High-level expression of *Pinus sylvestris* glutamine synthetase in *Escherichia coli*

Production of polyclonal antibodies against the recombinant protein and expression studies in pine seedlings

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Abstract In a previous work we reported the molecular characterization of a glutamine synthetase (GS; EC 6.3.1.2.) complementary DNA from a woody plant (Cantón et al. (1993) Plant Mol. Biol. 22, 819–828). The isolated cDNA (pGSP114) encoding a Scots pine (*Pinus sylvestris*) cytosolic subunit, has been subcloned into the expression vector pET3c to overproduce the GS polypeptide in *Escherichia coli* cells. The recombinant GS protein showed the same molecular size as a native Scots pine GS subunit. Antibodies against the pET3c-GSP114 encoded protein were raised in rabbits by injecting purified preparations and specificity was determined by immunoprecipitation of GS activity present in pine crude extracts. In spite of the antibodies were able to recognize both cytosolic and chloroplastic GS in tomato plants, they were unable to immunodetect chloroplastic GS in green cotyledons of pine seedlings and cytosolic GS was the unique recognized polypeptide. Unlike to that found in other plant species, cytosolic GS was strongly expressed in green tissues as determined by protein and Northern analysis. Our results suggest a key role for cytosolic GS in photosynthetic tissues of conifers.

Key words: *Pinus*; Woody plants; Glutamine biosynthesis; Recombinant protein; Protein expression

1. Introduction

Glutamine is a key amino acid in the intermediary metabolism of living organisms acting as a nitrogen donor in many biosynthetic reactions, intracellular and intercellular nitrogen transport and substrate for protein synthesis. Glutamine biosynthesis from glutamate and ammonia is catalyzed by glutamine synthetase (GS; EC 6.3.1.2), using ATP as an energy source and Mg^{2+} ion as a cofactor. In higher plants, GS activity is coordinated with the enzyme glutamate synthase (GOGAT; EC 1.4.1.14, 1.4.7.1) which catalyzes the transfer of the amido group from glutamine to 2-oxoglutarate generating two molecules of glutamate, one of them is cycled to the GS reaction and the other one is chaneled to biosynthetic nitrogen metabolism. The so-called GS/GOGAT metabolic pathway represents the main door for inorganic nitrogen entry into biological compounds, such as amino acids, nucleotides, chlorophyll and a variety of secondary plant metabolites [18].

GS, an octameric enzyme, exists in green plant tissues as two isoenzymes: GS1 localized in the cytosol and GS2 confined to the plastid [21]. GS isoforms are constituted of different size polypeptides which are encoded by homologous nuclear genes [19,31]. A number of cDNA clones have been isolated from a variety of plant species (reviewed by Forde and Cullimore [10]; Marsolier and Hirel [22]) which have been very helpful in defining a detailed molecular analysis of GS in plants. The expression of plant GS cDNAs in *E. coli* allowed the functional rescue of glnA defective mutants [9,29,1]. The availability of such specific gene probes has also allowed the study of in vivo expression of individual genes. Very recently GS promoters have been analyzed and regulatory elements involved in tissue-specific and metabolic regulation have been identified [4,23]. However, in woody plants, the knowledge of GS molecular properties and regulation of gene expression is much more limited, most likely due to the difficulties of working with these plants as experimental material. For instance, purification of enzymes from woody plants is a difficult task, particularly from conifer species which present lignified tissues and a high abundance of phenolics compounds [33]. An alternative to overcome this limitation is gene cloning and overproduction of the desired gene product.

We recently cloned and characterized a full-length cDNA encoding GS from Scots pine [7,8] and current work is devoted to study developmental and environmental regulation of this gene in developing seedlings. We are interested in the manipulation of glutamine biosynthesis by the aplication of molecular biology techniques, such as chimeric gene construction, transformation and differential expression analysis in transgenic woody plants. Our work is aimed to modify endogenous glutamine levels and to study the effect of this modification on amino acid metabolism, biomass production and tree growth rate.

As part of our research program, we have made a genetic construct to overexpress Scots pine GS cDNA in *E. coli* and produce large amounts of recombinant protein, which has been subsequently used to raise a high titer GS antiserum in rabbits. The antibody has been shown to recognize GS enzyme and it has been employed to assess protein distribution in pine tissues. This molecular tool will be helpful in the biochemical analysis of nitrogen metabolism in coniferous trees and other woody plant species.

