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Expression analysis of a cytosolic glutamine synthetase gene in cotyledons of Scots pine seedlings: developmental, light regulation and spatial distribution of specific transcripts

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Abstract

The expression of a cytosolic glutamine synthetase (GS1; EC 6.3.1.2) gene was examined in cotyledons of Scots pine seedlings. Light strongly stimulated GS1 mRNA accumulation during development. Similarly, steady-state levels of GS1 transcripts increased in dark-grown seedlings transferred to light and decreased in dark-adapted seedlings. Light/dark adaptation affected *rbcS* and *lhcb2* mRNA levels and chlorophyll contents in the same manner. Light-grown seedlings in the presence of the herbicide norflurazon showed a drastic decrease in mRNA for GS and photosynthetic proteins, whereas the effect of the herbicide on mitochondrial β-ATP synthase mRNA was limited. These results indicate that factors associated with developing chloroplasts could be required for maximal GS1 gene expression during seedling development. The level of GS polypeptide, determined by immunoblot, was up-regulated during seedling development in the light or dark. However, the levels of the polypeptide detected were unaltered by the light/dark adaptation treatments. The analysis of GS1 mRNA association with polysomes indicated that the discrepancies between GS protein and mRNA levels are not a result of a differential translational rate of the transcript in darkness relative to light. Two GS isoproteins with different isoelectric point were resolved by two-dimensional PAGE in light- and dark-germinated plants. The relative abundance of one of them was markedly affected by light and correlated with the observed changes in GS mRNA, suggesting that the other form is not a product derived from the detected transcript. *In situ* hybridization of cotyledon sections showed the presence of GS1 mRNAs in mesophyll and phloem cells confirming gene expression in photosynthetic tissues. High levels of transcript were detected also in meristematic cells of apical primordia. These data suggest a dual role for the GS1 gene associated with chloroplast development/activity and glutamine biosynthesis for nitrogen mobilization during early growth of Scots pine.

Introduction

Glutamine synthetase (GS, EC 6.3.1.2) catalyses the incorporation of ammonium to glutamate for glutamine biosynthesis, the first step in inorganic nitrogen incorporation into amino acids. In higher plants, GS is responsible for the assimilation of ammonium coming from the soil, nitrogen fixation or nitrate reduction (primary sources). In addition, GS has a function in the reassimilation of ammonium released during several metabolic processes (secondary sources), including protein and amino acid catabolism, photorespiration and the biosynthesis of phenylpropanoids. Because of its central role in plant nitrogen metabolism, the

biochemistry and molecular biology of GS has been extensively studied (see Lam *et al.*, 1996; and Lea *et al.*, 1990, for comprehensive reviews).

The native GS holoenzyme is an octamer with a molecular mass of about 330–380 kDa and exists in plants as two isoforms with different biochemical properties and subcellular localization: GS1 is localized to the cytosol and GS2 is confined to the plastid (McNally and Hirel, 1983). GS isoforms are the protein products of a family of nuclear genes that have been characterized in a range of angiosperm species (Cullimore *et al.*, 1984; Tingey *et al.*, 1988; Sakamoto *et al.*, 1989; Peterman and Goodman, 1991). With regard to their physiological role in plant metabolism, GS1 genes are preferentially expressed in non-photosynthetic tissues such as developing seeds, roots, and nodules where they are involved in the biosynthesis of glutamine required for seed germination and early growth, root development or the incorporation of symbiotically fixed dinitrogen (Hirel *et al.*, 1987; Walker and Coruzzi, 1989; Temple *et al.*, 1995). In healthy, photosynthetically competent green tissues, GS2 is the predominant form which is involved in the assimilation of ammonium released in nitrate reduction and photorespiratory glycine decarboxilation whereas GS1 is a minor represented enzyme (Edwards and Coruzi, 1989). However, GS1 expression is enhanced in photosynthetic tissues under several physiological conditions such as natural senescence (Kamachi *et al.*, 1992; Buchanan-Wollastron and Ainsworth, 1997), response to pathogen attack and herbicide treatment (Pérez-García *et al.*, 1995, 1998), or water stress (Bauer *et al.*, 1997). In all of these cases the assumed function of GS1 has been to generate glutamine for nitrogen transport to sink tissues. In the last few years, GS1 and GS2 promoters have been analysed and regulatory elements involved in tissue specificity and metabolic regulation have been identified (Brears *et al.*, 1991; Marsolier *et al.*, 1993; Tjaden *et al.*, 1995).

Most studies have focused on herbaceous annual crop plants and much less work has been done on perennials. Recently, molecular studies have been reported in woody plant models. Thus, a number of cDNA clones encoding glutamine synthetase have been isolated from pine seedlings by different strategies (Cantón *et al.*, 1993; Elmlinger *et al.*, 1994; Kinlaw *et al.*, 1996). The absence of N-terminal chloroplast transit peptide and homology analysis revealed that all isolated cDNA clones encode GS1 polypeptides, and expression analysis showed that GS1 gene is actively expressed in developing pine seedlings. These molecular data suggest that glutamine biosynthesis occurs in the cytosol of pine cells, at least during the early phases of plant growth. This hypothesis was supported by the immunocytochemical detection of GS1 in mesophyll and phloem cells of pine seedlings (García-Gutiérrez *et al.*, 1998).

