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Spatial and temporal expression of two cytosolic glutamine synthetase genes in Scots pine: functional implications on nitrogen metabolism during early stages of conifer development

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Summary

Ammonium assimilation during the initial stages of Scots pine growth involves two cytosolic glutamine synthetase (GS, EC: 6.3.1.2) isoenzymes encoded by separate genes, GS1a and GS1b. GS1a was most exclusively expressed in photosynthetic tissues of the seedling whereas GS1b was expressed ubiquitously showing higher levels in non-photosynthetic tissues such as root and hypocotyl. Temporal expression analysis has shown that when germination starts GS1b is the predominant form in the embryo, however, its relative abundance in the tissue decreased in the postgerminative stages when green cotyledons are developed. In contrast GS1a was present at a low level in the embryo but its abundance increased markedly during germination and seedling growth. These data suggest that GS1a and GS1b genes display different and non-redundant roles in the nitrogen metabolism of conifers. The precise localization of individual transcripts by *in situ* hybridization strongly supports this possibility. GS1 gene products are mainly expressed in different cellular types: GS1a in chlorophylic parenchyma and GS1b in the vascular bundles of all tissues examined in the seedling. Our data support that glutamine biosynthesis in pine seedlings follows a different pattern related to angiosperms involving two cytosolic GS proteins: one of them a typical cytosolic GS which may be involved in the generation of glutamine for N transport and a second cytosolic GS generating amino donors for the biosynthesis of major N compounds in photosynthetic tissues, a closer role to angiosperm chloroplastic GS. The results are discussed with regard to recent studies on N mobilization and metabolism during the initial stages of conifer development.

Keywords: N metabolism, cytosolic glutamine synthetase, conifer, gene expression, gene family, *in situ* hybridization.

Introduction

Ammonium assimilation is a well known metabolic process in angiosperms catalysed by the enzyme glutamine synthetase (GS, EC: 6.3.1.2). The metabolic requirement of GS activity to assimilate ammonium in plants is fulfilled by a number of isoforms expressed in specific organs and developmental stages (see Ireland and Lea, 1999 for a recent review). With regard to the subcellular localization, there are two different classes of GS in angiosperms, GS1 in the cytosol and GS2 in the chloroplasts (McNally and Hirel, 1983). In most plants examined chloroplastic GS (GS2) is encoded by a single nuclear gene whose expression is restricted to mesophyll photosynthetic cells (Lam *et al.*, 1996) where it is responsible for the assimilation of ammonium derived from reduction of nitrate and photorespiration. This late metabolic function has been demonstrated in barley mutants defective in GS2 where the remnant cytosolic GS activity is insufficient for the reassimilation of photorespiratory ammonia (Wallsgrove *et al.*, 1987). The cytosolic isoform (GS1) is encoded by a small gene family whose members are differentially expressed during development or in response to external stimuli (Lam *et al.*, 1996). Unlike GS2, the physiological

role of individual genes encoding the cytosolic isoform (GS1) remains unclear but recent reports indicate they may be involved in primary assimilation of ammonium from the soil (Sakakibara *et al.*, 1996), reassimilation of N mobilized during senescence (Buchanan-Wollaston and Ainsworth, 1997), in response to pathogen attack (Pérez-García *et al.*, 1998), herbicide treatment (Pérez-García *et al.*, 1998) or water stress (Bauer *et al.*, 1997).

Much less is known about N assimilation in woody plants and particularly in gymnosperms. In pine seedlings and other conifers the cytosolic isoform is so far the only form detected (Avila *et al.*, 1998; Cánovas *et al.*, 1998) and functional expression of GS2 gene has not yet been demonstrated in a reliable way (Cánovas *et al.*, 1998). Therefore conifers represent an interesting model to investigate the physiological role of GS1 isoforms in plants. Histochemical and immunocytochemical localization has demonstrated the presence of GS1 protein in the cytoplasm of mesophyll and vascular cells of pine suggesting a dual role for cytosolic GS in developing seedlings of conifers (García-Gutiérrez *et al.*, 1998). However it is unknown whether unique or different gene products undertake glutamine biosynthesis in both cell types. We have previously reported the existence of two GS1 isoforms in pine seedlings, GS1a and GS1b, with different chromatographic behaviour and composed of distinct polypeptides similar in size (Avila *et al.*, 1998). The pine GS1 isoprotein has been probed recently to be encoded by separate genes, evolutionary divergent but interestingly closely linked in the pine genome (Avila *et al.*, 2000).

In order to gain further insights on the function of GS1 in plants it is important to determine whether individual members of the gene family are involved in redundant metabolic pathways or they have independent and specific physiological roles. To achieve this objective GS1 isogene expression should be studied at mRNA and protein level but the experimental analysis is quite complex because: (i) specific mRNAs extracted from the same tissue can be expressed in different cell types and; (ii) individual GS1 polypeptides are usually difficult to separate and characterize independently.

In this paper a comparative study of GS1a and GS1b expression is reported. The results strongly suggest that GS1 isogene products play different and non-redundant metabolic roles in *Pinus sylvestris*.

