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FOCUS PAPER

Ammonium assimilation and amino acid metabolism in conifers

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Abstract

Conifers are the most important group of gymnosperms, which include tree species of great ecological and economic importance that dominate large ecosystems and play an essential role in global carbon fixation. Nitrogen (N) economy has a special importance in these woody plants that are able to cope with seasonal periods of growth and development over a large number of years. As N availability in the forest soil is extremely low, efficient mechanisms are required for the assimilation, storage, mobilization, and recycling of inorganic and organic forms of N. The cyclic interconversion of arginine and the amides glutamine and asparagine plays a central role in the N metabolism of conifers and the regulation of these pathways is of major relevance to the N economy of the plant. In this paper, details of recent progress in our understanding of the metabolism of arginine and the other major amino acids glutamine, glutamate, aspartate, and asparagine in pine, a conifer model tree, are presented and discussed.

Key words: Arginine, asparagine, aspartate, compartmentation, glutamate, glutamine, N metabolism, pine.

Introduction

The availability of nitrogen (N) in natural soils frequently limits plant growth and development. Consequently, during evolution, plants have developed regulatory mechanisms and symbiotic relationships to increase the efficiency of N uptake and utilization (Lea and Azevedo, 2006). The economy of reduced N has a special importance in trees that have to go through seasonal periods of growth and development over many years. Conifers, the most important group of gymnosperms, include tree species of great ecological and economic importance that dominate large ecosystems and play an essential role in global carbon fixation. Unlike many other plants, conifers have a strong preference for ammonium over nitrate as a N source (Kronzucker *et al*., 1997). Moreover, ammonium uptake in conifer roots is enhanced in micorrhizal fungal associations, whereas nitrate uptake is not affected to any great extent (Kronzucker *et al*., 1997; Chalot *et al*., 2006). Conifers are also able to take up organic N in the form of amino acids such as arginine and glutamate (Ohlund and Nasholm, 2004; Persson *et al*., 2006). In fact, there is increasing evidence that arginine is synthesized by mycorrhizae and translocated to intraradical mycelium where it is degraded to provide ammonium that is then assimilated by the plant cell (Chalot *et al*., 2006).

It is well known that N fertilization and atmospheric deposition in conifer forests dramatically increase the arginine content of needles and wood (Schneider *et al*., 1996; Nordin *et al*., 2001) and it has been proposed that this N-rich amino acid could reflect the N status of trees better than other parameters utilized in herbaceous plants (Edfast *et al*., 1996). Long-term deposition of N is carried out in the form of storage proteins in seeds and vegetative organs that are particularly rich in arginine and the amide amino acids (Muntz *et al*., 1998). The mobilization of this stored material provides the constituents for early and seasonal tree growth and development (Cantón *et al*., 2005). The cyclic interconversion of arginine and the amides glutamine and asparagine therefore plays a central role in the N metabolism of conifers and its regulation is critical to maintain the N economy of these long-living plants (Fig. 1). In this paper, recent advances in the metabolism of arginine and the other major amino acids glutamine, glutamate, aspartate, and asparagine in pine, a conifer model tree, are reviewed.

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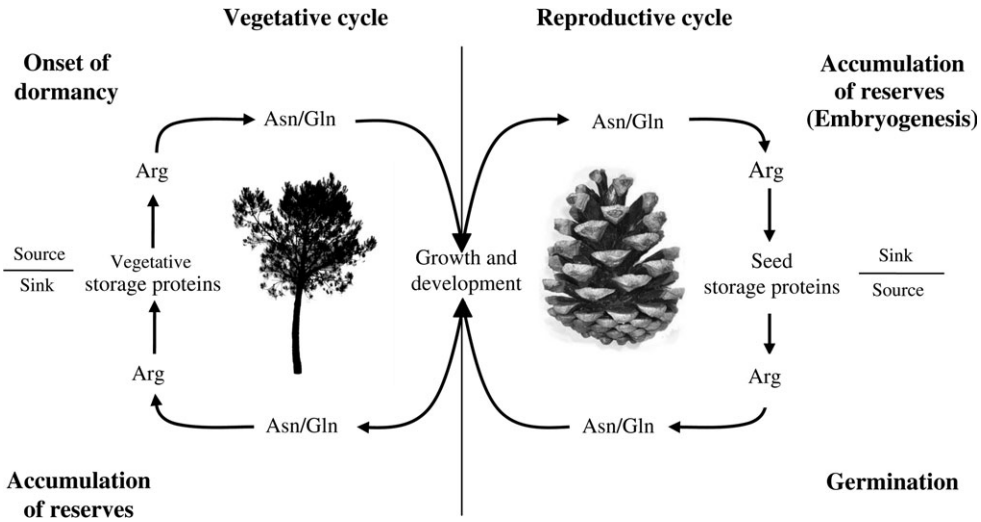

Fig. 1. Interconversions of key amino acids, arginine, glutamine, and asparagine during the reproductive and vegetative cycles of pine trees.