Unlike angiosperms, it is well documented that pine and other gymnosperm species synthesize photosynthetic pigments and develop chloroplasts even when grown in darkness (Bogdanovic, 1973; Mariani *et al.*, 1990). The light-independent synthesis of LHCIIb and other chloroplast polypeptides such as the large and small subunits of Rubisco have also been reported (Yamamoto *et al.*, 1991; García-Gutiérrez *et al.*, 1994). However, the extent of this ability is variable among conifers, even among *Pinus* species. Thus, whereas *P. pinaster* and *P. thunbergii* (Cánovas *et al.*, 1991; Mukai *et al.*, 1991; Garciá-Gutiérrez *et al.*, 1998) exhibit a strong photomorphogenic program in darkness, *P. sylvestris* requires light for full pigment synthesis and chloroplast development (Fernbach and Mohr, 1990; Kasemir *et al.*, 1990).

These differences could explain discrepancies found in pine GS regulation. The accumulation of GS activity and protein have been shown to be light-independent in developing *P. pinaster* seedlings (Cánovas *et al.*, 1991) and stimulated by light in the cotyledonary whorl of *P. sylvestris* seedlings (Elmlinger and Mohr, 1992). Furthermore, Elmlinger *et al.* (1994) recently reported that changes in GS transcript level were controlled by light through the phytochrome photoreceptor, and the effect was attributed to transcriptional regulation of a GS2 gene. However, because GS2 gene expression has not been detected in conifer seedlings at cellular, isoenzyme, polypeptide and mRNA levels (Cánovas *et al.*, 1991; Cantón *et al.*, 1993, 1996; García-Gutiérrez *et al.*, 1998), the molecular identity of GS transcripts remains unclear. The aim of this work was to get insight into the function of a GS1 gene by clarifying the expression pattern at mRNA level in cotyledons of *P. sylvestris* seedlings. Here, we report an expression analysis using specific probes derived from the previously isolated full-length GS1 cDNA (pGSP114) (Cantón *et al.*, 1993). The results show an unusual pattern of expression for this pine GS1 gene with regard to other cytosolic GS genes in plants.

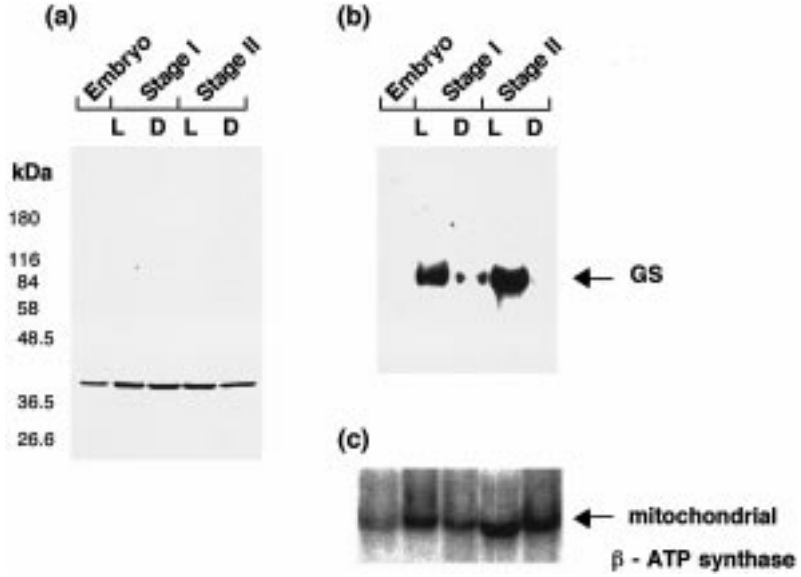

Figure 1. Western and northern blot analysis of GS gene products in cotyledons from developing seedlings. Total soluble polypeptides were prepared from embryo and cotyledons from light-grown (L) and dark-grown (D) seedlings, separated by SDS-PAGE (15 μg of protein per lane), electrotransferred onto nitrocellulose membranes and immunoprobed with GS antibodies. The molecular mass (kDa) of the protein markers is indicated on the left (a). Total RNA was extracted from embryo and cotyledons of light- and dark-grown seedlings and 20 μg from each sample were analysed by northern blot performed using specific pine cytosolic GS (b) and tobacco mitochondrial β-ATP synthase cDNA probes (c). In this experiment, a specific 3′-untranslated sequence derived from the previously isolated cDNA was used as molecular probe to avoid the possibility of cross-hybridization with other GS transcripts.

Materials and methods

Plant material and growth conditions

Pinus sylvestris seeds were provided by ICONA (Instituto Nacional para la Conservación de la Naturaleza, Madrid, Spain). After two days in aerated water, seeds were transferred to aluminium containers with vermiculite. Seed germination and growth of pine seedlings were carried out at 25 °C under continuous fluorescent white light (240 μmol photons m^{-2} s^{-1}) or complete darkness with three black plastic bags in a dark room. Cotyledons from seedlings grown for 15 days (1.5 cm in length) were used in all experiments unless otherwise specified. In experiments in which norflurazon (NF) was used, seedlings were grown under the same continuous light conditions except they were watered with a 0.1 mM NF (Sandoz, Basel, Switzerland).

