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Two different modes of early development and nitrogen assimilation in gymnosperm seedlings†

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Summary

Light-independent chloroplast development and expression of genes encoding chloroplast proteins occur in many but not all species of gymnosperms. Early development in maritime pine (*Pinus pinaster*) seedlings was strongly light-independent, whereas *Ginkgo biloba* seedlings exhibited a typical angiosperm-like morphogenesis with differentiated patterns in light and dark. In pine, chloroplast polypeptides were undetectable in the seed embryo and accumulated in cotyledons of both light- and dark-grown plants in good correlation with light-independent chlorophyll synthesis. In contrast, chlorophyll and chloroplast proteins were only detected in light-grown ginkgo. Pine cytosolic glutamine synthetase (GS) and ferredoxin glutamate synthase (Fd-GOGAT) were present at low levels in the seeds and accumulated at comparable amounts in light- and dark-grown seedlings. Fd-GOGAT was also barely detectable in the seeds of ginkgo and only accumulated in green plants with mature chloroplasts. In *G. biloba* seeds and etiolated plants only cytosolic GS was identified, while in light-grown seedlings this molecular form was present at low abundance and choroplastic GS was the predominant isoenzyme. The above results have been confirmed by immunolocalization of GS protein in pine and ginkgo plantlets. In pine, GS was present in the peripheral cytoplasm of mesophyll cells and also in the phloem region of the vascular bundle. Immunocytochemical analysis showed that the labelling of mesophyll and phloem cells was only cytoplasmic. In developing ginkgo, GS antigens were present in the chloroplasts of mesophyll parenchyma cells of leaflets and green cotyledons. In contrast, a weak labelling of GS was observed in the parenchyma and phloem cells of non-green cotyledons enclosed in the seed coat. Taking all this into account, our data indicate the existence of two different modes of GS and GOGAT regulation in gymnosperms in close correlation with the differential response of plants to light. Furthermore, the results suggest that glutamine and glutamate biosynthesis is confined to the chloroplast of mesophyll cells in species with light-dependent chloroplast, development whereas compartmentation would be required in species with light-independent plastid development.

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†This paper is dedicated to the memory of Professor Jacobo Cárdenas.

Introduction

Light, the source of energy for photosynthesis, is also the main environmental factor involved in early events of plant growth and development (Deng, 1994). In angiosperms, dark-germinated seeds develop as etiolated seedlings with long hypocotyls, unexpanded cotyledons or primary leaves and lack photosynthetic pigments. When etiolated plants are exposed to light, chlorophyll biosynthesis is induced and proplastids develop to chloroplasts (Harpster and Apel, 1985) This differentiation process involves the co-ordinated expression of both nuclear and plastid genomes (Tobin and Silverthorne, 1985).

In contrast to angiosperms, pine and other gymnosperm species accumulate chlorophyll and develop chloroplasts in a light-independent manner (Bodganovic, 1973; Mariani *et al.*, 1990). In the last few years, the synthesis of the light-harvesting chlorophyll a-b protein (LHCIIb) and the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase has been reported in dark-grown pine seedlings (Cánovas *et al.*, 1993; Mukai *et al.*, 1991; Yamamoto *et al.*, 1991). Nitrogen, which is required for such a developmental process in the dark, should be supplied during seed germination by degradation of storage proteins to their structural units, the amino acids, which are used directly in protein synthesis or subsequently deaminated. Ammonium released in protein catabolism is reassimilated into organic nitrogen by the sequential action of the enzymes glutamine synthetase (GS) (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.7.1; 1.4.1.14). The products of the GS/GOGAT metabolic pathway, glutamine

and glutamate, can be used as nitrogen donors for the biosynthesis of major nitrogen compounds required for early plant growth, such as amino acids, nucleotides and chlorophylls.

The biochemistry and molecular biology of GS and GOGAT has been studied extensively in the last decade (reviewed by Lam *et al.*, 1996; Lea *et al.*, 1990), mainly due to the key role of these enzymes in the nitrogen metabolism of higher plants. GS exists in angiosperms as two isoforms easily resolved by anion exchange chromatography and native electrophoresis: GS1, located in the cytosol, and GS2, associated to the plastids (McNally and Hirel, 1983). The genetic basis of the GS isoform variety is a small family of nuclear genes that are differentially expressed during plant development and in response to different external stimuli (Cullimore *et al.*, 1984; Tingey *et al.*, 1988). Two molecular forms of glutamate synthase can also be found in plants, differing in their respective electron donors for enzyme catalysis: NADH-GOGAT and ferredoxin (Fd)-GOGAT (Suzuki and Gadal, 1984), which are encoded by distinct genes (Gregerson *et al.*, 1993; Sakakibara *et al.*, 1991). Fd-GOGAT is a chloroplast-located enzyme (Botella *et al.*, 1988) and represents the predominant molecular form in green tissues. In etiolated seedlings, the enzymes GS2 and Fd-GOGAT are barely abundant but their steady state levels accumulate developmentally following plant illumination (Gálvez *et al.*, 1990; Suzuki *et al.*, 1987). This is a consequence of gene transcriptional activation (Becker *et al.*, 1992; Sakakibara *et al.*, 1991).

Most studies on nitrogen-assimilating enzymes have been developed in annual crop plants, and the information available for woody plants is much more limited, particularly at the molecular level. We have previously reported the expression of GS and GOGAT genes in dark-grown pine seedlings (Cánovas *et al.*, 1991; García-Gutiérrez *et al.*, 1995). In this paper comparative studies have been performed in light- and dark-grown gymnosperms including several conifers and *Ginkgo biloba* plants. The results obtained indicate the existence of two different modes of GS and GOGAT regulation in gymnosperms, depending on the differential response of plants to light.

