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Forcing expression of a soybean root glutamine synthetase gene in tobacco leaves induces a native gene encoding cytosolic enzyme

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Abstract

Glutamine synthetase (GS; EC 6.3.1.2) is present in different subcellular compartments in plants. It is located in the cytoplasm in root and root nodules while generally present in the chloroplasts in leaves. The expression of GS gene(s) is enhanced in root nodules and in soybean roots treated with ammonia. We have isolated four genes encoding subunits of cytosolic GS from soybean (*Glycine max* L. cv. Prize). Promoter analysis of one of these genes (GS15) showed that it is expressed in a root-specific manner in transgenic tobacco and *Lotus corniculatus*, but is induced by ammonia only in the legume background. Making the GS15 gene expression constitutive by fusion with the CaMV-35S promoter led to the expression of GS in the leaves of transgenic tobacco plants. The soybean GS was functional and was located in the cytoplasm in tobacco leaves where this enzyme is not normally present. Forcing this change in the location of GS caused concomitant induction of the mRNA for a native cytosolic GS in the leaves of transgenic tobacco. Shifting the subcellular location of GS in transgenic plants apparently altered the nitrogen metabolism and forced the induction in leaves of a native GS gene encoding a cytosolic enzyme. The latter is normally expressed only in the root tissue of tobacco. This phenomenon may suggest a hitherto uncharacterized metabolic control on the expression of certain genes in plants.

Introduction

Glutamine synthetase (GS; EC 6.3.1.2.) assimilates ammonia into organic matter and is present in the cytosol and chloroplasts of most higher plants [for reviews, see 30, 34]. Roots and root nodules generally contain a cytosolic GS (GSr and GSn, respectively), while leaves contain a chloroplastic GS isoform (GS2); the latter can be distinguished from GSr by ion-exchange chromatography [18, 20]. A tissue-specific GS isoform is present in root nodules of certain legumes

[3, 13]. GS is an octameric enzyme composed of 40 kDa subunits in the cytoplasmic enzyme and 45 kDa subunits in the chloroplastic form [27, 38].

Why GS occurs in two different compartments and why certain higher plants lack cytosolic GS, while in others it can represent up to 70% of the total leaf GS activity [30], is not understood. Furthermore, the nature of internal or external factors that control the expression of the respective GS genes encoding a particular isoenzyme remains to be determined. Biochemical [2, 46] and genetic [45] studies have demonstrated that chloroplastic GS is responsible for ammonia assimilation during photorespiration [26] and that the enzyme is more active during the daytime [12, 20]. A number of higher plants do not contain any leaf cytosolic enzyme while the C4 plants which lack photorespiratory metabolism, contain high cytosolic GS activity [18, 31]. In barley chloroplastic GS mutants, remnant cytosolic GS activity is insufficient for the reassimilation of photorespiratory ammonia [45].

In roots and root nodules, cytosolic GS is involved in primary assimilation of ammonia produced either by nitrate reduction or as a result of nitrogen fixation [see 13, 17, 34]. An increasing number of studies have shown that GS is encoded by a multigene family and each isoenzyme may comprise one of several gene products [3, 7, 8, 15, 38, 44]. The expression of each gene appears to be tissue-specific [3, 8, 14, 38] or regulated by external factors such as light [12, 38], nitrogen availability [17, 29, 42], senescence [24], and symbiotic association of *Rhizobium* [13, 15]. We have isolated several soybean genes encoding cytosolic GS in roots and root nodules. One of these genes was introduced in tobacco after the coding region of this gene was linked with a cauliflower mosaic virus (CaMV) 35S promoter. This resulted in the synthesis of functional soybean GS in tobacco leaf cytoplasm. We observed that expression of the soybean GS gene in tobacco leaves forced a native tobacco gene encoding cytosolic GS to be concomitantly expressed in leaves, where this gene is not normally expressed.

Materials and methods

Isolation of cDNA and genomic clones of cytosolic GS from soybean and tobacco

A cDNA clone encoding cytosolic GS was isolated from a tobacco (*Nicotiana tabacum* L. cv. Xanthi) seedling λgt10 library kindly provided by Dr. Palme (Max-Planck-Institut für Züchtungsforschung, Köln, Germany). The tobacco library was screened by plaque hybridization [28] using the ^{32}P-labelled insert of *Nicotiana plumbaginifolia* GS cDNA [39]. The *N. tabacum* GS cDNA insert was subcloned in a Bluescript vector (Stratagene, LaJolla, Ca) using the *Eco* RI sites. Sequence comparison of the tobacco GS cDNA showed 95% homology with that of *N. plumbaginifolia*, including the 3′ and 5′ non-coding regions [39; see also Fig. 3A]. A full-length cDNA clone from soybean (*Glycine max* L.cv. Prize) nodules was isolated by complementation in *Escherichia coli* as described [29]. Soybean genomic DNA was isolated from embryonic axes according to the procedure described by Paszkowski *et al.* [35]. After partial digestion with *Sau*3A, DNA fragments ranging from 15 to 20 kb were isolated by centrifugation on a 5 to 20% sucrose gradient and ligated into the *Bam* HI site of λEMBL3 vector. Soybean GS genomic clones were isolated using pGS20 as probe [17], and GS15 clone was analyzed in detail. Restriction maps of the tobacco and soybean GS cDNA clones are presented in Fig. 1.