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2. Materials and methods

2.1. Chemicals, enzymes

All biochemicals were either from Sigma (USA) or Boehringer Mannheim (Germany). Prestained molecular size markers were provided by Sigma. Restriction and DNA modifying enzymes were purchased from Boehringer. Sequencing primers T7 and SP6 were obtained from Promega Corp. (USA).

2.2. Plant material

Scots pine (*P. sylvestris*) seeds were provided by Servicio de Material Genético, ICONA (Instituto de Conservación de la Naturaleza), Estación 'El Serranillo', Guadalajara (Spain). Seeds were soaked overnight under aeration, then germinated in trays with vermiculite at 22°C. Three-week-old seedlings were used for tissue print experiments.

2.3. Plasmids and bacterial strains

Vector pGEM3Z and JM109 strain were purchased from Promega. Expression vector pET-3c and BL21 (D3) strain were kindly provided by Dr. E. Rodríguez-Bejarano.

2.4. Genetic construction, cloning and transformation

A full-length cDNA encoding the cytosolic GS of *Pinus sylvestris* was excised from the plasmid pGEM 3Z (Promega), where it was previously cloned, by overnight *Eco*RI digestion and agarose gel purification. The resulting 1.4 kb cDNA fragment including the GS open reading frame, a 35 nucleotide non-coding 5′ region and a 365 nucleotide untranslated 3′ region was inserted in the *Nde*I site of pET 3c expression vector [30] after blunt-ending of both, vector and insert, by treatment with the Klenow fragment of the *E. coli* DNA polymerase I. This construct (Fig. 1) was used to transform *E. coli* strain BL21 (DE). Transformed cells were selected by ampicillin (100 μg/ml)/chloramphenicol (170 μg/ml) resistance and plasmid preparations were carried out by the alkaline lysis method [2]. Recombinant plasmids with appropriated insert orientation in respect to T7 promoter were detected by restriction analysis and DNA sequencing by the dideoxy termination chain method [28]. All standard methods concerning restriction enzyme digestion, ligation, and transformation of bacterial cells were as described by Sambrook et al. [27].

2.5. Growth of bacteria and induction of recombinant GS synthesis

Cultures of transformed *E. coli* cells were grown at 37°C in LB medium supplemented with ampicillin until the culture reached an OD at 600 nm of 0.6. At this point the production of the recombinant protein was induced by the addition of IPTG (isopropylthio-β-D-galactoside) to a final concentration of 0.1 mM, and incubation was maintained at the same conditions for an additional period of 3 h. Cells were harvested, pelleted by centrifugation at $3000\times g$ for 15 min and subsequently resuspended in SDS-PAGE loading sample buffer. Total proteins present in the bacterial extracts were resolved by SDS-PAGE.

2.6. Extraction and purification of recombinant GS polypeptide

E. coli cells were resuspended in GS grinding buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) in a ratio of 1:50 buffer to culture volume. Cell lysis was carried out by sonication using a Branson sonifier-250 (Branson Ultrasonics, USA) equipped with a microprobe. Three 2 min pulses were applied at intensity level 2, separated by intervals of 30 s. The resulting extract was clarified by centrifugation at $20\,000\times g$ and insoluble material re-extracted with 1 M KCl in GS extraction buffer. Supernatant was discarded and inclusion body pellet was resuspended in 4 M urea, 0.1 M 2-mercaptoethanol, and gently shaken for 12 h at 4°C to complete protein solubilization. After centrifugation at $10\,000\times g$ for 30 min the supernatant containing GS polypeptide was extensively dialyzed against extraction buffer. Solubilized GS was purified by preparative gel electrophoresis as described [12].

2.7. Antibody production

Polyclonal antibodies against pine cytosolic GS were raised in New Zealand white rabbits. The animals were immunized by the application, in the back, of multiple intradermal injections of purified protein preparations (200 μg) emulsified with Freund's complete adjuvant (GIBCO Laboratories, USA). Forty days later the animals were boosted with the same amount of protein emulsified with incomplete Freund's adjuvant. Ten days later blood was collected from the ear marginal vein, allowed to clot overnight at room temperature and the serum recovered by centrifugation at $5000\times g$ for 5 min. Immunospecifity was determined by immunodifussion test analysis [25]. The GS antiserum was divided in aliquots and stored at −80°C until use.

