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Molecular and enzymatic analysis of ammonium assimilation in woody plants

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Abstract

Ammonium is assimilated into amino acids through the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT) enzymes. This metabolic pathway is driven by energy, reducing power and requires the net supply of 2-oxoglutarate that can be provided by the reaction catalysed by isocitrate dehydrogenase (IDH). Most studies on the biochemistry and molecular biology of N-assimilating enzymes have been carried out on annual plant species and the available information on woody models is far more limited. This is in spite of their economic and ecological importance and the fact that nitrogen is a common limiting factor for tree growth. GS, GOGAT and IDH enzymes have been purified from several woody species and their kinetic and molecular properties determined. A number of cDNA clones have also been isolated and characterized. Although the enzymes are remarkably well conserved along the evolutionary scale, major differences have been found in their compartmentation within the cell between angiosperms and conifers, suggesting possible adaptations to specific functional roles. The analysis of the gene expression patterns in a variety of biological situations such as changes in N nutrition, development, biotic or abiotic stresses and senescence, suggest that cytosolic GS plays a central and pivotal role in ammonium assimilation and metabolism in woody plants. The modification of N assimilation efficiency has been recently approached in trees by overexpression of a cytosolic pine GS in poplar. The results obtained, suggest that an increase in cytosolic GS might lead to a global effect on the synthesis of nitrogenous compounds in the leaves, with enhanced vegetative growth of transgenic trees. All these data suggest that manipulation of cytosolic GS may have consequences for plant growth and biomass production.

Key words: Gymnosperms, nitrogen assimilation, nitrogen recycling, transgenic trees, woody angiosperms.

Introduction

Ammonium and nitrate ions are present in the soils of boreal forests although they are usually available in low abundance (Martin and Lorillou, 1997). As a result, trees have developed mycorrizhal associations to increase the efficiency of nitrogen assimilation (Martin and Lorillou, 1997). In fact, N availability is a common limiting factor for tree growth and development. In conifer forests, low soil pH, high residual content of lignin and other secondary plant products in the soil limit nitrification. Consequently, ammonium is the predominant source of N for tree nutrition (Berg, 1986). It is well documented that conifers, unlike herbaceous plants, have a preference for ammonium over nitrate as the N source (Bedell *et al.*, 1999). Plant metabolic activities also release ammonium through processes such as protein/nucleic acid breakdown and subsequent amino acid/nucleotide catabolism, photorespiration and, the biosynthesis of phenylpropanoids. A product of the phenylpropanoid pathway is lignin, the second most abundant organic compound in the biosphere after cellulose. Lignin constitutes a major fraction in wood and therefore ammonium released in lignin biosynthesis is of quantitative importance during the life of a tree. Primary nitrogen assimilation from the soil and reassimilation into biomolecules are crucial processes

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for plant N economy, however, in many woody plants N assimilated from soil may not be utilized immediately, but can be stored for use during the next growing season (Coleman *et al*., 1994). Thus, for woody plants, the study of ammonium metabolism is of particular significance for the understanding of fundamental tree biology.

The incorporation of ammonium into the pool of N-containing molecules is first catalysed by the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Fig. 1). In this metabolic pathway GS (EC 6.3.1.2) catalyses the amidation of glutamate to generate glutamine at the expense of ATP hydrolysis. The second enzyme, GOGAT (EC 1.4.7.1; 1.4.1.14), is responsible for 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 and Lea, 1980). This N assimilatory pathway is driven by energy and reducing power derived from photosynthesis or from the catabolism of protein and carbon reserves. Glutamate and glutamine are the N donors for the biosynthesis of major N compounds in plants including other amino acids, nucleic acids bases, polyamines, and chlorophylls. For instance, N can be channelled to the biosynthesis of aspartate and asparagine catalysed by aspartate amino transferase (AspAT) (EC 2.6.1.1) and asparagine synthetase (AS) (EC 6.3.5.4), respectively (Fig. 1). Recent molecular studies using defective mutants supported the *in vivo* roles of AspAT in N metabolism (Schultz *et al*., 1998). In most plants the amides glutamine or asparagine are important vehicles for N transport between source and sink tissues.

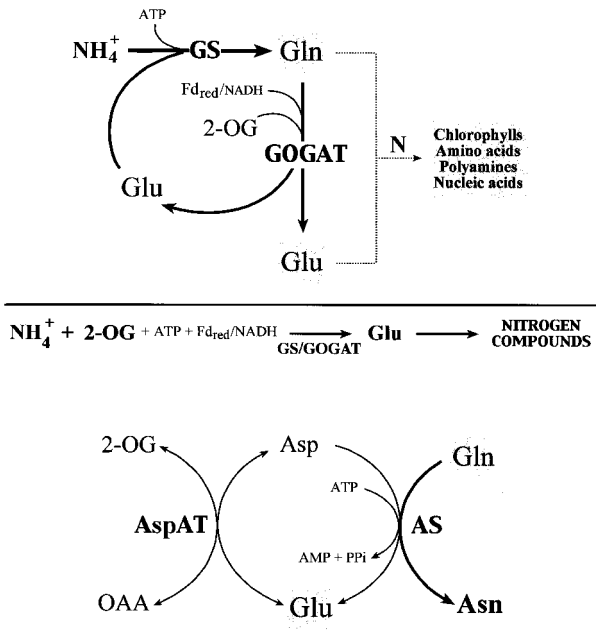

Fig. 1. The glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. The amino acids glutamine and glutamate are the main N donors for the biosynthesis of nitrogen compounds. Net biosynthesis requires the supply of energy, reducing power and 2-oxoglutarate as the carbon skeleton. In the lower part of the figure is shown the biosynthesis of aspartate and asparagine as an example of glutamine and glutamate utilization.

The stoichiometry of the GS/GOGAT cycle clearly shows that the incorporation of ammonium for the net synthesis of glutamate requires the supply of 2-oxoglutarate (Fig. 1). This oxoacid is provided by the reaction catalysed by NAD^+- or $NADP^+$-dependent isocitrate dehydrogenase (IDH) (EC 1.1.1.41; 1.1.1.42).

In addition to GS, GOGAT and IDH, another enzyme potentially involved in ammonium metabolism is NADH-glutamate dehydrogenase (GDH) (EC 1.4.1.2). GDH can catalyse the reductive amination of 2-oxoglutarate and the reverse catabolic reaction of oxidative deamination of glutamate. GDH is located in the mitochondrial matrix where it is mainly responsible for glutamate catabolism under carbon and N-limiting conditions (Stewart *et al*., 1995; Aubert *et al*., 2001). The enzyme may also function in the direction of glutamate biosynthesis when ammonium is highly abundant (Melo-Oliveira *et al*., 1996). The controversial role of GDH in the assimilation of ammonium has been discussed (Miflin and Habash, 2002).

The biochemistry and molecular biology of N-assimilating enzymes in plants has been extensively studied and recent comprehensive reviews are available (Lam *et al*., 1996; Temple *et al*., 1998; Ireland and Lea, 1999; Gálvez *et al*., 1999). However, most studies have focused on annual plants, thus available information concerning nitrogen assimilation in woody models is much more limited, particularly at the molecular level (Cánovas *et al*., 1998). This is in spite of the economic and ecological importance of these plants. In this paper the current status of research on ammonium assimilation and metabolism in woody plants is reviewed. In the first section the molecular characterization of the pathway in different woody plants is presented as well as subcellular localization in angiosperms and gymnosperms. In the second section, gene expression analysis and distribution in different cellular types is discussed with regard to the functional roles of the N-assimilating enzymes. Initial studies on genetic manipulation addressed to increase growth rate in trees are also presented. The final section includes future prospects for N assimilation studies in woody plants and potential applications.