Results

Chlorophyll synthesis during primary development of P. pinaster *and* G. biloba *seedlings*

Many gymnosperms are able to develop the greening process in the total absence of light (Bogdanovic, 1973; Mariani *et al.*, 1990), therefore showing a light-independent pattern of morphogenic development compared with most angiosperm species.Figure 1(a) shows the similarity in the morphological aspect of maritime pine (*Pinus pinaster*) seedlings grown in light or dark conditions. This gymnosperm species presents a strong light-independence in its primary development. In contrast, *G. biloba*, the unique survivor of *Gingkofita*, exhibited a typical angiosperm-like primary development with clear well-defined patterns of morphogenesis in the light and in the dark (Figure 1b, L and D). To characterize further differences in early development between both gymnosperm species, chlorophyll synthesis was examined in developing seedlings. As occurs in other conifer species (Cánovas *et al.*, 1993; Mariani *et al.*, 1990; Shinohara *et al.*, 1992), substantial amounts of chlorophyll were synthesized in dark-grown maritime pine whereas no such pigment accumulation was evident in dark-grown ginkgo (results not shown). These findings agree well with earlier ultrastructural studies (Rascio *et al.*, 1984) that demonstrated a limited differentiation of internal plastid membranes (prolamellar bodies) in *G. biloba* growing in darkness.

Changes in polypeptide pattern during seed–seedling transition in P. pinaster *and* G. biloba

During seed germination, carbon and nitrogen reserves in the seeds are mobilized to provide the precursors for the construction of the photosynthetic apparatus. As shown in Figure 2(a, gel), a dramatic change occurred in the pattern of total proteins during maritime pine seed–seedling transition that corresponded to storage protein breakdown and biosynthesis of chloroplast polypeptides. However, no major differences were observed in polypeptide profiles in light- and dark-grown maritime pine seedlings (Figure 2a gel, compare L and D). As molecular markers of this developmental process, LSU, SSU and LHCIIb protein levels were assessed by immunoblot analysis with pine antibodies raised against the chloroplast proteins (García-Gutiérrez *et al.*, 1993). Chloroplast polypeptides, which were undetectable in the seed embryo, accumulated in cotyledons of both light- and dark-grown plants (Figure 2a, Western). These results correlated well with the light-independent chlorophyll synthesis described above. Similar results have been reported in dark-grown *P. thunbergii* seeds (Mukai *et al.*, 1991). Moreover, light-independent LHCIIb synthesis and assembly of major pigmented complexes in the dark have been demonstrated in *P. palustris* (Cánovas *et al.*, 1993). The light-independent accumulation of chloroplast polypeptides has been shown to be regulated at transcriptional level (Kojima *et al.*, 1992; Yamamoto *et al.*, 1991).

In a similar experiment to that described above with pine seeds, changes in total polypeptides were investigated in light- and dark-grown ginkgo seeds. Figure 2(b, gel) shows that total polypeptide profiles were quite different in light- and dark-grown plants, in close agreement with the differences described above in primary development and chlorophyl accumulation (Figure 1b). As shown in

Figure 1. Effect of light- in the early development of gymnosperm seedlings.
Pinus pinaster and Ginkgo biloba seeds were germinated and grown for 25 and 40 days, respectively, in light (L) or dark (D) without any exogenous nutrient.
(a) *Pinus pinaster*; (b) *Ginkgo biloba.*

Figure 2(b, Western) chloroplast polypeptides that were absent in the seeds were only detected in light-grown plants, suggesting that LSU, SSU and LHCIIb synthesis in ginkgo is a light-regulated process. Chinn and Silverthorne (1993) reported that *Lhcb* mRNA was expressed at low levels in dark-grown plants, with increasing mRNA contents

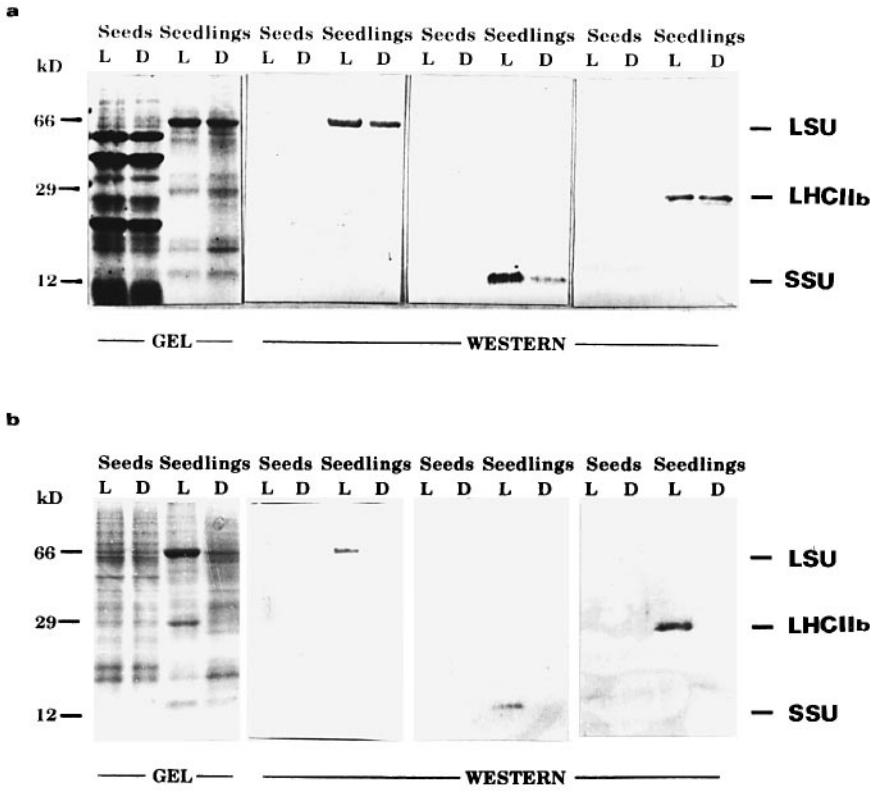


Figure 2. Steady-state levels of LSU, SSU and LHCIIb polypeptides in the seed–seedling transition of gymnosperms.
Total proteins were extracted from maritime pine and ginkgo seeds or seedlings, separated by SDS–PAGE, and either stained with Coomassie blue or electrotransferred to nitrocellulose membranes and immuno-decorated with specific antibodies. L, light; D, Dark. The electrophoretic mobility and molecular size (kDa) of protein markers are indicated on the left. The positions of immunoreactive bands are displayed on the right. The same amount of protein (30 μg) was loaded per lane except for maritime pine seeds, for which the loading was higher (100 μg).
(a) *Pinus pinaster*; (b) *Ginkgo biloba.*

in light-grown conditions, like that found in angiosperms. However, the transition period from etiolated to greened plants in gingko was longer than in most angiosperms and the stems of etiolated seedlings failed to become fully green (Chinn *et al.*, 1995).