2.8. SDS-PAGE and Western blotting

Protein samples were dissolved in loading buffer (30 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 0.5 M 2-mercaptoethanol), boiled for 5 min and subjected to electrophoresis in 12.5% (w/v) SDS-polyacrylamide gels [15]. Polypeptides were visualized by 1 h staining with 1% (w/v) Coomassie brillant blue R-250, 10% (v/v) acetic acid, 40% (v/v) methanol, and overnight extensive destaining in the same solution without the dye. For Western analysis gels were electroblotted to nitrocellulose, 0.45 mm pore size (Schleicher & Schüell, Germany) according to Towbin et al. [32] and processed for immunolabeling as described in Cánovas et al. [5] using anti-GS serum. Immunocomplexes were revealed using an avidin-biotin peroxidase kit (Vector Laboratories, USA). The following prestained proteins were used as molecular size markers: α-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa).

2.9. Tissue printing

Tissue print immunolocalizations were performed by following the protocol described by Ye and Varner [34]. Pine hypocotyls were sectioned (1 mm thick cuttings) using a scalpel and printed on nitrocellulose membranes 0.45 mm pore size (Schleicher & Schüell) pretreated with 0.2 M $CaCl_2$ for 20 min and dried with paper towels. Best results were obtained removing excess plant juice and tissue debris with paper tissues and gently pressing by hand the seedling sections onto the filters for a period of 25–45 s. Nitrocellulose strips were washed out with phosphate-buffered saline (PBS; 140 mm NaCl, 3 mM KCl, 5 mM Na_2HPO_4, pH 7,4) and air dried for 45 min. The quality of tissue transfers was examined by staining the printings with 2% (w/v) Ponceau S in 30% (v/v) trichloro acetic acid, 30% (w/v) sulfosalicylic acid, for 30 min at room temperature. For localization of GS protein nitrocellulose membranes were washed 3 times in 0.3% (v/v) Tween 80 in PBS, then blocked in 1% (v/v) periodic acid for 30 min to inactivate endogenous plant peroxidases. Immunolabeling was performed with anti-GS antibodies as described in the Western blotting protocol.

2.10. Northern analysis

RNA extraction was carried out as previously described by Cantón et al. [7]. About 10 μg of total RNA was separated in a 1.0% (w/v) agarose gel, transferred to nylon membranes (Nytran, Schleicher & Schüell) and hybridized with pine GS cDNA. The ^{32}P-labeled 3′ non-coding region of the pGSP114 cDNA was used as a hybridization probe. Equal amounts of total RNA were loaded per lane, as determined by OD at 260 nm, and normalized by staining with ethidium bromide.

2.11. Enzyme assay and protein determination

GS activity was determined by the transferase assay as described by Cánovas et al. [6]. Protein determinations were carried out using the dye-binding method of Bradford [3] with bovine serum albumin as standard.

2.12. Chlorophyll determination

Pigments were extracted from pine hypocotyls in 80% (v/v) acetone. Total chlorophyll content in the samples was determined as described by Graan and Ort [14].

3. Results and discussion

3.1. Production of pine GS protein in E.coli

A full-length cDNA clone (GSP114) encoding cytosolic Scots pine GS was inserted in the *Nde*I restriction site of pET3c expression vector to generate pET3c-GSP114. (Fig. 1, left panel). The correct orientation of the cDNA insert with respect to the T7 promoter was determined by restriction mapping and DNA sequencing (results not shown). Gene con-

