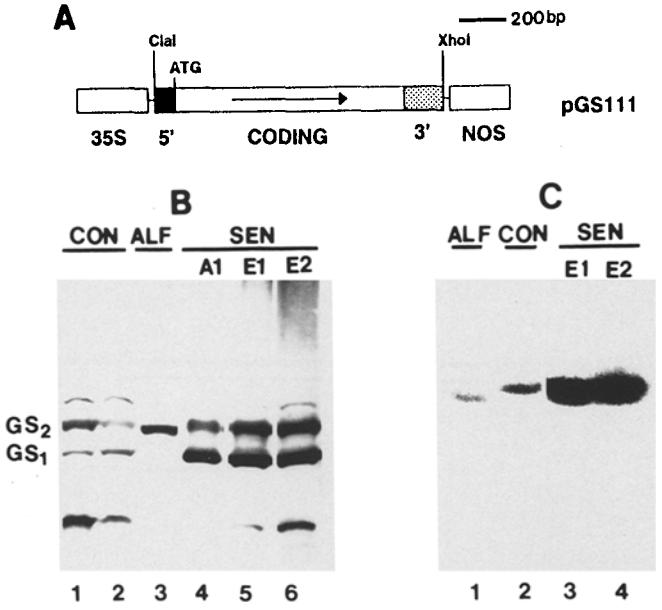

Fig. 2A–C. Analysis of tobacco plants transformed with a sense alfalfa GS_1 gene. **A** The sense construct pGS111. The full-length GS cDNA (DasSarma et al. 1986) was inserted in the sense orientation between the CaMV 35S promoter and the nopaline synthase (NOS) terminator into the polylinker of pMON316. The positions of the 5′ and 3′ untranslated regions and translation start codon (ATG) are indicated. **B** Western blot analysis of total soluble leaf protein extracts (200 μg/lane) from control tobacco (CON), alfalfa (ALF) and three independent tobacco sense transformants (SEN). The samples were separated by SDS-PAGE, transferred to nitrocellulose and the filter probed with pea seed GS antibody. The positions of GS_1 and GS_2 polypeptides are indicated. The plants used in this analysis were approximately 2–3 weeks post-planting in the soil. **C** Total soluble leaf protein (1 mg/lane) from alfalfa (ALF) control tobacco (CON) and sense tobacco transformants (SEN) were separated by native PAGE. The gel was then stained for GS activity using the transferase assay. The plants used in this analysis were approximately 6–8 weeks post-planting in the soil

jected to Western analysis. As seen in Fig. 1C, in all cases the higher molecular weight hybrid-selected translation (HST) product (38–39 kDa) comigrated with the major root GS isoform and the minor GS isoform from leaf (GS_1). No HST product migrating like the GS_2 precursor in the leaf was detected. While we do not know what the major lower molecular weight (34–35 kDa) HST product represents, we cannot rule out the possibility that it is the result of incomplete synthesis of the GS_1 polypeptide.

Taken together, our results suggest that the GS cDNA used for the antisense GS constructs encodes a GS_1 polypeptide that is expressed predominantly in the roots and nodules and forms a minor component in leaves.

Constitutive expression of the GS_1 gene in the sense orientation results in an increase in GS_1 protein in transgenic tobacco

The entire alfalfa GS_1 cDNA containing the coding region and the 5′ and 3′ flanking regions was placed behind the 35S promoter in the sense orientation (Fig. 2A) and introduced into tobacco. Western blot analysis of crude leaf extracts from young transformed (R_0) and control plants showed that plants with the GS_1 sense construct contained elevated levels of GS_1 and GS_2 polypeptides (Fig. 2B, lanes 4–6). The GS_1 polypeptide, however, showed a relatively greater increase. Of course, one cannot rule out the possibility of protein saturation on the blot in the case of the GS_2 protein. However, the GS_2 in transformed tobacco did not show any forms with an electrophoretic mobility similar to that of alfalfa GS_2 polypeptides, confirming that the introduced gene indeed codes for GS_1. While transformants E1 and E2 showed comparable levels of the GS proteins, transformant A1 showed a slightly lower level of GS proteins. An immunoreactive protein band with higher electrophoretic mobility than authentic GS was detected in all tobacco lanes, but at variable levels. The crude leaf extracts were also subjected to native gel electrophoresis and stained for GS activity. As seen in Fig. 2C, in the transformants a novel band of GS activity, in addition to the endogenous band of activity, was localized in a region comigrating with alfalfa leaf GS activity. An increase in activity was also observed in the region of endogenous tobacco leaf GS activity. Since the major GS activity in leaves is due to GS_2, an increase in the level of the endogenous GS activity band in the GS_1 sense transformants can be attributed to an increase in GS_2 protein.