Construction of the chimeric 35S-GS15 gene

A fragment containing the coding region and 3′ end of GS was isolated from λGS15 after digestion of the purified phage DNA by *Sal*I and partial digestion by *Eco* RI, and was subcloned into pBlueScript. The 5′ end *Eco* RI site is located 65bp upstream from the translation initiation codon ATG [29], and the coding region spans up to 4 kb downstream. The location of the 3′ end of this gene was determined using the shortest cDNA clone (300 bp) isolated from a soybean nodule cDNA library [17]. The *Xba* I/*Sal* I frag-

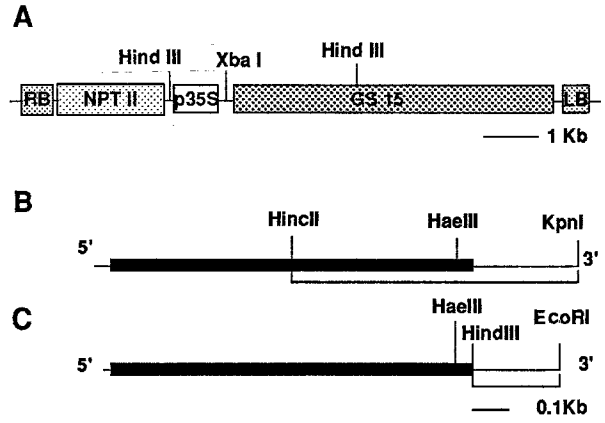

Fig. 1. A. CaMV 35S promoter fused with GS15 coding region to obtain 35S-GS15 in pBI121 [23]. RB and LB represent the right and left borders of the T-DNA. NPTII, neomycin phosphotransferase gene with nopaline synthetase promoter and terminator confering kanamycin resistance. B and C. Restriction maps of pGS20 from soybean [29] and cytosolic GS cDNA clone from tobacco indicating coding (thick line), non-coding (thin line) regions and positions of the restriction sites used to subclone specific fragments. The square brackets below each map indicate the fragments that were sub-cloned into pSPT for the S1 nuclease protection experiment (see Fig. 3).

ment containing the coding region of λGS15 was removed from pBluescript and subcloned into *Xba* I/*Eco* RI-cut (devoid of GUS) pBI121 [23] by blunt-ending at the *Eco* RI site to obtain p35S-GS15.

Plant transformation and regeneration

The pBin vector containing the appropriate construct in *E. coli* DH5 was transferred to *Agrobacterium tumefaciens* (strain LBA4404) *via* triparental mating using pRK2013 as a helper [10]. Kanamycin-resistant tobacco plants were regenerated during leaf disk transformation [22], transferred to soil, and watered daily with complete Hoagland solution [21] for four weeks, after which time they were harvested for protein and RNA extraction. Root and leaf tissues from independently transformed plants were analyzed for the level of GS transcripts. Four independently transformed plants expressing high levels of soybean GS were further analyzed for the phenomena reported here.

Isolation of poly(A)$^+$ mRNA and northern blot analysis

Poly(A)$^+$ mRNA isolation and northern blot analysis were carried out as described earlier [17]. Soybean and tobacco cytosolic GS cDNA probes were labeled with ^{32}P-dCTP, and hybridization was carried out at 65 °C in 50 mM Tris-HCl, pH 7.6, 2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll (40 000), 0.2% SDS, 0.1% sodium pyrophosphate, 6% NaCl, and 0.1 mg/ml denatured calf thymus DNA. Final washes were carried out in 0.1 × STE (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA), 0.1% SDS at 65 °C, and X-ray film was exposed to filters at – 80 °C.

S1 nuclease mapping of RNA

S1 protection experiments were carried out according to Coruzzi *et al.* [6]. The 3′ end of the tobacco cDNA clone (*Hin*d III-*Eco* RI fragment; Figs. 1C and 3A) was subcloned into pSPT18, whereas a *Hin*c II-*Kpn* I fragment of pGS20 from soybean was subcloned into pSPT19 (Fig. 1B) and both inserts were sequenced [36]. ^{32}P-labelled single-stranded tobacco and soybean RNA probes were synthesized *in vitro* using SP6 polymerase (Transprobe SP kit, Pharmacia) and ^{32}P-UTP (Amersham). Poly(A)$^+$ mRNA (1 μg) was used in each experiment.

