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Research

Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis

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Summary

• Here, embryo-specific patterns of glutamine synthetase (GS) genes were studied for the first time using pine somatic and zygotic embryogenesis as model systems.
• *GS1a* expression was absent in zygotic embryos whereas it was detected in the cotyledons of somatic embryos at late developmental stages along with transcripts for photosynthesis genes and arginase. These findings suggest that germination was initiated in maturing somatic embryos.
• *GS1b* transcripts were found mainly in procambial cells in both zygotic and somatic embryos. Expression of the *GS1b* in procambial cells before the differentiation of mature vascular elements indicated that this gene could be useful as a molecular marker for early stages of vascular differentiation in pine. Accordingly, a correlation was found between the quality of somatic embryos generated from three different cell lines and the pattern and level of *GS1b* expression.
• Our data suggest that *GS1a* and *GS1b* genes play distinct functional roles in the biosynthesis and mobilization of seed nitrogen reserves. Furthermore, the results presented may have potential application for improving conifer somatic embryogenesis.

Key words: conifer, embryogenesis, germination, glutamine synthetase (GS), photosynthesis, procambium.

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Introduction

Glutamine and glutamate are the key donors of nitrogen (N) for the biosynthesis of the major N compounds in plants, including other amino acids, nucleic acid bases, polyamines and chlorophylls. The enzyme glutamine synthetase (GS) (EC 6.3.1.2) catalyses the amidation of glutamate to generate glutamine at the expense of ATP hydrolysis, and the enzyme glutamate synthase (GOGAT) (EC 1.4.7.1; 1.4.1.14) catalyses the reductive transfer of amide N to 2-oxoglutarate for the generation of two molecules of glutamate, one of which is recycled for glutamine biosynthesis (Miflin & Lea, 1980). The biochemistry and molecular biology of the GS/GOGAT cycle has been extensively studied because of the key role that these enzymes play in plant growth and development (Lam *et al.*, 1996). In the photosynthetic tissues of many angiosperms, GS2, a plastid-located isoform of GS, is responsible for the assimilation of ammonium derived from nitrate reduction and photorespiration (Lam *et al.*, 1996; Ireland & Lea, 1999). A cytosolic isoform, GS1, is the predominant enzyme in roots and less abundant in green tissues with the exception of C4 plants, that exhibit low photorespiratory activity (Lam *et al.*, 1996; Ireland & Lea, 1999). The GS isoenzymes and their encoding genes have been isolated and characterized in pine, a model tree for molecular studies in conifers. Although these proteins are highly evolutionary conserved, major differences

have been found in their cellular compartmentalization between angiosperms and gymnosperms, suggesting possible adaptations to specific functional roles. The chloroplastic isoform (GS2) has not been detected in conifers even when using a number of molecular approaches (Cánovas *et al.*, 1998). However, two cytosolic GS have been identified and named as GS1a and GS1b. These isoforms exhibit different molecular and kinetic properties and are differentially expressed during germination and early seedling growth (Ávila *et al.*, 1998; Cantón *et al.*, 1999). In pine, two separate genes encode GS1 holoenzymes, *GS1a* and *GS1b* (Ávila Sáez *et al.*, 2000; de la Torre *et al.*, 2002). *GS1a* expression is associated to green tissues and partly induced by light whereas *GS1b* is constitutively expressed and associated to vascular tissue (Ávila *et al.*, 2001). Recently, *cis*-regulatory elements and *trans*-acting factors involved in the transcriptional regulation of pine *GS1* genes have been reported (Gómez-Maldonado *et al.*, 2004a,b).

A number of studies have shown the importance of N compounds, particularly glutamine, for the proliferation and maturation of somatic embryos in different species (Bozhkov *et al.*, 1993; Khlifi & Tremblay, 1995; Higashi *et al.*, 1998; Sreedhar & Bewley, 1998). Therefore, a balanced N supply and metabolism seem to be critical for plant embryogenesis. In addition, Higashi *et al.* (1998) have shown in carrot that the pattern of expression of *GS* genes during somatic embryo development resembled that during zygotic embryo development. Furthermore, the GS/GOGAT cycle has been revealed as the preferential pathway for N assimilation during somatic embryogenesis of white spruce (Joy *et al.*, 1997). However, very little is still known about N metabolism in embryonic tissues of conifers, the function of key genes, how these genes are expressed and how they are regulated during both zygotic and somatic embryo development. This knowledge is of major importance in understanding the biochemical regulation of plant embryo development and may also have practical implications for improving somatic embryogenesis. The major advantages of using somatic embryogenesis in conifers are that a large number of cloned plants can be obtained in a short time and that cryopreservation of embryogenic cultures allows storage of the genotypes with propagation ability (Hogberg *et al.*, 1998).

In this work we analyse the expression pattern of two *GS* genes during zygotic and somatic embryogenesis of *Pinus pinaster* and *Pinus sylvestris*. Our results indicate that the expression of the *GS1a* and *GS1b* genes is developmentally regulated and associated to chloroplast development and vascular tissue differentiation, respectively. Expression of these genes early in development suggests additional roles for GS1a and GS1b apart from those they fulfil in ammonium reassimilation during photosynthesis and N translocation. In this paper we discuss the possibility of two differential roles for these genes in seed storage protein metabolism. Furthermore, expression of the *GS1b* gene in vascular tissue before the differentiation of mature vascular elements indicates that it can be used as a molecular marker for early stages of vascular tissue differentiation in embryos and seedlings of pine.

Materials and Methods

Plant material

Pinus pinaster Aiton. and *P. sylvestris* L. seeds from Sierra Bermeja (Málaga, Spain) were provided by the Consejería de Medio Ambiente (Junta de Andalucía) and kept at 4°C until they were used. To grow the plants, pine seeds were imbibed for 3 d and then sown on wet vermiculite. Seedlings were collected at different developmental stages for subsequent *in situ* hybridization experiments.