Constitutive expression of the GS_1 gene in the antisense orientation results in a decrease in GS_1 and GS_2 proteins

The alfalfa GS_1 cDNA was placed in the antisense orientation distal to the 35S promoter (Fig. 3A) and introduced into tobacco. Crude total soluble protein extracts from leaves of alfalfa, young transformants (R_0) (with antisense GS_1 constructs) and control tobacco plants were subjected to SDS-PAGE followed by immunoanalysis with GS antibody (Fig. 3B). Transformants C2, D1 and D3 all showed a reduction in the level of GS_1 protein when compared to the control (Fig. 3B, lanes 4–6). A decrease in the level of GS_2 polypeptide was also observed in transformant D1 and to a very small extent in C2. The transformant D3, however, showed a slightly elevated level of GS_2 polypeptide. The results indicate that antisense alfalfa GS_1 constructs are effective in lowering the level of endogenous GS_1 protein levels and, to some extent, GS_2 protein in the heterologous plant, tobacco. Immunoreactive protein bands, one migrating faster and one migrating more slowly than the authentic GS proteins were present in most lanes containing tobacco proteins. The levels of these proteins appeared to be independent of the GS_1/GS_2 levels in the plant. The nature of these protein bands is not known at this stage. It is likely, however, that the small molecular weight immunoreactive products represent specific proteolytic products of GS.

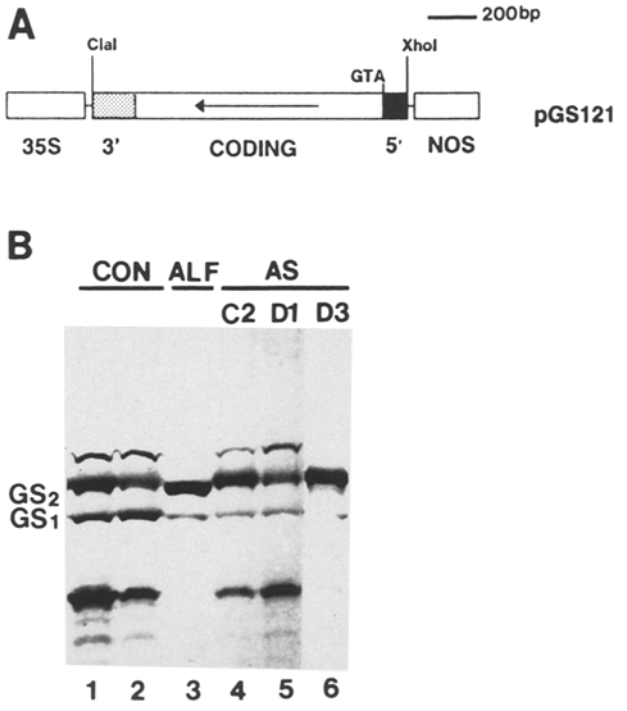

Fig. 3A and B. Analysis of tobacco plants transformed with an antisense alfalfa GS_1 gene. **A** The antisense construct pGS121. The full-length GS cDNA (Das Sarma et al. 1986) was inserted in the antisense orientation between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator into the polylinker of pMON316. The positions of the 5′ and 3′ untranslated regions and GTA codon are indicated. **B** Western blot analysis of total soluble leaf protein extracts (200 µg/lane) from control tobacco (CON), alfalfa (ALF) and three independent tobacco antisense transformants (AS). The samples were separated by SDS-PAGE, transferred to nitrocellulose and the filter probed with pea seed GS antibody. The positions of GS_1 and GS_2 polypeptides are indicated. The plants used in this analysis were approximately 2–3 weeks post-planting in the soil