Protein extraction, fractionation, enzymatic assay and western blotting

Protein extraction and western blot analysis were carried out as described [17] using tobacco GS antibodies [19]. Proteins were vizualized using peroxidase-conjugated goat anti-rabbit antibodies [40]. For DEAE-Sephacel chromatography, fresh leaves or roots (10 g) were homogenized with a mortar and pestle in 100 ml of 35 mM Tris-HCl pH 7.6, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM DTT, and 1 g Polyclar (acid-washed). The brei was filtered through two layers of cheesecloth and centrifuged at 45 000 × *g* for 30 min. The supernatant was layered directly

onto a DEAE-Sephacel column, 91 cm × 10 cm (Pharmacia, Uppsala) that had previously been equilibrated in the homogenizing buffer without Polyclar AT. Proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in 100 ml of the equilibrating buffer. The flow rate was adjusted to 20 ml/h and 2 ml fractions were collected. GS activity was assayed in each fraction as described [33].

Isoelectric focusing

Isoelectric focusing was carried out as described by Hirel *et al.* [19], followed by western blot analysis according to Towbin *et al.* [40].

Preparation of tobacco genomic DNA and Southern blot analysis

Tobacco DNA was prepared from leaf tissue as described by Dallaporta *et al.* [9]. For Southern blot analysis, 10 μg of *Hin*d III-digested genomic DNA was electrophoresed in 0.7% agarose gel, transferred to GeneScreen (DuPont, Boston, MA), and hybridized with a ^{32}P-labelled *Hae* III-*Kpn* I 3′ fragment of pGS20 (Fig. 1B).

Tissue fixation and protein-A gold immunolabelling

Tissue fixation and protein-A gold immunolabelling were carried out as described [5, 41].

Results

Constitutive expression of the soybean root cytosolic GS gene in leaves of transgenic tobacco plants

The soybean gene encoding cytosolic GS (GS15) induced in response to ammonia [29] was made constitutive by replacing its promoter region with CaMV-35S promoter. A transcription fusion was made in a binary vector as shown in Fig. 1A, and tobacco and *Lotus* plants were transformed using *Agrobacterium*-mediated transformation [22, 29]. Expression of this chimeric gene was examined in transgenic plants after selection and regeneration on kanamycin. Eleven independently transformed plants were assayed for the expression of GS activity.

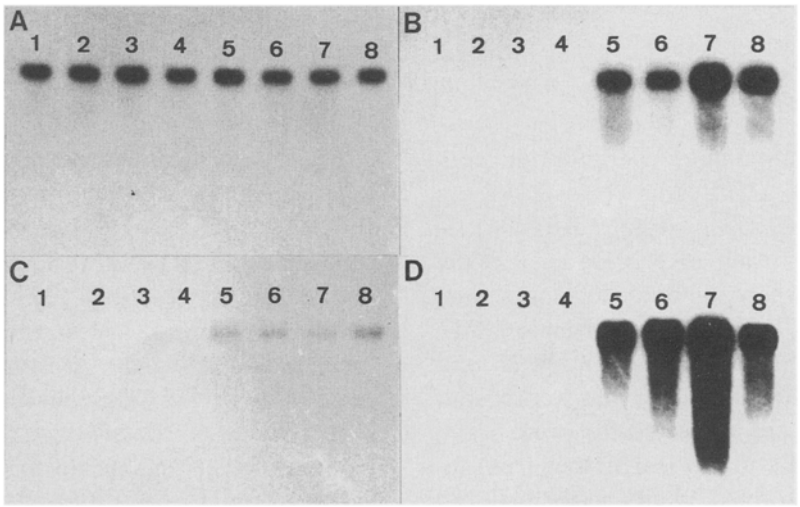

Fig. 2. Expression of cytosolic GS mRNA in tobacco and transgenic tobacco containing soybean GS gene. Panels A and B contain root poly(A)$^+$ mRNA and panels C and D contain leaf poly(A)$^+$ mRNA (1 μg each). Hybridization was with the *Hin*d II-*Eco* RI fragment (Fig. 1C) of the tobacco GS cDNA probe (A and C) and the *Hin*c II-*Kpn* I fragment (Fig. 1B) of the soybean GS cDNA probe (B and D). Lanes 1 and 2, poly(A)$^+$RNA from untransformed plants; lanes 3 and 4, RNA from plants transformed with pBI121; lanes 5 to 8, RNA from four independently transformed plants expressing 35S-GS15.