Characteristics of GS, GOGAT and IDH

Annual plants

The metabolic requirement for GS activity in ammonium assimilation in plants is fulfilled by GS isoforms expressed in specific organs and at specific developmental stages

(see Cren and Hirel, 1999; Ireland and Lea, 1999, for recent reviews). With regard to the subcellular localization, two different classes of GS have been reported in angiosperms: GS1 in the cytosol and GS2 in the chloroplasts. In the leaves of many plants GS2 is the predominant isoform where it is located in mesophyll photosynthetic cells, whereas GS1 is generally less abundant in photosynthetic tissues. The exceptions include C_4 plants, which exhibit low photorespiratory activity, and C_3 plants under certain physiological conditions, such as senescence and during response to biotic or abiotic stresses (Cren and Hirel, 1999). GS1 is the predominant isoform in roots and other non-photosynthetic tissues (Ireland and Lea, 1999), where it is located in cells of the vascular bundles (Edwards *et al*., 1990; Dubois *et al*., 1996). However, GS2 has also been identified in roots of legume plants grown in the presence of nitrate (Woodall and Forde, 1996), which could represent a physiological adaptation to nitrate-rich soils. In addition, nodule-specific GS1 is involved in post-N-fixation ammonium assimilation (Ireland and Lea, 1999). The complete amino acid sequence of cytosolic and plastidic GS subunits has been deduced from the corresponding cDNAs isolated from many annual plant species including major crops (Cren and Hirel, 1999; Ireland and Lea, 1999). In most plants examined, chloroplastic GS (GS2) is encoded by a single nuclear gene and its functional role is the assimilation of ammonium derived from the reduction of nitrate and from photorespiration (Lam *et al*., 1996). Cytosolic GS (GS1) exists as a variable number of isoforms which are encoded by a small gene family whose members are differentially expressed during development, or in response to external stimuli (Ireland and Lea, 1999). Unlike GS2, the physiological roles of individual genes encoding cytosolic isoforms (GS1) are unclear, but recent reports indicate that GS1 may be involved in a variety of processes, including the primary assimilation of ammonium from the soil (Sakakibara *et al*., 1996), N recycling and translocation between source and sink tissues in the plant, and reassimilation of N mobilized during senescence (Cren and Hirel, 1999).

In plants, glutamate synthase (GOGAT) occurs as two distinct molecular forms which differ with respect to the source of reductant for enzyme catalysis: NADH-GOGAT and ferredoxin (Fd)-GOGAT. Both enzymes display different physico-chemical, immunological and regulatory properties and are encoded by separate genes (Temple *et al*., 1998; Ireland and Lea, 1999). Fd-GOGAT is an iron–sulphur flavoprotein, plastid-located and represents the predominant molecular form in photosynthetic tissues although its presence has also been reported in roots and nodules (Lam *et al*., 1996; Temple *et al*., 1998). In most plants analysed, Fd-GOGAT is encoded by a single gene, however, in *Arabidopsis* two genes have been characterized (Coschigano *et al*., 1998): *GLU1* is exclusively expressed in the leaf and is light-regulated, whereas *GLU2* is expressed in leaves and roots and is not regulated by light. The expression pattern of the genes and the physiological characterization of defective mutants support a role of GS2 and Fd-GOGAT in the assimilation of ammonium derived from the reduction of nitrate and from photorespiration (Coschigano *et al*., 1998; Ireland and Lea, 1999). NADH-GOGAT, also an iron–suphur flavoprotein, is present at low abundance in leaves, but it is more abundant in non-photosynthetic tissues such as roots and nodules, where it is located in non-chlorophyllous plastids (Temple *et al*., 1998). The structure of the alfalfa gene encoding NADH-GOGAT has been reported (Temple *et al*., 1998) and its expression is restricted to root nodules where it plays a significant role in the assimilation of ammonium derived from symbiotic N_2 fixation (Trepp *et al*., 1999). The localization of GS1 and NADH-GOGAT proteins in the root vascular bundles of rice supports the possibility of a co-ordinated function in the assimilation of ammonium in roots (Ishiyama *et al*., 1998).

Plant IDHs differ in the pyridine nucleotide they use as co-substrate and also in their localization in the cell. The mitochondrial NAD^+-dependent IDH is the enzyme involved in the Krebs cycle, while $NADP^+$-dependent IDH exists in different subcellular compartments, including the cytosol, chloroplasts, peroxysomes, and mitochondria. Cytosolic $NADP^+$-IDH is the most active IDH enzyme in both angiosperms and gymnosperms, and it has been suggested to be the main enzyme involved in providing carbon skeletons for N assimilation when large amounts of 2-oxoglutarate are required (Chen and Gadal, 1990). Therefore, the supply of 2-oxoglutarate through a cytosolic pathway involving aconitase and $NADP^+$-IDH represents an alternative route to the Krebs cycle enzymes, for providing carbon skeletons for ammonium assimilation and the biosynthesis of glutamate and glutamine (Chen and Gadal, 1990; Gálvez *et al*., 1999) (Fig. 2). However, and regardless of its origin, mitochondrial or cytosolic, 2-oxoglutarate must be transported into the chloroplast for glutamate biosynthesis. Thus, it is possible that transport of metabolites across the membranes could be a limiting step in ammonium assimilation rather than 2-oxoglutarate biosynthesis (Gálvez *et al*., 1999). The biosynthesis of aspartate and asparagine is also dependent of carbon provision in the form of oxalacetate (Fig. 2). Therefore, the supply of carbon skeletons for amino acid biosynthesis requires the flow of carbon metabolites into the Krebs cycle to avoid depletion of intermediates. This requirement must be met by increasing carbon flux via glycolysis and phosphoenolpyruvate carboxylation. The metabolism of these intermediary compounds tightly links nitrogen assimilation and carbon metabolism.

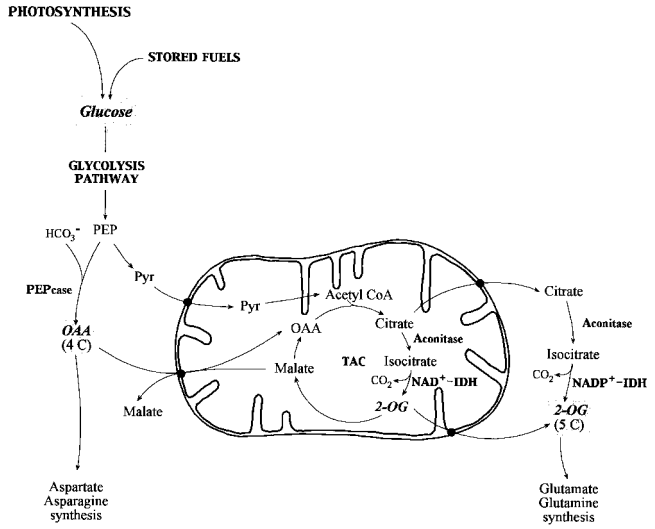

Fig. 2. Carbon flux and the central role of plant mitochondria in the provision of carbon skeletons for ammonium assimilation and amino acid biosynthesis. NAD^+-IDH, NAD^+-dependent isocitrate dehydrogenase; $NADP^+$-IDH, $NADP^+$-dependent isocitrate dehydrogenase; OAA, oxalacetate; PEP, phosphoenolpyruvate; PEPcase, phosphoenolpyruvate carboxylase; Pyr, pyruvate; 2-OG, 2-oxoglutarate; TAC, Krebs cycle.