Changes in GS and GOGAT polypeptides during seed–seedling transition in P. pinaster *and* G. biloba

Steady-state levels of GS and GOGAT polypeptides during seed germination were also determined by Western blotting analysis (Figure 3). In concordance with other chloroplast polypeptides examined, pine Fd-GOGAT was undetectable in the seeds and accumulated at comparable levels in light- and dark-grown seedlings (Figure 3a). Although relative GS abundance in the seeds was higher than Fd-GOGAT, a related pattern of expression was observed and cytosolic GS polypeptide accumulated during seed–seedling transition in darkness (Figure 3a).

In a parallel experiment, the presence of GS and Fd-GOGAT polypeptides was investigated in light- and dark-germinated *G. biloba* seeds. Figure 3(b) shows that Fd-GOGAT was barely detectable in the seeds, as occurs in maritime pine, and only accumulated in green plants with mature chloroplasts. The presence of GS protein was also immunorevealed using the same leaflet extracts. As Figure 3(b) shows, seeds and dark-grown plants contained a single GS immunoreactive band that co-migrated with pine cytosolic GS (GS1), which was included in the experiment as a control (results not shown). However, total protein extracts from light-grown ginkgo leaflets presented two polypeptides; one of them was identical in size to the major GS band detected in etiolated plants but quantitatively less represented. The additional polypeptide, of about 45 kDa, was the predominant GS molecular form in green tissues. These results suggest that in etiolated *G. biloba* GS1 is the major GS form, but when etiolated plants are exposed to light and chloroplast development takes place, chloroplastic GS accumulates in a light- dependent manner.

A survey of chloroplast proteins and GS/GOGAT polypeptides in light- and dark-grown conifers

In order to investigate further light-independent expression of chloroplast proteins and the pattern of GS/GOGAT polypeptides, additional experiments were conducted to determine their relative abundances in other conifer species, including stone and Scots pines, pinsapo fir and larch. As seen in Figure 4, LSU, SSU and LHCIIb polypeptides accumulated in the dark in all cases examined. However, their abundance was lower in pinsapo fir and larch than in pines. Similarly, Fd-GOGAT polypeptide was also shown to be present in all dark-grown conifers examined, whereas only cytosolic GS polypeptide could be detected in light- and dark-developed plants (Figure 4).

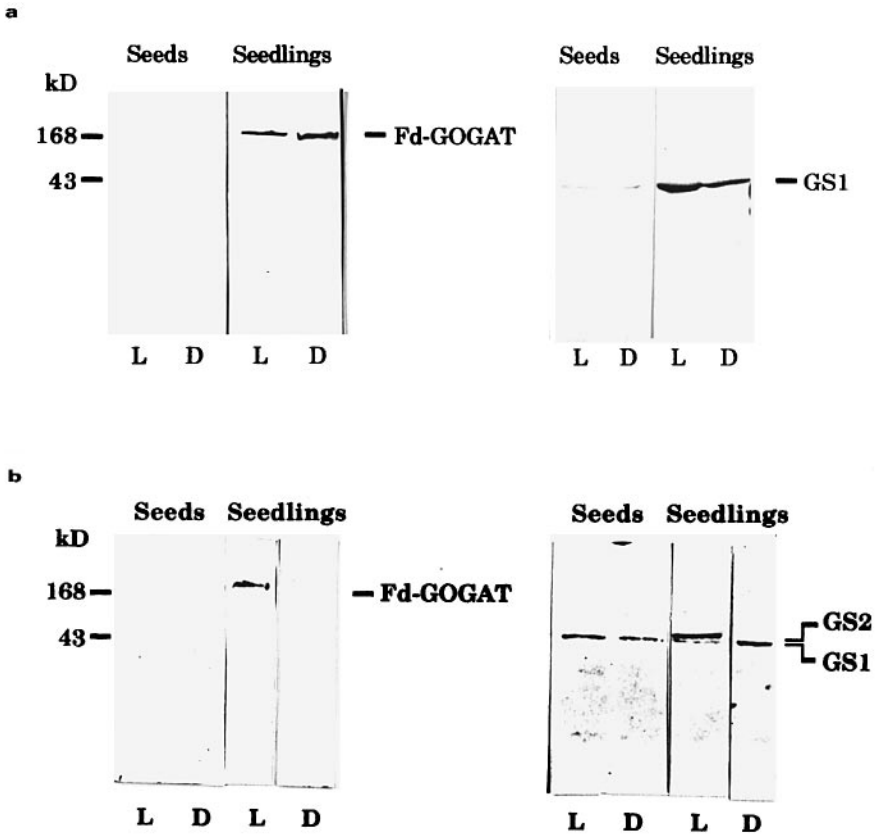


Figure 3. Immunoblots showing GS and GOGAT polypeptides in the seed–seedling transition of gymnosperms.
Total proteins were extracted from maritime pine and ginkgo seeds or seedlings, separated by SDS–PAGE, and the relative abundance of GS and GOGAT polypeptides analysed by using specific antibodies raised against pine GS and Fd-GOGAT. L, light-, D, Dark.
(a) *Pinus pinaster*; (b) *Ginkgo biloba.*

Cellular and subcellular localization of GS protein in P. pinaster *and* G. biloba *seedlings*

To determine the tissue and intracellular localization of GS, an immunogold labelling on cotyledons and/or leaflets of pine and gingko was performed. First, the immunolocalization of GS was studied at light-microscopy (LM) level. After silver enhancing, the zones decorated by the gold particles became blue-green to white under bright-field and epipolarized light. In pine cotyledons, the anti-GS antibodies recognized some antigens present in the light-peripheral cytoplasm of mesophyll cells, whereas observable organelles, plastids or nuclei, were unlabelled (data not shown; detailed below using transmission electron microscopy). A very strong labelling was also observed in cells located in the phloem region of the vascular bundle. Due to the strong intensity of labelling observed in phloem cells, it was difficult to acertain under light- microscopy that labelling was restricted to the cytosolic compartment and that organelles were free from labelling. In order to resolve this question, an immunolocalization of GS by transmission electron microscopy (TEM) was undertaken.

The TEM immunolocalization of the mesophyll cells further confirmed that light-labelling was observed in the cytoplasm and that chloroplasts were absolutely free of labelling (Figure 5a,b).