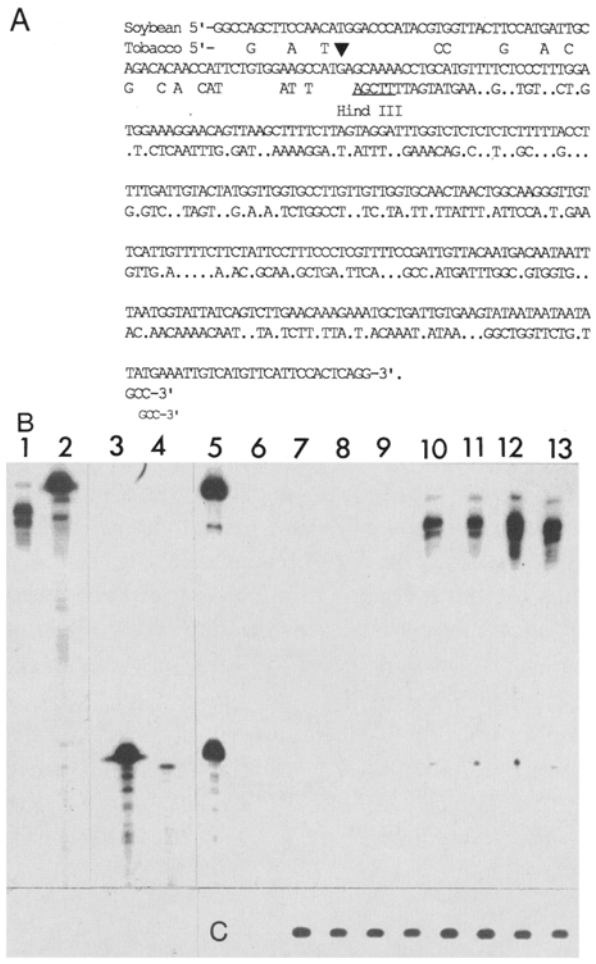

Fig. 3. A: Comparison of the 3′-end sequences of the soybean GS cDNA (pGS20) and a tobacco cytosolic GS cDNA. The nucleotide sequence of the tobacco cDNA clone is indicated as it differs from pGS20. The *Hin*d III restriction site used for subcloning is underlined, and the stop codon is shown by an arrowhead. The *Hin*d III-*Eco* RI fragment of the tobacco cDNA (Fig. 1C) was subcloned in pSPT18 and used as a specific antisense RNA probe in the S1 protection experiments to demonstrate the induction of native cytosolic GS mRNA in p35S-GS15 transformed plants. B: S1 protection assay on poly(A)$^+$ RNA showing induction of native cytosolic GS transcript in leaves of transgenic tobacco plants expressing soybean GS constitutively. Soybean and tobacco RNA probes were generated by subcloning respective GS fragments in pSPT19 vector followed by SP6 transcription and poly(A)$^+$ RNA (1 μg each) was used for hybridization. Lane 1: S1-protected fragment of 859 nucleotides (including 12 bp polylinker sequence) generated by soybean antisense RNA probe (871 nt) shown in lane 2. Lane 3: Undigested tobacco probe of 297 nt). Lane 4: S1 protected fragment with tobacco probe using tobacco root RNA. Lane 5: Mixture of undigested soybean and tobacco antisense RNA probes. Lanes 6 and 7: RNA from untransformed plants. Lanes 8 and 9: RNA from plants transformed with pBI121. Lanes 10 to 13: RNA from plants transformed with p35S-GS15. C: Tobacco leaf poly(A)$^+$ RNA (1 μg) hybridized with a ^{32}P-labelled insert of the β-subunit of the mitochondrial ATP-synthetase [4] to show that equal amounts of RNA were used in each S1-protection experiment (lanes 6–13).

The level of expression of soybean cytosolic GS mRNA in transgenic tobacco plants was determined by northern blot assay using the ^{32}P-labelled *Hinc* II-*Kpn* I fragment (Fig. 1B) of the cDNA clone pGS20 [17, 29]. Fig. 2B and D (lanes 5 to 8) show the amount of soybean GS transcript produced in roots and leaves of four independently transformed tobacco plants. No hybridization of the soybean GS probe was observed with RNA from roots or leaves of untransformed tobacco plants (Figs. 2B and D, lanes 1 and 2) or in plants transformed with pBI121 (lanes 3 and 4). The amount of tobacco cytosolic GS mRNA present in root and leaf tissues of transgenic plants was also determined using the 3′ non-coding region of the tobacco GS cDNA clone (Fig. 1C). No tobacco cytosolic GS transcript was detected in leaves of control plants (Fig. 2C, lanes 1 to 4), while abundant GS transcript was present in roots (Fig. 2A).