N mobilization, storage and recycling

Germination and seedling growth

The specific pathways and the regulation of enzymes involved in the mobilization of N in vegetative tissues of adult trees are still unknown, but considerable progress has recently been made in our understanding of how seed-stored N is recycled during germination and post-germinative growth. In pine, the majority of storage proteins in the seed are contained in the megagametophyte, a maternally derived tissue surrounding the embryo (Stone and Gifford, 1997). Storage proteins are extremely rich in arginine, the amino acid with the highest N content. Thus, arginine accounts for nearly half of the N reserves in the megagametophyte of loblolly pine (King and Gifford, 1997) and a similar abundance has been found in other conifers (Cánovas *et al*., 1998). These data suggest that arginine represents the major source of N for the biosynthesis of N compounds in the developing seedling. After the hydrolysis of proteins during germination, arginine is catabolized to produce ornithine, ammonium, and carbon dioxide by the sequential action of arginase (EC 3.5.3.1) and urease (EC 3.5.1.5) enzymes (Todd and Gifford, 2002). Thus, arginase activity, protein and mRNA are predominantly localized in the expanding cotyledons, which remain in contact with the megagametophyte throughout early seedling growth. Ornithine is further metabolized to other amino acids (Slocum, 2005), whereas ammonium is recycled through the glutamine synthetase (GS, EC 6.3.1.2)/glutamate synthase (GOGAT, EC 1.4.7.1) pathway (Cánovas *et al*., 1998) to generate glutamine and glutamate, the N donors for the biosynthesis of all major N compounds needed for plant growth (Ireland and Lea, 1999). For instance, N can be channelled to the biosynthesis of aspartate and asparagine catalysed by aspartate aminotransferase (AAT, EC 2.6.1.1) and asparagine synthetase (AS, EC 6.3.5.4). These metabolic reactions are particularly relevant in the mobilization of N stored in the conifer seed since asparagine is the most abundant amino acid in developing pine seedlings, accounting for about 70% of the total free amino acid pool (King and Gifford, 1997).

Of the enzymes described above, GS plays a key role in plant N metabolism. There are two major isoenzymes of GS located in different subcellular compartments and displaying non-overlapping roles. 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 (Ireland and Lea, 1999). GS1, a cytosolic isoform, is the predominant enzyme in roots and non-photosynthetic tissues and much less abundant in green tissues (Ireland and Lea, 1999). GS1 seems to be involved in the primary assimilation of ammonium from the soil and the recycling of ammonium released through metabolic processes other than photorespiration (Ishiyama *et al*., 2004; Cantón *et al*., 2005). A number of reports in the past few years supported a role of GS1 in plant development, growth, and biomasss production (Fuentes *et al*., 2001; Jing *et al*., 2004). Recent genetic and molecular approaches have shown that GS may be a key component of plant N use-efficiency and yield (Hirel *et al*., 2001; Tabuchi *et al*., 2005; Martin *et al*., 2006). All these reports suggest that cytosolic GS (GS1) plays a central and pivotal role in N metabolism that is essential for N use-efficiency in higher plants. Conifer trees represent a good experimental model to study the function of GS1, as no evidence for a plastid GS2 in conifers has been obtained in biochemical, molecular, and microscopical analyses (García-Gutiérrez *et al*., 1998) and, therefore,

GS1 must be involved in amino acid biosynthesis in both photosynthetic and non-photosynthetic cells. Phylogenetic analysis suggests that plant GS2 evolved from a duplicated GS1 around the time of land plant evolution (Avila-Sáez *et al*., 2000) and it is tempting to speculate that GS in photosynthetic cells was moved from the cytosol (GS1) to the chloroplast (GS2) when plants were exposed to the present oxygen levels in the atmosphere, perhaps as an adaptive mechanism to overcome the high levels of ammonium released during photorespiration. There are two GS1 isoenzymes in pine GS1a and GS1b, which have different expression patterns, molecular and kinetic properties (Avila *et al*., 1998, 2001*a*; de la Torre *et al*., 2002). GS1b, is highly abundant in pine hypocotyls and roots, although is also present at low levels in the cotyledons. In all these tissues, gene expression is associated with vascular bundles (Avila *et al*., 2001*a*; Suárez *et al*., 2002) suggesting that GS1b plays a role in N transport and translocation as reported for GS1 in angiosperms (Ireland and Lea, 1999). Thus, Avila *et al.* (2001*a*) proposed a catalytic cycle between GS1b and AS to synthesize asparagine in the vasculature of cotyledons for N transport to other parts of the seedling. GS1b expression is up-regulated by gibberellin during germination and the advanced stages of development when the megagametophyte reserves are exhausted (Gómez-Maldonado *et al*., 2004*b*).

In contrast, GS1a expression is associated with the development of the chloroplast and enhanced by light, suggesting that its physiological role may be similar to that of GS2 in angiosperms (Cantón *et al*., 1999). Furthermore, the high level of expression of plastidic ferredoxin (Fd)-dependent GOGAT suggests that a functional GS1a/Fd-GOGAT cycle is operative in pine photosynthetic cells (García-Gutiérrez *et al*., 1998; Suárez *et al*., 2002). NADH-GOGAT (EC 1.4.1.14) is only present in low amounts in green pine tissues (García-Gutiérrez *et al*., 1995), where it may have an essential role in vascular tissues (Lancien *et al*., 2002; Lea and Miflin, 2003). In both types of cells the GS/GOGAT pathway requires carbon skeletons in the form of 2-oxoglutarate and a candidate enzyme involved in its metabolic origin is $NADP^+$-dependent isocitrate dehydrogenase (ICDH, EC 1.1.1.42) (Hodges *et al*., 2003). In pine, a correlation in the abundance of ICDH, GS, and Fd-GOGAT was found during the early germination stages and chloroplast biogenesis (Palomo *et al*., 1998), suggesting that this enzyme has a role in the provision of 2-oxoglutarate for glutamate biosynthesis.