Pinus sylvestris somatic embryogenesis

Embryogenic cultures were initiated in 2000 from the immature ovules collected from open-pollinated trees growing in a seed orchard in central Sweden (Brunsberg). The ovules were cultured on the modified DCR medium (Gupta & Durzan, 1985), with increased concentrations of KNO_3 (8.1 mM) and $CaCl_2 \cdot 2H_2O$ (1.16 mM) and $Ca(NO)_3 \cdot 2H_2O$ was omitted. This medium was supplemented with 2,4-dichlorophenoxyacetic acid and benzyladenine at 9.0 μM amd 4.4 μM, respectively, as well as with 3% (w : v) sucrose and 0.35% (w : v) gelrite. The established cell lines were cryopreserved in liquid nitrogen, thawed and grown in the same conditions as at initiation. For embryo maturation the lines were pretreated for 4 wk on plant growth regulator-free modified DCR medium and then transferred to DCR maturation medium containing 7.5% (v : v) polyethylene glycol (PEG-4000), 60 μM abscisic acid and 3% (w : v) maltose.

Pinus pinaster somatic embryogenesis

Embryogenic cultures were initiated in 2000 from AFOCEL seed lot obtained by controlled pollination. Embryogenic lines were cultivated as described by Ramarosandratana *et al.* (2001) and cryopreserved within 6 months after culture initiation. They were thawed and regrown every year in order to maintain a good embryogenic potential of the plant material. Maturation treatments were performed by inoculating filter paper disks that were previously laid down on Petri dishes containing solidified maturation medium (DCR medium with 6% (w : v) sucrose gelled with 0.9% (w : v) gelrite (Sigma, St Louis, Mo, USA) with suspension of the peripheral parts of embryogenic callus (10%, w : v) in liquid maturation medium (for further details see Ramarosandratana *et al.*, 2001).

RNA extraction and Northern blot assay

Total RNA from embryogenic cultures was extracted as previously described (Chang *et al.*, 1993). Ten micrograms of

each RNA sample were loaded on denaturing agarose-formaldehyde gel (1.2% (w : v) agarose; 2.2 M formaldehyde) and then blotted onto nylon filters. cDNA probes for pine *GS* genes *GS1a* (EMBL accession number X69822) and *GS1b* (EMBL accession number AJ005119) were radiolabelled with [α-^{32}P]dCTP by random priming (Amersham Laboratory, Buckinghamshire, UK) according to Sambrook & Russell (2001). It has previously been determined that these probes specifically recognize mRNAs for GS1a and GS1b in *P. sylvestris* and *P. pinaster* (Cantón *et al.*, 1993; Ávila Sáez *et al.*, 2000; F. R. Cantón, C. Ávila & F. M. Cánovas, unpublished). Filters were incubated at 65°C overnight in hybridization buffer (6 × standard saline citrate (SSC), 5× Denhardt's (Sambrook & Russell, 2001), 1% (w : v) sodium dodecyl sulphate (SDS) and 10 μg ml^{-1} salmon sperm (DNA) supplemented with probe. Washes were carried out at 65°C in 1 × SSC and 0.1% (w/v) SDS. As a loading control ribosomal RNA was stained on the filters with methylene blue.

In situ hybridization

Tissue was fixed and paraffin-embedded as described by Cantón *et al.* (1999). The full-length cDNAs of pine *GS1a* (1423 bp fragment of clone pGSP114; EMBL accession number X69822) and *GS1b* (1451 bp fragment of clone pGSP15; EMBL accession number AJ005119) were subcloned in Bluescript SK+ plasmids (Stratagene, Cedar Creek, TX, USA) and used for the synthesis of sense and antisense riboprobes. A comparison of the *GS1a* and *GS1b* sequences was described in Ávila Sáez *et al.* (2000). In addition to GS, the following pine cDNAs were used for RNA synthesis: *rbcS* (338-bp fragment of clone 1H09; EMBL accession number AJ878075) coding for the rubisco small subunit, *psbO* (732 bp fragment of clone 1H12; EMBL accession number AJ878076) coding for the 33 kDa protein of Photosystem II (PSII) and *arginase* (1366 bp fragment of clone ARS20; GenBank accession number AF130440) kindly provided by Prof. D. J. Gifford. Plasmids were linearized with the appropriate restriction enzymes and used as templates for RNA probe synthesis with digoxigenin-UTP (Roche Farma SA, Barcelona, Spain) and T3 or T7 RNA polymerases according to Langdale (1993). *In situ* hybridizations on zygotic and somatic embryos and root tissue sections were carried out as previously described (Cantón *et al.*, 1999).

Results

GS expression analysis in zygotic embryos and roots

Previous studies with pine seedlings in our laboratory had shown that the expression of the *GS1b* gene could be detected in the vascular tissue all along the plant (Ávila *et al.*, 2001). In agreement, when gene expression was examined in 2-d imbibed and dry mature zygotic embryos of *P. pinaster* (Fig. 1a,b) and *P. sylvestris* (data not shown), the *GS1b* transcripts were found exclusively localized in the embryonal vascular system (procambium), a thin strand of narrow and tightly packed cells running from cotyledons down to root pole. The presence of GS1b message indicates that pine seeds have the ability for rapid production of the enzyme at the initial stages of seedling growth. No apparent difference was observed between dormant (dry) and very early germinating (imbibed) embryos (Fig. 1a,b).