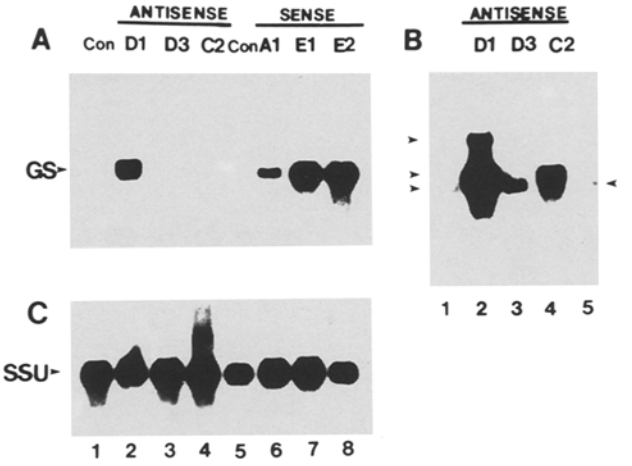

Fig. 4A–C. Analysis of the GS transcripts in the leaves of the transformed tobacco plants. **A** Poly(A)$^+$ RNA (2 µg/lane) from controls (lanes 1 and 5), three independent transformants containing antisense GS (lanes 2–4) and three containing the sense GS construct (lanes 6–8) were subjected to Northern analysis using probe 3 of the GS cDNA (Fig. 1B). **B** Longer exposure of lanes 1–5. **C** The same blot probed with the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit

GS sense and antisense transcripts accumulate to levels several-fold higher than the endogenous tobacco GS mRNA

Poly(A)$^+$ RNA from the leaves of plants transformed with the sense and antisense constructs and control plants was subjected to Northern blot analysis using a double-stranded GS gene fragment from pGS100 (Fig. 4). The accuracy of RNA loading was verified by probing the RNA blots with a rRNA gene probe (data not shown) and a Rubisco small subunit probe (De Rocher et al. 1991). As seen in Fig. 4A (lanes 2, 3, 4, 6, 7 and 8), both sets of transformants (containing either the sense or antisense GS constructs) exhibited higher levels of hybridization with the GS gene probe compared to the control (lanes 1, 5). The results suggest that both the sense and antisense transformants were accumulating high levels of the corresponding GS transcript. While the transformants with the sense GS constructs appeared to contain more GS transcripts than the transformants with the antisense GS constructs, a high degree of plant to plant variation in the level of GS transcripts was observed in both sets of transformants. Thus, sense GS transformant A1 appeared to have about a 10-fold lower level of GS transcripts than transformants E1 and E2 (based on the hybridization signal, Fig. 4A) and the antisense GS transformant D1 similarly appeared to have a 10- to 20-fold higher level of antisense GS transcript than the transformant D3. The antisense GS transcripts appeared to migrate as three different molecular weight species (Fig. 4B). This can probably be accounted for by the choice of polyadenylation sites and/or length of poly(A) tails.

There appears to be a correlation between the level of the sense GS transcripts and the increase in GS_1 polypeptide, and between the steady-state level of antisense GS transcript and the reduction in GS polypeptides in the transformants. However, the correlation is not entirely quantitative. The sense transformants E1 and E2, in spite of an approximately 10-fold higher level of GS transcripts compared to transformant A1, showed only a slight increase in the GS_1 polypeptide (Fig. 2B).

No decrease in endogenous GS mRNA in plants with antisense GS constructs

To check specifically for changes in the levels of the endogenous GS mRNA in the antisense plants, poly(A)$^+$ RNA from antisense GS and control plants was subjected to Northern analysis using the strand-specific riboprobe targeted against the endogenous sense GS transcripts. The RNA loadings were the same as in Fig. 4. As seen in Fig. 5A, no difference in hybridization to the riboprobe was observed between antisense GS plants and control plants. Our results suggest that the antisense alfalfa GS transcript does not lower the steady-state level of the endogenous GS transcripts in the heterologous plant, tobacco. Since a significant decrease in