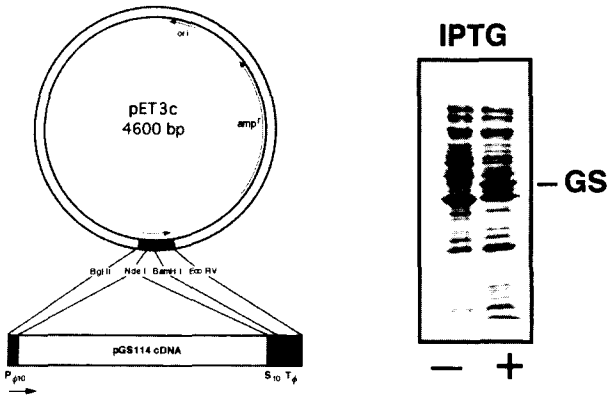

Fig. 1. Expression of *Pinus sylvestris* GS cDNA in *E. coli*. Physical map of the plasmid pET3c-GSP114 for the expression of pine GS cDNA (left panel). The cDNA encoding pine GS subunit (pGSP114) was inserted into the *NdeI* site of vector pET3c under transcriptional control of Pf10 promotor of bacteriophage T7. The resulting genetic construction was introduced in *E. coli* strain BL21(DE3) and transformed cells grown in the presence, or in the absence of IPTG. Total proteins were extracted, resolved by SDS-PAGE and stained with Coomassie Blue (right panel). Thirty micrograms of protein were loaded per lane. The position of GS polypeptide is indicated on the right side of the panel.

structions were transfected to *E. coli* and cell cultures developed in the presence or in the absence of IPTG. Crude extracts of total proteins from cell cultures in both growth conditions were separated by SDS-PAGE and polypeptides visualized by staining with Coomassie brillant blue. As shown in Fig. 1 (right panel), protein extracts from IPTG-induced *E. coli* cells contained an abundant polypeptide with an apparent molecular size of about 41 kDa that was not present in non-induced cells. The relative abundance of this band was variable among different tested clones, but as average roughly represented 15–20% of total protein in bacterial extracts, as determined by gel densitometric analysis. These results clearly demonstrate that the expression of the 41 kDa band depended on the IPTG induction of bacterial cultures.

The following experiments were carried out to determine the identity of the IPTG-induced protein, as well as the distribution of pET3c-GSP product in the soluble and/or insoluble fractions of broken *E. coli* cells. Inclusion bodies were isolated from small-scale cell cultures, solubilized in 4 M urea and protein extracts extensively dialyzed. Polypeptides in solubilized and initial soluble extracts were separated by SDS-PAGE and the presence of GS protein visualized by immunoblot analysis, using an heterologous polyclonal antiserum raised against tobacco GS [11] which was previously shown to recognize pine GS [6]. A strong immunoreactive signal was only present in solubilized insoluble fraction (results not shown) and therefore large-scale protein extraction for GS purification was carried out with the insoluble cell fraction. The expression of recombinant GS protein as insoluble aggregates has been previously reported by Bennet and Cullimore [1] who also showed that the degree of solubility of the expressed polypeptide was dependent on expression temperature.

3.2. *Purification and characterization of the pET3c-GSP114 expression product*

To further characterize the translational product accumulating in recombinant cells after induction with IPTG, the 41 kDa band was purified from bacterial crude extracts by preparative SDS-PAGE [13]. The purified protein was compared with protein extracts derived from Scots pine cotyledons and bacterial lysates (Fig. 2, gel). The electrophoretic mobility of