In pine seedlings, *GS1a* expression has been associated to green tissues in both hypocotyl and cotyledons and enhanced by light concomitantly with increased expression of photosynthesis-related genes (Cantón *et al.*, 1999). As expected, since embryos do not have green photosynthetic tissues, *GS1a* expression could not be detected in pine zygotic embryos (Fig. 1c). During early germination, however, the *GS1a* gene is expressed in seedlings (Cantón *et al.*, 1999; M. J. Pérez-Rodríguez, unpublished).

In order to determine the earliest developmental stage when *GS1b* expression was initiated in zygotic embryos, *P. pinaster* seed cones were collected from May to July at 1-wk intervals. Samples were analysed microscopically to identify the developmental stages of the embryos. Figure 2 shows three developmental stages named T3, T4 and T6, according to

Fig. 1 Expression analysis of *GS* in pine mature zygotic embryos. Longitudinal tissue sections of 2-d-imbibed (a) and dry (b) mature zygotic embryos of *Pinus pinaster* were used for *in situ* hybridization. *GS1b* expression is detected in procambial cells from cotyledons to root pole. Longitudinal section of seed hybridized to *GS1a* probe (c). Bar, 1 mm.

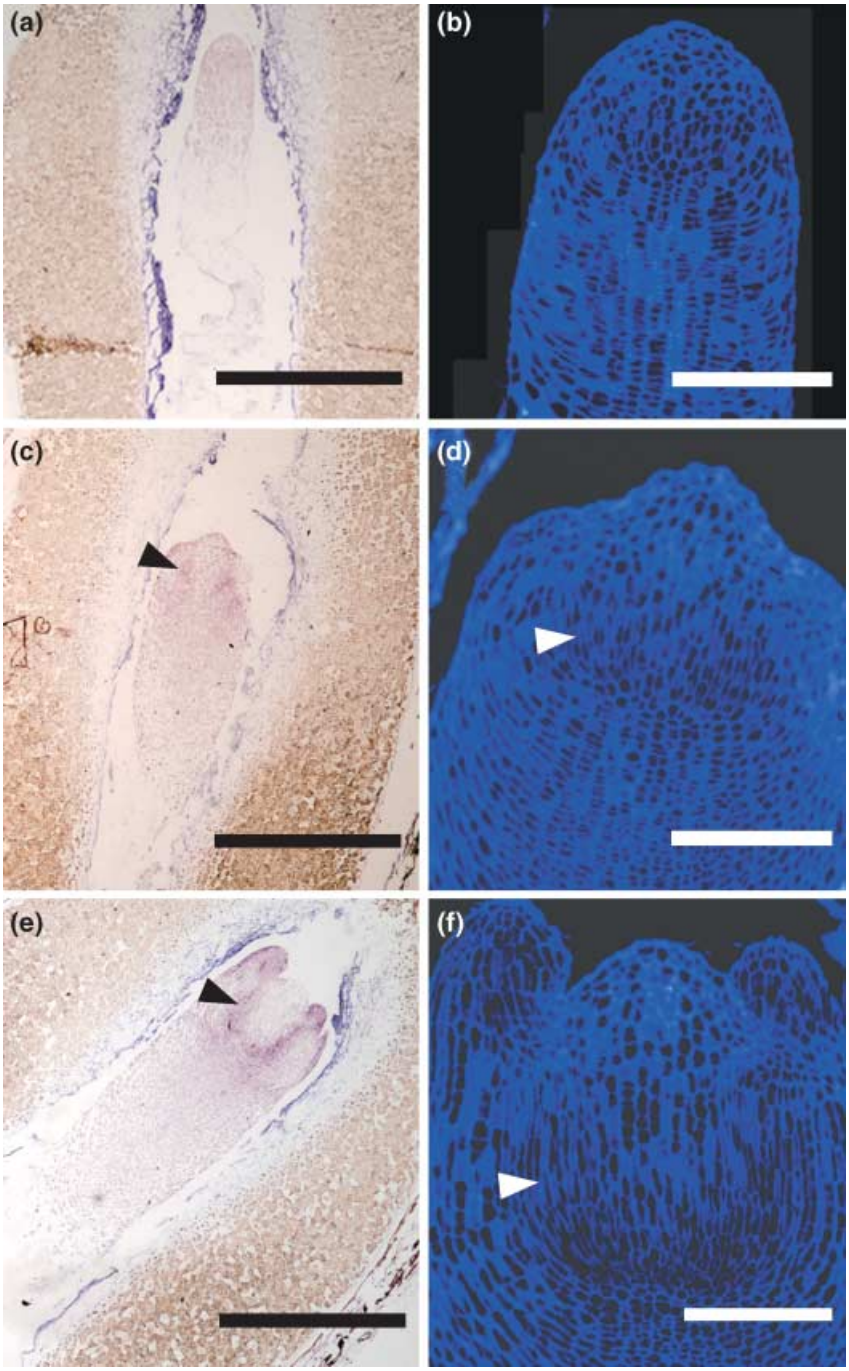

Fig. 2 Localization of *GS1b* expression in developing zygotic embryos. Longitudinal sections of *Pinus pinaster* embryos were used for *in situ* hybridizations at the transition to filamentous to cotyledonary stages (stages T3 and T4) (a,c) and at the cotyledonary stage (stage T6) (e). The signal was first detected at stage T4 in vascular cells (c, arrowhead) and showed more strongly in cotyledonary embryo (e, arrowhead). Right panels correspond to magnifications of left panels and show calcofluor-stained tissue observed under UV light. Embryo vascular tissue is detected at the T3 stage as a strand of roughly elongated cells located in the centre of the embryo (b). At the T4 stage (d) procambial cells look clearly elongated (arrowhead) and later in stage T6 the vascular system is organized like a central cylinder with branches into the cotyledons (f, arrowhead). Bar, 1 mm (left panels), 200 μm (right panels).