Woody angiosperms

GS has been characterized from angiosperm woody species including apple (*Malus domestica*) (Titus and Kang, 1982), avocado (*Persea americana*) (Loulakakis *et al*., 1994), grapevine (*Vitis vinifera*) (Loulakakis and Roubelakis-Angelakis, 1996), rubber tree (*Hevea brasilensis*) (Pujade-Renaud *et al*., 1997), black walnut (*Juglans nigra*) (Simonson and Twigg, 1999), hybrid poplar (*Populus tremula*×*P. alba*) (Gallardo *et al*., 1999), and the root nodules of alder (*Alnus glutinosa*) in symbiosis with *Frankia* (Hirel *et al*., 1982; Guan *et al*., 1996) (Table 1). Available biochemical data and the molecular characterization of GS cDNA clones indicate the existence of chloroplastic and cytosolic isoenzymes possibly encoded by a small gene family as reported for herbaceous angiosperms.

Two complementary DNA (cDNA) clones encoding Fd-dependent glutamate synthase have been characterized from grapevine and the deduced amino acid sequence of the enzyme is significantly similar to the respective sequences of other plant Fd-GOGATs (Loulakakis and Roubelakis-Angelakis, 1997). The existence of two Fd-GOGAT genes in the grapevine genome was inferred by Southern blot analysis using the isolated cDNAs as molecular probes (Loulakakis and Roubelakis-Angelakis,

Table 1. *Molecular characterization of genes/proteins involved in ammonium assimilation in woody plants*

Species	Name	Subcellular location	Subunit size (kDa)	cDNA accession no.	Reference
Angiosperms					
Vitis vinifera	GS2	Chloroplast	43–44		Loulakakis *et al*., 1996
	GS1:1	Cytosol	39	X94320	Loulakakis *et al*., 1996
	GS1:2	Cytosol	39	X94321	Loulakakis *et al*., 1996
	Fd-GOGAT	Chloroplast		X98542	Loulakakis and Roubelakis-Angelakis, 1997
				X98541	Loulakakis and Roubelakis-Angelakis, 1997
Malus domestica	GS1?	Cytosol?			Titus and Kang, 1982
	NADH-GOGAT				Titus and Kang, 1982
Persea americana	GS2	Chloroplast	44		Loulakakis *et al*., 1994
	GS1	Cytosol	41		Loulakakis *et al*., 1994
Hevea brasilensis	GS1	Cytosol	39–40	AF003197	Pujade-Renaud *et al*., 1997
Juglans nigra	GS2	Chloroplast		AF169795	Simonson and Twigg, 1999
Alnus glutinosa	GS1	Cytosol	43	Y08680	Hirel *et al*., 1982; Guan *et al*., 1996
Eucalyptus globulus	$NADP^+$-IDH	Cytosol	42	U80912	Boiffin *et al*., 1998
Populus tremula×*P. alba*	GS2	Chloroplast	45		Gallardo *et al*., 1999
	GS1	Cytosol	40		Gallardo *et al*., 1999
Gymnosperms					
Ginkgo biloba	GS2	Chloroplast	45		Garcia-Gutiérrez *et al*., 1998
	GS1	Cytosol	41		Garcia-Gutiérrez *et al*., 1998
	Fd-GOGAT	Chloroplast	168		Garcia-Gutiérrez *et al*., 1998
Pinus sylvestris	GS1a	Cytosol	41	X69822	Cantón *et al*., 1993; Elmlinger *et al*., 1994
	GS1b	Cytosol	41	AJ005119	Avila *et al*., 1998
	Fd-GOGAT	Chloroplast	168	X79933	Garcia-Gutiérrez *et al*., 1995
	$NADP^+$-IDH	Cytosol	46		Palomo *et al*., 1998
Pinus pinaster	GS1	Cytosol	43		Cánovas *et al*., 1991
	Fd-GOGAT	Chloroplast	168		Garcia-Gutiérrez *et al*., 1995
	PII[a]	Cytosol?	26		Cánovas, Avila, Cantón, unpublished result
Pinus banksiana	GS	Cytosol?	40–44		Vézina and Margolis, 1990
Pseudotsuga menziesii	GS	Cytosol	56–64		Bedell *et al*., 1995
Picea abies	NAD(P)-IDH	Mitochondria			Cornu *et al*., 1996

[a]Data derived from characterization of the cDNA.

1997). It is unknown whether or not they are equivalent to the two Fd-GOGAT genes in *Arabidopsis*. NADH-GOGAT activity was extracted from the bark tissue of the apple, partially purified and its kinetic parameters determined although the enzyme has not been characterized at the molecular level (Titus and Kang, 1982).

Recently, a cDNA clone encoding $NADP^+$-dependent IDH from an eucalypt (*Eucalyptus globulus*) has been reported (Boiffin *et al*., 1998). The deduced protein lacks an amino terminal transit peptide and shows the highest similarity to plant cytosolic IDH. The enzyme was preferentially localized in the epidermis and vascular elements of the root, where its relative abundance was enhanced by ectomycorrhizal colonization. These findings suggest a role of the enzyme in providing carbon skeletons for the assimilation of N translocated from the fungal partner.

Gymnosperms

The enzymes characterized in gymnosperms are summarized in Table 1. GS has been purified from needles and roots of jack pine (*Pinus banksiana*) and from roots of Douglas fir (*Pseudotsuga menziesii*), and its physicochemical and kinetic properties have been determined (Vézina and Margolis, 1990; Bedell *et al*., 1995). With regard to the subcelullar localization of the enzyme, in the photosynthetic tissues of pine seedlings and other conifers, only cytosolic isoforms of GS (GS1) have been identified (Cánovas *et al*., 1991; Cantón *et al*., 1993, 1996; Avila *et al*., 1998). The chloroplastic isoform (GS2) has not yet been detected by using a number of different molecular approaches including separation of isoforms by ion-exchange chromatography, Western blot analysis, and screening and random sequencing of clones in pine cDNA libraries. The localization of the GS protein exclusively in the cytosol of photosynthetic and non-photosynthetic pine cells was demonstrated by immunocytolocalization (García-Gutiérrez *et al*., 1998). These data indicate that glutamine biosynthesis occurs in the cytosol of pine cells, not only during the initial stages of pine development, but also in pine trees (Avila *et al*., 2000). However, the presence of GS2 has been reported in the leaves of ginkgo (*Ginkgo biloba*), a non-coniferous gymnosperm (García-Gutiérrez *et al*., 1998).