RNA isolation and northern blot analysis

Total RNA was prepared as previously described (Cantón *et al.*, 1993). Samples (20 μg) of total RNA were denatured in 10 μl of a solution containing 10 mM MOPS (morpholinopropanesulfonic acid) buffer pH 7, 0.5 mM EDTA, 2.5 mM sodium acetate, 3.3% (v/v) formaldehyde and 25% (v/v) formamide by incubation for 5 min at 65 °C. For loading in the agarose gel, 1 μl of a 3% (w/v) Ficoll 40 and 0.1% (w/v) bromophenol blue solution was added. RNA was fractionated on a 1.0% (w/v) agarose gel, 0.66 M formaldehyde in MOPS. RNA was viewed by ethidium bromide staining and transferred onto Nylon membranes (Schleicher and Schuell, Dassel, Germany). cDNA probes were radiolabelled with [α-^{32}P]dCTP using the random primer system from Amersham (Buckinghamshire, UK). A 3′-end non-coding fragment from a cDNA encoding the *P. sylvestris* cytosolic GS (Cantón *et al.*, 1993) was used for the hybridizations, unless otherwise specified. As a control for photosynthetic nuclear gene expression, two cDNAs corresponding to *lhcb2* and *rbcS* genes from *Pinus thunbergii* were used (Yamamoto *et al.*, 1988a,b). A mitochondrial β-ATP synthase probe from *Nicotiana plumbaginifolia* of 1.25 kb (Boutry and Chua, 1985) was used as a control representative of nuclear genes encoding nonphotosynthetic proteins. Hybridizations were performed at 42 °C in 50% (v/v) formamide, 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5× Denhardt's solution, 0.1% (w/v) SDS and 100 μg/ml

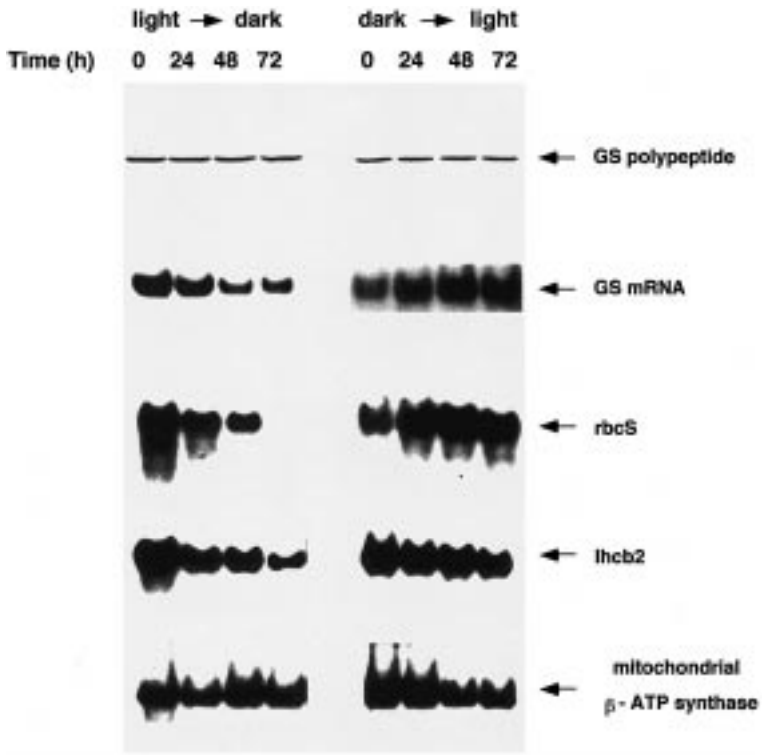

Figure 2. Effect of light/dark transitions on the steady-state levels of GS gene products and mRNAs of nuclear genes encoding photosynthetic and mitochondrial proteins. *Pinus sylvestris* seeds were germinated and developed in continuous light or dark until cotyledons became 1.5 cm in length (15–20 days after sowing). Proteins (15 μg of total soluble protein per lane) and mRNAs (20 μg of total RNA per lane) corresponding to 0, 24, 48 and 72 h after light-dark or dark-light transfer were separated by electrophoresis, transferred to filters and processed for either western and northern blot analysis. GS antibodies and cDNA probes were as described in Figure 1. For photosynthetic gene mRNA level evaluation, cDNAs encoding for Rubisco small subunit (*rbcS*) and type II apoprotein of Photosystem II light-harvesting complex (*lhcb2*) from *Pinus thunbergii* were used as molecular probes.

salmon sperm DNA. Filters were washed twice in 6× SSPE/0.1% SDS at room temperature and 1× SSPE/0.1% SDS at 37 °C.

Isolation of polysomes

Polysomes were isolated from 15 g of cotyledons by ultracentrifugation on a sucrose pad, according to Adamska *et al.* (1991) with the modifications introduced by Mittler and Zilinskas (1994). Polysome preparations were resuspended in 1% (w/v) triisopropylnaphthalene sulfonic acid, 10 mM 2-mercaptoethanol, 6% (w/v) *p*-aminosalicylic acid, 1% (w/v) NaCl and 6% (v/v) phenol and extracted as previously described for total RNA (Cantón *et al.*, 1993).

Protein extraction and western blot analysis

Soluble proteins from cotyledons were extracted in 50 mM Tris pH 8.0, 2 mM EDTA, 10 mM 2-mercaptoethanol. Protein concentrations were determined by the Bradford (1976) method, resolved by SDS-PAGE, electroblotted and GS polypeptides immunodetected as described (Cánovas *et al.*, 1991), with specific antibodies (Cantón *et al.*, 1996).

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional PAGE was carried out as described by O'Farrel (1975). Isoelectrofocusing (IEF) was performed at 200 V for 2.5 h in gels containing 2.5% (v/v) carrier ampholytes (Pharmalyte pH 3–10 from Sigma Chemical Co, St Louis, MO), 4% (w/v), 9.2 M urea. The second dimension was performed by mounting the IEF gel strips containing focalized proteins on 12.5% (w/v) polyacrylamide-SDS slab gels for one-dimensional SDS-PAGE. After electrophoretic separation proteins were blotted onto nitrocellulose membranes for immunodetection as described above.