the nine-stage system described by Ciavatta *et al.* (2001): T3 and T4 correspond to transition from filamentous to cotyledonary stages (Fig. 2a–d); T6 corresponds to cotyledonary stage (Fig. 2e,f). *GS1b* transcripts were found in the cells of the procambium and the expression was first detected at T4, the late transition to cotyledonary stage (Fig. 2c), just after morphological differentiation of vascular cells could be distinguished (Fig. 2b,d). This transition stage is considered as the beginning of embryo maturation and can be biochemically distinguished by the accumulation of storage proteins (Hakman *et al.*, 1990; Chatthai & Misra, 1998). The expression level of the *GS1b* gene increased as embryo maturation proceeded (Fig. 2e,f). Post-fertilized ovules were also isolated from *P. sylvestris* seed cones, beginning at the end of June (*c.* 3 wk after fertilization) until the end of July (7 wk after fertilization) at 1-wk intervals. *In situ* hybridization analysis revealed a similar

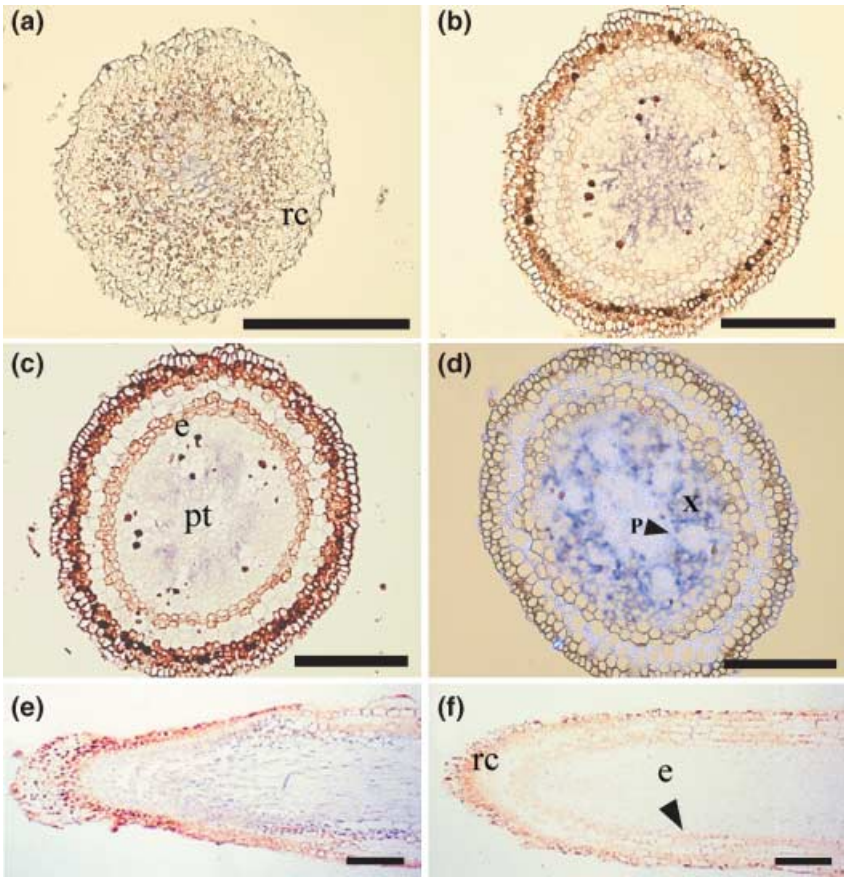

Fig. 3 Localization of *GS1b* expression in developing roots of pine seedlings. Roots of *Pinus pinaster* were cross-sectioned from their tips upwards (a–d) and *GS1b* mRNA detected by *in situ* hybridization. Expression signal shows as a purple colour in the centre of the root apical meristem (a,b) and in the vascular bundles at later developmental stages (c,d). Localization of *GS1b* transcripts in longitudinal sections of roots; specific antisense RNA probe (e), control sense strand (f). P, Phloem; X, xylem; pt, pith; rc, root cap; e, endodermis. Bar, 200 μm.

pattern of *GS1b* expression in *P. sylvestris* compared with *P. pinaster* (data not shown).

It has been previously reported that pine embryonal vascular system do not contain mature elements (Berlyn, 1967). Therefore, *GS1b* expression must certainly be linked to very early stages of vascular tissue differentiation during embryo development. However, to further test this assertion, we examined the expression pattern of the *GS1b* gene in the root tip of pine seedlings. The process of differentiation in the root is a continuous one that proceeds steadily toward the apex without discontinuities (Steve & Sussex, 1989). *Pinus pinaster* root tips embedded in paraffin were cross-sectioned and *GS1b* expression was analysed as shown in Fig. 3. A signal was observed in the procambium of the root apical meristem as a small purple circle surrounded by the root cup (Fig. 3a). In the upper tissue sections, *GS1b* expression was seen as patches around the pith that allowed the vascular strands to be envisaged (Fig. 3b,c). The hybridization signal was finally restricted to differentiated xylem and phloem tissues (Fig. 3d). The exclusive localization of *GS1b* transcripts in the vascular cylinder was demonstrated in longitudinal sections of the root (Fig. 3e). These results confirmed that the expression of the *GS1b* gene was indeed associated with very early stages of vascular tissue differentiation.