In cotyledons of Scots pine (*Pinus sylvestris*) two GS isoforms, GS1a and GS1b, have been reported (Cantón *et al*., 1993; Avila *et al*., 1998) which exhibit differential chromatographic behaviours and are composed of subunits of a similar size, but different charge. GS1a is predominant in pine cotyledons (Cantón *et al*., 1993), while GS1b is a minor form whose relative amount increases following phosphinothricin (PPT) treatment (Avila *et al*., 1998). PPT is a structural analogue of glutamate that behaves as a powerful and irreversible inhibitor of GS activity. Full-length cDNA clones encoding these two cytosolic isoforms have been isolated and the deduced amino acid sequences analysed (Cantón *et al*., 1993; Elmlinger *et al*., 1994; Avila *et al*., 2000). GS1a and GS1b polypeptides lack N-terminal presequences confirming their assembly into cytosolic oligomeric enzymes. This inference is supported by comparative analysis with the GS amino acid sequences from angiosperms. Interestingly, GS1a contains amino acid residues characteristic of only the GS2 polypeptide, including cysteine residues usually absent in the cytosolic polypeptides. By using site directed mutagenesis it was determined that Cys249 is involved in enzyme stability (A García-Gutiérrez, FM Cánovas, unpublished results). *GS1a* and *GS1b* genes are closely linked in the genome, supporting a proposed origin of *GS* isogenes by adjacent gene duplication. *GS1b* is more similar to cytosolic GS from angiosperms than to *GS1a*. The study of phylogenetic relationships between plant *GS* genes suggest the possibility that angiosperms might have received an ancestral *GS1* gene more closely related to the *Pinus GS1b*, whereas *GS1a* is unique to gymnosperms (Avila *et al*., 2000). An extension of this study, including analysis of GS sequences from plants, algae and prokaryotes, showed that *GS2* possibly evolved from a duplicated *GS1* gene long before the gymnosperms/angiosperms divergence (Fig. 3). Although expression of a *GS2* gene has not been detected in conifers, these findings are consistent with the presence of a GS2 enzyme in the gymnosperm *G. biloba* (García-Gutiérrez *et al*., 1998).

Fd-GOGAT has also been characterized in gymnosperms. As found in angiosperms, Fd-GOGAT is a single polypeptide of about 168 kDa in pine (*P. pinaster* and *P. sylvestris*), pinsapo fir (*Abies pinsapo*), larch (*Larix decidua*) and ginkgo (García-Gutiérrez *et al*., 1995, 1998). Several cDNA clones encoding the C-terminal region of pine Fd-GOGAT were isolated from an expression library from *P. sylvestris* seedlings by screening with affinity-purified antibodies against the enzyme (García-Gutiérrez *et al*., 1995). The pine polypeptide contains three conserved cysteine residues involved in an iron–sulphur cluster and a flavin mononucleotide binding site consisting of two putative domains conserved in other plant Fd-GOGATs (Temple *et al*., 1998). Comparison of the pine amino acid sequence with monocot and dicot sequences available in the data bank, revealed that the primary structure of Fd-GOGAT is remarkably well conserved in angiosperms and gymnosperms, in spite of the evolutionary distance (García-Gutiérrez *et al*., 1995). Results derived from Southern blot analysis are consistent with the existence of more than one gene for Fd-GOGAT in the pine genome (García-Gutiérrez *et al*., 1996). NADH-GOGAT activity has also been detected in young pine seedlings (Elmlinger and Mohr, 1991;

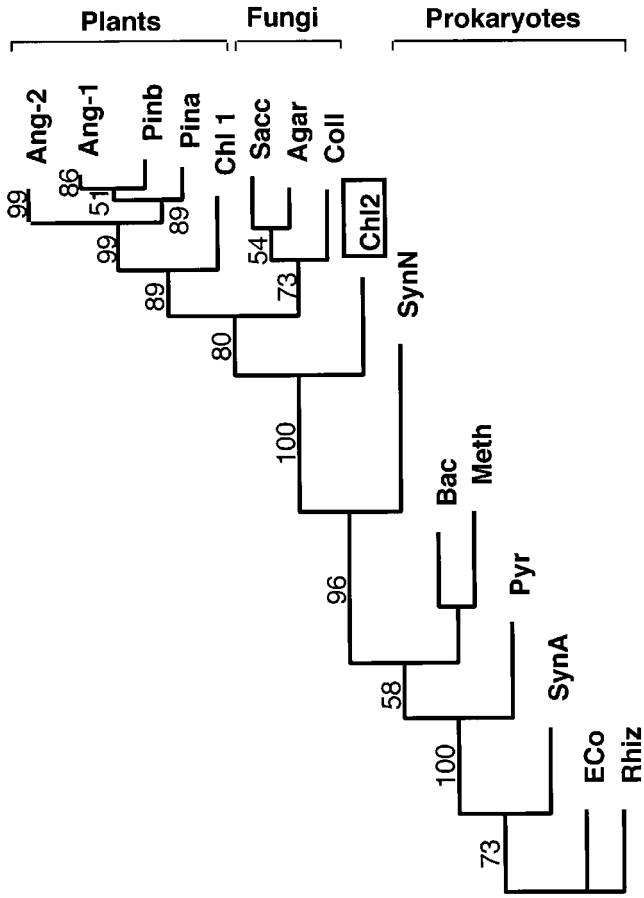

Fig. 3. Phylogenetic analysis of GS sequences (redrawn from Avila *et al.*, 2000). To examine explicitly the phylogenetic relationships between the GS sequences a cladistic parsimony analysis was performed. The length of the branches is proportional to the number of changes along the branches. Note that *Chlamydomonas* GS2 sequence (boxed) is unrelated to the angiosperm GS2 gene being the sister of all plant and fungal sequences. Ang-1, angiosperm GS1; Ang-2, angiosperm GS2; Pina, *Pinus sylvestris* GS1a; Pinb, *Pinus sylvestris* GS1b. Algae: Dun, *Dunaliella salina*; Chl1 and Chl2, *Chlamydomonas reinhardtii*. Fungi: Agar, *Agaricus bisporus*; Col, *Colletotrichum gloeosporoides*; Sacc, *Saccharomyces cerevisiae*. Prokaryotes: Bac, *Bacillus subtilis*; Eco, *Escherichia coli*; Meth, *Methanococcus maripaludis*; Pyr, *Pyrococcus furiosus*; Rhiz, *Rhizobium leguminosarum*; SynA and SynN, *Synechocystis* sp.

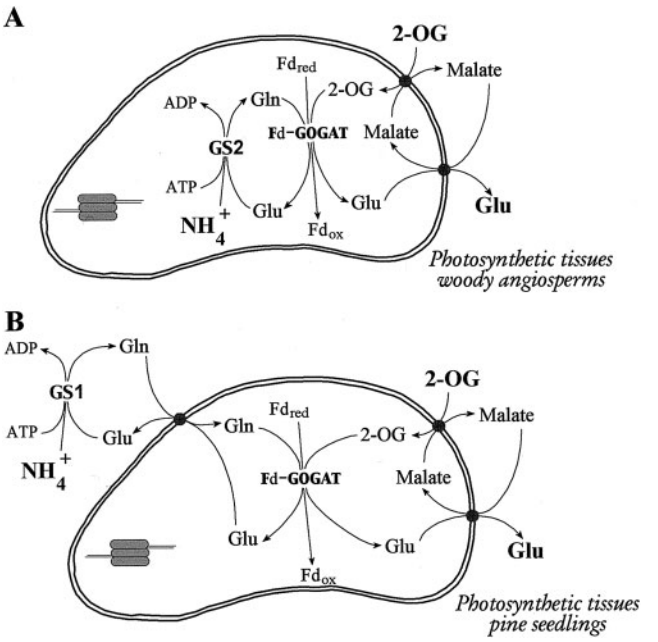

Fig. 4. Compartmentation of the GS/GOGAT cycle in the photosynthetic tissues of woody plants. (A) In woody angiosperms, ammonium is assimilated inside the chloroplast by GS2/Fd-GOGAT cycle and therefore glutamine and glutamate are generated in the same subcellular compartment. (B) In pine and other conifers, ammonium is assimilated in the cytosol and therefore glutamine and glutamate biosynthesis occur in separate compartments. The separation implies the translocation of glutamine from the cytosol to the chloroplast for glutamate synthesis.