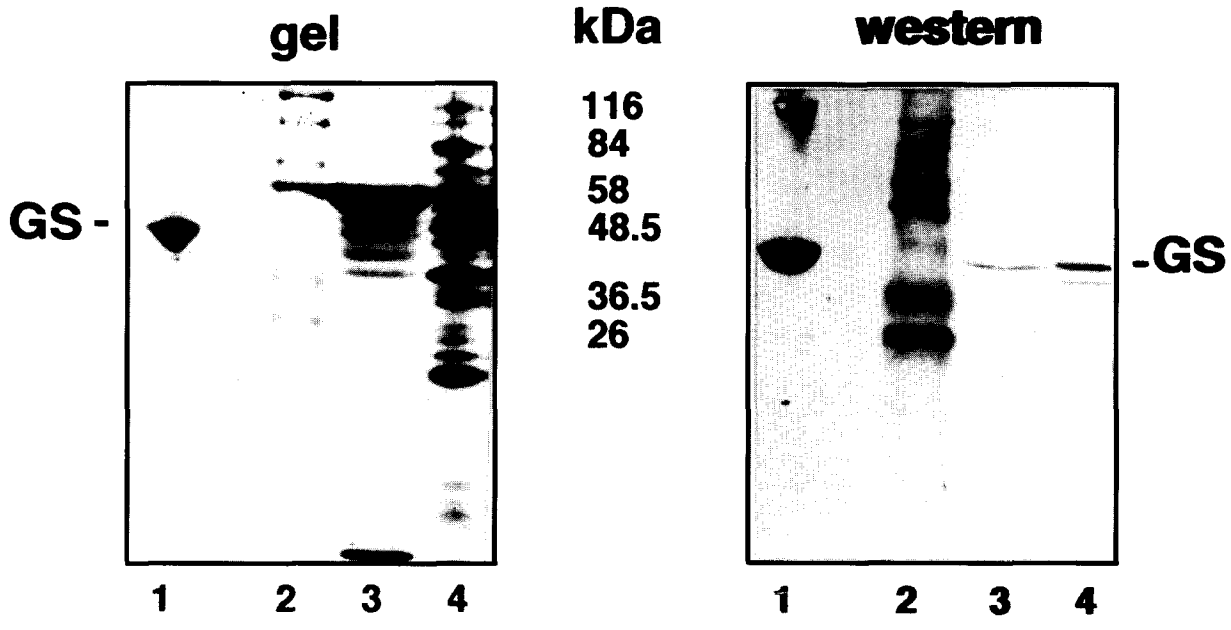

Fig. 2. Characterization of recombinant GS polypeptide. Protein samples were separated by SDS-PAGE and either, stained with Coomassie Blue (gel) or electroblotted onto nitrocellulose membranes and immunoprobed with an antiserum raised against tobacco GS[11] (Western). Lane 1, GS purified from IPTG-inducted recombinant BL21(DE3) cells (5 μg); lane 3, Scots pine crude extract (40 μg); lane 4, crude extract from BL21(DE3) cells expressing pETc-GSP114 (40 μg). A mixture of protein polypeptides of known sizes, was resolved in lane two, and their corresponding molecular masses are indicated in kDa. The position of the GS protein band (41 kDa) is also marked on both sides of the figure.

GS polypeptides was visualized by immunodetection on nitrocellulose filters (Fig. 2, Western). Immunoblot analysis showed that the size of the cross-reacting GS polypeptides