The TEM immunolocalization also showed that the heavy labelling in cells of the phloem zone was only cytoplasmic. An overview of this cell type showed a strong labelling in the cytoplasm (Figure 5c), whereas the organelles, including the mitochondria, remained unlabelled. Few, dense mitochondria with poor organization of inner membrane have been described frequently in phloem companion cells of angiosperms (Dubois *et al.*, 1996). Vacuoles and cell walls also remained unlabelled. Higher magnifications (Figure 5d,e) confirmed the cytosolic localization of GS. Control experiments performed with normal rabbit serum instead of anti-GS serum confirmed the specificity of immunolocalization (Figure 5f).

In developing ginkgo seedlings, the anti-GS antibodies clearly recognized antigens present in mesophyll parenchyma cells of leaflets (Figure 6a, arrows). Higher magnifications (Figure 6b) indicated that this labelling was restricted to the plastids, whereas observable nuclei and other cell constituents were unlabelled. In developing ginkgo seedlings, the cotyledons were still partially enclosed in the seed coat and we found different patterns of GS immunolocalization depending on the zone studied. In the uncovered region of cotyledons located near the primary axis of the seedling, we found a strong labelling of GS in parenchyma cells (Figure 6c). Higher magnifications (Figure 6d) clearly indicated that this labelling was restricted to the plastids of these cells and that nuclei or

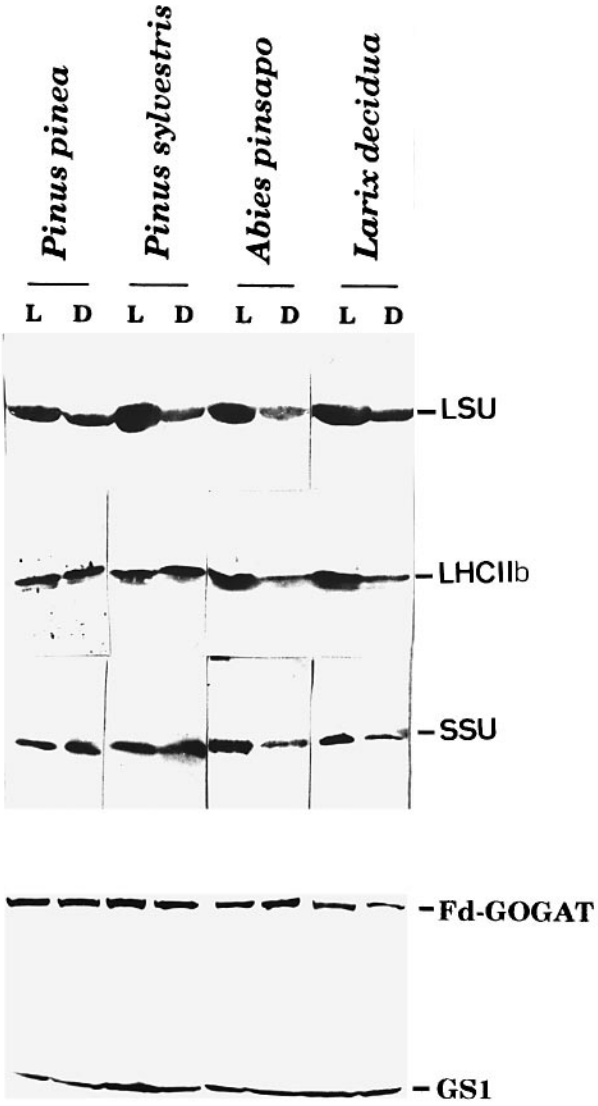


Figure 4. Western blot analysis of LSU, SSU, LHCIIb, GS and GOGAT polypeptides in cotyledons of several conifer species.
Total proteins were extracted from light- (L) and dark-grown (D) seedlings and the relative abundance of photosynthetic proteins and polypeptides of nitrogen-assimilating enzymes was visualized by Western blotting using specific antibodies as described above. About 30 mg of protein was loaded in each well.

other cell components remained unlabelled. In contrast, inside the distal part of cotyledons enclosed within the seed coat, only a weak labelling of GS could be observed in the parenchyma cells (Figure 6e). On the other hand, a detailed examination of the vascular bundle of cotyledons showed a weak labelling in some cells interspersed in the phloem zone, and we assumed that these cells were the phloem companion cells (Figure 6c, thin arrows). Higher magnifications (Figure 6f, arrows) confirmed the existence of this labelling, which appeared in the cytoplasmic compartment but was non-uniformly distributed, preventing us from excluding the localization of GS inside small-sized organelles. The controls run with normal rabbit serum instead of anti-GS serum confirmed the specificity of labelling observed; only a weak background signal was observed (Figure 6g, arrows). Since at a light-microscopic level it was not possible to observe the intracellular localization of GS in phloem cells (cytosol or small organelles) conclusively, we decided to undertake a TEM immunolocalization of GS.

The TEM immunolocalization showed that the light-heterogeneous labelling seen in the phloem companion cells under light microscopy was due to a very dense concentration of GS in particular zones of the cytoplasm (Figure 7a, arrows). Higher magnifications indicated that the labelling was localized on weakly contrasted and poorly preserved organelles (Figure 7b and c), which were indeed proplastids. The poor preservation was probably due to the light-fixation, as commonly used for immunocytochemistry studies and/or degeneration/senescence of cotyledons. Our classical method using osmium fixation and araldite embedding also indicated that these poorly preserved organelles were proplastids (results not shown). These types of plastids have been described in *G. biloba* (Camefort and Schaeffer, 1965; Cecch-Fiordi and Maugini, 1972). Only an unsignificant labelling was observed in the cytosol, the vacuole or the cell walls. Control experiments performed with normal rabbit serum instead of anti-GS serum confirmed the specificity of these immunolocalizations (Figure 7d).

Discussion

In this work we have studied the effect of light in the accumulation of chloroplast protein markers and GS/GOGAT polypeptides in germinating seeds of several gymnosperm species.

It is well established that many gymnosperms are able to synthesize chlorophyll, develop chloroplast-like plastid and express photosynthesis-related genes in darkness (Alosi *et al.*, 1990; Bodganovich, 1977; Mariani *et al.*, 1990; Yamamoto *et al.*, 1991). It is reasonable to postulate that this is the consequence of a developmental programme with limited, environmental interactions. However, the extent of this light-independent pattern of development varies among plant species. Thus, in conifers, *P. pinaster* (Cánovas *et al.*, 1991; this work) and *P. pinea* (Drumm-Herrel and Mohr, 1992) show a strong ability to follow a photomorphogenic programme in the absence of light, while Scots pine, larch and pinsapo fir present a more limited ability and light is required for full pigment accumulation and chloroplast development (Kasemir *et al.*, 1990; Mariani *et al.*, 1990).