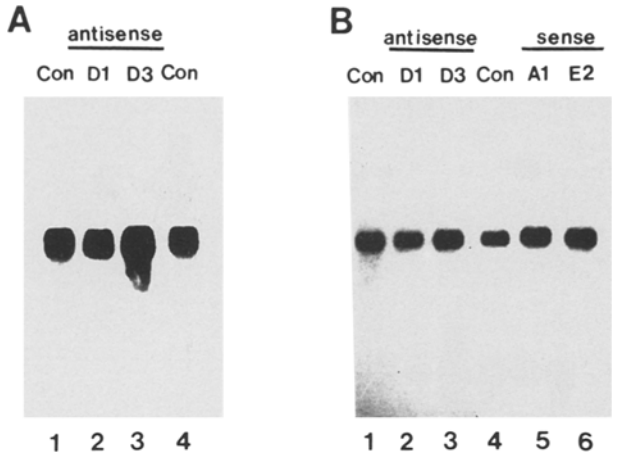

Fig. 5A and B. Analysis of the endogenous tobacco GS transcript levels in the leaves of transformants containing the antisense construct. **A** Analysis of the GS_1 transcript levels in the transformed tobacco plants. Poly(A)$^+$ RNA (2 μg/lane) from the leaves of controls (lanes 1 and 4) and two plants transformed with the antisense GS constructs (lanes 2 and 3) were subjected to Northern analysis using an antisense strand-specific riboprobe to the coding region of the alfalfa GS cDNA. **B** Analysis of the GS_2 transcript levels in the transformed tobacco plants. Poly(A)$^+$ RNA (2 μg/lane) from controls (lanes 1 and 4), two independent transformants containing antisense GS (D1 and D3, lanes 2–3) and two containing the sense GS construct (E1 and A1, lanes 5–6) were subjected to Northern analysis using a 1.5 kb *Eco*RI fragment from pGS185 that encodes a pea GS_2 cDNA (Tingey and Coruzzi 1987)

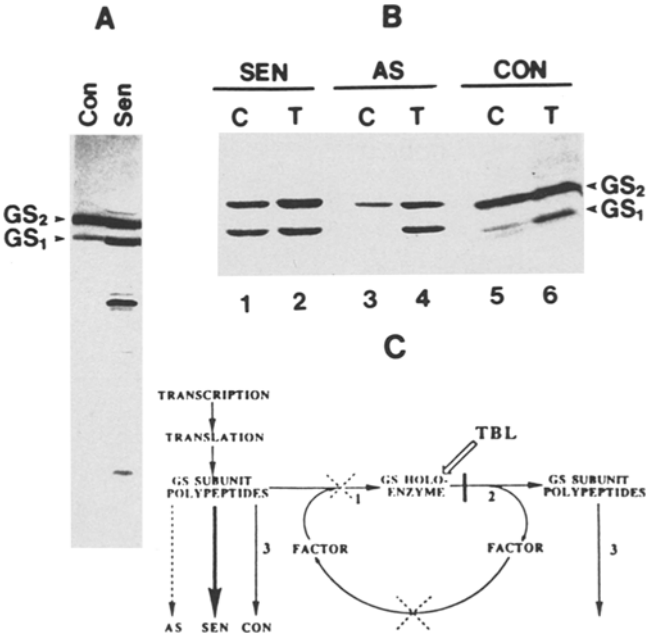

Fig. 6A–C. Analysis of possible regulatory control of GS holoenzyme assembly. **A** Western blot analysis of total soluble leaf protein extracts (200 μg/lane) from mature control tobacco (CON) and a mature sense transformant (SEN). The samples were separated by SDS-PAGE, transferred to nitrocellulose and the filter probed with pea seed GS antibody. The positions of GS_1 and GS_2 polypeptides are indicated. **B** Excised leaves from control tobacco (CON), sense transformant E2 (SEN) and antisense transformant D1 (AS) were incubated overnight in infusion buffer (10 mM D,L-malic acid, 10 mM K_2HPO_4, pH 4.1; Grabau et al. 1986) in the presence and absence of the GS inhibitor tabtoxinine-β-lactam (TβL; 25 μM). Total soluble protein extracts (100 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and the filter probed with pea seed GS antibody. The positions of GS_1 and GS_2 polypeptides are indicated. **C** Proposed model for the post-translational regulation of the assembly of the GS holoenzyme. Processes labeled 1, 2 and 3 denote assembly, turnover and proteolysis, respectively. *Thin arrows* represent normal rates of turnover, *dashed arrows* represent reduced rates, while *thick arrows* represent highly enhanced rates. The site of action of TβL is denoted as a *thick cross bar*, while the inhibitory effects of the blockage on GS assembly are represented by *broken crosses*. AS, SEN and CON represent antisense, sense and control plants, respectively

GS proteins is observed in the GS_1 antisense plants, it is likely that inhibition occurs at the level of translation.