Results

Spatial distribution of pine GS1 gene expression

We have previously found that GS1a is the predominant isoform in pine cotyledons with regard to GS1b which is much less abundant (Avila *et al.*, 1998; Avila *et al.*, 2000). To gain information on the metabolic roles of the GS1 isoforms in pine, we decided to study their expression patterns in different tissues at mRNA and protein level. Thus, we have further characterized GS1a/GS1b expression in pine seedlings analysing the steady-state levels of individual transcripts and their correlation to GS1a/GS1b polypeptides in different sections of the seedling (Figure 1a). Seven different parts of the seedling were considered: cotyledon (C), upper hypocotyl (US), lower hypocotyl (LS), and four root sections R1 (connection with hypocotyl), R2, R3 and R4 (root tip). To test the presence of GS1a and GS1b isoenzymes, proteins extracted from the different sections were firstly separated by 2D-PAGE and then detected by western blot knowing the predicted isoelectric point of both polypeptides (Avila *et al.*, 2000). As shown in Figure 1(b), GS1a protein was present in cotyledons, upper hypocotyl and to a less extent in lower hypocotyl. However GS1b protein was present in all seven sections of the seedling and it was the only isoform found in the root. In hypocotyls, the GS1b spot was more abundant in the lower rather than in the upper part and hardly detectable in cotyledons. When we analysed transcript level of both GS genes in the same samples (Figure 1c) our results were in good agreement with the spatial distribution of the protein isoenzymes. GS1a mRNA level was undetectable in all four sections taken in the roots, showing its highest level in cotyledons. GS1b mRNA was higher in roots and the lower section of the hypocotyl in good correlation with the observed GS1b protein abundance. The GS1b transcript was also present in cotyledons, however, the presence of the polypeptide was weakly detectable at least by 2-D electrophoresis from crude extracts. These results indicate a precise distribution of both isoenzymes along the seedling that may have a physiological significance.

Temporal distribution of pine GS1 expression

In order to further investigate the expression of GS1 isogenes, individual mRNAs and polypeptides were also examined in the embryo and developing seedlings (Figure 2). Separation by 2D electrophoresis and western blotting showed that GS1b protein was predominant in the embryo but its relative abundance decreased in the seedlings (Figure 2a). In good agreement with previous results obtained in our laboratory (Cantón *et al.*, 1999), GS1a was present at low levels in the embryo and accumulated in the seedling stages. The abundance of GS1a and GS1b transcripts was studied in the same samples in which the polypeptides were immunodetected (Figure 2b). A comparative analysis showed a good correlation between the presence of the mRNAs and the corresponding proteins suggesting that GS1 isogene expression is regulated mainly at the transcriptional level.

Next, we examined the relative amounts of both transcripts in the embryo and differentiated organs of the