GS expression analysis during somatic embryo development

Another goal of our work was to examine the expression pattern of GS genes during somatic embryogenesis and compare the results with those obtained in zygotic embryos. Pine somatic embryogenesis process encompasses two main phases: proliferation and maturation. We first analysed the expression of the *GS1a* and *GS1b* genes by Northern blot at the proliferation stage. Four cell lines of *P. pinaster* were chosen to represent significant differences with regard to the macromorphology (based on visual aspects: white, spiky, low necrosis) and their ability to proliferate. The GS1a message was barely detectable in all the four cell lines (not shown). By contrast, GS1b transcripts were highly abundant (Fig. 4) with increased levels of gene expression for lines XYB and PN661, which displayed higher proliferation rate compared with the other two lines. Similar results were found when GS expression was analysed in *P. sylvestris* embryos. These data show that *GS* genes are expressed in embryogenic cultures and suggest that glutamine biosynthesis is required very early during pine somatic embryogenesis.

Differentiation and maturation of somatic embryos was stimulated on maturation medium containing abscisic acid (ABA). The expression pattern of both *GS* genes was then

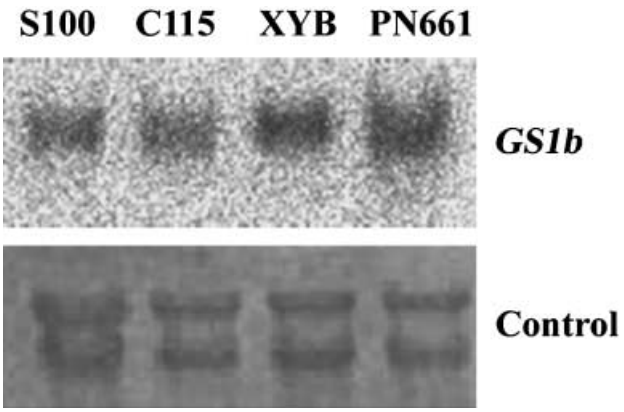

Fig. 4 Expression analysis of GS transcripts by Northern blot in embryonal suspensor masses. RNA extracted from four different cell lines of *Pinus pinaster* (S100, C115, XYB and PN661) were used to compare relative abundance of transcripts. Staining of ribosomal RNA was used as a loading control.

investigated in cotyledonary embryos obtained after 9 wk of contact with ABA by *in situ* hybridization (Fig. 5a,b). The expression of the *GS1b* gene was present in the embryonal vascular system, demonstrating that there was no difference in the expression pattern between zygotic and somatic embryos (compare Figs 1a,b and 5b). However, interestingly, somatic embryos exhibited accumulation of *GS1a* transcripts in the cotyledons (compare Figs 1c and 5a). This finding prompted us to examine whether genes associated with chloroplast development were also expressed in somatic embryos. Thus, we analysed the expression pattern of two nuclear genes, *rbcS* and *psbO*, coding for photosynthetic proteins: the stromatic rubisco small subunit, a soluble polypeptide located in the chloroplast stroma, and the 33 kDa protein, a marker for thylakoid membrane of PSII. Spatial distribution of these transcripts matched the expression pattern of the *GS1a* gene (Fig. 5c,d). Moreover, when observed under the light microscope, these embryos showed some greening during maturation (data not shown). Zygotic embryos undergo a desiccation-induced dormancy programme that separates embryogenesis from postgerminative development (Thomas, 1993). By contrast, our results indicate that chloroplast development had been initiated in pine cotyledonary somatic embryos before they were artificially brought to a dormant state, suggesting that the germination programme might be activated during somatic embryo maturation. We next examined the expression of a gene encoding for the enzyme arginase in maturing pine embryos (Fig. 6). Arginase is induced in the shoot pole of the seedling during germination of *P. taeda* where it is responsible for metabolizing the amino acid arginine released through the breakdown of storage proteins (Todd *et al.*, 2001). We found a high expression level for the gene *arginase* from the cotyledons to the root pole in somatic embryos (Fig. 6a). By contrast, zygotic embryos showed no hybridization signal (Fig. 6b).

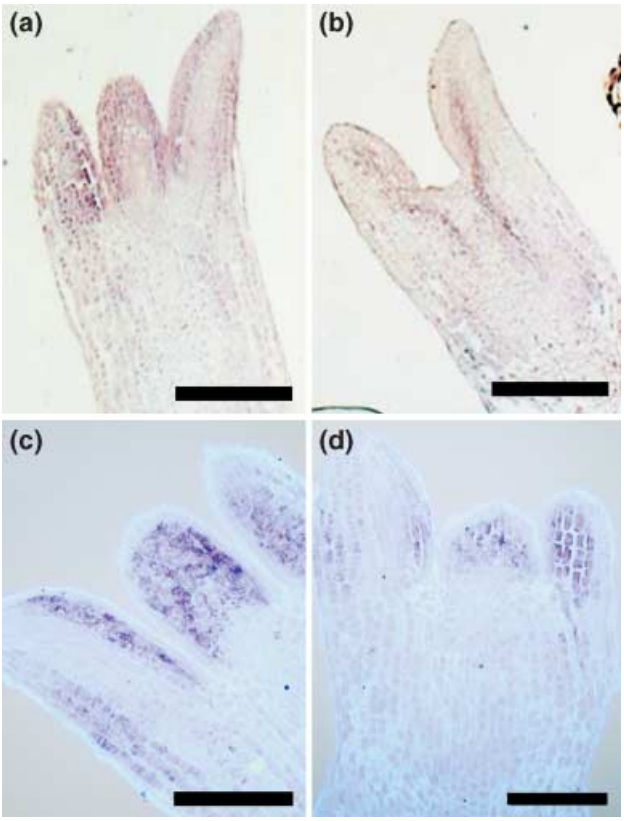

Fig. 5 Expression patterns of *GS1a*, *GS1b* and nuclear genes encoding photosynthetic proteins in pine somatic embryos. Cross-sections of cotyledonary embryos (*Pinus sylvestris*) were analysed by *in situ* hybridization. The *GS1a* gene (a) was expressed in cotyledons whereas *GS1b* expression (b) was observed in procambial cells. Transcripts for *rbcS* (the small subunit of rubisco) (c) and *psbO* (the 33 kDa protein of photosystem II (d) were located in the cotyledons. Bar, 500 μm (a), 200 μm (c,d).