No change in the steady state levels of GS_2 mRNA in the transformants

To check if overexpression or downregulation of GS_1 affects the level of GS_2 transcripts, leaf poly(A) RNA from the transformants and control plants was subjected to Northern blot analysis using a double-stranded GS_2 gene probe from pea (Tingey et al. 1988). The blot was probed with a rRNA gene probe to standardize loads (data not shown). As seen in Fig. 5B, while the antisense transformant D1 (lane 2) showed a slightly lower level of hybridization, the sense GS_1 transformants (lanes 5 and 6) showed no difference compared to the control (lanes 1 and 4). The results suggest that the pea GS_2 probe does not hybridize to the alfalfa or tobacco GS_1 transcript. Furthermore, the results also suggest that overexpression of GS_1 sense or GS_1 antisense transcripts does not significantly affect the level of GS_2 transcripts.

Assembly of GS subunits into a holoenzyme is under regulatory control

The steady-state level of GS_1 protein in the GS_1 overexpressing transformants did not show a direct correlation with the steady-state level of the corresponding transcript. Furthermore, analysis of the GS protein profile in crude leaf extracts from older GS_1 overexpressing plants has consistently shown the presence of higher levels of small molecular weight immunoreactive products compared to controls (Fig. 6A). These small molecular weight immunoreactive proteins most probably represent GS breakdown products. Taken together, these results suggest that GS_1 subunits synthesized in excess of a certain threshold level are degraded.

Hence we propose that the measured level of GS subunits, particularly in older plants, is not a true measure of synthesis, since this does not take GS turnover into account. To obtain a true measure of GS synthesis, we analyzed the effect of in vivo stabilization of GS by tabtoxine-β-lactam in control plants and transformants with either the sense or antisense GS constructs. Tabtoxinine-β-lactam (TβL), an irreversible inhibitor of GS en-

zyme, binds to the GS holoenzyme and prevents its dissociation into subunits, thus preventing its turnover (Temple et al. 1990). As seen in Fig. 6B, uptake of TβL by the leaves resulted in higher levels of accumulation of GS proteins compared to untreated tissue in all three cases. However, while the leaf from the plant with the antisense GS_1 construct showed about a fivefold increase in the level of GS_1 and GS_2 proteins as a result of TβL treatment, the TβL-treated leaf from the GS_1 overexpressing plants showed only a slight increase in GS_1 and GS_2 levels over the untreated control. The increase in GS levels in TβL treated control leaf over untreated leaf was intermediate between that obtained for the GS_1 antisense and the GS_1 overexpressing plants. Irrespective of the levels of GS proteins in the untreated samples, after TβL treatment the levels of GS subunits in the GS_1 antisense and GS_1 overexpressing plants were identical. This would imply that the amount of GS holoenzyme that is stabilized by TβL is about the same in both the GS_1 antisense and the GS_1 overexpressing plants. Taken together, the results suggest that the assembly of GS subunits into a holoenzyme is a rate-limiting step.

Manipulation of the level of GS protein in tobacco affects total GS activity and total protein content

While the antisense GS_1 transformant showing the highest level of GS_1 antisense RNA (D1) exhibited visible symptoms of nitrogen deficiency, the GS_1 overexpressing plants (E1 and E2) were visibly greener than control plants. This would imply that manipulation of the GS protein level affects overall plant performance. As seen in Fig. 7B, the GS_1 overexpressing plants exhibit about a 45% increase in total soluble protein over control, the GS_1 antisense plants (D1 and E3) exhibit a 40% decrease in total soluble protein. Furthermore, while the GS_1 depressed plants (D1 and E3) exhibit a 40% decrease in GS activity, the GS_1 overexpressing plants exhibit a 25% increase in GS activity (Fig. 7A). The weak antisense plants (C2 and D3) did not show any significant difference in GS activity and total soluble protein compared to the control. In these measurements, GS activity was calculated based on fresh weight, since the sense and antisense plants showed substantial changes in total soluble protein. This is despite the fact that, in older tissues, the transformants did not show such a large difference in GS protein levels compared to the control.

Modulation of GS protein levels affects phosphoenolpyruvate carboxylase (PEPC) and hydroxypyruvate reductase (HPR) levels in tobacco leaves

To check specifically if modulation of GS levels affects other related plant processes, we analyzed relative levels of PEPC and HPR in the most strongly affected antisense (D1) and sense (E2) plants. PEPC catalyzes the