Lignification

Conifer trees divert large amounts of carbon into the biosynthesis of phenylpropanoids particularly to generate lignin, an important constituent of wood. Although lignin does not contain N, the phenylpropane skeleton required in this metabolic pathway is provided by the deamination of phenylalanine (or tyrosine) catalysed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). Ammonium ions are released in this reaction and are returned to metabolism by active N recycling via the GS/GOGAT pathway (van Heerden *et al*., 1996). Moreover, cells undergoing lignification also consume very large numbers of methyl groups generated through C1 metabolism associated with glycine and serine catabolism (Mouillon *et al*., 1999). Consequently, large amounts of ammonium will be released by glycine oxidation during active lignin biosynthesis and evidence to support the existence of this metabolic pathway in trees has recently been reported (Cantón *et al*., 2005). In conclusion, the existence of such mechanisms of N reassimilation in lignifying cells is critical to maintain high rates of lignification without affecting the N economy of the plant.

Recent reports have provided insights into the nature of the enzymes involved in the reassimilation of ammonium in conifer vascular tissues. The abundance and precise localization of *GS1b* gene expression in the vascular bundles of pine seedlings support a role for GS1b in N recycling associated with lignin biosynthesis in xylem cells (Suárez *et al*., 2002; Cantón *et al*., 2005). In fact, *GS1b* transcripts and polypeptides accumulate in developing xylem cells where the activities of PAL and the enzymes involved in C1 metabolism are high, and therefore ammonium is liberated. The levels of Fd-GOGAT activity, protein, and mRNA are very low in pine stems and roots (García-Gutiérrez *et al*., 1995) and therefore NADH-GOGAT could well be the enzyme involved in the reassimilation of ammonium in xylem cells. This assumption is supported by the presence of expressed sequence tags (ESTs) for NADH-GOGAT in a database from pine xylem wood (http://pinetree.ccgb.umn.edu). Other enzymes such as glutamate dehydrogenase (GDH, EC 1.4.1.2), AAT and AS have recently been reported to be present and/or expressed in developing vascular cells (Fontaine *et al*., 2006; Cañas *et al*., 2006; de la Torre *et al*., 2006) and therefore could participate in the recycling process.

Embryogenesis

Only a few studies have been carried out on N metabolism during conifer embryogenesis. Recent studies in zygotic and somatic embryos of pine have shown that the GS1b isoform plays an essential role during conifer embryogenesis, whereas *GS1a* expression appears to be correlated with the proliferation ability of embryonic cell lines (Pérez-Rodríguez *et al*., 2006). The results are consistent with the previously reported expression of genes encoding distinct GS isoforms in somatic embryos of carrot (Higashi *et al*., 1998) and confirm the physiological importance of glutamine biosynthesis during plant embryogenesis. In mature zygotic embryos, *GS1b* expression was restricted to a strand of elongated cells conforming

the embryo vascular system (procambium) in agreement with a proposed role for the GS1b isoenzyme in intercellular and inter organ N transport (Suárez *et al*., 2002). In developing zygotic embryos, *GS1b* expression was also located in procambial cells being first detected at the initiation of the maturation phase (within filamentous to cotyledonary stages), just after embryonal vascular tissue could be distinguished (Pérez-Rodríguez *et al*., 2006).

A major metabolic event during seed maturation is the accumulation of reserves at late embryogenesis in 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 Norway spruce at the transition stage from a filamentous to a cotyledonary embryo (Hakman *et al*., 1990). Furthermore, during white spruce somatic embryo development, a switch in amino acid metabolism was demonstrated, which involved increases in glutamine, glutamate, and arginine (major components of seed storage proteins in conifers) and a decrease in alanine. 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 (Joy *et al*., 1997). 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 the GS1b isoenzyme in the synthesis of storage proteins, the main sink for amino acids during embryo maturation (Pérez-Rodríguez *et al*., 2006).

A general overview of different pathways of N metabolism in pine tissues is shown in Fig. 2.

Transcriptional regulation of glutamine biosynthesis

As previously discussed, several lines of evidence indicate that cytosolic glutamine synthetase (GS1) plays a crucial role in the N metabolism of conifer trees and, therefore, the reaction catalysed by this enzyme may be a major step in controlling growth and development. In pine, GS1a and GS1b isoforms exhibit differential kinetic properties according to the function of the catalysts in the cells where they are located (de la Torre *et al*., 2002). The mechanisms that underlie the differential expression of GS isoforms in photosynthetic and vascular cells involve the interaction between tissue-specific transcriptional factors and functional motifs in the *GS1a* and *GS1b* promoters. It has been found that the structures of *GS1a* (Avila *et al*., 2001*b*) and *GS1b* (Gómez-Maldonado *et al*., 2004*b*) promoters are largely unrelated and the distinct expression patterns displayed by both genes within the plant body are consistent with this finding. *GS1a* promoter contains AT-rich repeated sequences similar to those previously described in light-regulated genes such as *rbcS*, *cab*, and *GLN2* (Avila *et al*., 2001*b*). Functional analyses have confirmed that the upstream region of the gene contains regulatory sequences implicated in the response to light and showed that the interaction of these sequences with regulatory elements is required for transcriptional activity (Gómez-Maldonado *et al*., 2004*a*).