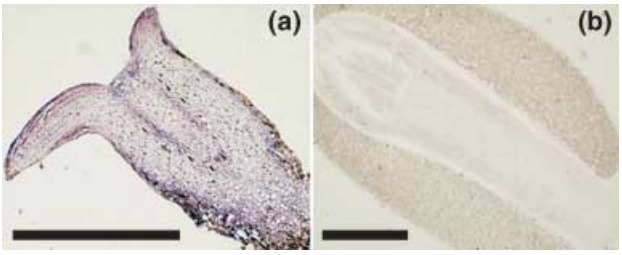

Fig. 6 *Arginase* expression analysis in mature zygotic and somatic embryos of pine. *In situ* hybridization of the *arginase* gene in mature somatic (a) and zygotic (b) embryos of *Pinus pinaster*. No expression was detected in zygotic embryos. By contrast, a conspicuous hybridization signal was observed in somatic embryos. Bar, 1 mm.

GS expression and vascular development

As mentioned above, *GS1b* expression was clearly associated with vascular tissue differentiation during both embryo and seedling development. In addition, the expression of *GS1b* in vascular cells before the differentiation of mature vascular

elements indicated that this gene could be useful as molecular marker for early stages of vascular differentiation in pine. In order to test this hypothesis, we examined *GS1b* expression in different quality somatic embryos at proliferation and maturation phases. Three *P. pinaster* cell lines, DE606, NM626 and PN654, of different genotypes were selected for producing somatic embryos. The performance of these lines was valued according to yield and morphology. Yield was estimated by the number of mature embryos produced per gram fresh weight of embryogenic callus. Concerning morphology, cotyledonary embryos were considered normal if possessing four or more cotyledons, elongated hypocotyl, no signs of hypotrophy along the hypocotyl axis, germinability and good *ex vitro* growing performance. For number of embryos produced, line DE606 was considered the best (215 cotyledonary embryos per gram fresh weight of embryogenic callus) followed by line NM626 (80 embryos per g) and finally line PN654 (two embryos per g). For morphological criteria, cell line DE606 produced better quality embryos than line NM626. The PN654 embryos showed more developmental abnormalities than either DE606 or NM626 embryos, and would never grow further to generate somatic plants.

Results obtained from the analyses of *GS1b* expression in precotyledonary and cotyledonary somatic embryos of all the three cell lines were consistent with those obtained by morphological analysis (Fig. 7). Like in zygotic embryos, *GS1b* expression was restricted to the vascular system in cotyledonary (Fig. 7e,j,l) and developing (Fig. 7b,d,g,i,k) somatic embryos. However, *GS1b* transcripts were much more difficult to detect in NM626 than in DE606 embryos and indicated deficient vascular development, particularly at the embryo root pole, even at the cotyledonary stage (Fig. 7, compare e and j). In PN654 embryos, *GS1b* expression was remarkably low even at the cotyledonary stage (Fig. 7l)**.** Therefore, vascular pattern formation shown by *GS1b* hybridization signal, correlated with the morphological characteristics of the different somatic embryos.

Discussion

In this study a detailed molecular analysis of two genes involved in glutamine biosynthesis has been performed in pine somatic and zygotic embryos. Results obtained from Northern analyses carried out with embryogenic cultures (Fig. 4), are consistent with those previously reported for genes encoding distinct GS isoforms in carrot somatic embryos (Higashi *et al.*, 1998) although, unlike carrot cultures, pine embryogenic cultures expressed the *GS* gene associated with chloroplast development at a much lower level than that associated with N transport. Both works, however, indicate the importance of glutamine biosynthesis during the first stages of somatic embryogenesis.

To gain further insights into the expression pattern of these genes during pine embryogenesis, the distribution of *GS1a* and *GS1b* transcripts was precisely analysed by *in situ* hybridization in specific cell types of both zygotic and somatic embryos (Figs 1, 2, 5 and 7). *GS1a* expression was undetectable in the zygotic embryo (Fig. 1c) but induced early in germination and restricted to cotyledons (unpublished). In developing seedlings the expression was also present in the cotyledons where it is developmentally regulated and enhanced by light, in good correlation with the expression of photosynthesis-related genes (Cantón *et al.*, 1999). Furthermore, *GS1a* mRNA is located in the cells of the chlorophyllic parenchyma, suggesting a role for this gene in the assimilation of the ammonium released during the photosynthetic process (Ávila *et al.*, 2001). We have found that the presence of *GS1a* transcripts in somatic cotyledonary embryos was also correlated with the expression of two nuclear genes encoding photosynthetic proteins: *rbcS* and *psbO* (Fig. 5a,c,d). These findings strongly suggest that during pine somatic embryogenesis chloroplast development was indeed initiated. In angiosperms, the accumulation of gene products associated with photosynthesis is regulated by light (Tobin & Silverthorne, 1985). However, it is well documented that pine and other gymnosperms are able to develop functional chloroplasts even when growing in darkness (Oku *et al.*, 1974; Mariani *et al.*, 1990). Thus, in several pine species the light-independent synthesis of LHCIIb and other chloroplast polypeptides such as rubisco large and small subunits have been reported (Yamamoto *et al.*, 1991; Cánovas *et al.*, 1993; Cantón *et al.*, 1999). In addition to light, other factors such as nutritional status also affect chloroplast development (Mullet, 1988). Therefore, the composition of the culture medium during the somatic embryogenesis process, particularly the carbohydrate to N ratio, may represent a key factor responsible for the observed expression pattern. This assumption is supported by the report on enhanced photosynthetic gene expression in *Arabidopsis* at high levels of both sugar and N (Martin *et al.*, 2002). Thus, the expression of *GS1a* and photosynthesis-related genes during somatic embryogenesis could in part be triggered by light exposure and/or nutrient availability.