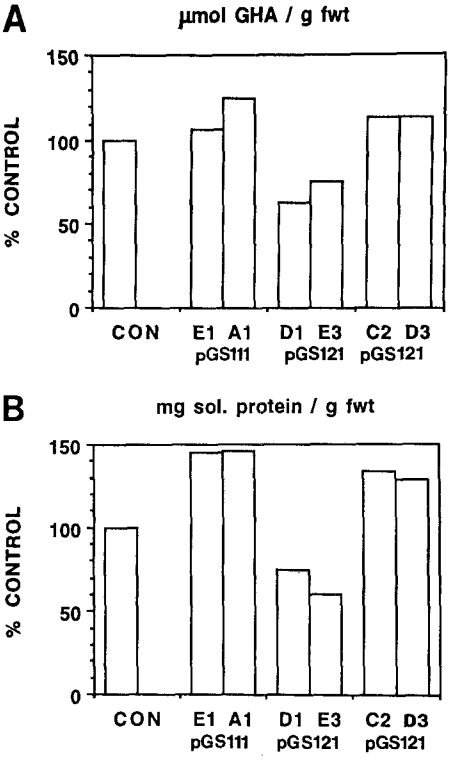

Fig. 7A and B. Analysis of leaf GS activity and total protein levels in the leaves of sense and antisense transformants. The fifth leaf from similarly aged mature control tobacco plants (CON), transformants containing both the sense (pGS111 E1 and A1) and transformants containing the antisense construct (pGS121 D1, E3, C2 and D3) were assayed as crude extracts for total leaf GS activity and total soluble protein. **A** GS activity based on fresh weight (µmol GHA/g fwt). **B** Total soluble protein (mg soluble protein/g fwt) is represented as the percentage difference over control values

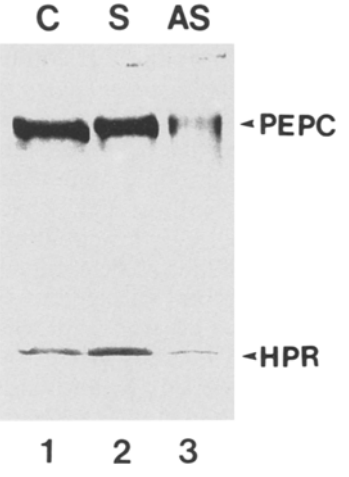

Fig. 8. Analysis of phosphoenolpyruvate carboxylase (PEPC) and hydroxypyruvate reductase (HPR) levels in the leaves of the sense and antisense transformants. Total soluble leaf protein (200 µg/lane) from mature control tobacco (C), sense transformant E2 (S) and antisense transformant D1 (AS) was fractionated by SDS-PAGE followed by Western blot analysis using antibodies to PEPC and HPR

anaplerotic fixation of CO_2 to form the precursor for the GS/GOGAT cycle and HPR catalyzes the conversion of hydroxypyruvate to glycerate in the photorespiratory cycle. Western blot analysis was performed on total soluble proteins from the control, a GS_1 antisense (D1) and a GS_1 overexpressing plant (E2) using a PEPC antibody and an HPR antibody (Fig. 8). While the GS_1 antisense plants exhibit decreased levels of both proteins, the GS_1 overexpressing plants exhibit only a very minor or no increase in the level of these proteins over the control.

Discussion

The results presented in this paper demonstrate that the full-length alfalfa GS_1 gene, when transcribed in an antisense orientation from the 35S promoter, is capable of downregulating both GS_1 and GS_2 in tobacco leaves. Besides demonstrating that the antisense RNA approach is effective in silencing GS gene expression, our results also show that a heterologous antisense GS_1 transcript is effective in inhibiting both GS_1 and GS_2 gene expression. Most of the reports on successful inhibition of target gene expression using the antisense RNA approach have utilized a homologous gene (van der Krol et al. 1988a; 1990a; Smith et al. 1990; Quick et al. 1991; Stockhaus et al. 1990). There are only a few reports on partial inhibition using heterologous antisense RNA (van der Krol et al. 1988b; Visser et al. 1990; Schuch et al. 1990). The antisense approach has also been shown not to be effective for certain genes (van der Krol et al. 1990b; Delauney et al. 1988). It is well established that a higher degree of sequence homology is maintained among the same GS isoform from different sources than among the different GS isoforms (Pesole et al. 1991; Tingey and Coruzzi 1987). We would thus predict that alfalfa GS_1 antisense RNA is more homologous to tobacco GS_1 than to the GS_2 genes. The alfalfa GS_1 coding sequence shares 81% homology at the nucleotide level to the GS_1 coding sequence of *Nicotiana plumbaginifolia* (Tingey and Coruzzi 1987). Five different regions that are highly conserved among the different GS isoforms were identified, based on multiple alignment (Pesole et al. 1991). It is interesting to note that while the alfalfa GS_1 gene did not hybridize to immobilized GS_2 mRNA of either tobacco or alfalfa, as determined by Northern analysis or analysis of hybrid selected translation products, the antisense alfalfa GS_1 transcript was capable of downregulating GS_2 in tobacco. While the mechanism of inhibition of gene expression by antisense RNA is not known, one of the proposed models assumes the mediation of hybrid formation between the mRNA and antisense RNA molecules (van der Krol et al. 1988b). If the mechanism is as proposed, our results would suggest that formation of short duplexes along the length of the GS transcript with the antisense alfalfa GS_1 RNA is sufficient for effective inhibition of expression of the GS genes in tobacco.