The regulation of pine GS1b by gibberellins seems to be related to specific transcription factors interacting with a pyrimidine-rich box (GA box) (Gómez-Maldonado *et al*., 2004*b*). However, it cannot be ruled out that other elements present on the promoter region could be involved. In fact, elements with sequence homology to GARE and CARE elements (Cercós *et al*., 1999) have been found in the promoter region of *GS1b*, although their functionality has not yet been tested. The proximal region of the *GS1b* promoter contains AC elements (Gómez-Maldonado *et al*., 2004*b*), which are frequently found in the promoters of genes encoding enzymes involved in vascular development and lignin biosynthesis. These *cis*-acting elements are likely to function as targets for transcription factors whose role is to co-ordinate gene expression, spatial and temporally. Thus, a co-ordinated regulation of the phenylpropanoid pathway by some R2R3 MYB proteins that bind AC elements has been proposed (Martin and Paz-Ares, 1997). The relative importance of the AC motif as a regulatory element has been demonstrated with the sequencing of the *Arabidopsis thaliana* genome, where 29% of the genes involved in the biosynthesis of precursors of lignin contained AC elements and some of them have been tested functionally (Raes *et al*., 2003). In pine, two members of the R2R3-MYB family of transcription factors that are expressed in lignifying cells were shown to bind the *GS1b* promoter (Gómez-Maldonado *et al*., 2004*c*) and activate its transcriptional activity. Furthermore, these two MYB proteins were most abundant in the cells where the *GS1b* gene was actively transcribed. As stated above, lignification is a process with high demands of N recycling and GS1b is the major enzyme involved in N recycling in pine vascular cells. These findings suggest a simple mechanism to co-ordinate lignin biosynthesis and the reassimilation of ammonium in the same cell-types via transcriptional regulation (Fig. 3). Interestingly, this is one of the few examples that show how primary and secondary metabolism can be co-regulated. In fact, the existence of a cross-talk between primary and secondary metabolism has recently been demonstrated (Rohde *et al*., 2004; Scheible *et al*., 2004). Other transcriptional regulators, for instance, Dof factors, might also be involved in lignin production (Rogers *et al*., 2005). Dof factors are unique to plants and have been found associated with diverse gene promoters, suggesting that they may be involved in a variety of different types of signal-responsive and/or tissue-specific gene expression in plants. Recently, metabolically