Tissue fixation, dehydration and embedding

Pine cotyledons were fixed in 1% (v/v) freshly prepared glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7 for 3 h at room temperature. Plant tissue was vacuum-infiltrated for 10 min once an hour. The samples were then washed twice in the buffer, dehydrated in an ethanol series (final concentration 100% ethanol) and treated with increasing concentrations of xylene. Without removing the xylene, 5 pellets of paraffin were added, cotyledons were then incubated at room temperature for 3×30 min, left overnight and finally incubated at 42 °C for 1 h. After adding 5 more pellets of paraffin, the pieces of tissue were incubated at 42 °C for 2×4 h and left overnight at 60 °C. Six changes of 8 h each were made with molten paraffin and the blocks were made after a final change of 2 h in fresh paraffin at 60 °C. Paraffin-embedded cotyledons were sliced with disposable blades in a Leitz microtome (Ernst Leitz, Midland, Ontario, Canada) and paraffin was removed in xylene. The sections were aligned on slides treated with poly-D-lysine and kept in an incubator at 37 °C . RNA conservation was confirmed by acridine orange staining. Control sections were stained with blue toluidine in order to check the tissue morphology.

In situ *hybridization*

Before hybridization the tissue was treated as described by Langdale (1992) with the following modifications. Tissues were treated with 1 μg/ml proteinase K (at 37 °C) for 30 min, washed with 2 mg/ml glycine for 5 min and post-fixed with 4% paraformaldehyde for 20 min. The sections were immersed in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min. Samples were allowed to air-dry for 3–4 h before proceeding to the next stage. Hybridization was performed in a humidified box for 24 h at 55 °C. The probe was denatured by warming at 65 °C for 5 min and diluted in hybridization buffer (4× SSC, 10× Denhardt's solution, 0.5 μg/ml DNA from herring sperm, 10% dextran sulfate). Labelled probe (1–3 ng) was applied per section. Slides were subsequently washed twice in 1× SSC/0.1% SDS (w/v), twice in 0.2× SSC/0.1% SDS (w/v) (at 55 °C), 2× SSC/2× SSC with 10 μg/ml RNAse (at 37 °C), and twice in 2× SSC. Hybridized transcripts were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase according to the manufacturer's directions (RNA colour kit for non-radioactive *in situ* hybridization, Amersham) with the following changes. The samples were placed in block solution (3% w/v blocking reagent in Tris-buffered saline) for 1 h. Incubation with antibody was performed at 4 °C overnight. The required colour development time was 1.5–2.5 h depending on the target abundance. Samples were then dehydrated, infiltrated with xylene for 2×10 min and mounted in Eukit. Photographs were taken with a Nikon automated camera on a Nikon microscope under bright-field illumination, using Fuji Sensia 100 ASA films. A full-length cDNA of *P. sylvestris* cytosolic GS (Cantón *et al.*, 1993) and a 393 bp *Sal*I fragment containing 3′-untranslated region of this cDNA were subcloned separately in Bluescript SK+ plasmids (Stratagene) and used to make riboprobes. cRNA probes were labelled with digoxigenin-11-UTP (Boehringer) in a standard *in vitro* transcription reaction (Langdale, 1992).

Results

Developmental regulation of GS1 gene expression

To investigate the expression of the *P. sylvestris* cytosolic GS gene during the initial stages of plant development, we analysed the abundance of GS protein and its corresponding mRNA in the embryo of imbibed seeds and the cotyledons of seedlings germinated in continuous light or dark. GS protein level was determined by western blot analysis at three developmental stages (Figure 1a): embryo, seedling stage I (1 week after imbibition) and seedling stage II (2 weeks after imbibition). Only one band with an apparent molecular mass of 41 kDa (GS1) was detected in all stages analysed and its relative abundance increased in the initial phases of seed germination independent of whether the seedlings were provided with white light or not (Figure 1a, compare embryo and seedling stages, L and D). These results in *P. sylvestris* are in good agreement with earlier observations in *P. pinaster* (Cánovas *et al.*, 1991).

To determine whether GS1 protein accumulation was accompanied by a parallel increase of GS1 mRNA levels, total RNA was isolated from the same developmental stages for which we had evaluated protein and the abundance of GS mRNA was determined by northern blot analysis. We used a specific 3′-untranslated sequence derived from the previously isolated cDNA (Cantón *et al.*, 1993) as a molecular probe to avoid the possibility of cross-hybridization with other GS transcripts. All presented northern blots were rehy-

bridized with the full-length cDNA and identical results were found (data not shown). The results shown in Figure 1b indicate that GS1 message was barely detectable in the embryo, but its steady-state level increased early during seedling development in both light and dark-grown conditions (stage I). It should be noted, however, that the GS1 mRNA level was higher in light-grown than in dark-grown seedlings; this difference was particularly pronounced in the cotyledons of stage II plants (Figure 1b, stage II, compare L and D). As a control, the same filter was hybridized with a mitochondrial β-ATP synthase probe (Figure 1c). The level of this transcript changed during germination but it was not affected by light in the same manner as the GS mRNA level.

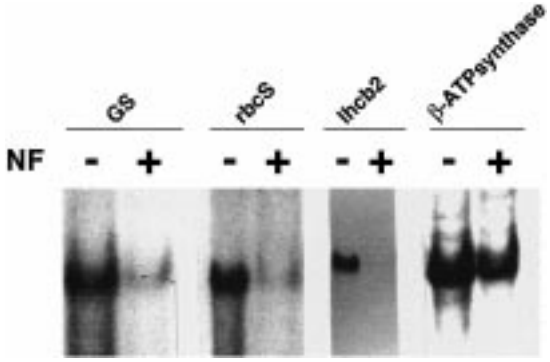

Figure 3. The effect of norflurazon on the levels of nuclear gene transcripts. Total RNAs extracted from 1.5 cm cotyledons of light-grown seedlings treated with (+) or without (−) norflurazon were isolated and hybridized with 3′-end GS probe. The same filter was stripped and rehybridized with specific probes for *rbc*S, *lhcb2* and mitochondrial β-ATP synthase transcripts. A 20 μg portion of RNA was loaded per lane.