The steady-state level of antisense alfalfa GS_1 transcript in the leaves of transgenic tobacco was substantially higher than the endogenous GS mRNA level in the leaves of the control tobacco. We also observed that the level of antisense GS_1 transcript was indicative of the extent of inhibition. However, in spite of the accumulation of high levels of alfalfa GS_1 antisense transcripts and a decrease in the level of GS_1 and GS_2 polypeptides, no change in the level of endogenous steady-state GS mRNA was observed in these plants. This is somewhat in contrast to the more thoroughly investigated systems involving the chalcone synthase genes of petunia (van der Krol et al. 1988b; 1990a, b) and the polygalacturonase gene of tomato (Smith et al. 1990). In these cases involving homologous antisense RNA, the antisense gene transcript did not accumulate to high levels and no correlation between the antisense transcript level and the extent of inhibition was observed. Furthermore, in these and other well investigated systems (Delauney et al. 1988; Cannon et al. 1990; Oeller et al. 1991), inhibition of expression by antisense RNA has always been accompanied by a decrease in the level of the target gene message. In some of these systems (van der Krol et al. 1988a, b; Mol et al. 1990), it has been proposed that the antisense RNA forms a duplex with the corresponding endogenous sense RNA and the duplex is susceptible to degradation by RNases, resulting in the loss of the target mRNA. RNA (antisense or sense) in excess of the duplex then accounts for the remaining intact antisense or sense RNA, as the case may be. The relatively high steady-state level of antisense alfalfa GS_1 RNA, together with the absence of any effect on the endogenous GS transcript in our system, would imply that the heteroduplex is stable. The heteroduplex consisting of duplexed regions intermingled with single-stranded loops, could very well behave like RNA molecules with extensive secondary structure. This heteroduplex would differ in its physical properties from a duplex formed between homologous strands. It is likely that short duplexes, flanked by nonaligned sequences along the length of the heteroduplex, stabilize the hybrid. Eguchi (1991) has shown that an RNA double helix is stabilized by the presence of terminal unpaired bases.

A substantial lowering in the level of GS polypeptides without an accompanying decrease in the level of endogenous GS transcript in the GS_1 antisense plants, suggests that the inhibition of expression occurs at the translational level. It is likely that the stable heteroduplex formed between tobacco GS transcripts and the antisense alfalfa GS_1 transcripts is not accessible to the translational machinery. The heteroduplex is either not able to exit the nucleus or the duplex does not allow for ribosome binding and interaction with the required translation factors.

Our results suggest that the alfalfa GS_1 antisense RNA, when produced in a constructive manner downregulates both GS_1 and GS_2, in spite of the fact that the level of GS_2 transcripts far exceeds that of GS_1 (Edwards et al. 1990). This is contradictory to the general belief that antisense inhibition requires a large excess

of antisense RNA (van der Krol et al. 1988b). However, the molecular analysis of the mechanism by which the alfalfa GS_1 antisense RNA inactivates GS_1 and GS_2 is complicated by the fact that the two genes are expressed in different cell types (Edwards et al. 1990; Brears et al. 1991) and the local concentration of each transcript type is not known. This implies that steady-state levels of antisense GS_1 RNA measured by Northern blot analysis cannot reflect the ratio of antisense to sense RNA in each cell type. Of course, it remains unresolved whether the decreased expression of GS_2 in the GS_1 antisense plants is a direct effect of antisense inhibition or an indirect effect resulting from the poor overall performance of the plants.