García-Gutiérrez *et al.*, 1995), but no molecular studies of the enzyme have yet been performed.

NADP$^+$-IDH has been purified and characterized from Scots pine (*P. sylvestris*). Only one form of cytosolic localization was detected in green cotyledons with molecular and kinetic properties similar to those described for NADP$^+$-IDHs in angiosperms (Palomo *et al.*, 1998). These data suggest that the enzyme is well conserved in plants and could play similar physiological roles in angiosperms and gymnosperms. Expression studies in different pine tissues during early development, suggest that in addition to providing 2-oxoglutarate for glutamate biosynthesis, NADP$^+$-IDH may have other, as yet unknown, biological roles. NAD$^+$-IDH and NADP$^+$-IDH activities have also been studied in mitochondria purified from Norway spruce (*Picea abies*) seedlings (Cornu *et al.*, 1996). Both enzymes were detected in the mitochondrial matrix fraction, but at different abundances, NAD$^+$-IDH activity was about 2-fold more abundant than NADP$^+$-IDH. Furthermore, kinetic differences in substrate affinities were observed. No further characterization at the molecular level has yet been undertaken.

The characterization of GS, GOGAT and IDH in angiosperm woody plants indicates that these enzymes may play similar physiological roles to those found in annual herbaceous plants. Thus in photosynthetic tissues, glutamine and glutamate synthesis is catalysed by the GS2/Fd-GOGAT cycle located within the chloroplast (Fig. 4A), although 2-oxoglutarate should be provided by cytosolic or mitochondrial IDHs. In the seedlings of the gymnosperm *g. biloba*, the subcellular localization of GS and GOGAT enzymes is similar to that found in angiosperms (Fig. 4A). However, in conifers, glutamine biosynthesis occurs in the cytosol and Fd-dependent glutamate synthase is a soluble enzyme located in the chloroplast stroma (García-Gutiérrez *et al.*, 1995) (Fig. 4B). The separation of glutamine and glutamate biosynthesis in different subcellular compartments implies

not only the compartmentation of the GS/GOGAT cycle but also implies that glutamine must be transported from the cytosol into the plastid for glutamate production. Recent studies indicate the existence of a translocator in the chloroplast membranes of *P. pinaster*, that may be responsible for the import of glutamine into the organelle in antiport with glutamate (MG Claros, FM Cánovas, unpublished data). It has been hypothesized that the distribution of glutamine biosynthesis in different cellular compartments may be associated with etiolation in seedlings (García-Gutiérrez *et al*., 1998). Chloroplast development in conifers is far less regulated by light than in ginkgo or in angiosperms. Conifers would present an ancestral pathway of seedling development in plants, while etiolation appeared later in evolutionary linneages (García-Gutiérrez *et al*., 1998). According to this hypothesis, glutamine and glutamate biosynthesis would be confined to the chloroplast of mesophyll cells in species with light-dependent chloroplast development, whereas compartmentation between cytosol and chloroplast could be required in species, with light-independent plastid development.

Expression studies as an approach to identify functional roles of individual enzymes

Effect of nitrogen nutrition and light

Expression analysis in grapevine revealed that two *GS1* isogenes are highly expressed in roots and to a lower extent in shoots and leaves (Loulakakis and Roubelakis-Angelakis, 1996). Both genes are differentially affected by exogenously supplied ammonium, whereas nitrate had no effect (Loulakakis and Roubelakis-Angelakis, 1996). However nitrate stimulated Fd-GOGAT expression (Loulakakis and Roubelakis-Angelakis, 1997). These data are in agreement with previous reports in annual herbaceous plants, indicating a role of specific members of the *GS1* gene family in the assimilation of externally supplied ammonium in co-ordination with NADH-GOGAT (Ireland and Lea, 1999). The increase of Fd-GOGAT and GS2 expression in maize roots in response to exogenously supplied nitrate (Redinbaugh and Campbell, 1993) suggest a role of the plastid-located GS/GOGAT cycle in the assimilation of ammonium derived from nitrate reduction. By contrast to that found in angiosperm woody plants, N nutrition either as nitrate or ammonium has a limited role in the regulation of GS and Fd-GOGAT in developing seedlings of maritime (Cánovas *et al*., 1991; García-Gutiérrez *et al*., 1995) and Scots pine (Elmlinger and Mohr, 1991, 1992).

Light is another external stimulus regulating N-assimilating enzymes in plants. Thus, the expression of genes for GS2 and Fd-GOGAT is light-regulated, whereas for the GS1 isoenzymes and NADH-GOGAT little effect of illumination, if any, has been described (Lam *et al*., 1996). Light enhancement of grapevine Fd-GOGAT expression was observed at the mRNA and enzyme activity level, but the transcript was also present at lower abundance in dark-grown plants (Loulakakis and Roubelakis-Angelakis, 1997). Light strongly stimulated *GS1a* mRNA accumulation during the development of Scots pine cotyledons (Cantón *et al*., 1999). *GS1a* transcripts increased in dark-grown seedlings transferred to light and decreased in dark-adapted seedlings in a similar way to mRNAs for photosynthesis genes such as *rbcS* and *lhcb2*. Functional expression analysis of the *GS1a* promoter in transgenic *Arabidopsis*, indicates it contains regulatory sequences involved in the response to light (Avila *et al*., 2001*b*). Regulation of conifer GS by light appears to be exerted by coaction of the phytochrome and cryptochrome photoreceptors (Elmlinger *et al*., 1994). In *P. sylvestris* seedlings, light is a major factor controlling the accumulation of Fd-GOGAT (Elmlinger and Mohr, 1991). However, *P. pinaster* seedlings accumulated Fd-GOGAT activity, polypeptide and transcript in a light-independent manner (García-Gutiérrez *et al*., 1995), suggesting a differential requirement of light among pine species for Fd-GOGAT accumulation during early seedling development.