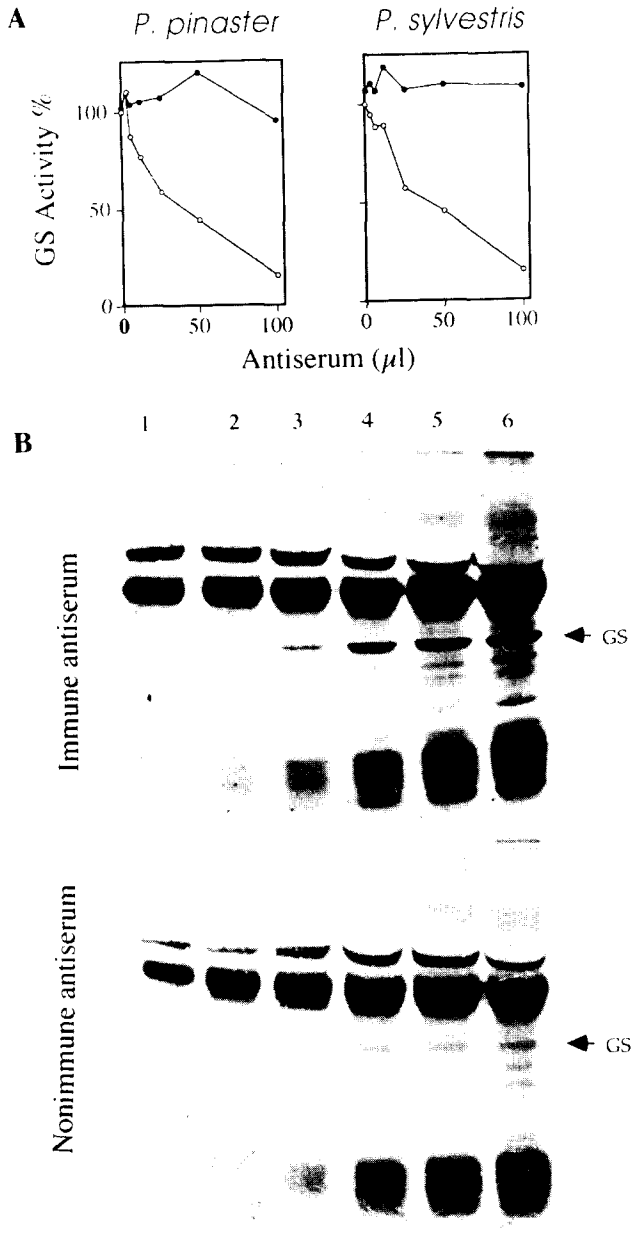

Fig. 3. Immunoprecipitation of pine GS enzyme by the antiserum raised against the recombinantly expressed GS protein. (A) Soluble proteins were extracted from Scots (*P. sylvestris*) and maritime (*P. pinaster*) pine cotyledons and aliquots containing the same level of GS activity (0.1 nkat/ml) were added with increasing volumes of rabbit antiserum. After incubation at 4°C for 4 h, samples were centrifuged at 10000×*g* for 30 min, and GS activity determined in the supernatants by the transferase assay. (○) GS antiserum, (●) nonimmune antiserum. (B) GS-antibody immunocomplexes recovered in the pellets were washed out with PBS, their components resolved by SDS-PAGE and immunoblotting accomplised by using immune and non-inmune antisera. Lanes 1–6 correspond in the order given to samples with increasing antiserum amounts in the *P. sylvestris* GS activity immunoprecipitation experiment (A). The migration of the 41 kDa GS polypeptide is marked on the right. The extremely abundant 55 and 25 kDa polypeptides correspond to the heavy and light subunits of γ-immunoglobulins.

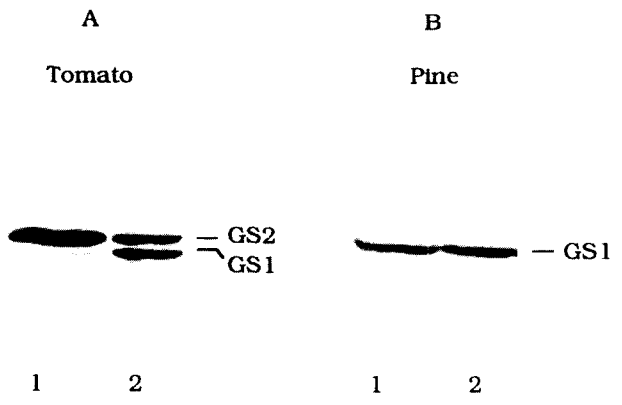

Fig. 4. Immunoblot analysis of tomato and pine extracts using pine GS antibody as a probe. Soluble proteins were extracted from tomato leaflets and pine cotyledons, resolved by SDS-PAGE and the presence of GS polypeptides examined by Western blotting. (A) Healthy tomato leaflet (lane 1) and tomato leaflet infected with *Pseudomonas syringae* pathovar tomato (lane 2) [24]; a dilution of the antiserum 1:1000 was used. (B) *P. pinaster* (lane 1) and *P. sylvestris* (lane 2); a dilution of the antiserum 1:10000 was used.

(Fig. 2, lanes 1, 3 and 4) was the same in all samples examined, indicating the similar nature of the recombinant and wild-type GS proteins. In addition, the results of the Western also confirmed that the purified protein band corresponded to the overexpressed GS polypeptide initially present in crude extracts (Fig. 2, lane 4). The lower (and minor represented) cross-reacting band detected in lane 4, may either correspond to an alternative expressed product of the recombinant plasmid or to a degradation product of the intact GS polypeptide in *E. coli*. In the context of the first hypothesis, it is interesting to point out that final preparations of GS purified from needles and roots of jack pine seedlings also contained two GS polypeptides different in size, the smaller one being the minor represented [33]. The identical molecular size of wild-type (Fig. 2, lane 3) and intact recombinant GS (Fig. 2, lane 4) strongly suggested that translation of the gene construction starts in the first ATG of the cDNA and therefore recombinant GS subunit is expected to be constituted by 357 amino acid residues [7]. However, the pine sequence contains a second in-frame ATG codon at 93 bp that would define an additional open reading frame which would encode a polypeptide of 326 amino acids (about 3 kDa smaller in size) [20]. Regarding the second hypothesis, it is well documented that endogenous GS in *E. coli* is marked by oxidative modification becoming susceptible to proteolytic degradation [16,17,26]. The possibility that such a mechanism could recognize the heterologous GS protein should also be considered. Further work is required to determine the molecular basis of pine GS subunit heterogeneity.