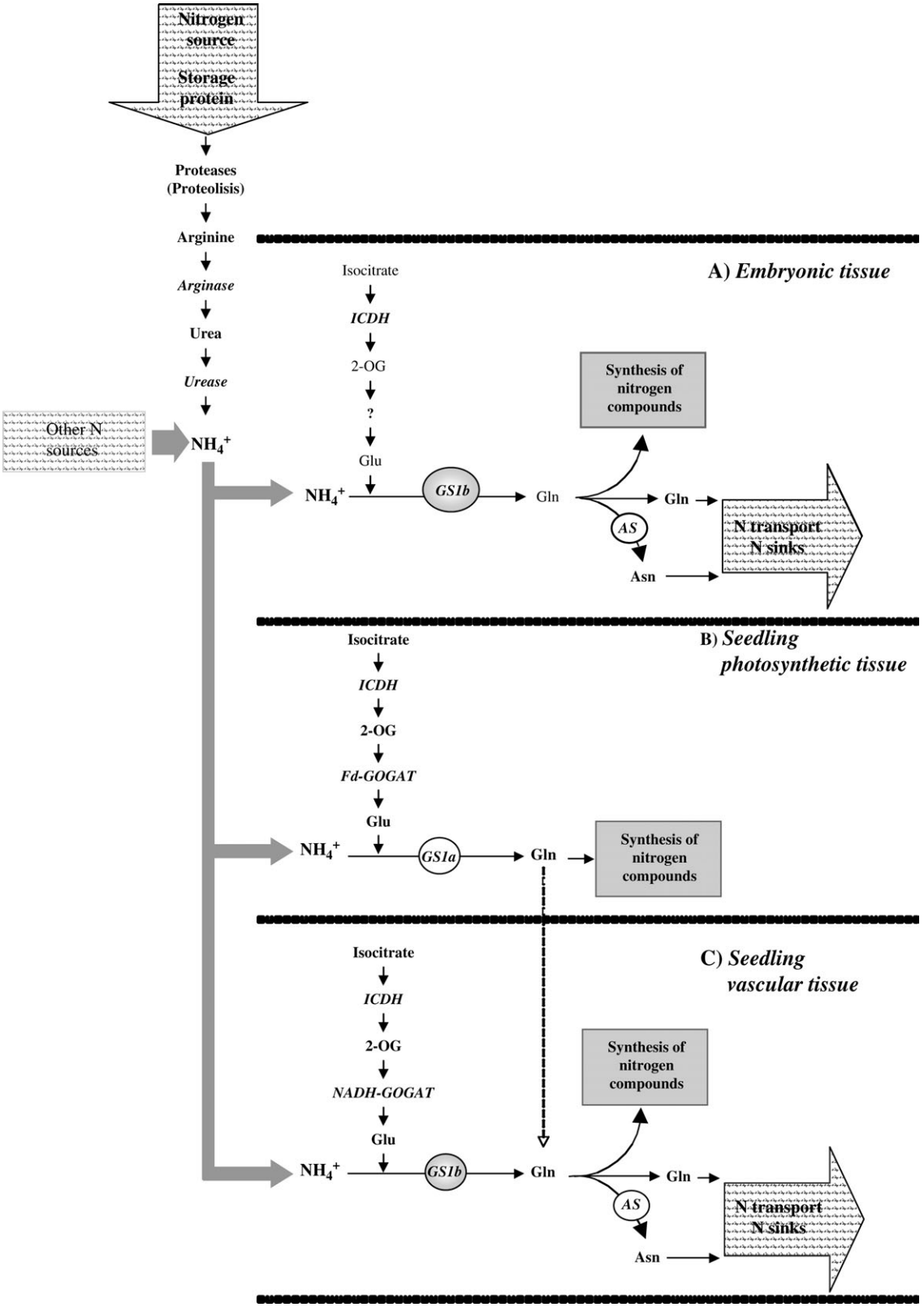

Fig. 2. Metabolic pathways involved in nitrogen metabolism in different pine tissues. Enzyme abbreviations: AS, asparagine synthetase; Fd-GOGAT, ferredoxin-dependent glutamate synthase; GS1a, cytosolic glutamine synthetase a; GS1b, cytosolic glutamine synthetase b; ICDH, isocitrate dehydrogenase; NADH-GOGAT, NADH-dependent glutamate synthase.

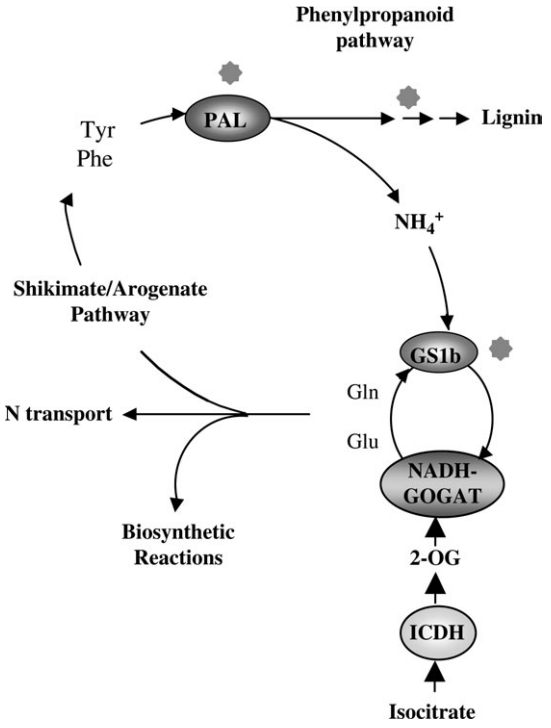

Fig. 3. Co-ordinated regulation of lignin biosynthesis and nitrogen recycling. Metabolic steps regulated by Myb proteins (star). Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; GS1b, cytosolic glutamine synthetase b; NADH-GOGAT, NADH-dependent glutamate synthase; ICDH, isocitrate dehydrogenase.

engineered *Arabidopsis* plants with Dof 1 from maize showed improved N assimilation and growth under low-N conditions (Yanagisawa *et al*., 2004), suggesting that the transcription factor could be a key regulator in the co-ordinated gene expression involved in carbon-skeleton production for N assimilation.

Asparagine metabolism and the reallocation of stored nitrogen

Following the mobilizaton of storage proteins in the megagametophyte, the released N in the developing seedling may be incorporated into new proteins or stored for later use. The reallocation of stored N from the megagametophyte into the developing seedling may play a crucial role in conifer development, modulating the flux of N in relation to the demand. This demand will be dependent on the rate of cell division and expansion, but also includes a requirement for a pool of N to support later development, once the reserves are exhausted and before the seedling becomes fully autotrophic.

The mobilization of N reserves in the megagametophyte during germination causes an increase in the concentrations of free amino acids in the developing seedling. As described above, arginine, glutamate, and glutamine are the most abundant free amino acids at the very early stages of germination (King and Gifford, 1997). However, soon after germination and following radicle emergence, a pronounced change in the amino acid composition is observed, which is characterized by a remarkable increase in the concentration of asparagine. In the seedlings of loblolly pine, free asparagine accounts for about 70% of the free amino acid pool and becomes the most abundant free amino acid in the seedling (King and Gifford, 1997). Asparagine is a key metabolite for transport of N in plants (Sieciechowicz *et al*., 1988; Lea *et al*., 2007). Several chemical properties make asparagine a convenient vehicle for N transport: it is more soluble than ureides and more stable than glutamine and it is also a more efficient N carrier than glutamine because it has a higher N/C ratio. As a N transport molecule, asparagine appears to be targeted toward sink tissues where there is a requirement for large amounts of mobilized N (Sieciechowicz *et al*., 1988). There is now considerable evidence that glutamine-dependent AS is the main enzyme used for the synthesis of the asparagine in higher plants (Lea *et al*., 2007). In particular, during seed germination in angiosperms, AS plays a major role in the mobilization of N resources, when storage proteins are converted to asparagine for transport to growing apices (Kern and Chrispeels, 1978; Lea *et al*., 2007). It is therefore also possible that AS is utilized to reallocate the mobilized N for later processes of seedling development.