Dark-grown conifer seedlings represent a closed heterotrophic system in which the extent of pigment synthesis and chloroplast development could be related to the abund-

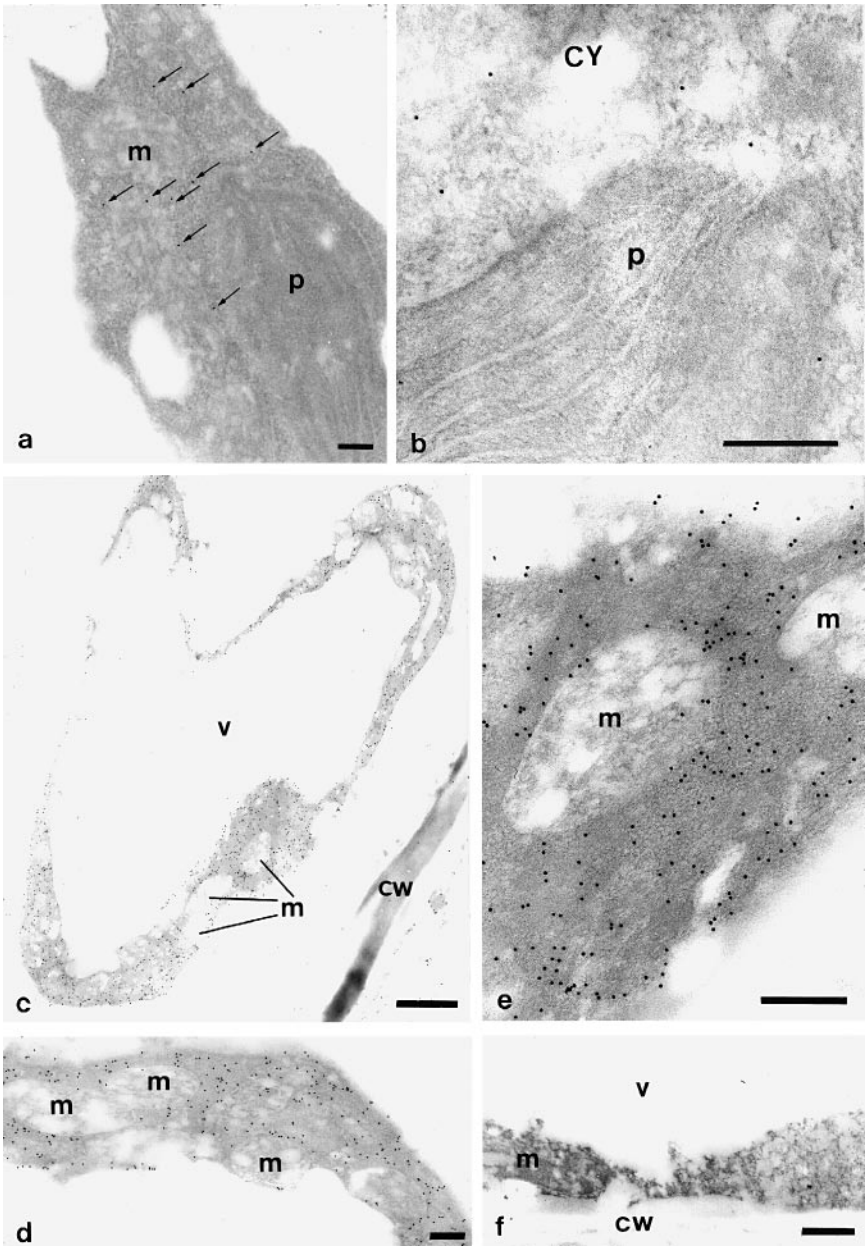
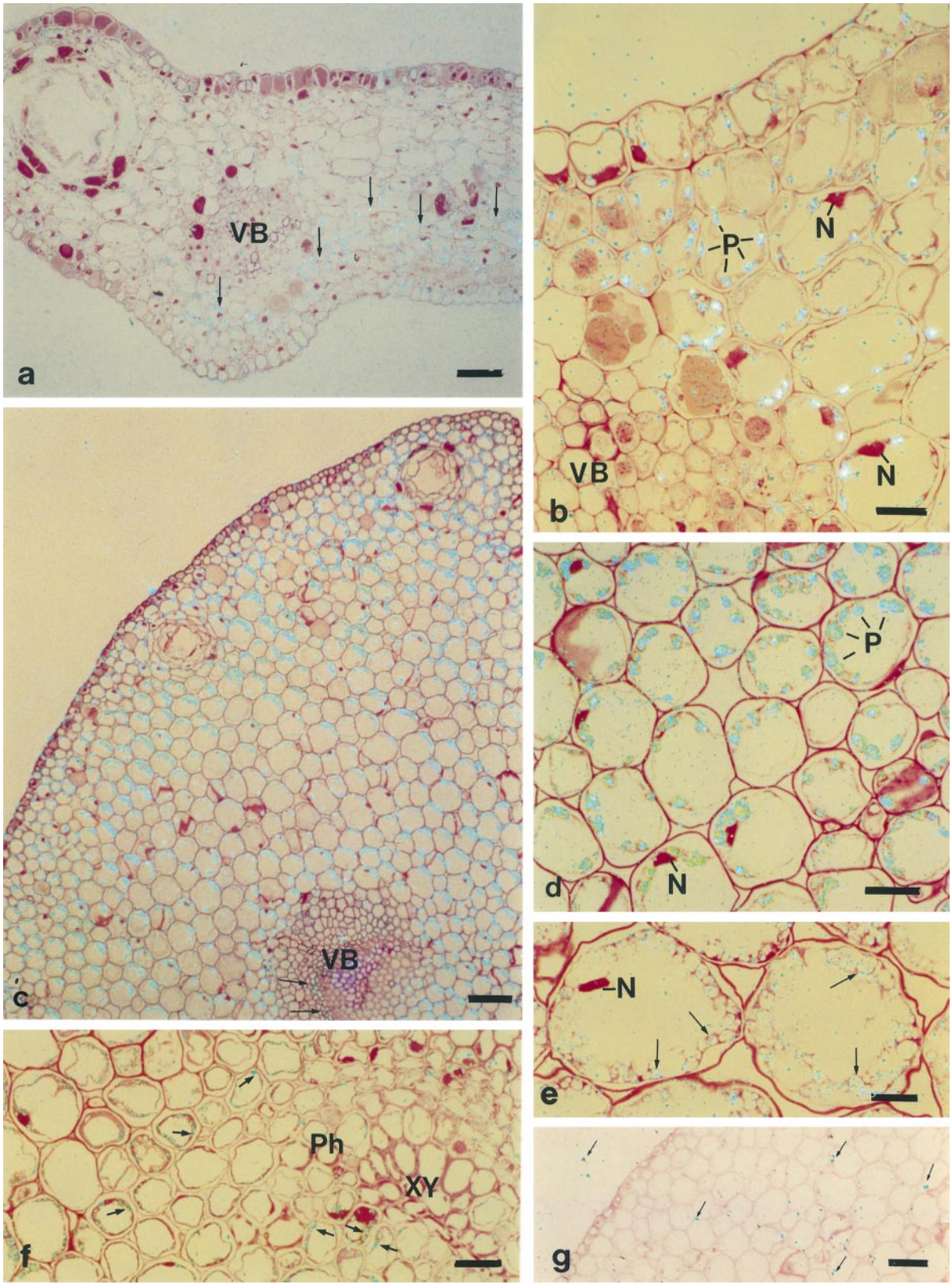