Light regulation of GS1 gene expression

Since the above results suggested an influence of light on the developmental regulation of Scots pine GS1 expression, we decided to investigate whether the levels of GS1 protein and mRNA could be affected by short-term light-dark transitions. Seedlings germinated in continuous light or darkness (stage II in Figure 1) were transferred either to dark or to light for 72 h. GS1 polypeptide and GS1 mRNA levels were monitored after the periods of time indicated. As shown in Figure 2, the levels of GS protein were not significantly affected during the course of the treatments. However, GS mRNA levels were very sensitive to light/dark transitions. When light-adapted seedlings were exposed to dark for increasing time intervals, a progressive decrease in GS mRNA level was observed (Figure 2, left panel). Conversely, GS1 mRNA accumulation was induced when dark-grown seedlings were transferred to continuous light (Figure 2, right panel), and 48 h of continuous illumination was required to reach maximal levels of GS1 mRNA.

Parallel to the changes in GS1 mRNA levels, the chlorophyll contents showed a 1.6-fold decrease 72 h after transfer from light to darkness, and a 2.3-fold increase after 72 h of light exposure (data not shown). The correlation between GS1 mRNA and chlorophyll levels prompted us to examine whether light alters also the levels of transcripts of two nuclear genes encoding photosynthetic proteins, the Rubisco small subunit (*rbcS*) and a protein of the light-harvesting complex of photosystem II (*lhcb2*). The light-independent expression of *rbcS* and *lhcb* genes in conifers has been extensively characterized in previous studies (Yamamoto *et al.*, 1988a, b, 1991) and represent good molecular markers to study chloroplast development. As shown in Figure 2 (left panel, *rbcS, lhcb2),* both genes are highly expressed in stage II (15 days after imbibition) light-grown seedlings but the abundance of these mRNAs declined when plants were transferred to darkness, similar to the observed reduction in GS mRNA level under the same experimental conditions. This reduction was particularly accentuated for the *rbcS* transcript, and it was almost undetectable after 72 h of dark treatment (Figure 2, left panel, *rbcS*). As occurs with GS mRNA, transcripts for both photosynthesis genes accumulated in the cotyledons of stage II (15 days after imbibition) dark-grown seedlings (Figure 2, right panel), although the steady-state levels were lower than in light-grown seedlings (Figure 2, compare time 0 h in left and right panels). Upon the transfer of dark-grown seedlings to the light, a drastic increase in *rbcS* transcript level was observed, once more in close agreement to that observed for GS mRNA (Figure 2, right panel). In contrast, the *lhcb2* mRNA level did not increase after the shift to light, and even seemed to decrease with extended light treatment. The levels of a transcript from a nuclear gene encoding a mitochondrial β-ATP synthase were not affected in the same manner as the GS1, *lhcb2* and *rbcS* transcripts (Figure 2, β-ATP synthase, left and right panels).

The dependence of GS1 expression on functional chloroplasts

To investigate whether the coordinated accumulation of GS1 and nuclear photosynthetic gene mRNA lev-

els during seedling development require a developed chloroplast, we carried out experiments with the herbicide norflurazon (NF) to obtain cotyledons with plastids severely damaged by photooxidation. The morphogenesis of light-grown pine seedlings treated with the herbicide proceeded normally, except that chlorophylls were apparently absent in the cotyledons and hypocotyl (data not shown). This effect on the chlorophyll contents in NF-treated seedlings was light-dependent and greening of NF-treated seedlings grown in the dark was not apparently affected, suggesting that it was a result of the photooxidative damage of the carotenoid-free plastids. The mRNA levels of GS1, *rbcS* and *lhcb2* and the mitochondrial β-ATP synthase in cotyledons of NF-treated seedlings were compared with those in untreated seedlings. As depicted in Figure 3, the levels of all of these transcripts were affected in herbicide-treated seedlings. However, the levels of GS1 and the photosynthetic gene transcripts (particularly the *lhcb2* transcripts) were more severely decreased than the level of the transcript encoding the non-photosynthetic protein.

GS1 mRNA and GS1 protein correspondence

To determine whether the discrepancies between GS protein and transcript levels described above could reflect a differential efficiency in mRNA translation in light and dark growing conditions, polysome-associated mRNAs were isolated from stage II seedlings and analysed by northern blot. A similar difference in mRNA levels between seedlings grown in the light or the dark was found in both total RNA and polysomal fractions (data not shown). These findings indicate that GS1 transcripts are not preferentially associated with polysomes in the dark, suggesting therefore a low translational rate of the transcript in darkness relative to the light.

GS1 protein and mRNA discrepancies could also be explained by the fact that the cDNA probe is gene-specific whereas the antibody may detect more than one GS gene product. In order to explore this possibility two-dimensional gel electrophoresis with subsequent western blot analysis was performed to distinguish different GS polypeptides and to study how light/dark treatments affected their relative abundance (Figure 4). When separated by size and charge at least two GS1 isoproteins differing in isoelectric point (pI) (acidic and basic forms) were clearly resolved in the cotyledons of light- and dark-grown seedlings (Figure 4, time 0 h, arrows). Interestingly, the basic spot (pI about 6.5) was the predominant GS1 polypeptide in the light but much less abundant in the dark (Figure 4, compare time 0 h in both treatments). Furthermore, when light-adapted plants were exposed to dark for increasing time intervals, a progressive decrease in its relative abundance was apparent (Figure 4, compare panels from 0 to 72 h). After the transfer of dark-adapted plants to light the same GS isoprotein increased in abundance to be the predominant polypeptide at the end of the treatment (Figure 4, compare panels from 0 to 72 h). The existence of two polypeptides with opposing accumulation patterns may account for the constant protein level observed in Figures 1 and 2.

