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Review

Molecular aspects of nitrogen mobilization and recycling in trees

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Abstract

Plants have developed a variety of molecular strategies to use limiting nutrients with a maximum efficiency. N assimilated into biomolecules can be released in the form of ammonium by plant metabolic activities in various physiological processes such as photorespiration, the biosynthesis of phenylpropanoids or the mobilization of stored reserves. Thus, efficient reassimilation mechanisms are required to reincorporate liberated ammonium into metabolism and maintain N plant economy. Although the biochemistry and molecular biology of ammonium recycling in annual herbaceous plants has been previously reported, the recent advances in woody plants need to be reviewed. Moreover, it is important to point out that N recycling is quantitatively massive during some of these metabolic processes in trees, including seed germination, the onset of dormancy and resumption of active growth or the biosynthesis of lignin that takes place during wood formation. Therefore, woody plants constitute an excellent system as a model to study N mobilization and recycling. The aim of this paper is to provide an overview of different physiological processes in woody perennials that challenge the overall plant N economy by releasing important amounts of inorganic N in the form of ammonium.

Introduction

Nitrogen availability is a particular challenge for plant survival. In natural soils N is often a significant factor limiting plant growth and development, so different metabolic regulations and interactions have been evolved to guarantee the strict economy of this essential nutrient during the plant life cycle. N assimilated into biomolecules can be released back to inorganic nitrogen (ammonium) in different physiological contexts in the plant cells (Miflin and Lea 1980). In some particular cases ammonium release is a process intrinsic to a metabolic pathway, as is the case for photorespiration or the biosynthesis of phenylpropanoids; in others, the release of ammonium is part of a general process to remobilize N from plant storage material to metabolic sinks in developing organs.

High amounts of ammonium are released during the regeneration of 3-phosphoglycerate in the photorespiratory pathway (Keys et al. 1978). In fact, the conversion of glycine to serine in the C2 cycle is probably the most important metabolic process that liberates ammonium in photosynthetic cells. It may represent an order of magnitude more N than the amount corresponding to primary assimilation through nitrate reduction (Miflin and Lea 1980). The importance of N recycling in this process is so great that mutants with reduced activity of the enzymes involved in the reassimilation of photorespiratory ammonium are unable to grow under ambient CO_2 conditions (air), exhibiting severe synthoms of N deficiency.

However, these mutants are perfectly viable when plants are transferred to high CO_2 conditions that suppress photorespiration (Somerville and Ogren 1980; Wallsgrove et al. 1987).

In addition to photorespiration, the metabolism of phenylpropanoids is also an important source of ammonium in plant cells. N is removed from phenylalanine or tyrosine, as the ammonium ion, via the reaction catalyzed by the enzyme phenylalanine-ammonia lyase (PAL). A product of the phenylpropanoid pathway is lignin, the second most abundant organic compound in the biosphere after cellulose. In cells with a high rate of phenylpropanoid synthesis, such as those undergoing lignification, the amount of liberated ammonium can be so extensive that an efficient system of N recycling must exist to avoid severe N deficiency. As lignin constitutes a major fraction of wood this N recycling is of crucial importance for the growth and development of woody plants (Suárez et al. 2002).

In different periods during the life cycle, plants have to deal with the mobilization of material from nutrient reserves to provide developing organs with the required amounts of C and N for growth. In these crucial periods when a large number of metabolites are required to support rapid growth, a tight regulation must exist to coordinate the expression of genes involved in reserve mobilization with those encoding enzymes that are able to use the mobilized material. Germination of seeds is one of these processes involving mobilization and reassimilation of large amounts of storage material. Following seed germination, seed storage proteins break-down and amino acid hydrolysis release enormous amounts of ammonium, which in turn must be reassimilated to synthesize all N-containing molecules required for plantlet growth (Limami et al. 2002). Plants have also evolved mechanisms to store nutrients in excess and/or recycle N that might otherwise be lost from various causes, for instance during leaf senescence or pathogenic attack. Vegetative storage proteins (VSPs) are proteins that accumulate in vegetative tissues such as leaves, stems and, depending on plant species, tubers, when excess resources are available and serve as a temporary reservoir of amino acids for use in subsequent phases of growth and development (Stepien et al. 1994).