Figure 5. TEM immunolocalization of GS in pine seedlings.
(a, b) Immunolocalization of GS in the mesophyll cells. A light-labelling (arrows) was observable in the cytoplasm (CY) whereas chloroplast (p) and mitochondria (m) were absolutely unlabelled; bar = 0.1 μm.
(c–e) Immunolocalization of GS in the vascular bundles. A strong labelling was observed in the cytoplasm, whereas mitochondria (m), vacuoles (v) and cell walls (cw) remained unlabelled. (c) Overall view of a phloem cell; bar = 1 μm. (d, e) Higher magnifications confirming the cytosolic localization of GS in these cell types; bar = 0.1 μm.
(f) Control section incubated with normal rabbit serum; no labelling was observed. cw, cell wall; v, vacuole; m, mitochondria; bar = 1 μm.

ance of nitrogen and carbon reserves in the seed. In this respect, the relationships between storage protein abundance (nitrogen richness) and light- independent chlorophyll synthesis have still not been examined. In conifers, storage proteins are extremely rich in amino acids with a high nitrogen-carbon ratio, such as arginine (Allona *et al.*, 1994; Feirer, 1995). Moreover, in pine seedlings, when storage proteins are degraded following seed germination, arginine and the amides glutamine and asparagine have been reported as the most abundant amino acids (Feirer, 1995). The activity of the GS/GOGAT pathway is crucial in nitrogen recycling during seed–seedling transition, providing glutamine and glutamate for the biosynthesis of pigments and photosynthetic proteins involved in autotrophic plant growth. In dark-grown conifer seedlings the expression of GS and GOGAT genes is required to provide the essential amino donors for chloroplast biogenesis. For instance, glutamate production inside the plastid is a necessary step for the biosynthesis of 5-aminolevulinic acid, the precursor of the tetrapyrrole ring of chlorophyll (Von Wettstein *et al.*, 1995). The amount of chlorophyll in darkness is a limiting factor for pigment–protein assembly (photosystem complexes and LHC) in the thylakoid membranes (Shinohara *et al.*, 1992). Moreover, Mukai *et al.* (1991) have suggested that activation of nuclear *Lhcb* and *rbc* genes could be conditioned to the biosynthesis of chlorophyll in developing chloroplasts in darkness. Light-independent expression of GS and GOGAT genes has


VB
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been proved during early development of pine seedlings (Cánovas *et al.*, 1991; Cantón *et al.*, 1996; García-Gutiérrez *et al.*, 1995).

In this study we show that the same expression pattern of GS and Fd-GOGAT polypeptides was present in several coniferous gymnosperm species (Figure 4). In all cases examined, only one GS polypeptide similar in size to the cytosolic GS in maritime pine was immunodetected, suggesting that glutamine biosynthesis in conifers occurs in the cytosol. This molecular-based assumption was confirmed by immunolocalization of GS using light and electron microscopy (Figure 5). Histochemical and immunocytochemical examination of cross-sections from cotyledons demonstrated that GS protein was present in the cytoplasm of mesophyll cells and no immunoresponse associated to the plastids was observed (Figure 5a and b). Functional analysis of pea GS promoters in trangenic tobacco revealed that GS2 was mainly expressed in mesophyll cells containing chloroplasts, whereas GS1 expression was restricted to phloem cells (Edwards *et al.*, 1990). These findings strongly support the involvement of GS2 in the assimilation of ammonium derived from photorespiration and nitrate reduction. In contrast, results shown in this paper indicate an important physiological role for cytosolic GS in conifer photosynthetic metabolism, as suggested previously by other lines of evidence (Cantón *et al.*, 1996). However, further work is needed to show whether or not conifer GS1 can assume the physiological role of GS2 in angiosperms. In this work, we also show the presence of cytosolic GS in phloem cells (Figure 5c–e) which agrees well with that found by others in angiosperms (Carvalho *et al.*, 1992; Pereira *et al.*, 1992). The physiological role of GS in this cell type has been proposed to be the biosynthesis of glutamine for nitrogen translocation to other parts of the plant. Thus, the presence of pine GS1 in mesophyll and phloem cells suggests different metabolic