Induction of native cytosolic GS gene in transgenic tobacco plants expressing soybean GS constitutively

In four transgenic tobacco plants exhibiting high levels of soybean GS gene expression, the amount of tobacco GS mRNA in roots was similar to the amount in untransformed control plants (Fig. 2A, lanes 5 to 8). In leaves of these plants, expression of native cytosolic GS mRNA was observed (Fig. 2C, lanes 5 to 8). The two types of GS could be distinguished either by species-specific probes (Fig. 2) or by S1 mapping using a specific GS fragment from tobacco and soybean (Fig. 3). Figure 3A shows sequences of the 3′ ends of the tobacco and soybean GS cDNA clones, respectively, beginning at a common *Hae* III site 68 bp upstream of the termination codon. Only 33%

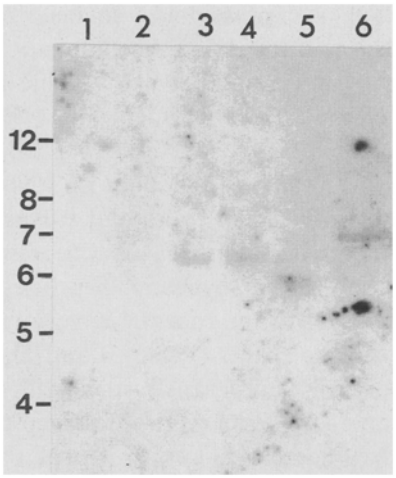

Fig. 4. Southern blot analysis of transformed tobacco plants. Genomic DNA (10 μg) was digested with *Hind* III and probed with the *Hae* III-*Kpn* I 3′-end fragment of pGS20 cDNA (see Fig. 1B). Lane 1, DNA from untransformed plant; lane 2, plant transformed with 35S-GUS [23]; lanes 3, 4, 5 and 6, four independently transformed plants with p35S-GS15. The sizes of marker DNA fragments are in kb.

homology exists in the 3′ non-coding ends of tobacco and soybean GSs, which is insufficient for cross-hybridization. Thus, the fragment of tobacco GS cDNA used as a probe clearly discriminated between soybean and tobacco transcripts (Fig. 2). To further confirm the specificity of the tobacco probe in the northern blot experiment, S1 protection assay was carried out using ^{32}P-labelled antisense RNA produced from the same soybean- and tobacco-specific cDNA fragments (Fig. 1B and C). Induction of the native cytosolic GS transcript in leaves of transformed plants can be clearly seen in Fig. 3B, corresponding to the 287 bp protected tobacco fragment (lanes 10–13, cf. lane 4). The 859 bp protected fragment found only in leaves of transgenic plants expressing the soybean GS gene served as a control (lanes 10–13, cf. lane 4). Similar induction results were observed in four independently transformed plants, suggesting that this was not a chance event but a reflection of some physiological control on gene expression. That an equal amount of RNA was used in each lane is demonstrated (Fig. 3C) by the hybridization of β-subunit of ATP synthase [4].

Southern blot analysis of 4 independently transformed plants expressing high levels of GS revealed (Fig. 4) random integration of the p35S-GS15 construct in the tobacco genome. Lanes 3 to 6 show that a DNA fragment of approximately 6 kb hybridized with the 3′ end of GS probe, confirming that the 3′ end of GS (3.5 kb from *Hind* III site to the left border of T-DNA; see Fig. 1A) was integrated at different sites into the genome.

Synthesis and activity of soybean cytostolic GS in transgenic tobacco plants

To determine if the soybean GS transcript in transgenic tobacco produced a functional enzyme, we analyzed GS enzyme activity in leaf and root tissues. The data presented in Figs 5 and 6 suggest that soybean GS mRNA indeed produced a functional cytosolic enzyme in leaves of trans-

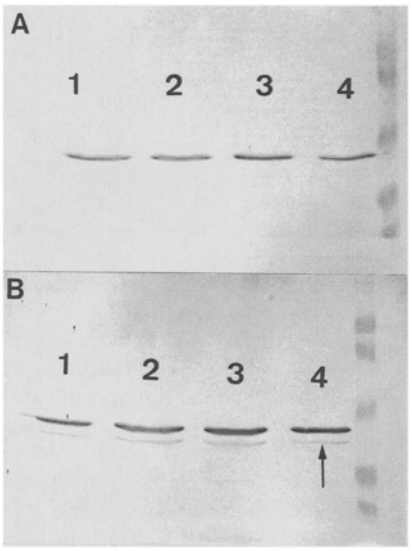

Fig. 5. Western blot analysis of GS subunits in tobacco plants transformed with p35S-GS15. A: Lanes 1 and 2: untransformed plants; Lanes 3 and 4: plants transformed with pBI121; B: Lanes 1 to 4: plants transformed with p35S-GS15. The arrow indicates the position of the extra protein band corresponding to one of the soybean cytosolic GS subunits (M_r 40 kDa) [17]. The larger protein band M_r 45 kDa) corresponds to the chloroplastic GS subunit [39] (see also Fig. 5A). The bands in the lane on the right side of each panel correspond to the position of protein markers (Bio-Rad Laboratories). Their respective molecular weights are 110, 84, 47, 33 and 24 kDa starting from the top of the panel.