Glutamine synthesized in developing pine cotyledons is consumed in the biosynthesis of glutamate in the plastid to support the development and synthesis of the components of the photosynthetic machinery (Cánovas *et al*., 1998; Cantón *et al*., 1999; Avila *et al*., 2001*a*), but it is also used to a large extent for the synthesis of asparagine (Cañas *et al*., 2006). The high content of free asparagine observed after germination is not homogenously distributed throughout the pine seedling and most of this increase results in the accumulation of the amino acid in the hypocotyl (Cañas *et al*., 2006). A cDNA encoding AS has recently been cloned from pine (*P. sylvestris*) seedlings and specific polyclonal antibodies were raised against the recombinant protein expressed in *E. coli*. Using molecular techniques, the expression of the corresponding gene, named *PsAS1*, has been analysed during germination and early seedling development. *PsAS1* mRNA and polypeptide are absent in dormant embryos. However, *PsAS1* gene expression started along with embryo elongation, suggesting that high levels of AS are required when the N storage material has been mobilized in the seed and there is active cell proliferation and development of essential structures within the organs of the seedling. The temporal and spatial pattern of *PsAS1* expression during germination matched the increase in asparagine concentration observed in loblolly and Scots pines (King and Gifford, 1997; Cañas *et al*., 2006). The *PsAS1* gene was expressed mainly in hypocotyls in high

amounts, whereas the levels of transcript and protein were much lower in cotyledon and radicles. The correlation between amino acid concentration and *PsAS1* expresion during germination, suggests that the product of this gene plays a specific role in the accumulation of N as free asparagine in hypocotyls. Furthermore, the analysis of AS polypeptides by combining 2D-electrophoresis and western-blot detection suggested that the product of *PsAS1* is the main enzyme responsible for asparagine synthesis during the early stages of development. Moreover, in contrast to previously reported data on AS regulation in angiosperms (Coruzzi, 2003; Herrera-Rodríguez *et al*., 2006), the spatial and temporal regulation of *PsAS1* expression together with the limited effect of light on the overall distribution of transcripts, indicates that the accumulation of high concentrations of free asparagine in pine hypocotyls is a developmentally regulated process.

The low abundance of AS protein detected in the cotyledons, in comparison with that observed in hypocotyls, suggests that most of the asparagine produced in the seedling is not synthesized in the cotyledons. Accordingly, glutamine generated in the vascular cells of cotyledons must be transported to the hypocotyl, where it will be used as a substrate in the glutamine-dependent reaction catalysed by AS. To support this high rate of asparagine synthesis, the glutamate released in the AS reaction will need to be transaminated to aspartate by AAT activity. Taken together, these results suggest the existence of a GS1b/AS pathway for transferring the excess of free N in the cotyledons into asparagine in the hypocotyls. In this process, part of the N in the form of glutamine is recaptured in the soluble storage pool of asparagine.

The metabolic fate of asparagine in young pine trees has recently been investigated. *PsASPG*, a gene encoding asparaginase (EC, 3.5.1.1), has been identified by cloning a cDNA from developing seedlings of Scots pine (Cañas *et al*., 2007). *PsASPG* was overproduced in *E. coli* and the purified recombinant protein exhibited asparaginase activity. The phylogenetic analysis of the amino acid sequence of the pine enzyme, together with sequences from other plants and organisms, clearly indicated that it belongs to the K^+-dependent subfamily of plant asparaginases. In contrast to K^+-independent asparaginases, the K^+-dependent enzyme in *Arabidopsis* showed a strict specificity for asparagine and it had no activity toward β-aspartyl dipeptides (Bruneau *et al*., 2006), suggesting an exclusive functionality as an asparaginase. Furthermore, the kinetic parameters of the *Arabidopsis* K^+-dependent enzyme further suggest that it may metabolize asparagine more efficiently under conditions of high metabolic demand for N.

The transcriptional profile of the *PsASPG* gene, during germination and seedling development, strongly indicates that this gene plays a role in mobilizing the N reallocated in hypocotyls as free asparagine. However, it appears that this asparagine is mainly destined to support specific process of development. Thus, the expression of the *PsASPG* gene correlated spatially and temporally with the occurrence of a highly lignified vascular system and *PsAPG* transcripts were located in cells of the cambial region (Cañas *et al*., 2007). When the levels of *PsAS1* and *PsASPG* transcripts were analysed during seedling development, it was observed that the expression of both genes was temporally co-ordinated to control the reallocation of N from seed storage proteins toward the hypocotyl. Therefore, the asparagine accumulated in the hypocotyl appears to support the establishment of a fully developed secondary vascular system in the hypocotyl, immediately after seed storage material is exhausted.

The biosynthesis of aspartate and derived amino acids

There is a major metabolic demand for aspartate in the cytosol as a substrate for asparagine biosynthesis in the reaction catalysed by AS and in the plastid as a precursor of the metabolic pathway leading to the biosynthesis of the essential amino acids lysine, threonine, isoleucine, and methionine (Azevedo *et al*., 2006). Aspartate is generated by transamination from glutamate in the reversible reaction catalysed by AAT, a pyridoxal 5′-phosphate-dependent enzyme (Ireland and Lea, 1999). Members of the *AAT* gene family in plants encode different AAT polypeptides that are assembled into dimeric isoenzymes distributed in several subcellular compartments such as the cytosol, mitochondria, plastid, and peroxisome (Schultz and Coruzzi, 1995; Wilkie and Warren, 1998). The amino acid sequences of similar AAT isoenzymes have been identified in loblolly and maritime pines (F de la Torre, FM Cánovas, unpublished data). In fact, the sequences of all AAT polypeptides in angiosperms and gymnosperms are quite well conserved, suggesting a possible origin from a common ancestor (Winefield *et al*., 1995; Wadsworth, 1997; F de la Torre, FM Cánovas, unpublished data). Members of the *AAT* gene family have been well characterized in *Arabidopsis* (Coruzzi, 2003), however, the isolation of the genes was performed by RT-PCR using degenerate oligonucleotides spanning highly conserved stretches of *E. coli* and *S. cerevisiae* AAT (Schultz and Coruzzi, 1995), and this approach possibly prevented the isolation of phylogenetically unrelated *AAT* genes. Recently, sequences of a prokaryotic-type *AAT* (*PT-AAT*) gene have been identified in a bank of ESTs from maritime pine (*P. pinaster*) (De Santis *et al*., 2005). Interestingly, orthologous single copy genes are present in the sequenced genomes of *Arabidopsis*, rice, and poplar indicating that *PT-AAT* is widely distributed in angiosperms and gymnosperms. The amino acid sequence of this novel AAT is unrelated to the eukaryotic AATs previously characterized in plants and animals, but similar