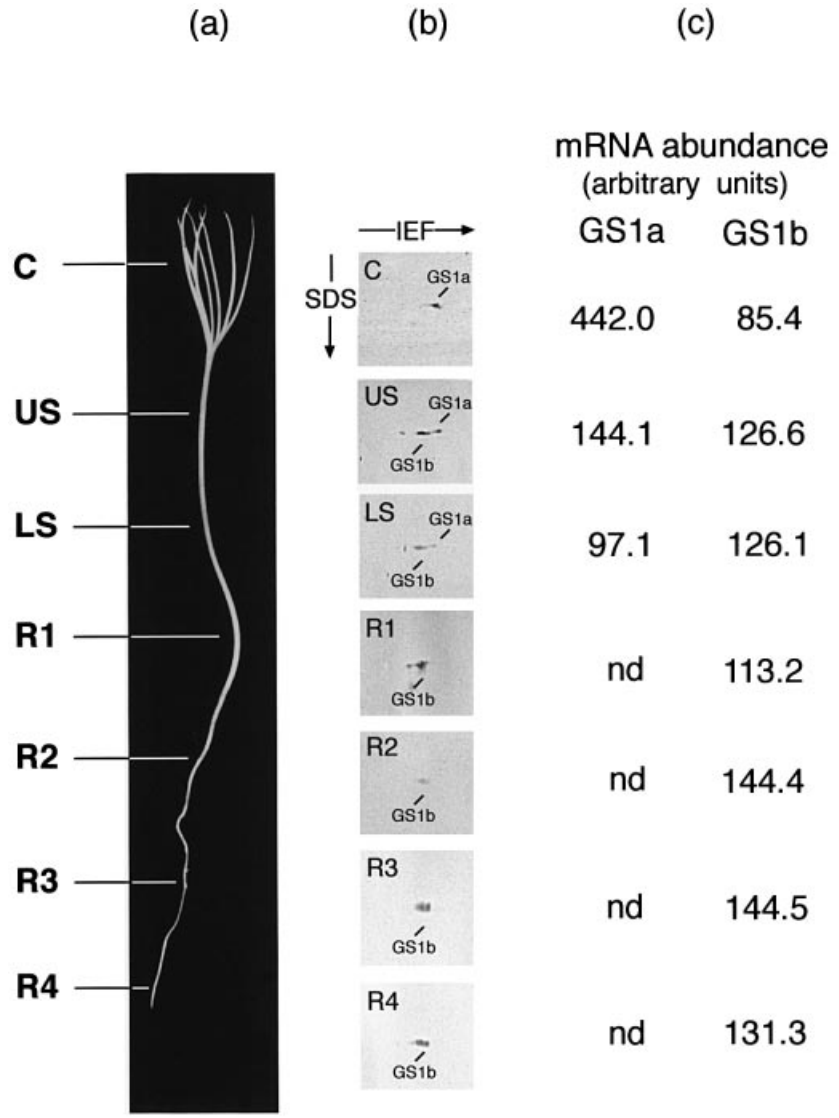

Figure 1. Spatial distribution of GS1 isoproteins and mRNAs in sections of *P. sylvestris* seedlings.
(a) Schematic representation of a pine seedling (15 days after imbibition, DAI) where the considered sections are indicated: (C) cotyledons (US) upper hypocotyl (LS) lower hypocotyl (R1) interconnection root-shoot (R2) root (R3) root (R4) root tip. (b) Separation and identification of GS isoenzymes present in all seven considered sections. Total soluble proteins were extracted, separated by 2D-electrophoresis and GS spots revealed by Western blotting using specific antibodies (Cantón *et al.*, 1996). A total amount of 50 µg of protein was loaded by gel. The presence of GS1a and GS1b polypeptides is indicated by the arrows. (c) GS1a and GS1b transcript abundance in sections of the seedling. Total RNA was isolated and the level of both transcripts determined by slot-blot hybridization using the 3′-non-coding regions of the cDNA clones pGSP114 (Cantón *et al.*, 1993) and pGSP15 (Avila *et al.*, 2000) as specific molecular probes. Quantification of the radioactive signals was carried out using a Bioimaging analyser BAS-1500 (Fujifilm). The results were normalized against the constitutive expression of the ribosomal protein. Total RNA was isolated at different stages of seedling development, slot-blotted and probed.

developing seedling (root, hypocotyl and cotyledon). As shown in Figure 2(c) (germination) GS1b mRNA is highly abundant in dry embryos and its steady-state level increased immediately with development. In contrast, GS1a transcript was very low at the three embryonic stages, indicating that gene expression is unaffected by imbibition and germination. In the roots of developing seedlings GS1b transcript level was almost constant keeping a high level in all three stages tested, whereas GS1a transcript was undetected in the same samples (Figure 2c, root). In developing hypocotyls both GS1 transcripts were detected with increasing levels for GS1a transcript (Figure 2c, hypocotyl). In cotyledons, GS1b transcript decreased with the age of the plant, however, GS1a transcript was increasing at the same time with at least a fivefold higher level than GS1b for the last examined stage (Figure 2c, cotyledon).

Localization of GS1 transcripts in the embryo

The precise localization of GS1a and GS1b transcripts was compared by *in situ* hybridization of adjacent transversal sections along the embryo structure (Figure 3). The observed expression patterns were very similar to that found by protein and standard RNA analysis. GS1a expression was very low in cotyledons, hypocotyl and radicle. GS1b transcripts were detectable throughout the embryo structure but expression was mostly located within the hypocotyl. As shown in Figure 3 (II, panel b) strong labelling was found in majority of the epidermis and central parenchyma cells except in those related with the formation of the first vascular elements where the signal was weaker. *In situ* hybridization of sequential sections taken from the hypocotyl axis (data not shown) confirmed that the strongest expression of GS1b was