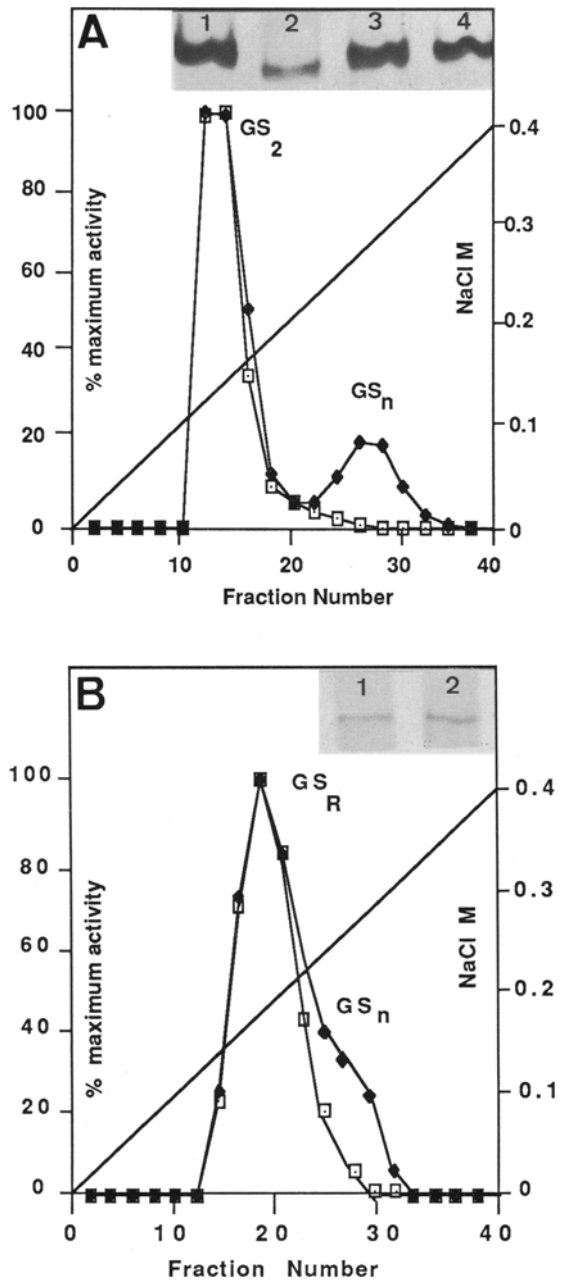

Fig. 6. DEAE-Sephacel chromatography of GS activity in leaf (A) and root (B) extracts from untransformed and transformed (35S-GS15) tobacco plants. Leaf extract from untransformed plants □—□. Leaf extract from p35SGS15-transformed plants ■—■. The top right-hand corner in panel A shows a western blot of the corresponding peaks of GS activity (see Materials and methods). A. Lanes 1 and 3: first peak of GS activity in untransformed and 35S-GS15 transformed plants, respectively, corresponding to the tobacco chloroplastic GS subunit (GS2, see lane 4). Lane 2: second-peak present only in 35S-GS15-transformed tissue corresponding to soybean cytosolic GS subunit (GSn). Lane 4: purified chloroplastic GS from untransformed tobacco plants. B. GS elution profile in root samples from control and transformed tobacco. Insert shows a western blot of cytosolic GS activity (GSr) in roots of untransformed tobacco (lane 1) and transformed plants (lane 2) where GS activity is represented by a mixture of GSr and GSn.

genic tobacco. Cytosolic and chloroplastic GS isoenzymes can be identified either by determining the size of their respective subunits by SDS-PAGE, or isoelectric focusing followed by western blot assay, or by separating isoenzyme activities with ion-exchange chromatography. Chloroplastic and cytosolic GS enzymes are generally eluted at different ionic strengths, which may vary according to the plant or the organ examined [see 30]. Furthermore, GS can function as a homomer or a heteromer [13, 16], and it has been shown in *Phaseolus vulgaris* that cytosolic form of this enzyme consists of three different subunits, α, β and γ [8]. Figure 5 shows a western blot of a crude protein extract from leaves of control and transformed plants immunoreacted with tobacco chloroplastic GS antibodies. In leaves of plants over-expressing soybean GS mRNA, an extra protein band with a molecular mass of 40 kDa was detected (Fig. 5B, lanes 1 to 4). This protein corresponds to a cytosolic GS that is not normally expressed in tobacco leaves [31]. The large protein band of 45 kDa, detected in both control and transformed plants (Fig. 5A and B), corresponds to the chloroplastic GS subunit. In root tissue, it is difficult to distinguish pre-existing cytosolic GS protein from the new protein expressed in transgenic plants, because the subunit molecular mass of both proteins is about 40 kDa and no epitope-specific antibody is available. The total amount of GS protein was found to be increased in transformed plants, however (Fig. 5B).