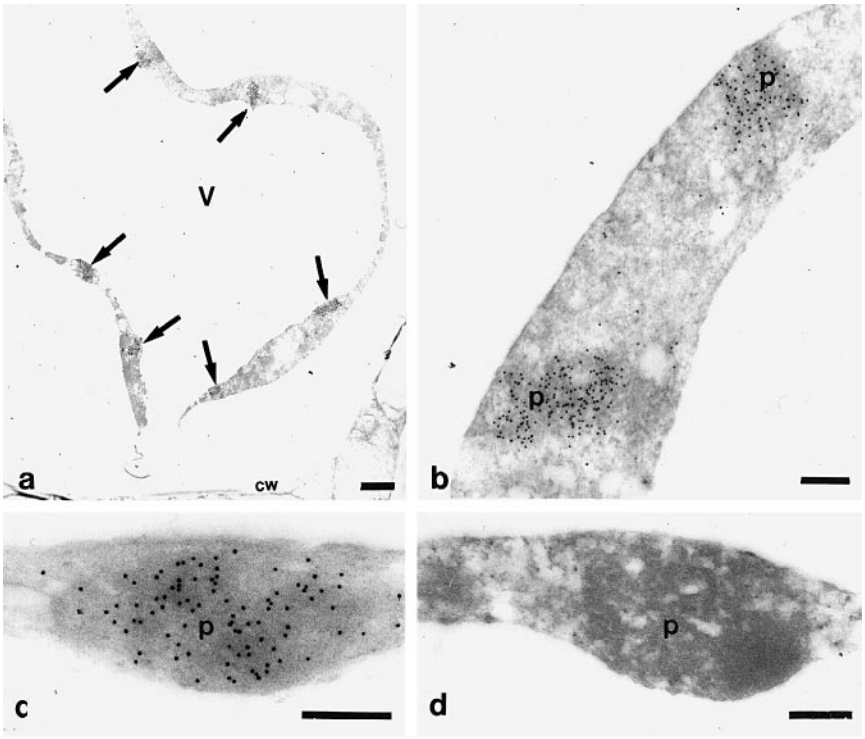

Figure 7. Immunolocalization of GS at electron microscopy level. A strong labelling was observed on the lightly contrasted proplastids (p) seen in the phloem cells, whereas cytoplasm and cell walls (cw) remained unsignificantly labelled.
(a) View of a phloem cell. Arrows indicate region of high GS density; bar = 1 μm. V, vacuole.
(b, c) Higher magnifications indicating the plastid (p) localization of GS in these cell types; bar = 0.1 μm.
(d) Control sections incubated with normal rabbit serum, no labelling was observed. P, plastid; bar = 0.1 μm.

Figure 6. Immunolocalization of GS in ginkgo seedlings.
Histological immunolocalization by immunogold labelling followed by silver enhancing. Sections 2 mm thick were cut out from cotyledons and leaflets, immunolabelled, stained by fuschin and examined under bright field and epipolarized light microscopy.
(a, b) First leaves of ginkgo seedlings. A strong labelling was observed in the numerous plastids of mesophyll cells (arrows). VB, vascular bundle; P, plastid; N, nuclei; (a) bar = 200 μm. (b) bar = 50 μm.
(c–f) Cotyledons of ginkgo seedlings.
(c, d) Strong labelling in plastids of parenchyma cells located in the proximal (near the seedling axis) zone of the cotyledons, a sparse labelling was also detected in some cells interspersed in the phloem region of vascular bundles (thin arrows). VB, vascular bundle; N, nuclei; P, plastid; (c) bar = 200 μm. (d) bar = 50 μm.
(e) Weak labelling in the parenchyma cells located in the distal (enclosed in the seed coat) zone of the cotyledons. N, nuclei; bar = 30 μm.
(f) Higher magnification of a vascular bundle in a proximal zone of the cotyledons. A sparse labelling (arrows) was observed in the phloem cells. Ph, phloem; XY, xylem; bar = 50 μm.
(g) Control sections incubated with normal rabbit serum. Only a weak and very sparse back-ground labelling was observed (arrows); bar = 200 μm.

roles for these cytosolic enzymes in developing trees. However, it remains unclear whether or not these enzymes are the products of the same or separate genes.

It is worthwhile noting that localization of GS in the cytosol of pine cells implies glutamine transport through the chloroplast membrane because Fd-GOGAT is located in the chloroplast stroma, as occurs in other photosynthetic organisms, and therefore glutamate synthesis is confined to the plastid (García-Gutiérrez *et al.*, 1995). A glutamine translocator, which is also responsible for glutamate export to the cytosol, has been described in spinach leaves (Yu and Woo, 1988), but its operativity in pine cells have yet to be demonstrated.

Although we were unable to detect expression of the *GS2* gene in conifer seedlings at cellular, isoenzyme, polypeptide and mRNA levels (Cánovas *et al.*, 1991; Cantón *et al.*, 1993; this work), a phylogenetic analysis carried out with pine GS and angiosperm GS sequences suggested that GS1/GS2 divergence occurred before angiosperm/gymnosperm separation and therefore GS2 gene should be present in gymnosperms (Muñoz-Chapuli *et al.*, unpublished results). The detection of a GS2 polypeptide in *G. biloba* leaves (Figure 3b) is consistent with this finding. However, these results do not exclude the possibility that conifer GS2 is expressed at low levels in the whole seedling or that its expression is restricted to specialized cells and can vary markedly in other physiological and/or developmental situations. In fact, the variability in the proportions of GS isoforms in photosynthetic tissues is already known (MacNally *et al.*, 1983). Thus, in Solanaceae plants, such as tobacco, potato or tomato, GS2 is the predominant form in the leaves and GS1 only is expressed in vascular tissue (Cánovas *et al.*, 1984; Carvalho *et al.*, 1992; Pereira *et al.*, 1992). Senescence and pathogen or chemical stresses alter the normal expression pattern, GS2 is repressed and GS1 is induced (Kawakami and Watanabe, 1988; Pérez-García *et al.*, 1995).

From this and other studies, it can be inferred that chloroplast development and expression of genes encoding chloroplast proteins in gymnosperms are far-less regulated by light than in angiosperms. This ability is present, to a greater or lessor extent, in many but not all the gymnosperms. An exception is *G. biloba*, a living fossil that presents light-dependent chloroplast development and chlorophyll synthesis (Figure 1; Chinn and Silverthorne, 1993, 1995; Rascio *et al.*, 1984). The accumulation of LHCIIb and Rubisco polypeptides during seed–seedling transition in illuminated plants (Figure 2) and their absence in the dark, suggest that the expression of photosynthesis genes is light-regulated in *G. biloba*. This agrees with a recent report by Chinn *et al.* (1995) in which light-regulated expression of individual *Lhcb* mRNAs was demonstrated. The above findings indicate that primary development in *G. biloba*, as occurs in angiosperms, can follow two alternative ways depending on whether light is present or not. In *Arabidopsis*, a number of genes involved in this pattern of development has been identified in the last few years (Chory, 1993; Deng, 1994). The role of these genes is repressing photomorphogenesis in the absence of light, because mutations that inactivate them lead to the pattern of development observed in the light (Wei *et al.*, 1994). It has been suggested that photomorphogenesis is the default pathway of early developmental process in plants, while etiolation appeared later in evolutionary lineages, as an adaptation of land plants to darkened habitats (Wei *et al.*, 1994). According to this hypothesis, conifers would present an ancestral pathway of seedling development while *G. biloba* would have acquired a dark-adaptative developmental programme.