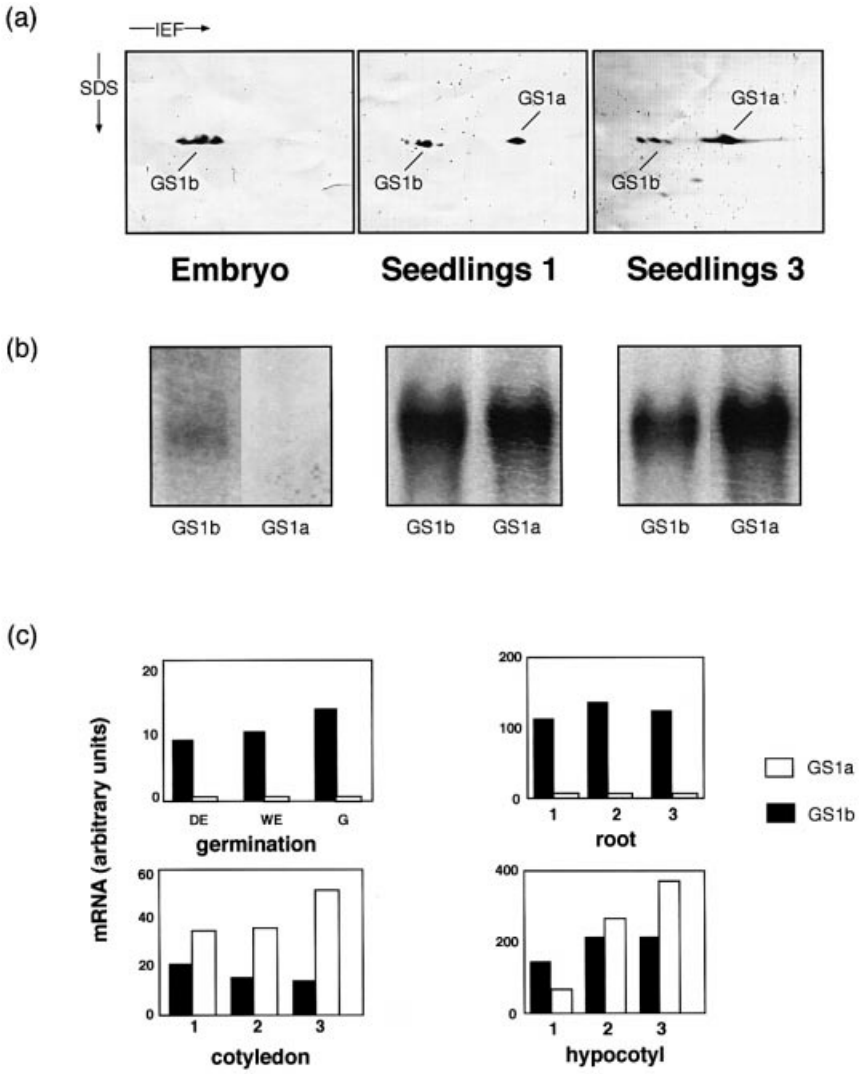

Figure 2. Comparative analysis of GS1a and GS1b gene expression in the embryo and developing seedlings of *P. sylvestris*.
(a) Western blotting analysis of GS1 isoforms separated by 2D electrophoresis. Proteins were extracted from embryos, seedling stage 1 (5 DAI), seedling stage 3 (10 DAI) and processed as described in Figure 1. (b) Northern blotting analysis of GS1a and GS1b messages. Total RNA was isolated at the same stages in which the GS1 proteins were analysed and the presence of the GS1 mRNAs revealed by hybridization with the corresponding cDNA probes. (c) Steady-state levels of GS1a and GS1b transcripts during development. Total RNA was extracted from embryos and different organs of developing seedlings, slot-blotted and probed with 3′ specific cDNAs for GS1a and GS1b as described in Figure 1. Germination: (DE) dry embryo (WE) wet embryo and (G) germinating seed (2DAI). In the seedling, samples taken from root, hypocotyl and cotyledon were performed at three developmental stages: 1 (5 DAI), 2 (7DAI), 3 (10 DAI)

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located in the medular region and showed that transcript abundance was decreasing in direction to the apical and basal areas of the embryo (Figure 3 I, III).

Localization of GS1a and GS1b transcripts in the seedling

The precise localization of GS1a and GS1b gene expression was also determined in different organs of the seedlings at several developmental stages. Significant findings are shown in Figure 4. In developing green cotyledons both transcripts were present although a preferential distribution depending on the cellular-type was observed with complementary expression patterns (Figure 4, I, compare (a) and (b) panels). GS1a expression was found largely associated to parenchymatic cells and appeared to be very weak in the vascular system. In contrast, the abundance of GS1 transcripts was restricted to cells of the vascular bundles. No expression signals were seen in the epidermic cells independently of the used probe.

A comparative study of transcript localization in the hypocotyl again showed contrasting patterns of expression for the GS1 isogenes. In the upper part of the organ, GS1a was strongly expressed in the chloroplast containing cells of the cortical parenchyma (Figure 4, II, panel a), but labelling was very weak in the central part of the section. GS1b expression was lower than GS1a the highest response being preferentially found in the region where the vascular bundles were present (Figure 4, II, panel b). The abundance of GS1a transcript was very low in the hypocotyl basal part, close to the root, while GS1b was most exclusively restricted to vascular cells (results not shown).

GS1b was the only transcript detected in root sections (Figure 4, III, compare (a) and (b) panels), the expression of GS1a was absent in some of the root cellular-types. GS1b mRNAs were detected in low abundance in cells of the