Cellular distribution and developmental regulation

Expression of N-assimilating enzymes has been examined in detail during conifer seed germination and initial stages of seedling development. In loblolly pine (*Pinus taeda*), the breakdown of storage proteins during germination coincides with the accumulation of free amino acids in the seedling, particularly arginine, which is the predominant vehicle for N transport from the megagametophyte to the embryo (King and Gifford, 1997). Protein breakdown is accompanied by a marked increase in arginase activity. Urease activity has also been reported in pine seedlings confirming that arginine catabolism is an important source of ammonium during the early growth of pine seedlings (Todd *et al*., 2001). The roles of the two *GS1* genes (*GS1a* and *GS1b*) in N flow from the seed to the developing seedling have been recently reported for Scots pine (Avila *et al*., 2001*a*). *GS1b* is the functional gene at the early stages of germination providing the organic N necessary for *de novo* protein biosynthesis and is possibly related to the loss of seed dormancy (Schneider and Gifford, 1994). High levels of GS1b expression in the medullar region of the hypocotyl precedes formation of the first vascular elements suggesting that GS1b functions in N translocation in developing seedlings. By contrast, GS1a, Fd-GOGAT (García-Gutiérrez *et al*., 1995) and NADH-GOGAT (A García-Gutiérrez, FM Cánovas, unpublished data) expression is very low in the embryo and presumably the

corresponding genes are not involved in glutamate biosynthesis in the embryo.

In seedlings, expression of *GS1a* is restricted to tissues containing chloroplasts, including cotyledons and the upper part of the hypocotyl (Cantón *et al*., 1993). Furthermore, in Scots pine *GS* gene expression in these tissues is strongly stimulated by light (Elmlinger *et al*., 1994; Cantón *et al*., 1999). These data support a role for GS1a in the generation of amino donors for the biosynthesis of major N compounds in photosynthetic tissues: a role similar to the physiological role of chloroplastic GS2 in angiosperms. By contrast, GS1b is highly abundant in hypocotyls and roots, although it is also present at low levels in the cotyledons. In all these tissues GS1b is associated with the vascular bundles (Avila *et al*., 2001*a*). This expression pattern is quite similar to that found for GS1 in angiosperms (Edwards *et al*., 1990; Dubois *et al*., 1996) and suggests that GS1b plays an important role in N transport and translocation within the seedling. Moreover, the presence of GS1b in the xylem of pine trees (Avila *et al*., 2000; FM Cánovas, C Avila, FR Cantón, unpublished results), suggests a functional role of this GS isoform in the reassimilation of ammonium released from lignin biosynthesis during wood formation. In green cotyledons, where GS1a and GS1b coexist, they show distinct distribution patterns, further supporting differentiated functions in N metabolism (Fig. 5). GS1a expression in cotyledons is well correlated with the reported abundance of arginase (Todd *et al*., 2001), suggesting that GS1a plays a primary role

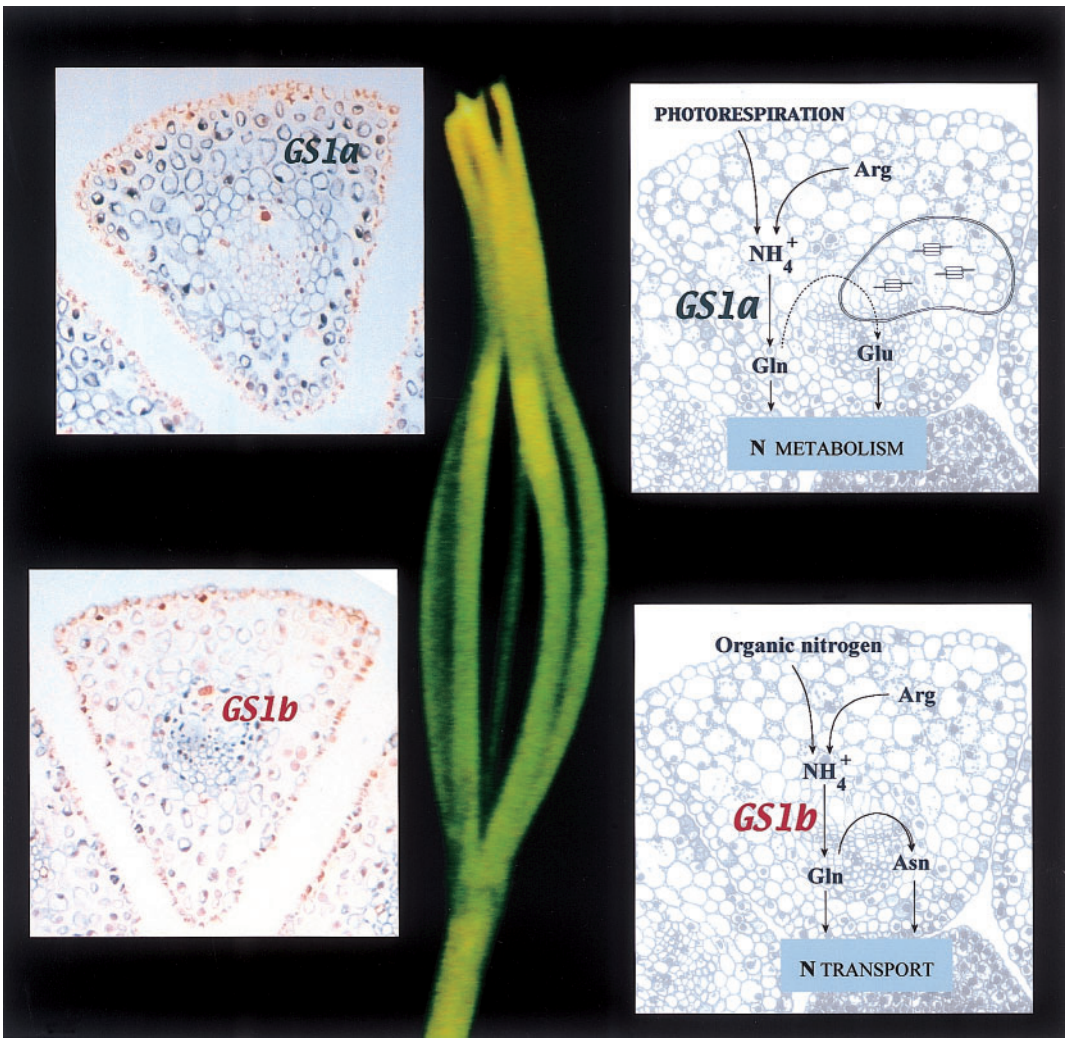

Fig. 5. Ammonium assimilation in pine seedlings at early stages of growing involves two cytosolic isoforms of glutamine synthetase encoded by separate genes and expressed differentially with development. The precise localization of *GS1* mRNAs is shown by *in situ* hybridization (left panels). GS1a expression increased during growth in green cotyledons where is located in photosynthetic cells. *GS1b* transcripts are predominant in non-photosynthetic tissues, but also present in the cotyledons where they are located in the vascular bundles. This pattern of expression suggests non-redundant roles for both isoforms (right panels). GS1a may play a role 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. The localization of GS1b mainly in vascular cells is consistent with a functional role of this isoform in the metabolism of amides for N transport to the growing parts of the plant.

in the reassimilation of ammonium released in arginine metabolism. GS1a may also function in reassimilation of ammonium released in photorespiration in photosynthetic cells of pine seedlings; a metabolic role that chloroplastic GS (GS2) assumes 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). High levels of expression of Fd-GOGAT and NADP-dependent IDH (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 asparagine in the later stages of seedling growth, it has been suggested that asparagine is the form of N that is transported from cotyledons to other parts of the pine seedling (King and Gifford, 1997). 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 asparagine 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), 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 for GS1b in N transport to sink tissues. Recently, using an antisense approach, Brugière *et al*. reported an essential role of GS1 located in phloem cells in the production of proline (Brugière *et al*., 1999). Nevertheless, proline abundance is very low in germinating pines (King and Gifford, 1997), therefore, GS1b does not appear to be involved in the biosynthesis of this amino acid following storage protein breakdown. Biochemical and molecular data suggest the existence of two glutamine cycles in developing pine seedlings (Fig. 5): (1) 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 cell growth and proliferation; and (2) a cytosolic GS1b/AS pathway involved in the biosynthesis of amides in vascular tissues for N transport to the growing apices.