to prokaryotic enzymes and particularly to cyanobacterial AATs. These findings suggest that PT-AAT, exclusively present in plants, might have arisen by endosymbiosis of ancestral cyanobacteria (de la Torre *et al*., 2006). In agreement with this hypothesis, the precursor polypeptide of PT-AAT contains a plastid targeting presequence that is processed *in planta* to a mature 45 kDa subunit, immunologically unrelated to the isoforms of AAT previously characterized, and that is located in the plastid stroma. All the above data indicate the coexistence of two different forms of AAT in the plastid: a eukaryotic-type (Coruzzi, 2003) and a prokaryotic-type (de la Torre *et al*., 2006). It will be important to establish whether these enzymes have distinct roles in plastid N metabolism.

The characterization of *Arabidopsis* mutants (Miesak and Coruzzi, 2002; Schultz *et al*., 1998) confirmed that the cytosolic isoenzyme is involved in aspartate biosynthesis in the light to generate a pool to be used for asparagine synthesis in darkness. However, mutants deficient in the plastidic isoenzyme had no visible phenotype except that increased glutamine concentrations were observed, either in plants growing in the light or dark (Miesak and Coruzzi, 2002; Schultz *et al*., 1998). These results and recent functional genomic analysis of the aspartate gene family in *Arabidopsis* strongly suggest that the plastidic isoform has a role in shuttling reducing equivalents (Liepman and Olsen, 2004) as proposed for the phylogenetically related mitochondrial and peroxisomal isoenzymes (Winefield *et al*., 1995; Wadsworth, 1997).

If the plastidic PT-AAT is derived from an endosymbiont, the physiological role in plants might be related to the function that the enzyme plays in cyanobacterial N metabolism. In cyanobacteria, where an incomplete tricarboxylic acid cycle exists, carbon skeletons in the form of 2-oxoglutarate are channelled through the GS/GOGAT cycle for ammonium assimilation and net glutamate biosynthesis (Muro-Pastor *et al*., 2005). In these photosynthetic organisms, AAT enzyme activity coupled to the GS/GOGAT cycle represents an important reaction providing a range of amino acids for protein synthesis and other metabolic pathways. PT-AAT could play a similar role in the chloroplasts of higher plants utilizing glutamate for amino acid biosynthesis inside the organelle. In fact, the *PT-AAT* genes are predominantly expressed in green tissues of pine, *Arabidopsis*, and rice suggesting a key role of the enzyme in N metabolism associated with photosynthetic activity. Moreover, this novel form of AAT accumulated in parallel with the chloroplast development that takes place during the initial stages of pine growth as previously described for the GS/GOGAT enzymes (Cánovas *et al*., 1998; García-Gutiérrez *et al*., 1995; Palomo *et al*., 1998). In keeping with this suggestion, it has been found that the affinity of PT-AAT for glutamate (K_m 1 mM) (de la Torre *et al*., 2006) is much higher than that reported for plastidic and other eukaryotic AATs, where the K_m values range between 10 mM and 30 mM (Taniguchi *et al*., 1995; Wilkie and Warren, 1998). It is important to remember that about 80% of the total amino N in the chloroplast stroma under conditions of steady-state photosynthesis is contained in glutamine, glutamate, and aspartate (Riens *et al*., 1991). The concentration of glutamate in the chloroplast ranges between 15 mM and 50 mM (Riens *et al*., 1991; Winter *et al*., 1994), well above the K_m value of PT-AAT for this substrate. By contrast, the concentration of 2-oxoglutarate has been estimated to be in the micromolar range and even lower in the chloroplast stroma because the GS-GOGAT cycle represents a strong sink (Weber and Flugge, 2002). Consequently, a high glutamate/2-oxoglutarate ratio in the stroma would drive biosynthetic transaminations favouring aspartate biosynthesis. In this metabolic scenario, the two forms of plastidic AAT will be saturated and the flux to aspartate biosynthesis very high through the catalytic action of both enzymes. However, under physiological conditions where glutamate abundance is low, only the isoenzyme of prokaryotic origin would ensure the biosynthesis of aspartate-derived amino acids and other N compounds. A main pathway of aspartate utilization is the biosynthesis of the aspartate-derived amino acids lysine, threonine, isoleucine, and methionine, all of which are required for protein synthesis and produced exclusively in the plastid (Azevedo *et al*., 2006). Interestingly, all these amino acids are essential in the nutrition of animals where the PT-AAT does not exist (de la Torre *et al*., 2006). In addition to amino acid biosynthesis, aspartate could have other metabolic fates inside the plastid. For example, a recent report indicates that it is also involved in the biosynthesis of pyridine nucleotides via quinolinate. Thus, early steps in the biosynthesis of NAD in plants also start with aspartate and take place in the plastid (Katoh *et al*., 2006).