Whatever the controlling factors are, our results suggest that during pine somatic embryogenesis some metabolic activities associated to germination could take place. In fact, when the expression of gene encoding for the enzyme arginase was analysed, a strong signal was detected all through the embryo (Fig. 6a). Nevertheless, our findings might not completely agree with those previously reported, since Todd *et al.* (2001) found that arginase activity was restricted to the shoot pole of *P. taeda* germinating embryos. Our results could argue in favour of an active synthesis-degradation pathway for the amino acid arginine during pine somatic embryogenesis. Since arginine is an important component of storage proteins in pine, this could reflect the simultaneous biosynthesis and breakdown of such proteins related to the processes of embryogenesis and germination, respectively. In agreement, during oil palm somatic embryogenesis, embryo maturation as well

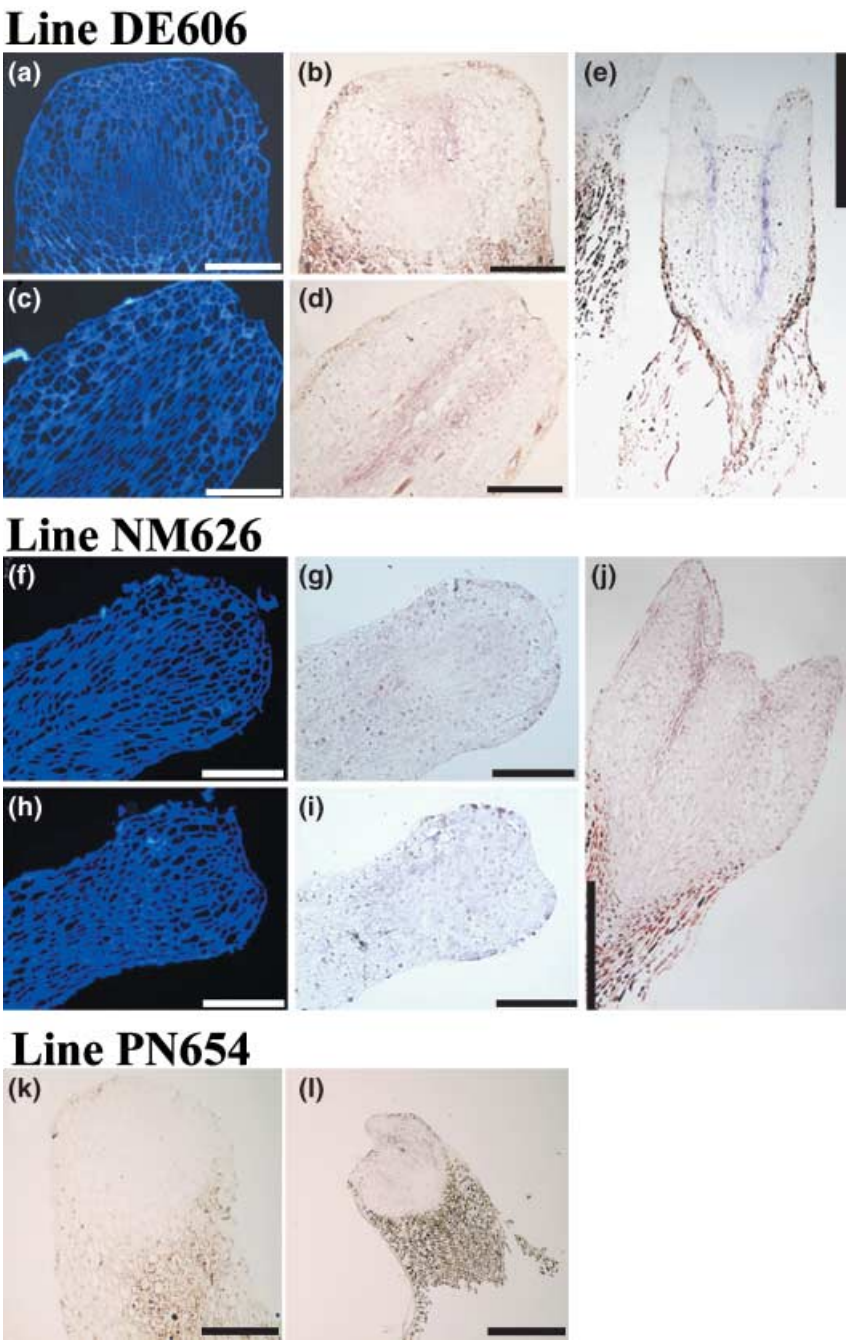

Fig. 7 Comparison of *GS1b* expression patterns in developing somatic embryos. *In situ* hybridization in precotyledonary (b,d,g,i,k) and cotyledonary (e,j,l) somatic embryos of *Pinus pinater* from cell lines DE606, NM626 and PN654. Hybridization signal can be observed as a purple colour under bright field microscope. In DE606 embryos *GS1b* expression was detected in the procambium of cotyledonary and precotyledonary stages (b,d,e). With respect to line NM626, *GS1b* expression was observed in cotyledonary embryos (j) but hardly detected in precotyledonary stages (g,i). Expression in PN654 embryos was undetectable at precotyledonary stages (k) and very weak at cotyledonary stage (l) showing severe distortions of the embryonal vascular system. The differentiation and organization of the vascular system of precotyledonary embryos from lines DE606 and NM626 can be observed by calcofluor staining under UV light (a,c,f,h). Bar, 200 μm (precotyledonary embryos; a–d,j,g,h,i,k), 500 μm (cotyledonary embryos; e,j,l).