Changes in gene expression in response to stress or senescence

The expression of GS1a in Scots pine appears to be dependent on factors associated with the integrity of developing chloroplasts (Cantón *et al*., 1999). In the presence of norfluorazon, a herbicide causing chloroplast damage by photo-oxidation as a result of carotenoid biosynthesis inhibition, light-grown plants showed a large decrease in the levels of *GS1a*, *rbcS* and *lhcb2* mRNAs, whereas the abundance of the mitochondrial β-ATP synthase was less affected. In tomato plants, infection by *Pseudomonas syringae* or treatment with the herbicide, phosphinothricin, a specific GS inhibitor, leads to chloroplast degeneration and apparent leaf chlorosis (Pérez-García *et al*., 1995, 1998*a*). When bacterial infection or herbicide treatment was carried out in the light, down-regulation of GS2 expression was observed and cytosolic GS1 appeared as the predominant GS polypeptide. These GS isoform replacements only occurred in illuminated leaves and were not observed during bacterial infection or during PPT treatment when photosynthetic activity was suppressed (Pérez-García *et al*., 1998*a*). This indicates that light-dependent factors are implicated in the regulation of expression of GS isoforms. The transient application of PPT to developing pine seedlings triggers the accumulation of the GS1b holoenzyme and, after separation by 2D-electrophoresis, specific induction of the GS1b polypeptide was observed (Avila *et al*., 1998). This response appears to be transcriptionally regulated (Avila *et al*., 2000) and is restricted to green tissues, suggesting a dependence on photosynthetic metabolism as reported for tomato (Pérez-García *et al*., 1998*a*). These data suggest that treatment with PPT provokes the loss of photosynthetic functions and subsequent chloroplast degeneration.

To study further the effect of the herbicide in conifers, the precise localization of *GS1b* transcripts was determined in sections of pine cotyledons using *in situ* localization. As shown in Fig. 6, general enhancement of expression was found in all cellular types, but the induction of GS1b expression was mainly localized in cells of the photosynthetic parenchyma.

These data are in agreement to the findings reported for annual plants. Pérez-García *et al*. demonstrated that the induced GS1 in response to PPT or bacterial infection was localized in mesophyll cells of tomato plants where it could play a role in the assimilation of N mobilized from chloroplast degradation (Pérez-García *et al*., 1998*b*). Similar results were found in senescing tobacco leaves, where a parallel induction of GS protein expression in the mesophyll cell cytosol was observed with the progression of leaf senescence (Brugière *et al*., 2000). In tomato leaves, the newly synthesized GS1 polypeptide was shown to be a novel GS isoform different in charge from the constitutive GS present in untreated tissue (Pérez-García *et al*., 1998*b*). In tobacco, the new GS1 protein was identified as the product of the *Gln1-3* gene, that is expressed in the cytosol of roots and flower cells (Dubois *et al*., 1996).

In addition to the central role of GS1 in N remobilization recent reports suggest the implication of other auxiliary enzymes in this process. For example, during natural senescence in tobacco, a strong correlation between proteolytic activity and both GS1 and GDH expression was observed (Masclaux *et al*., 2000). GDH may provide the required glutamate for glutamine biosynthesis when Fd-GOGAT activity is not present. In

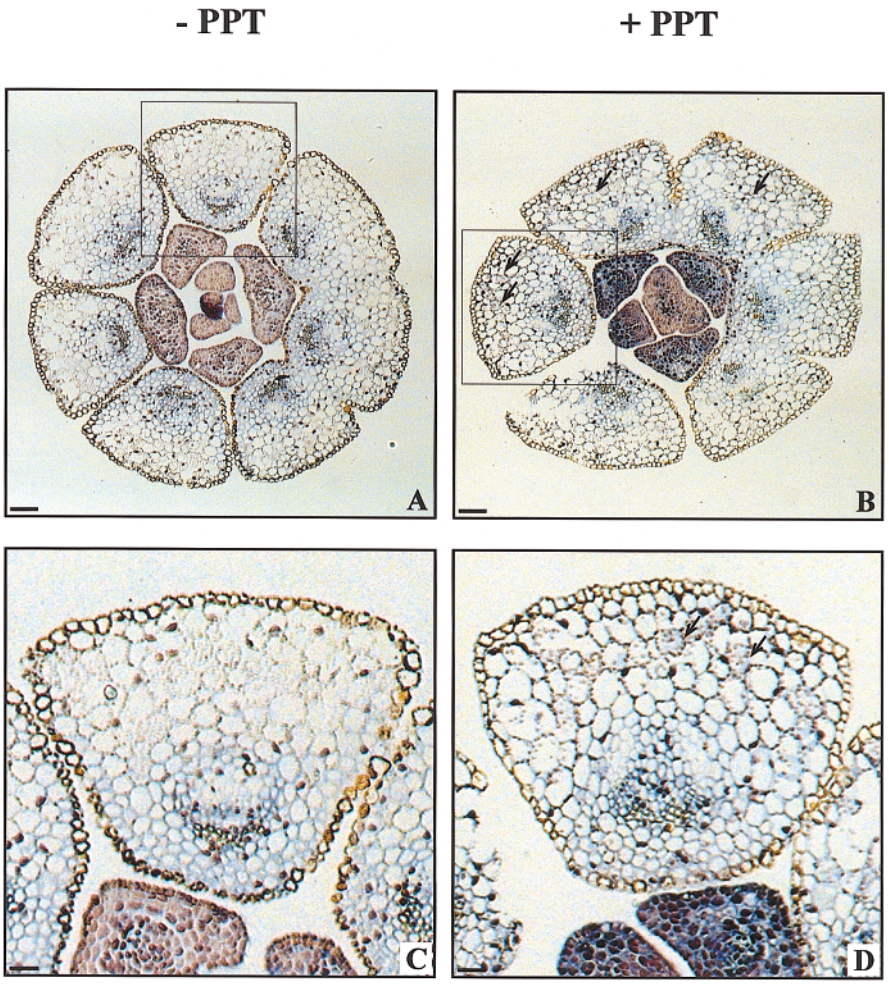

Fig. 6. Treatment of pine seedlings with phospinothricin triggers the induction of GS1b expression. The precise localization of *GS1b* transcripts was examined by *in situ* hybridization in the cotyledons of 3-week-old *Pinus sylvestris* seedlings. (A) Section of untreated plant. (B) Section of a plant 8 h after PPT treatment (Avila *et al*., 2000). Magnification in (C) and (D) show how GS1b expression is extended from the vascular bundles to the chlorophyll parenchyma in response to the herbicide. Scale bars represent 200 μm in (A) and (B), and 100 μm in (C) and (D).

tomato plants infected with *Pseudomonas*, the induction of asparagine synthetase (AS) has been observed in parallel with the induction of GS1 expression. This suggests the existence of a functional GS1/AS cycle (F Olea, A Pérez-García, A de Vicente, FM Cánovas, unpublished data), as proposed for pine seedlings (Avila *et al*., 2001*a*; this work). These findings further suggest that the metabolic status of the leaf, in particular the C/N ratio, could be involved in the control of expression of genes involved in ammonium assimilation. This co-ordinated GS1/AS pathway can be operative for the assimilation of ammonium under specific conditions, such as natural or induced senescence, carbon starvation (Chevalier *et al*., 1996), response to water stress (Bauer *et al*., 1997), or N fixation (Trepp *et al*., 1999). The existence of an excess of N and/or a limitation of carbon is common to all these conditions.