Cell-type distribution of GS1 transcripts

To further characterize GS1 expression in light-grown Scots pine seedlings the spatial pattern of GS1 transcripts was determined by *in situ* RNA hybridization on transverse sections of paraffin-embedded cotyledons (Figure 5). Digoxigenin-labelled riboprobes generated by *in vitro* transcription of cloned GS cDNA were used. Hybridization with the antisense probe revealed the presence of GS mRNA in sections from 1.5 cm long (stage II) pine cotyledons and there was no unspecific background in control sections hybridized with sense probes (Figure 5D, compare left and right panels). *In situ* hybridization experiments carried out in parallel with the 3′-untranslated region of the cDNA confirmed that labelling can be specifically attributed to only one gene product, GS1 mRNA (data not shown). Expression was most abundant in the stem apical primordia adjacent to the position where the cotyledons arose but it was also present in cotyledons (Figure 5D). Higher magnifications of a cotyledon area were used to localize GS mRNA to specific cell types (Figure 5E–G). GS transcripts were seen in mesophyll cells of the photosynthetic parenchyma but were clearly absent in the epidermis (Figure 5E). In addition, GS expression was observed in the phloem cells of the vascular tissue whereas no hybridization signal was present in xylem vessels (Figure 5F). A strong and specific GS labelling was detected in the meristematic cells of apical primordia (Figure 5G).

Discussion

In green tissues of angiosperms chloroplastic GS2 and ferredoxin dependent-glutamate synthase (Fd-GOGAT) are the main enzymes involved in Gln and

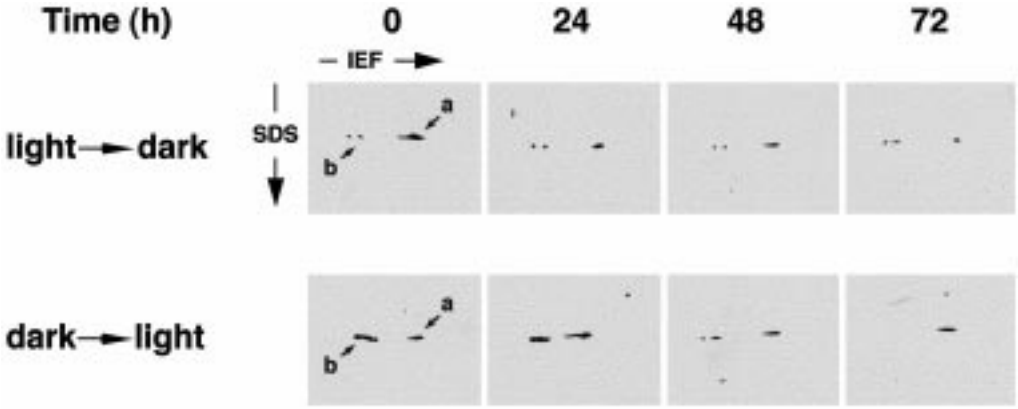

Figure 4. Two-dimensional polyacrylamide gel electrophoresis analysis of GS polypeptides during light-dark transition. *Pinus sylvestris* seeds were germinated, developed and treated exactly as described in Figure 2. At the indicated times of 0, 24, 48 and 72 h, proteins were extracted, separated by isoelectric focusing (IEF, pH 3–10) and SDS-PAGE (SDS), electroblotted onto nitrocellulose, and GS polypeptides revealed by immunobinding to GS antibodies. The positions of the major immunoreactive spots, a (basic) and b (acidic) is marked by arrows.

Glu biosynthesis, using ammonium provided by nitrate reduction and/or photorespiration. This biological role, associated with photosynthetic metabolism, provides insight into the way in which the GS2 gene is regulated: GS2 mRNA accumulation in etiolated pea leaves is stimulated as a result of the action of phytochrome as well as light-induced changes in chloroplast metabolism (Edwards and Coruzzi, 1990).

Our previous work showed that in cotyledons of developing *P. pinaster* seedlings GS and Fd-GOGAT polypeptides are accumulated in a light-independent fashion (Cánovas *et al.*, 1991; García-Gutiérrez *et al.*, 1995), and the available data indicate the cytosolic localization of the most abundant form of GS in cotyledons (Cánovas *et al.*, 1991; García-Gutiérrez *et al.*, 1998). No identification of a chloroplastic GS form in maritime green pine cotyledons has been reported so far. Early studies of the expression pattern of the *P. sylvestris* GS1 gene showed a specific accumulation of the mRNA in photosynthetic organs of the seedlings (Cantón *et al.*, 1993, 1996). Furthermore, high levels of GS protein are associated with chloroplast-containing tissues in *P. sylvestris* seedlings (Cantón *et al.*, 1996; Elmlinger and Mohr, 1992). Elmlinger *et al.* (1994) have reported a light-induced increase of GS transcripts levels in *P. sylvestris* seedlings and suggested that this could be a result of the accumulation of a GS2 transcript. However, a detailed study of GS expression has not been completed to clearly determine whether the increase of GS message abundance in response to light could be attributed to GS1 or GS2 expression.