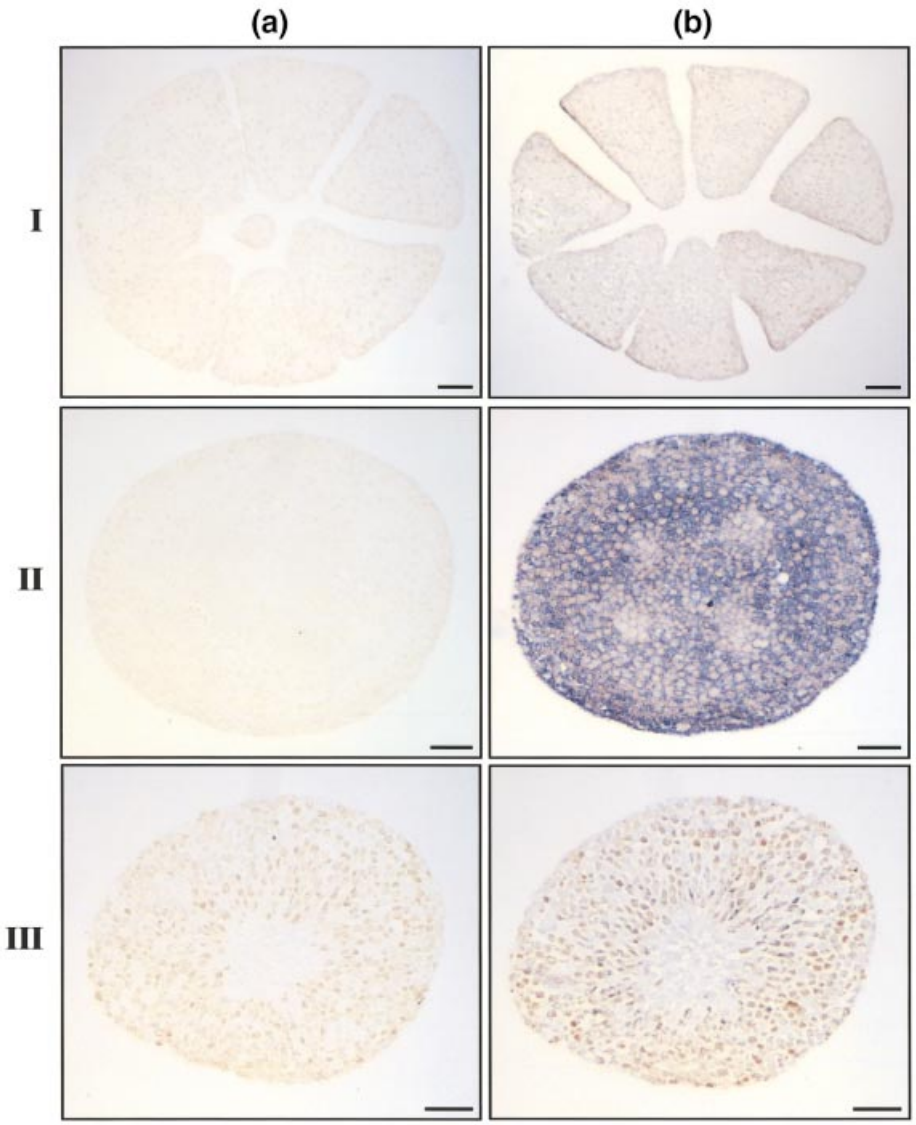

Figure 3. *In situ* hybridization analysis of GS expression in *P. sylvestris* embryo.
Transversal cross-sections through the embryo were used for localization of GS1a (left panels, a) and GS1b (right panels, b) mRNAs by *in situ* hybridization with antisense RNA probes (pGSP114 and pGSP15, respectively). In all cases the specificity of the signals was established by *in situ* hybridization of control sections with sense probes. (I) embryonary cotyledons; (II) hypocotyl; (III) radicle, a slight brown signal was observed following the fixation of the tissue and independent of the *in situ* hybridization procedure. Scale bars represent 200 μm.

cortical parenchyma but strong expression signals were found in the central vascular cylinder (Figure 4, III, panel b).

Discussion

In conifers, storage reserves within the seeds are present in both the embryo and the megagametophyte, a maternally derived haploid tissue (Biswas and Johri, 1997). The mobilization of protein vacuoles and lipids bodies during pine germination provide the required N, carbon, energy and reducing power for seedling formation and growth (Stone and Gifford, 1999). In this work we have investigated the functional roles of two GS1 genes (GS1a and GS1b) in the N flow from the seed to the developing seedling through two complementary experimental approaches: (i) a comparative study of the abundance of individual GS1 polypeptides and transcripts in the seed and different sections of the seedling and; (ii) a comparative study of the precise localization of GS1 transcripts in the germinating seed and different cell-types of the seedling.

Functional role of GS1 isoforms in the germinating embryo

The abundance of GS1b polypeptides and transcripts (Figures 2 and 3) in the embryo reflects the importance