It is worth noting that in *G. biloba* seedlings, as occurs with the products of photosynthesis genes, the expression pattern of nitrogen-assimilating enzymes is similar to that found in angiosperms (Becker *et al.*, 1992; Edwards and Coruzzi, 1989; Sakakibara *et al.*, 1991). Thus, in seeds and etiolated plants, no Fd-GOGAT and GS2 were detectable and GS1 is the most abundant molecular form, probably involved in the generation of glutamine for transport to developing tissues. After greening, GS2 and Fd-GOGAT accumulated in close correlation with a light- regulated pattern of development (Figure 3). Furthermore, the specific localization of GS was confirmed by immunocytochemical analysis of ginkgo green leaflets and cotyledons (Figure 6). As occurs in photosynthetic tissues of many angiosperms (Botella *et al.*, 1988; Carvalho *et al.*, 1992), the most abundant GS isoform (Figure 3b) is confined to the chloroplasts of mesophyll cells and proplastids (Figures 6 and 7). Taken together, the protein blot studies and the subcellular localization studies are consistent with the hypothesis that in this gymnosperm species, genes involved in nitrogen assimilation behave like their corresponding genes in angiosperms.

In conclusion, our results suggest that the distribution of glutamine biosynthesis in different cellular compartments may be associated with the etiolation response in the seedlings. Furthermore, differences in the expression of genes encoding nitrogen-assimilating enzymes in gymnosperms appear to be correlated with a differential response of plants to light. Further studies are needed to characterize genes involved in glutamine biosynthesis in these woody plants, and data derived from these studies will contribute to a better understanding of developmental and environmental control of gene expression in trees.

Experimental procedures

Plant material

Maritime pine (*P. pinaster* aiton) and *G. biloba* seeds were soaked for 3 days in sterile distilled water and then germinated in plastic

trays containing vermiculite, without any external addition of fertilizer. Germination and early growth of seedlings were performed in complete darkness or under white light (Sylvania F48T12/CW/VHO fluorescent lights, 200 mmol photons m^{-2} s^{-1}) at 22°C.

Chlorophyll determination

Chorophyll contents were estimated by the Arnon's method (1949)

Protein extraction and quantification

Conifer cotyledons and ginkgo leaflets were harvested at the corresponding developmental stages, frozen in liquid nitrogen and stored at –80°C until use. Tissue was ground with a mortar and pestle in liquid nitrogen and total proteins extracted in 10 mM Tris–glycine (pH 8.9), 5 mM 2-mercaptoethanol, 1 mM PMSF, 1% (w/v) SDS. The inclusion of SDS in the extraction buffer allowed the solubilization of total protein from the tissues, in this way preventing the underestimation of specific polypeptides. Protein concentration in the extracts was determined by the method of Zaman and Verwilghen (1979), using BSA as standard.

Gel electrophoresis and protein blot analysis

Proteins were analysed by denaturing electrophoresis in the presence of SDS using the discontinuous buffer system (Laemmli, 1970). After electrophoresis, separated polypeptides were either visualized by Coomassie blue staining or electrophoretically transferred to nitrocellulose membranes according to the method of Towbin *et al.* (1979). Immunolabelling was carried out essentially as described by Cánovas *et al.* (1991) using the antibodies raised against the following proteins: LSU, SSU, LHCIIb and Fd-GOGAT from *P. pinaster* (García-Gutiérrez *et al.*, 1993, 1995), recombinantly expressed GS1 from *P. sylvestris* (Cantón *et al.*, 1996), and GS2 from tobacco leaves (Hirel *et al.*, 1984). It has been demonstrated that GS antibodies cross-react with cytosolic and chloroplastic GS isoforms in different plant species.

Tissue fixation, dehydration and embedding

The explants were treated for immunolocalization of GS as described previously (Dubois *et al.*, 1994). Pine and ginkgo cotyledons were fixed in freshly prepared 1.5% (v/v) paraformaldehyde in phosphate buffer for 4 h at 4°C. Plant material was then dehydrated in a graded ethanol series [final concentration 80% (v/v) ethanol] then embedded in LR white resin (Polysciences, Warrington, USA). Polymerization was carried out in gelatin capsules at 50°C.

Immunolocalization

For immunoTEM study, ultrathin sections were mounted on 400-mesh nickel grids, allowed to dry at 37°C, and treated essentially as described previously (Dubois *et al.*, 1996). Sections were first incubated with 5% (v/v) normal goat serum in T1 buffer [0.05 M Tris–HCl buffer containing 2.5% (w/v) NaCl, 0.1% (w/v) BSA and 0.05% (v/v) Tween-20, pH 7.4] for 1 h at room temperature, then with anti-GS rabbit serum diluted 1:70 in T1 buffer for 6 h at room temperature. Sections were then washed five times with T1 buffer, twice with T2 buffer [0.02 M Tris–HCl buffer containing 2% (w/v) NaCl, 0.1% (w/v) BSA and 0.05% Tween-20, pH 8] and incubated with 10 nm colloidal gold–goat anti-rabbit immunoglobulin complex (Sigma, St Louis, MO) diluted 1:50 in T2 buffer for 2 h at room temperature. After several washes, grids were treated with 5% (w/v) uranyl acetate and observed with a Philips CM12 electron microscope at 80 kV.

For immunological studies with a light microscope, thin sections of 2 mm were floated on drops of sterile water on Biobond (British Biocell, Cardiff, UK)-coated slides and essentially the same procedure of labelling (described above) was performed, except that 1 nm instead of 10 nm colloidal gold–goat anti-rabbit immunoglobulin complex (British Biocell) was used. Labelling was silver enhanced as described by the supplier (British Biocell) and sections were back-stained with 1% (w/v) fuschine before miscroscopic observations under bright field plus epipolarized light. For both techniques, controls were run by omitting the primary antibody or by its substitution with normal rabbit serum.

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