It is not surprising that in the GS_1 sense tobacco plants, the alfalfa GS_1 subunits are not only synthesized but also assembled into an active enzyme. Eckes et al. (1989) had previously demonstrated the successful expression of this particular alfalfa gene in tobacco at the enzymatic level. What is, however, interesting is that an increase in GS_1 protein is also accompanied by an increase in GS_2 protein subunits and active GS_2 enzyme (Fig. 2). Since no increase in GS_2 transcript was observed in the GS_1 overexpressing transformants, the increase in GS_2 protein in these transformants can be attributed either to improved translation efficiency or to increased GS enzyme stability. Changes in the level of protein synthesis and turnover, in turn, can be attributed to the overall performance of the plants.

GS_1 genes are specifically expressed around the vascular tissue (Edwards et al. 1990; Brears et al. 1991). Based on that, it has been proposed that GS_1 protein functions in the synthesis of glutamine for nitrogen transport (Brears et al. 1991). Furthermore, based on the fact that GS_1 gene expression is enhanced in senescing rice leaves and is accompanied by a marked decrease in glutamate content, Kamachi et al. (1991) have also suggested that GS_1 functions in the synthesis of glutamine for transport from senescing leaves to growing tissue. How does modulation of GS_1 levels contribute to plant performance? Can improved performance by the GS_1 overexpressing plants be simply due to increased GS activity in the transport cells or does the GS_1 protein, now synthesized in the photosynthetic cells, assist in the assimilation of photorespiratory ammonia that diffuses out of the chloroplast? Similarly, is the poor performance of the GS_1 antisense transformants attributable to downregulation of GS_1 or GS_2 protein? These questions can only be addressed if the expression of these genes is modulated individually and in specific cell types. Such experiments are in progress.

A decrease in the steady-state level of HPR and PEPC in the GS_1 antisense plants and a minor increase in these proteins in the GS_1 overexpressing plants is probably reflective of the overall status of the plant. However, we cannot rule out the possibility that changes in these proteins are a direct outcome of GS levels. HPR catalyzes a step in the photorespiratory cycle and thus changes in the level of this protein probably are a result of changes in GS_2 levels. Similarly, changes in PEPC levels accompanying changes in GS levels might indicate that synthesis of an empty C-skeleton is somehow influenced by the plant's potential to load these C-skeletons with nitrogen. This interesting observation indeed merits further investigation.

Based on the analysis of GS protein levels in leaves of GS_1 overexpressing sense, and GS_1 antisense, plants incubated with the toxin TβL, it appears that the assembly of the GS holoenzyme is under some type of regulatory control (Fig. 6C). The toxin binds irreversibly to the GS holoenzyme (Langston-Unkefer et al. 1987), thus preventing its turnover (Temple et al. 1990). In its presence, the GS subunits that assemble into a holoenzyme are locked in and the subunits are not proteolytically degraded (Temple et al. 1990). If all the GS subunits that are synthesized in a plant are assembled into a holoenzyme, the GS_1 overexpressing plants should show relatively higher accumulation of GS_1 polypeptide in the presence of TβL, when compared to control or antisense plants. In our studies, however, irrespective of the plant background (transformed with sense or antisense GS constructs), the steady-state level of GS subunits in the presence of TβL was more or less similar (Fig. 6B). Based on these results, we propose that assembly of GS subunits into a holoenzyme requires a factor which is released when the enzyme is turned over and recycled (Fig. 6C). In the presence of TβL, the holoenzyme, along with the factor, is locked into the assembled form and the factor is no longer available for assembling more GS subunits. Blocking the GS holoenzyme turnover in the GS overexpressing plants will very rapidly inhibit the assembly process. Thus, over an extended period of time, blockage in turnover will result in the same amount of GS subunits being locked into a holoenzyme, irrespective of the concentration of available GS subunits (sense versus antisense plants). In this context, it is interesting to note that Hemon et al. (1990) showed that tobacco plants transformed with the *Phaseolus vulgaris* γGS coding sequence driven by the 35S promoter contained high levels of γGS; and the corresponding enzymatic activity in young plants, but very insignificant levels of γGS in mature plants. The γGS transcript level was, however, comparable in both young and mature plants. Our results also point to a similar phenomenon. While the younger transformants (sense and antisense) showed a dramatic difference in the level of GS protein when compared to the control (Figs. 2 and 3), the older plants did not show such substantial differences (Fig. 6B). Our interpretation of our data and those of Hemon et al. (1990) is that the post-translational assembly of GS polypeptides is probably under some form of developmental control. It is crucial that we understand this regulatory step if we wish to successfully modulate GS levels in plants.

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