Potential GS activity of the extra protein band in leaves of transformed tobacco plants (Fig. 5) was tested following DEAE-Sephacel chromatography of a crude leaf extract. Figure 6 shows the elution pattern followed by measurement of

GS (biosynthetic) enzymatic activity [33]. In leaves of control plants, a single peak of GS (GS2) activity eluted at 0.13 M NaCl was detected (Fig. 6A), corresponding to the chloroplastic enzyme. In plants transformed by p35S-GS15, an extra peak of GS activity was eluted at 0.25 M NaCl, representing about 25 to 30% of total GS activity and corresponding to root and root nodule cytosolic GS (GS_n). Western blot analysis of this fraction confirmed (Fig. 6, insert) the presence of an additional cytosolic GS protein (cf. Fig. 5). An extra peak of GS activity was also observed in root tissue by DEAE-Sephacel chromatography (Fig. 6B). Because this activity was eluted at the same ionic strength as that in leaves of transformed tobacco, suggests that it is represented by the soybean GS protein. Moreover, both activities exhibited a similar subunit composition of 40 kDa (Fig. 6B, insert).

We cannot rule out the possibility that part of the recombinant GS protein in leaves was produced from the induced native cytosolic message; however, the relatively low level of transcripts in leaves of transgenic plants compared to roots (Fig. 2A and C) suggests that contribution of native mRNA to the synthesis of new GS is probably very low. In isoelectric focusing experiments followed by western blotting (Fig. 7), we did not detect any polypeptides except that corresponding to the soybean GS. This could be due to the lack of translation of newly synthesized tobacco cytosolic GS mRNA, stability, or to the sensitivity limits of the detection. Nevertheless, this experiment demonstrated that cytosolic GS subunit synthesized in transgenic plants corresponds to the most abundant polypeptide found in soybean nodule GS [see 17].

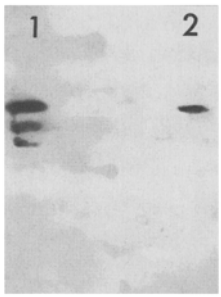

Fig. 7. Isoelectrofocusing and immunodetection of GS polypeptides of soybean cytosolic GS in transgenic tobacco. Lane 1: Soluble proteins extract from nodule (40 μg). Lane 2: (GSn) isolated by DEAE-Sephacel chromatography from p35S-GS15 transformed tobacco leaves (see Fig. 6A).

Subcellular localization of GS protein in transgenic tobacco plants

The presence of GS in both chloroplast and cytoplasm of transgenic tobacco was demonstrated by direct immunocytochemical localization. Antibodies against tobacco chloroplastic GS that react to both cytosolic and chloroplastic GS proteins [29], were used in this experiment. In leaves of control plants, gold particles appear over the chloroplast stroma only (Fig. 8), while tissue from transgenic tobacco shows both cytosolic and chloroplastic location of GS. Together with the previous data, this finding demonstrates that a functional soybean cytosolic GS can be expressed in a subcellular compartment of transformed tobacco leaves where there is normally no GS activity. No phenotypic effect on plant growth was apparent in plants producing GS in leaf cytoplasm [cf. 11].

Discussion

To understand the regulation of cytosolic GS in leaves of higher plants, a soybean cytosolic GS gene specifically expressed in roots and root nodules was expressed in root and leaves of transgenic tobacco under the control of a strong constitutive (CaMV-35S) promoter. In these experiments, interspecies splicing occurred normally, producing abundant soybean GS mRNA. A 1.5 kb poly(A)$^+$ mRNA was detected in roots and leaves which translated into an enzymatically active cytosolic soybean GS protein in transgenic tobacco leaves.

We observed concomitant expression of the gene encoding native cytosolic GS in transgenic tobacco leaves expressing soybean GS. This induction is unprecedented and may represent hitherto uncharacterized metabolic control of plant