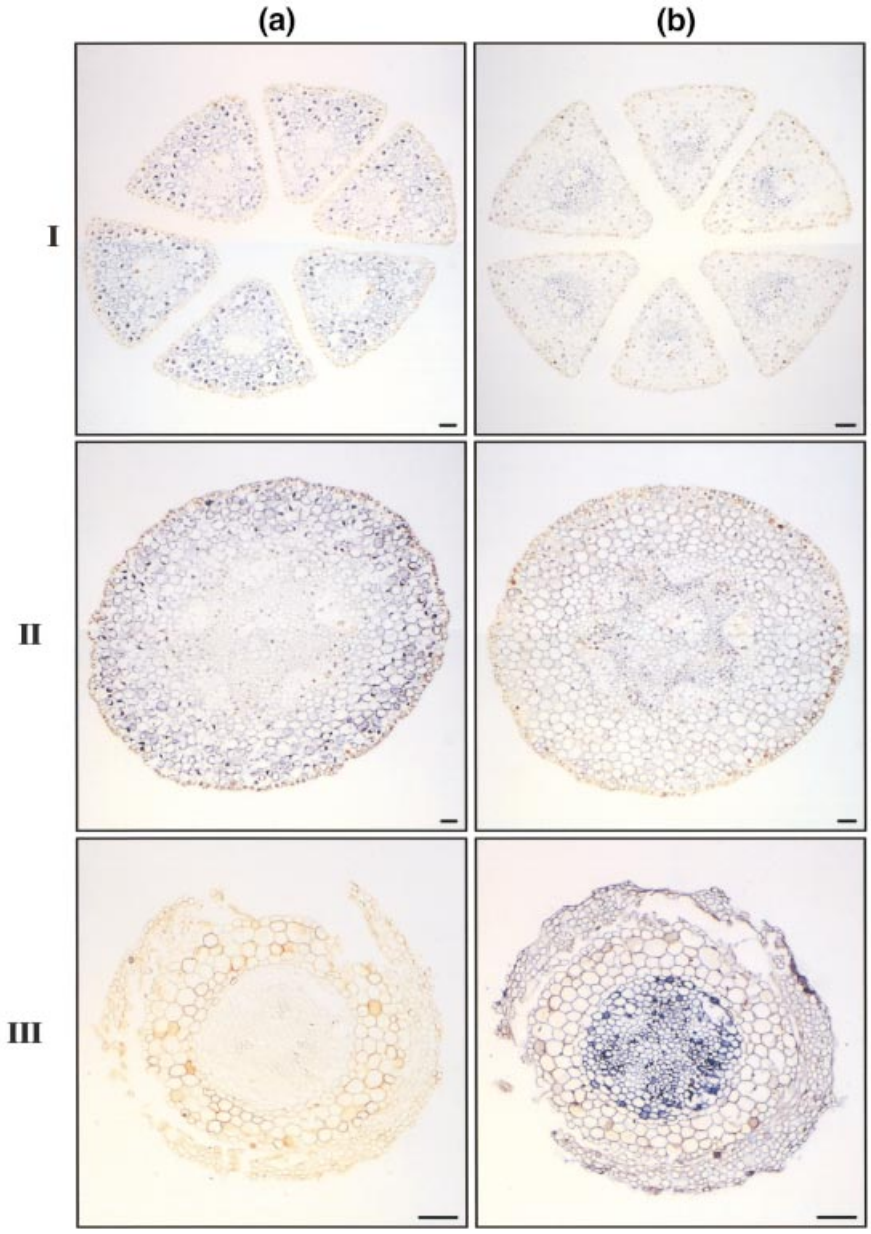

Figure 4. *In situ* hybridization analysis of GS expression in *P. sylvestris* seedlings. Transversal cross-sections through the seedlings were used for localization of GS1a (left panels, a) and GS1b (right panels, b) mRNAs by *in situ* hybridization with antisense RNA probes (pGSP114 and pGSP15, respectively). In all cases the specificity of the signals was established by *in situ* hybridization of control sections with sense probes. (I) cotyledons; (II) hypocotyl; (III) root. Scale bars represent 200 μm.

of this gene during the initial stages of germination of *P. sylvestris*. In this plant species protein hydrolysis begins in the embryo (Simola, 1974) and the comparative analysis of GS1a and GS1b expression indicates that GS1b is the functional gene in the reassimilation of ammonium released in the breakdown of embryo storage proteins at early stages of germination. Furthermore these data also suggest that the function of GS1b at this time may be important for the *novo* protein biosynthesis possibly related with the loss of seed dormancy (Schneider and Gifford, 1994). In contrast, GS1a is barely detectable in the embryo both at mRNA and protein levels (Figure 2a,b) suggesting no implication of this gene in the initial events of pine germination.

RNA and polypeptide analyses (Figure 2) indicate that GS1b is highly abundant in the embryo but do not provide information about the spatial distribution of the GS1b expression along the structure. However *in situ* hybridization analysis (Figure 3) revealed that the GS1b messenger was very low in the embryonary cotyledons, much higher in the embryo radicle and particularly abundant in the hypocotyl (Figure 3). These data are consistent with the fact that protein vacuoles are more rapidly depleted in the central and basal area of the embryo (Stone and

Gifford, 1997) probably because of the high demand of amino acids that takes place during radicle expansion and differentiation (root protrusion). It is important to note that the high level of GS1b expression in the medular region of the embryo hypocotyl (Figure 3) precedes the formation of the first vascular elements suggesting, therefore, that even at this early stage of plant development the function of GS1b is N translocation to other parts of the plant (roots and cotyledons of the developing seedlings). Effectively, no mature vascular elements are preformed in the embryos of *leucocyteinus* (Figure 3) unlike those found in other woody plants such as *Ginkgo* or *Populus* (Busse and Evert, 1999).

As occurs with GS1a (Figure 2), Fd-GOGAT (García-Gutiérrez *et al.*, 1995) and NADH-GOGAT (García-Gutiérrez *et al.*, unpublished data) are very low in the embryo and presumably these genes are not involved in glutamate biosynthesis in the tissue. An alternative source of glutamate for glutamine biosynthesis could be the reaction catalysed by glutamate dehydrogenase, an important enzyme during seed germination (Melo-Oliveira *et al.*, 1996). The supply of 2-oxoglutarate in this reaction could be provided by NADP isocitrate dehydrogenase, an enzyme absent in the megagametophyte but active in germinating embryos (Palomo *et al.*, 1998).