All these antecedents prompted us to study the expression pattern of the cytosolic GS encoding gene to gain insight into its physiological roles and the effect of light on its expression. GS1 protein is present in imbibed embryos of *Pinus sylvestris* (Figure 1a) where it could be involved in ammonium assimilation coming from mobilized nitrogen during seed germination, providing the nitrogen compounds required for the establishment of the autotrophic metabolism (i.e. chloroplast development and establishment of the photosynthetic apparatus). In addition to this initial role, when the cotyledons become photosynthetically active, GS will be responsible for ammonium assimilation from nitrate reduction and photorespiration. This increased demand of GS activity is well correlated with increased mRNA and protein levels in cotyledons from developing seedlings relative to the embryo (Figure 1, a and b panels). Northern blot data revealed that light strongly promotes GS1 mRNA accumulation during cotyledon development. Currently, the mechanism and photoreceptor(s) involved in this light response are unknown. A phytochrome-mediated activation of a gene for cytosolic GS during imbibition of photosensitive lettuce seeds has been reported (Sakamoto *et al.*, 1989), indicating that in angiosperms, some cytosolic GS genes could be regulated by light.

GS1 mRNA accumulates to moderate levels during early development of cotyledons in dark (Figure 1b), suggesting that the expression of this gene is partially under control of developmental factors. Similarly, dark-grown seedlings accumulate chlorophylls and transcripts of nuclear-encoded photosynthetic proteins (Figure 2). However, the results from experi-