as vigour of regenerated plantlets was improved by adding arginine to the maturation medium; arginine favoured the accumulation of the dominant storage protein content per dry weight (Morcillo *et al.*, 1999). The pattern of storage protein accumulation has been examined in somatic embryos of *Pinus strobus* during 16 wk of maturation treatment (Klimaszewska *et al.*, 2004). Interestingly, the protein content increased from weeks 6 to 9 and then gradually decreased from weeks 10 to 16. The reason for the reduction in protein content was not clear since no visible signs of germination were observed, however, gene expression or metabolic activities associated with germination were not analysed. According to our data, those results could be explained as a consequence of the onset of germination during maturation treatment. Precocious germination can prevent the expression of genes at the end of embryogenesis that prepare the embryo for subsequent developmental events (Goldberg *et al.*, 1994). In addition, precocious germination can bring about a reduction in storage protein accumulation in maturing embryos having a negative effect on plant survival (Klimaszewska *et al.*, 2004).

This is further confirmed by the beneficial impact of desiccation or other postmaturation treatments on embryo conversion into plantlets. These treatments slow down or abolish germination, allowing embryos to reach full morphological maturation (Misra *et al.*, 1993; Attree & Fowke, 1995).

According to previous works, the major amino acid residues of seed storage proteins in *P. taeda* and *P. pinaster* are arginine, glutamine and glutamate (Allona *et al.*, 1994; King & Gifford, 1997). Upon germination, these proteins breakdown in the megagametophyte and arginine diffuses to the shoot pole of the embryo. Then, by the catalytic activity of the enzymes arginase and urease, NH_4^+ ions are produced in the embryo cells (Todd *et al.*, 2001). It seems reasonable that the activity of the GS1a isoenzyme in the shoot pole of the germinating embryo would account for the reassimilation of that NH_4^+ into glutamine (Suárez *et al.*, 2002; this work).

The distribution of *GS1b* mRNA was also examined by *in situ* hybridization in developing and mature zygotic embryos. In mature embryos, the presence of the gene transcript was restricted to a strand of elongated cells conforming the embryo vascular system (procambium) (Fig. 1b). This result is in agreement with a proposed role for the GS1b isoenzyme in intercellular and interorgan N transport (Suárez *et al.*, 2002). However, in developing zygotic embryos, *GS1b* expression was also located in procambial cells, being first detected at the initiation of the maturation phase within stages T4 (filamentous) to T6 (cotyledonary), just after embryonal vascular tissue could be distinguished (Fig. 2c,d). A major metabolic event during seed maturation is the accumulation of reserves at late embryogenesis. This fact proves to be particularly relevant when considering N metabolism of conifer embryogenesis because of the important amount of seed storage protein that is accumulated in both the megagametophyte and the embryo itself. The initiation of this maturation phase (and accumulation of storage proteins) was established in zygotic and somatic embryos of *Picea abies* (Norway spruce) at the transition stage from filamentous to cotyledonary embryo (Hakman *et al.*, 1990). The importance of glutamine availability in the process has been previously reported in different species. Thus, the addition of glutamine as a N source to the maturation medium could promote somatic embryo maturation in carrot (Higashi *et al.*, 1998), enhance the synthesis of storage reserves in developing somatic embryos of alfalfa and oil palm (Sreedhar & Bewley, 1998; Morcillo *et al.*, 1999), improve the germinability of black spruce somatic embryos (Khlifi & Tremblay, 1995) and encourage their development (Finner *et al.*, 1989). Furthermore, during white spruce somatic embryo development, a switch in amino acid metabolism was shown which involved increases in glutamine, glutamate and arginine (major components of seed storage proteins in conifers) and a decrease in alanine (Joy *et al.*, 1997). In parallel, the activities of the GS/GOGAT enzymes also increased during the transition from filamentous to cotyledonary stages revealing the GS/GOGAT cycle as the preferential pathway for N metabolism. Collectively, the data above indicate that the biosynthesis of glutamine is active during pine embryo maturation and that such a synthesis is conducted through the activity of the enzyme GS. Therefore, the time- and space-specific expression patterns of *GS* genes might be critical for obtaining fully mature embryos. The induction of the *GS1b* gene detected at the initiation of embryo maturation suggests the participation of this gene in the synthesis of storage proteins, the main sink for amino acids during embryo maturation.

We also performed the analysis of the *GS1b* expression in precotyledonary (Fig. 7b,d) and cotyledonary (Figs 5b and 7e) somatic embryos. Like in zygotic embryos, a correlation was found between the development of the vascular system and the level of *GS1b* expression. The degree of similarity observed when comparing *GS1b* expression pattern in pine zygotic and somatic embryos suggests that, with regard to GS1b function and regulation, zygotic and somatic embryogenesis behave in the same manner.

To the best of our knowledge, the present work shows for the first time in plants that the expression of a *GS* gene is induced very early in vascular development, even before xylem or phloem elements can be distinguished (Figs 1 and 2). The association between *GS1b* expression and vascular differentiation has been also demonstrated in seedlings during root vascular development (Fig. 3). To test this further, we undertook the analysis of *GS1b* expression in embryos of different quality, based on morphological parameters (Fig. 7). The correlation shown between the morphology of the different embryos and the pattern and level of *GS1b* expression, confirmed that this gene is a suitable molecular marker for early vascular tissue differentiation in pine.

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