Functional role of GS1 isoforms in the seedlings

Most storage proteins in loblolly pine and other conifer seeds are located in the megametophyte (Stone and Gifford, 1999). In *P. sylvestris* protein mobilization in this tissue only proceeds when the bulk of protein reserves in the embryo is depleted (Simola, 1974) initiating the N flux to the developing seedling. This process occurs during and even after germination, because the cotyledons of the developing seedlings remain in contact with the seed coat until the reserves of the megametophyte are exhausted. The most abundant amino acid in the protein seeds of conifers is arginine (Allona *et al.*, 1992; Allona *et al.*, 1994; Leal and Misra, 1993) and also the predominant vehicle for N transportation from the megagametophyte to the embryo where it is a major component of the free amino acid pool (King and Gifford, 1997). Protein breakdown is accompanied by a marked increase in arginase activity and functionality of urease has also been reported in pine seedlings confirming that arginine catabolism is an important source of ammonium during the early growth of pine seedlings (Guitton, 1964; King and Gifford, 1997).

In seedlings, the expression of GS1a was restricted to tissues containing chloroplasts, including cotyledons and the upper part of the hypocotyl. Low levels of GS1a polypeptide/transcript were also found in the basal section of the hypocotyl while they were not seen in the root sections (Figures 1 and 2). This is consistent with previous data indicating that GS gene is actively expressed in green cotyledons (Cantón *et al.*, 1993; Elmlinger *et al.*, 1994), regulated by light and dependent on factors associated with developing chloroplasts (Cantón *et al.*, 1999). The function of GS1a associated to the chloroplast biogenesis and/or metabolic activity is confirmed by the *in situ* localization of the transcripts in cells of the chlorophyllic parenchyma present in cotyledons and the upper part of the hypocotyl (Figure 4). In contrast, GS1b is highly abundant in the hypocotyl and the root although it is also present at low levels in the cotyledons (Figures 1 and 2a). The results of *in situ* localization experiments demonstrate that GS1b gene product is associated to the vascular bundles in all tissues examined, including cotyledons, hypocotyl and roots (Figure 4). In fact, it is important to point out that the pattern of distribution of GS1b is quite similar to that found in the embryo and again suggests that the gene plays an important role in N transport or translocation within the plantlet.

In cotyledons and hypocotyls where GS1a and GS1b coexist, their distinct distribution patterns suggest differentiated functions in N metabolism. The localization of most of the GS1a expression in cotyledons is well correlated with the reported abundance of arginase activity in the same organ (King and Gifford, 1997), suggesting that GS1a plays a primary role in the reassimilation of ammonium released in arginine metabolism. When the photosynthetic apparatus is functional, another function for GS1a in pine seedlings would be the reassimilation of ammonium released in photorespiration, a metabolic role of chloroplastic GS (GS2) in angiosperms. In this context it is worth noting that arginine metabolism has been proposed to be involved in the N photorespiratory cycle (Ludwig, 1993). The high level expression of Fd-GOGAT and NADP-dependent isocitrate dehydrogenase (García-Gutiérrez *et al.*, 1995; Palomo *et al.*, 1998) suggest that a functional GS1a-GOGAT cycle is operative in green tissues of pine.

Based on the abundance of Asn in the later stages of seedling growth, King and Gifford (1997) suggested that Asn is the form in which N is transported from cotyledons to other parts of the seedling in pine as occurs in angiosperms (Kern and Chrispeels, 1978; Lea and Miflin, 1980). Asparagine biosynthesis in plants is a glutamine dependent reaction catalysed by the enzyme asparagine synthetase (Sieciechowicz *et al.*, 1988). Thus, the generation of glutamine for Asn biosynthesis could be undertaken by GS1b. The localization of GS1b in vascular cells, the same place where asparagine synthetase expression has been reported (Nakano *et al.*, 2000; Tsai and Coruzzi, 1990) is consistent with this hypothesis. Moreover, the abundance of GS1b in the hypocotyl and roots and the precise localization of GS1b transcripts in the central cylinder of the root further support such a role of the

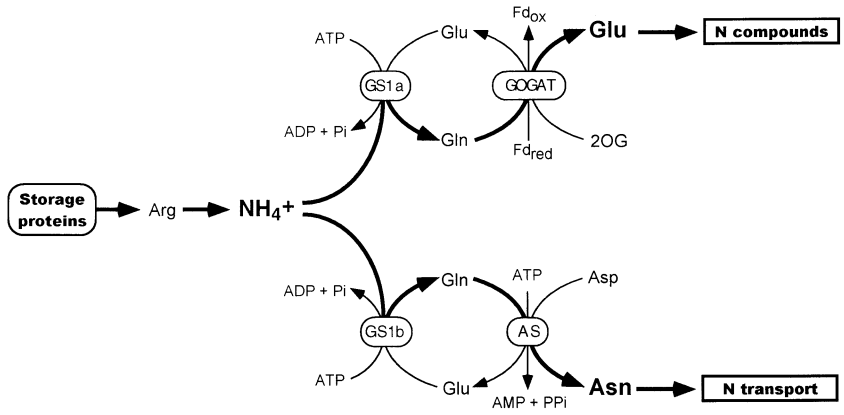

Figure 5. Schematic representation of the proposed glutamine cycles operative in pine seedlings.

enzyme in N transport to sink tissues. On the other hand the specific pattern of GS1b expression in the vascular elements is similar to those GS1 genes in angiosperms (Dubois *et al.*, 1996; Edwards *et al.*, 1990; Pereira *et al.*, 1992). Recently, Brugière *et al.* (1999) have reported an essential role of GS1 located in phloem cells in the production of proline by using antisense technology. Nevertheless, proline abundance is very low in germinating pines (King and Gifford, 1997), and therefore GS1b does not appear to be involved in the biosynthesis of this amino acid following storage protein breakdown.