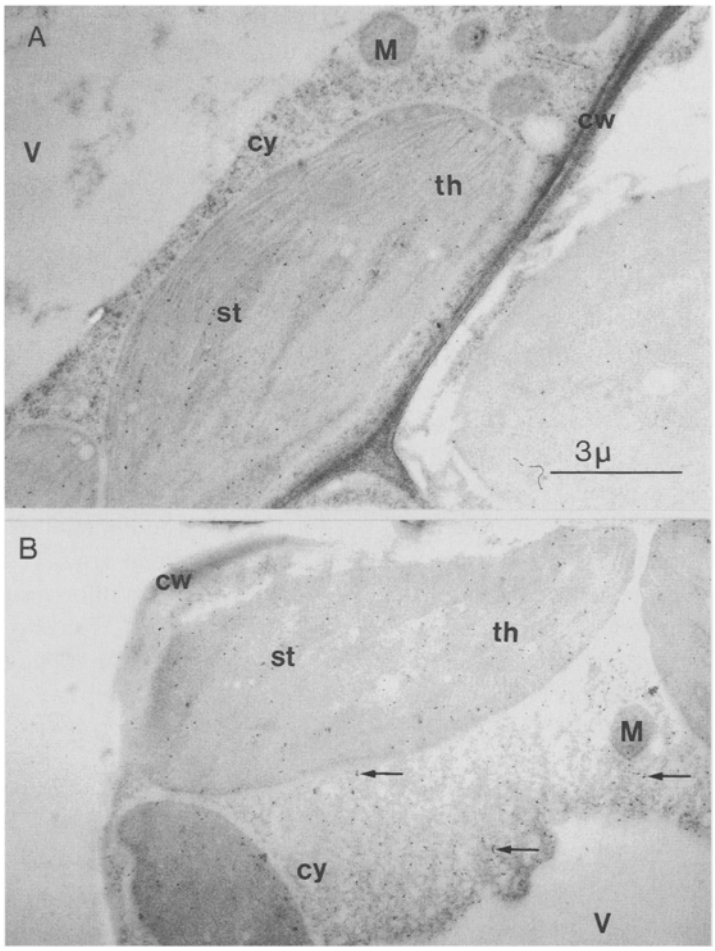

Fig. 8. Protein-A immunogold localization of GS in transgenic tobacco plants. A. Leaf section from untransformed plants. B. Section from plants transformed with p35S-GS15. Note the location of gold particles in the cytosol of transformed plants (arrows). cy, cytosol; cw, cell wall; mi, mitochondria; st, stroma; th, thylakoid; v, vacuole.

gene expression. One possible explanation of this phenomenon is that forced expression of soybean GS gene in a subcellular compartment of leaf, where it is not normally expressed, altered the regulation of native gene expression due to a change in metabolites [31]. Co-suppression of a homologous gene *in trans* has recently been observed [32]. In leaves of higher plants exhibiting no cytosolic GS activity, trace amounts of the corresponding mRNA are generally present [17, 39], but this RNA does not give rise to functional GS *in vivo* (see Figs. 5 and 6). Enhancing the expression of this cytosolic native GS mRNA apparently also did not produce any functional GS. Therefore, other factors acting at the translational or post-translational levels, including the stability of this protein, may be responsible for the regulation of leaf cytosolic GS synthesis and activity. In a recent paper describing over-expression of an alfalfa cytosolic GS gene in tobacco, a five-fold increase in total cytosolic GS activity was reported [11]. This increase in enzyme activity may be due to higher stability of the alfalfa enzyme in a heterologous environment, since the amount of transcript was similar in both studies. In contrast to the stability of cytosolic GS in most higher plants [20, 30], the soybean GS protein and enzyme activities in transgenic tobacco tissue in our studies were highly labile. The exact contribution of the new cytosolic GS to overall ni-

trogen metabolism in transgenic tobacco may be significant, in light of the fact that the native cytosolic GS gene is induced by this alteration. Moreover, the density of gold particles in the leaf cytosol of 35S-GS15 transformed plants was comparable to that in the chloroplast (Fig. 8), suggesting that a significant amount of soybean GS protein is present in cytoplasm, although the antibody may not react to these two proteins with the same efficiency.

Earlier studies suggest that metabolites involved in nitrogen assimilation may play a role in regulating the expression of cytosolic GS genes in soybean [17, 29, 42]. The forced expression of GS activity in leaf cytosol of the transgenic tobacco plants may have altered the nitrogen metabolism activating the transcription of otherwise silent native cytosolic GS gene(s). Because we obtained similar results in independent transformants, integration of soybean GS gene in the vicinity of a tobacco GS gene is probably not the reason for activation of the native gene. Metabolic control of gene expression by small organic or inorganic molecules has been discovered in an increasing number of studies [1, 25, 29, 37], and a change in the subcellular site of a specific enzyme is likely to alter cellular metabolism. In studies carried out on alfalfa GS gene expression in tobacco [11] or *Phaseolus* [16], no attempt was made to assess the level of native GS gene transcript in transgenic plants.

In the leaves of higher plants, ammonia availability in the cytosol may control nitrogen and carbon balance, leading to the regulation of the genes encoding key enzymes involved in nitrogen assimilation and those providing carbon skeletons. Most GSs are composed of different subunits. A combination of various subunits may be particularly suited to a specific physiological condition, producing optimum stable enzyme activity. Experiments are now in progress to express other soybean GS genes simultaneously or individually in tobacco and *Lotus* to obtain maximal enzyme activity and stability in various tissues. The appearance of nodule-enhanced GS in some legumes or elevated expression of root GS in others may also be related to the type of nitrogen metabolism (amides or ureides) and flow of carbon in different species, since the energy costs of these two types of metabolism are very different [see 43]. Constitutive expression of GS in roots may allow retrieval of residual ammonia from the rhizosphere. Such alterations may be particularly important in crops such as rice where many microbes in the paddy fix dinitrogen and may release ammonia in water.

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