3.3. Polyclonal antibody production and characterization

Antibodies were raised in rabbits against purified recombinant GS polypeptide. Preliminary experiments showed that the antiserum cross-reacted with crude preparations of soluble proteins from pine cotyledons as judged by double immunodiffusion tests (results not shown). However, to clearly demonstrate the monospecifity of the antibodies toward native GS enzyme we assayed their ability to inhibit the GS activity present in plant crude preparations. As shown in Fig. 3, 100 μl of the antiserum abolished about 90% of the GS activity

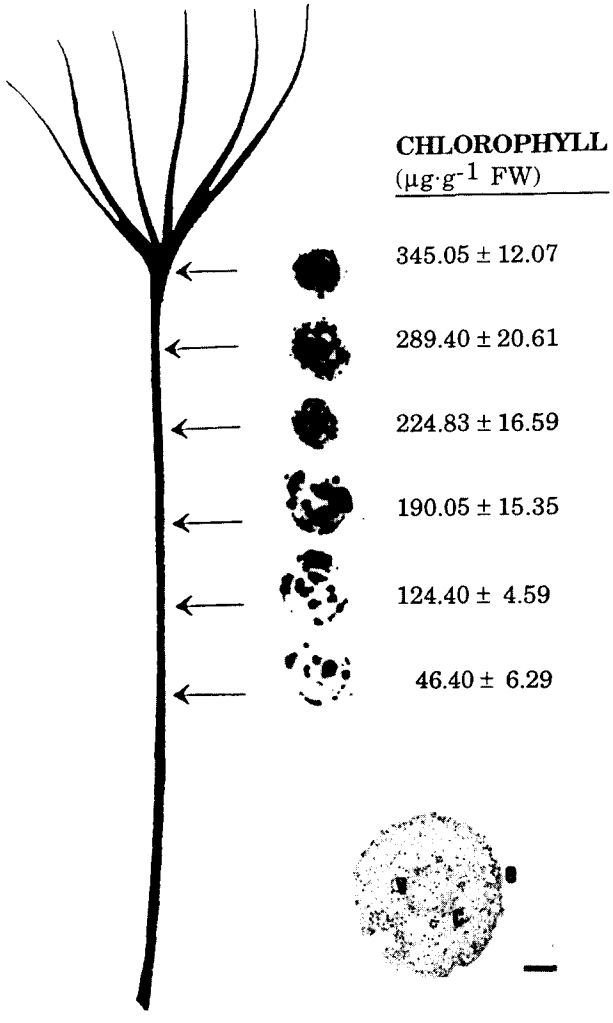

Fig. 5. GS distribution in Scots pine seedlings. Pine hypocotyls were sectioned along, printed onto nitrocellulose membranes and the presence of GS protein immunodetected by essentially following the same protocol described for the Western blot experiments. The position of the cuttings is indicated by the arrows. Total chlorophyll contents in the same areas where the tissue printings were perfomed are displayed on the right side of the figure. Two independent extractions were carried out assayed twice. A cross-section of pine stem is also included for anatomical comparison. Horizontal bar: 120 μm. v, vascular tissue; co, cortex; e, epidermis.

present in soluble extracts from maritime and Scots pine seedlings (Fig. 3A). No inhibition of GS activity was observed in the same experimental conditions but using non-immune serum (Fig. 3A). To confirm the above results, the presence of the GS polypeptide was investigated in the immunoprecipitates. Following the addition of increasing amounts of GS antiserum to the plant extracts, the inhibition of GS enzyme activity in the supernatants was concomitant with the accumulation of a 41 kDa polypeptide in the immunoprecipitates of the same protein extracts (Fig. 3B). No such accumulation of GS protein was observed in the immunoprecipitates by adding the non-immune serum (Fig. 3B).