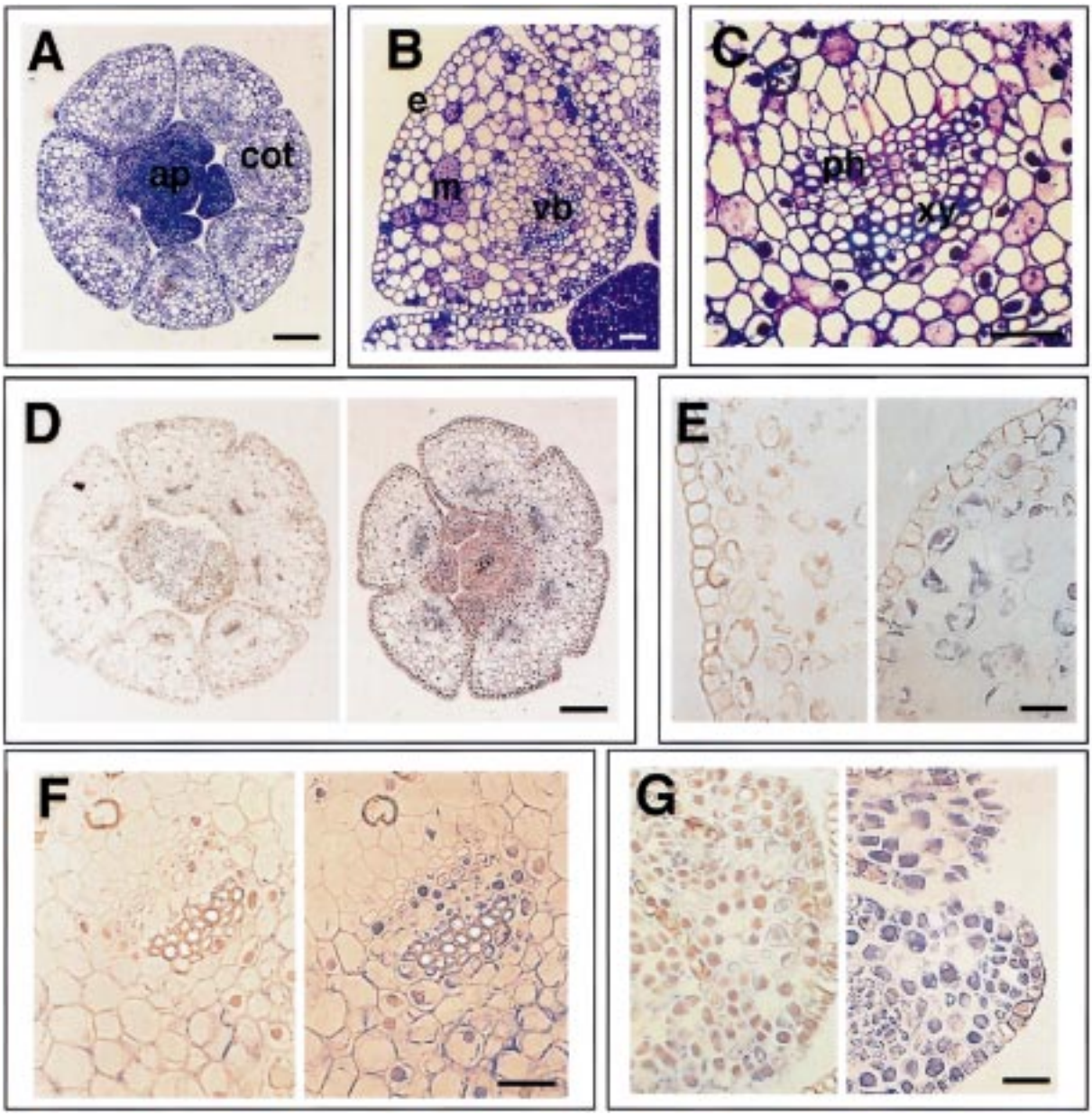

Figure 5. Tissue distribution of GS mRNA in developing pine seedlings analysed by *in situ* hybridization. Seedlings were grown in a regimen of 16 h light/8 h dark until reaching a cotyledon length of 1.5 cm. A to C. Control sections stained with blue toluidine to check structural integrity of the tissue prior to hybrydization. A. Light micrograph of transverse section (10 μm) through the basal zone of the cotyledon (cot) whorl which encloses the apical primordia (ap). B. From the outside, the cotyledon consists of an epidermal layer (e), several layers of mesophyll parenchyma cells (m) and vascular bundles (vb). C. A magnified view of the vascular bundles showing phloem (ph) and xylem (xy) cells. D to G. *In situ* localization of GS transcripts after hybridization with the antisense pGSP114 probe (right panels). Transverse sections were also hybridized with sense control probes (left panels). Note the absence of hybridization in the controls. Similar results were found when sections were hybridized with the 3′-untranslated region of the cDNA (results not shown). D. Bright-field micrograph showing an overall view from the cotyledon whorl section. GS transcripts were detected in the apical primordia and cotyledons. E. A magnified view of a cotyledon showing the accumulation of the GS transcripts in mesophyll cells and the absence of labelling in epidermis. F. A detail of the vascular bundle area showing the presence of GS transcripts in the phloem. No labelling was found in the xylem vessels. G. An intense hybridization signal was observed in the cells of the apical primordia. Bars represent 500 μm in A and D and 100 μm in B, C, E, F and G.

ments transferring plants from light to dark and vice versa clearly show that light is necessary for maximal expression of the GS1 gene as well as for two nuclear genes encoding photosynthetic proteins. The parallel kinetics of accumulation or decline of GS1 mRNA and mRNAs of chloroplast proteins could be as a result of a common regulatory mechanism (mediated by specific photoreceptors) or the requirement of factors associated with the presence of developing chloroplast for maximal promoter activity or a combination of both. One possible factor for GS gene induction could be the new ammonium released from photorespiratory activity. However, ammonium does not increase the GS protein levels in *Pinus pinaster* cotyledons (Cánovas *et al.*, 1991). Alternatively, the changes in GS and photosynthetic mRNA levels might be as a result of a senescence process or metabolic status associated with the light-dark transitions. Kamachi *et al.* (1992) reported a chloroplastic GS polypeptide level decline in parallel to other chloroplast enzymes during natural senescence of rice leaves.

A drastic decrease in mRNA for GS and photosynthetic proteins was observed in cotyledons with photodamaged chloroplasts by treatment with norflurazon (Figure 3). However, the effect on mRNA levels for a mitochondrial β-ATP synthase was limited. Under our experimental conditions, addition of NF did not alter the general morphology of seedlings in the light (data not shown), suggesting that the effect of herbicide treatment was mainly confined to the chloroplast, and the extent of the photooxidation outside the organella was limited. This result supports the hypothesis of a coordinated expression of the GS1 gene and chloroplast development in cotyledons of *P. sylvestris* seedlings.

In our initial expression studies there was clearly no correlation between the effect of light on GS protein and mRNA levels. While mRNA levels were dramatically altered depending on the light conditions, protein levels were barely affected. The sustained level of GS protein in the dark could be as a result of a higher translational rate of the mRNA relative to the light. The analysis of the polysomal RNA fraction showed that the GS mRNA was not preferentially translated in dark and suggests that this GS1 transcript has a limited contribution to the level of GS1 protein observed in dark-developed cotyledons. Alternatively, the expression of a different GS1 gene, divergent enough in sequence to hybridize with our probe under the conditions used, could not have been detected and more than one GS1 species (unresolved by one-dimensional gel electrophoresis) could contribute to GS protein population in Figures 1a and 2 (GS polypeptide). Two-dimensional gel analysis revealed the presence of at least two GS1 isoproteins (a and b) in cotyledons of light- and dark-grown seedlings (Figure 4). These findings are in agreement with a recent work from our laboratory reporting two GS1 isoforms in *P. sylvestris* seedlings (Avila *et al.*, 1998) which are composed of subunits similar in size but differing in charge. The isoelectric point of the basic GS1 form (pI 6.5) is close to the deduced value of the polypeptide encoded by the pGSP114 cDNA clone and to the corresponding expression product in *Escherichia coli* (Cantón *et al.*, 1996; Avila *et al.*, 1998). The relative abundance of this cytosolic GS was markedly affected by light and fairly well correlated with the observed changes in GS message in a similar fashion to that described for GS2 in angiosperms (Edwards and Coruzzi, 1989). All these data confirm an unusual pattern of expression for the conifer GS1 gene characterized in the present work when compared to cytosolic GS genes in other plant species. Our results also suggest the existence of other GS1 gene(s) in pine with different expression profiles and physiological roles. Current work in our lab is addressed to the isolation and characterization of such GS gene(s).

The spatial pattern of expression reported in this paper further supports a chloroplast-associated role of this GS1 gene. The high level of mRNA in the meristematic cells of apical primordia suggests an early requirement of this GS form for development of green tissues and differentiation of photosynthetic cells. The detection of GS message in mesophyll and phloem cells is consistent with the previously described distribution of GS1 protein in pine seedlings (García-Gutiérrez *et al.*, 1995), and this could indicate a double function of this gene associated with chloroplast development/activity and glutamine biosynthesis for nitrogen translocation to other parts of the seedling.

In summary, the higher mRNA levels of this cytosolic GS gene in light-developed cotyledons and its correlated expression pattern with genes encoding photosynthetic proteins suggest a specific role in glutamine biosynthesis and ammonium assimilation associated with chloroplast development/activity. In early seedling stages it may provide the required nitrogen for chloroplast development, and subsequently assimilate ammonium generated as a result of chloroplastic activity and synthesize glutamine for nitrogen transport in light.

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