In many trees the N present in leaves is mobilized during autumn and stored in perennial tissues to be remobilized at the beginning of the next growing season. Increased levels of N-assimilating enzymes such as GS and possibly GDH have been reported in apple (Titus and Kang, 1982), during the autumnal senescence of leaves before abscission. Although analysis of specific GS isoenzymes was not undertaken in this study, it is possible that cytosolic GS could be involved. Since this process is of great importance for overall N economy in trees, studies of N cycling and remobilization need to be re-examined by determining seasonal variations of gene expression for certain key enzymes. The activities and isoenzyme profiles of GS and GDH have been studied during development and ripening of avocado fruit (Loulakakis *et al*., 1994). No changes were apparent during fruit development. By contrast, steady-state levels of both enzymes were considerably altered during ripening. GDH expression increased during the ripening, whereas total GS activity declined (Loulakakis *et al*., 1994).

Hormonal regulation of ammonium assimilation in trees has been recently reported. In the latex of rubber tree, transcripts for the cytosolic GS accumulate in response to ethylene (Pujade-Renaud *et al*., 1997). Although initially the effect was observed in trees regularly submitted to tapping (wounding) (Pujade-Renaud *et al*., 1994), direct regulation by ethylene, independent of wounding, was also demonstrated. The observed induction required 6–12 h, a period possibly needed for transduction of the ethylene signal (Pujade-Renaud *et al*., 1997).

Modification of nitrogen metabolism in transgenic woody plants

A current focus in plant improvement is the increase in plant growth and biomass accumulation as a result of the modification of the expression of growth-related genes. In early studies of expression of chimeric genes encoding key N metabolism enzymes such as nitrate and nitrite reductase, GS and AS, no effect on the phenotype of the modified plants was observed (Foyer and Ferrario, 1994). Co-transformation with other genes should be considered in order to avoid the possible generation of new limiting steps in the metabolic pathway. Nevertheless, more recent studies have shown that characteristics of agronomic importance can be introduced in transgenic herbaceous plants by the expression of heterologous GS isoenzymes. Thus, a higher capacity for photorespiration (Kozaki and Takeba, 1996), stimulation of seedling growth (Migge *et al*., 2000), and increase in tolerance to salt stress (Hoshida *et al*., 2000) have been reported using engineered plants which overexpress chloroplastic GS2. Furthermore, an increase in biomass production and acceleration of development have been observed in leguminous plants which overexpress cytosolic GS1 (Vincent *et al*., 1997; Limami *et al*., 1999).

Poplar is considered a model in molecular investigations of forest trees because of its small genome size, easy vegetative propagation and *in vitro* culture, and its amenability to transformation via *Agrobacterium tumefaciens* (Klopfenstein *et al*., 1997). The existence of a number of fast-growing hybrid poplar clones also permits short-term field trials to obtain results in a relatively short period of time in comparison with other trees (Klopfenstein *et al*., 1997).

The modification of N assimilation efficiency has recently been approached in trees by the overexpression of pine GS1a in hybrid poplar (Gallardo *et al*., 1999). An advantage in using woody plants in studying enhanced nitrogen assimilation is that the effect of the transgene expression can be accumulated in the tissues/organs for a long period of time, because of the long life cycle of trees. Overexpression of pine GS affected levels of GS activity, and the contents of chlorophyll and protein, which suggest that up-regulation of cytosolic GS1 may lead to a global effect on the synthesis of nitrogenous molecules in poplar leaves. In addition, these changes were associated with modification of the phenotype, and a correlation between GS activity in young leaves and the vegetative growth was found (J Fu, R Sampalo, F Gallardo, EG Kirby, FM Cánovas, unpublished data). Alterations of the phenotype include a higher leaf number and leaf surface area, which could explain the enhanced vegetative growth. These results suggest that the efficiency of N utilization may be engineered in trees by the manipulation of glutamine biosynthesis. Field trial studies have been initiated to evaluate the economic interest of the genetically modified poplar lines.

Future prospects

Considerable knowledge has been gained over the last ten years on the molecular characteristics and molecular regulation of N-assimilating enzymes in woody plants, including angiosperm and gymnosperm species. This research has greatly contributed to our understanding of how inorganic N is assimilated and utilized in trees. However, the available information is still limited and efforts should be made to increase basic research on N metabolism and to integrate new advances in biotechnology to improve growth and development of economically important woody species. Although all new studies will contribute to this goal, the concentration of efforts in model trees, such as poplar for angiosperms and pine for gymnosperms, is advisable.

In future years, the availability of new molecular tools for biological studies of trees will permit characterization of new genes involved in N metabolism and determination of their specific physiological roles. Functional studies are now possible in woody plants because routine transformation protocols via *Agrobacterium* are available for poplar and rapid progress has been reported in the last few years for conifers. The use of somatic embryogenic cell lines is critical in conifers for the generation of transgenic trees. Somatic embryogenesis also represents a useful model to study developmental gene expression and the functional roles of the enzymes during embryo development (Filonova *et al*., 2000). Results obtained with woody plant systems will be compared with information derived from structural/functional genomic studies in *Arabidopsis* and specific roles will be investigated *in vivo* by functional rescue of isolated mutants. For example, genomic technologies have recently been used to study the effect of a variety of N regimes on plant metabolism (Wang *et al*., 2000). Results from this study indicate that changes in N supply influence not only expression of genes involved in N assimilation, but also

those involved in other metabolic pathways. Similar studies of gene expression at the organ or tissue levels are now feasible in tree models, with the existence of EST databases from poplar (http://www.forestbiotech.com) and loblolly and maritime pines (http://www.cbc.umn.edu; http://www.pierroton.inra.fr/Gemini). Studies on gene expression at the mRNA level will be complemented by proteomic analysis, including separation of proteins by 2D gel electrophoresis and sequence determination or mass spectrometry analysis of protease digestion products (Costa *et al*., 1999). Another promising line of research will be to study at the molecular level, the genetic basis of important traits, such as N use efficiency, grain yield, and height growth (Hirel *et al*., 2001). Genetic maps for poplar and pine have been established and now genes involved in N metabolism can be localized in the genome. The possible association of specific genes with quantitative trait loci (QTLs) are currently being investigated. The construction of defined BAC libraries for trees and the identification of individual clones using the isolated cDNAs and genomic clones as molecular probes, will accomplish the physical mapping of the regions of the genome where key genes are localized. This will allow molecular characterization of gene clusters involved in traits of interest in forestry and tree management.

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