The ability of the antiserum raised against the recombinant GS to specifically recognize GS isoproteins in evolutionary distant plant species, such as tomato and pine, was also studied by Western blotting analysis (Fig. 4). In mature green tomato leaves, chloroplastic GS is the predominant isoform, [5] and Fig. 4A (lane 1). However, we recently showed that when tomato leaflets are infected with *Pseudomonas syringae* pathovar tomato, GS2 and other chloroplast-located proteins disappear and GS1 accumulates in parallel with the progress of plant pathogenesis [24] (Fig. 4A, lane 2). These results clearly demonstrate the capacity of the antiserum raised against the gymnospermous GS to recognize GS isoenzymes in angiosperms. These findings are in accord with the fact that primary sequences of GS isoenzymes are highly conserved in higher plants [7,10]. By contrast, Fig. 4B also shows that protein extracts from pine cotyledons contained only one immunoreactive signal whose molecular size corresponded to the cytosolic polypeptide (GS1). These data are in good agreement with earlier reports from our laboratory showing that GS1 is the predominant GS isoform in pine seedlings [6–8]. Moreover, it is interesting to point out that the GS antiserum described in this work was able to recognize both GS1 and GS2 in tomato leaves but, it was unable to immunodetect specifically GS2 in an homologous system, confirming therefore that glutamine biosynthesis in conifer seedlings is localized in the cytosol.

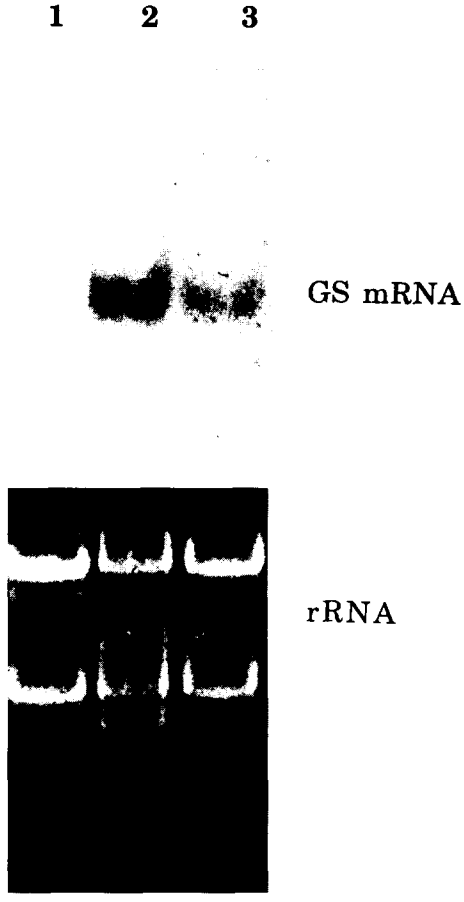

Fig. 6. Steady-state levels of GS mRNA in Scots pine hypocotyls. Total RNA was isolated from the radicle (lane 1), the apical hypocotyl (lane 2) and basal hypocotyl (lane 3) regions of pine seedlings and subjected to RNA gel blot analysis. Hybridization was performed with a radioactively labelled pine GS cDNA probe (3′ untranslated region of pGSP114). Each lane contained 10 μg of total RNA. An ethidium bromide-stained gel of the same samples is shown below as a control of RNA loadings.

3.4. GS protein expression in pine seedlings

Polyclonal antibodies generated against GS recombinant protein were also used to examine the spatial distribution of GS protein in pine hypocotyls by tissue printing. As Fig. 5 shows, the immunostaining was stronger within the upper than in the lower part of the seedling stem. The specificity of this staining was verified by controls in which similar tissue prints where treated with a rabbit non-immune serum. No immunoresponse was seen in these control experiments (results not shown). In Scots pine hypocotyls there is a progressive decrease in total chlorophyll content from the top (region adjacent to the cotyledons) to the bottom (region closer to the radicle) (Fig. 5). Accordingly, our results suggest that high levels of GS protein are associated to chloroplast-containing tissues in the seedling. These results are consistent with previous data obtained in our laboratory showing that GS protein and GS mRNA were more abundant in pine green tissues [6,7]. To further test this possibility we next examined the abundance of GS mRNA in total RNA preparations derived from radicles, as a control from a non-photosynthetic tissue, top and bottom regions of pine hypocotyls (Fig. 6). Again the experimental results suggest that GS gene corresponding to the isolated cDNA is highly expressed in chloroplast-containing tissues of *P. sylvestris* seedlings. These results indicate that a cytosolic GS, instead of a chloroplastic enzyme, plays a key physiological role in nitrogen metabolism of pine photosynthetic tissues.

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