Metabolic organization of N metabolism in conifer cells

It is interesting to consider the compartmentation of the metabolic pathways leading to the biosynthesis of the major amino acids in conifers, since comparative studies between gymnosperms and angiosperms might reveal differential features in the metabolism of plants during evolution. In the photosynthetic cells of most angiosperms ammonium is assimilated inside the chloroplasts by the GS2/Fd-GOGAT cycle and therefore glutamine and glutamate are generated in the same subcellular compartment. In pine and other conifers, ammonium is assimilated in the cytosol and therefore glutamine and glutamate biosynthesis occurs in separate compartments. Such a cellular compartmentation will require glutamine/glutamate exchange through the plastidic membrane and experimental

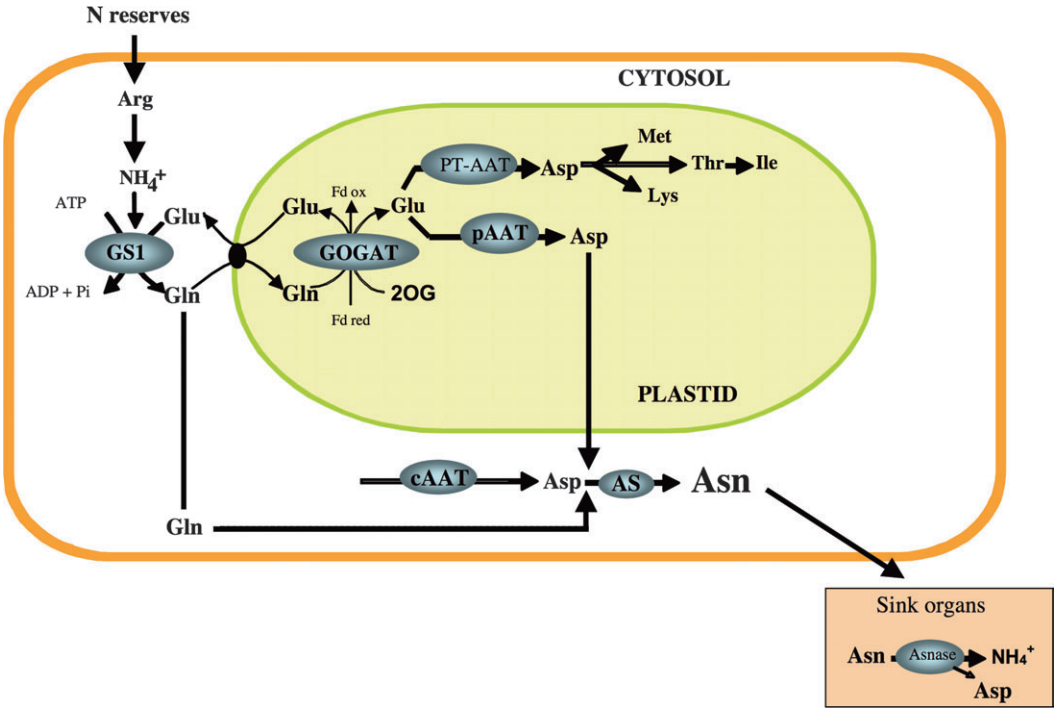

Fig. 4. Compartmentation of nitrogen metabolism in pine. Enzyme abbreviations: GS1, cytosolic glutamine synthetase; GOGAT, glutamate synthase; PT-AAT, prokaryotic type aspartate aminotransferase; pAAT, plastidic aspartate aminotransferase; cAAT, cytosolic aspartate aminotransferase; AS, asparagine synthetase; Asnase, asparaginase.

evidence for a putative translocator has been found in isolated chloroplasts from maritime pine cotyledons (MG Claros, FM Cánovas, unpublished data). As shown in Fig. 4, the biosynthesis of glutamine in the cytosol is coupled to asparagine metabolism in the same subcellular compartment (of the same or a different cell-type) for N storage and distribution within the plant. Asparagine metabolism via AS in source tissues and asparaginase in sink organs is critical for pine growth and development (Cañas *et al*., 2006, 2007).

Aspartate is synthesized from glutamate in the plastid and channelled through the aspartate metabolic pathway for the biosynthesis of lysine, threonine, isoleucine, methionine, and other essential N compounds. Consequently, metabolic processes for N storage and transport have their origin in the biosynthesis of the amides glutamine and asparagine located in the cytosol. On the other hand, glutamate and other amino acids required for protein biosynthesis and precursors of nitrogenous compounds are generated inside the plastid organelle. Interestingly, evolutionary analysis of the major enzymes involved in the pathways supports this metabolic organization. It is widely accepted that chloroplasts of higher plants originated from free-living cyanobacteria that became endosymbionts. Recent evolutionary analysis suggests that thousands of *Arabidopsis* genes have been transferred to the nuclear genome from a cyanobacterial endosymbiont (Martin *et al*., 2002). With regard to N metabolism, phylogenetic and functional analyses support that GOGAT and PT-AAT enzymes were acquired from the cyanobacterial ancestor of plastids and might therefore have the same evolutionary origin (Dincturk and Knaff, 2000; de la Torre *et al*., 2006). However, the enzymes involved in amide biosynthesis did not originate in the endosymbiont, and GS and AS genes in plants have eukaryotic origin (Suárez *et al*., 2002; Cañas *et al*., 2006). In this context, and in contrast to what is widely accepted, the subcellular distribution of metabolic pathways for ammonium assimilation and amino acid biosynthesis shown in Fig. 4 could represent a vestige of an early metabolic pathway. Later in evolution, glutamine biosynthesis could have moved to the chloroplast where the presence of the GS/GOGAT cycle would provide adaptive advantages to plants through enhanced photorespiratory ammonium assimilation inside the organelle.

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