The results reported here suggest the existence of two glutamine cycles in developing pine seedlings (Figure 5): (i) a GS1a/GOGAT pathway implicated in ammonium assimilation and glutamate biosynthesis in mesophyll cells where a high demand of N compounds would be required for photosynthetic cell growth and proliferation and; (ii) a GS1b/AS pathway involved in the biosynthesis of amides in vascular cells for N transport to the growing apices.

In conclusion, the evidence reported here indicates that pine GS1 gene family contains at least two isoforms that have unique and precise patterns of spatial and temporal expression suggesting they play distinct functional roles in N metabolism of conifers. It is worth of mentioning that such specific functions should be relevant not only in the seedling stage but also later during development of pine trees because GS1a and GS1b expression has also been detected in the needles of 2 years-old maritime pine (Avila *et al.*, 2000). In spite of the functional differences found in this work it is intriguing that the two GS1 genes are closely located in the pine genome (Avila *et al.*, 2000). Further work is needed to clarify the possible relationships between metabolic functionality and chromosomal localization.

Experimental procedures

Plant material

The Scots pine seeds (*P. sylvestris*) used in all experiments were from Servicio de Material Genético, ICONA (Instituto de Conservación de la Naturaleza, Madrid, Spain). Seeds were imbibed in deionized water for 12 h under continuous aeration then they were germinated in a plastic pot using vermiculite (Eurover, Europerlita, SA, Almeria, Spain) as support. Seedlings were grown in a controlled culture chamber (Ibercex H-900-B, ASL, SA, Madrid, Spain) at 24°C of temperature using a 16 h light/8 h dark photoperiod. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO; Koxka, Pamplona, Spain) at a flux density of 150 μmol m^{-2} s^{-1}. Plants were watered regularly but no nitrogen fertilization was added. Under these conditions of growth the endosperm of conifers supplies nitrogen and other nutrients to the developing seedling for a period of 2–3 weeks until seed reserves begin to be exhausted (Cánovas *et al.*, 1998; Flaig and Mohr, 1992). Samples were harvested at the same time in the light/dark cycle. Q3

RNA extraction, Northern and slot blot analysis

Total RNA was isolated from different pine tissues and developmental stages by using the phenol/SDS method (Ausubel *et al.*, 1987). For Northern analysis, total RNA (10 μg per lane) was separated on denaturing formaldehyde-agarose gels and blotted onto nylon filters which were pre-hybridized at 42°C in 50% Formamide, 5 × SSC, 5 × Denhardts, 50 mM sodium phosphate pH 6.5 and 100 μg ml^{-1} denatured salmon sperm DNA. Gel transfer and hybridization was performed essentially as described (Cantón *et al.*, 1993). A probe of 500 bp derived from the 3′ end of both GS clones was used in this study and the equivalence in RNA loading was analysed by hybridization with a cDNA encoding a ribosomal protein (Rbp4). Signal intensity in the slot-blots was quantified using the Bioimaging analyser BAS-1500 (Fujifilm España SA, Barcelona, Spain).

Two-dimensional electrophoresis and Western blot analysis

Two-dimensional gel electrophoresis was carried out exactly as described previously (Avila *et al.*, 1998). After second dimension gels were transferred to nitrocellulose membranes and immunodetection was performed following the method described by Cantón *et al.* (1996). Q4

Embryo and seedling preparation for in situ *hybridization*

In situ hybridization studies were carried out at the relevant early developmental stages. Cotyledons, hypocotyls and radicle from

developing seedlings were sliced off and vacuum was applied then to permit fixative penetration. Mature and germinated embryos were fixed without slicing or vacuum applying. Individual embryos or organ seedling stages were fixed in 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde in 100 mM phosphate buffer pH 7.4 for 3 h at room temperature. Fixed tissue was dehydrated with ethanol, cleared with xylene and embedded in paraffin as described previously (Cantón *et al.*, 1999). Embedded tissue was sliced into serial 8 or 10 μm sections with a Leitz microtome (Ernst Leitz, Midland, Ontario, Canada) and attached to microscope slides that were coated with poly D-lysine.

RNA in situ *hybridization*

Gene-specific antisense probes were synthesized from pGSP114 (accession number X13408) for GS1a gene and pGSP15 (accession number AJ005119) for GS1b gene. Preparation of digoxigenin-labelled riboprobes was performed according to the method of Langdale (1993). As a control, a single-stranded DIG-labelled sense probe was synthesized from the linearized template.

Preparation and hybridization with paraffin-embedded embryos or organs from seedling stages and immunological detection of the hybridized transcripts were carried out as given in Cantón *et al.* (1999). Sections were photographed with bright-field illumination using a light microscope (Nikon Optiphot, Nikon, Japan).

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