Whatever the source of ammonium release this N will go back into the pool of N-containing molecules as a result of the reactions catalyzed by the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Miflin and Lea 1980). The end products of this cycle, glutamine and glutamate, are the N donors for the biosynthesis of major N compounds in plants including other amino acids, nucleic acid bases, polyamines and chlorophylls. Therefore, the combined activities of these two enzymes play a key role in mobilization/recycling to respond to N demand during vegetative and reproductive growth and potentially contribute to N use efficiency.

The metabolic requirements for GS activity (EC 6.3.2.1) in plants are fulfilled by different GS isoforms. Two different classes of GS have been reported in angiosperms: GS1 in the cytosol and GS2 in the chloroplasts. In most plants GS2 is encoded by a single gene whereas GS1 isoforms are the products of a small family of nuclear genes that are differentially expressed during development and in response to different external stimuli (Cren and Hirel 1999; Ireland and Lea 1999). In many species GS2 is highly abundant in mesophyll cells of the leaf where it is involved in assimilation of ammonia from the reduction of nitrate, and in recycling the ammonium released during photorespiration. GS1 is a minor enzyme in leaves but is highly abundant in the vascular elements of roots, nodules, flowers and fruits. The proposed role for GS1 is the primary assimilation of ammonium and the biosynthesis of glutamine for N transport. In N-fixing symbiosis, nodule-specific GS1 isoforms are involved in the assimilation of ammonium derived from N_2 fixation (Ireland and Lea 1999). The localization of GS1 in the mesophyll cells has also been demonstrated (García-Gutiérrez et al. 1998; Pérez García et al. 1998) and a number of reports in the past few years supported a role for GS1 in plant development including chloroplast development (García-Gutiérrez et al. 1998; Suárez et al. 2002), senescence (Brugière et al. 2000), the response to biotic and abiotic stress (Pérez-García et al. 1998) and growth (Gallardo et al. 1999; Fuentes et al. 2001; Fu et al. 2003). GS1 genes have been found to co-localize with quantitative trait loci (QTL) for yield components in the plant genome (Hirel et al. 2001; Obara et al. 2001). All these recent 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.

Glutamate synthase (GOGAT) exists in plants as two distinct molecular forms which differ with respect to the source of reductant for enzyme catalysis: NADH-GOGAT (EC 1.4.1.14) and ferredoxin (Fd)-GOGAT (EC 1.4.7.1). Both enzymes are iron–sulphur flavoproteins displaying different physico-chemical, immunological and regulatory properties and are encoded by separate genes (Temple et al. 1998; Lea and Miflin 2003). Fd-GOGAT is the predominant molecular form in photosynthetic tissues where it is located in the chloroplast stroma of mesophyll cells. Biochemical and genetic analysis indicate that Fd-GOGAT plays an essential role, in coordination with GS2, in the reassimilation of photorespiratory ammonium. NADH-GOGAT is particularly abundant in the vascular bundles of roots and shoots as well as in nodules. The enzyme plays a significant role in the assimilation of ammonium derived from symbiotic N_2 fixation or nitrate reduction or from direct uptake (Temple et al. 1998; Lea and Miflin 2003). Its participation in the remobilization of N has recently been reported (Yamaya et al. 2001).

The role of the GS/GOGAT cycle and the functional compartmentation of GS activities in recycling ammonium in several crop plants has been extensively reviewed. However, recent progress on the regulation of these enzymes and their encoding genes in physiological contexts involving N-recycling in woody plants still need to be covered. This paper will present an overview of such processes in woody perennials, where mobilization and recycling of N are critical events, with special emphasis on those particularly characteristic of this group of plants. For example, lignification or the N storage in vegetative organs during dormancy become specially relevant as part of the plant general strategy to survive and complete its life cycle. Moreover, in some woody perennials such as trees, these processes are quantitatively massive so that woody plants constitute an excellent system as a model to study N mobilization and recycling.

Recycling of N released in photosynthetic metabolism

As with herbaceous plants, recycling of ammonium liberated in photorespiration is critical for woody perennials N economy. In herbaceous angiosperm C_3-type plants, the reassimilation of photorespiratory ammonium is located in the chloroplast, where glutamine synthetase (GS2) and ferredoxin-glutamate synthase have the available ATP and reducing power required for their enzymatic activities. The roles of these two enzymes determine the regulation pattern of their genes: both are induced by light through phytochrome and it has been shown that GS2 gene expression is also affected by photorespiratory activity (Edwards and Coruzzi 1989; Coschigano et al. 1998). Biochemical and molecular characterization of GS and GOGAT from several woody angiosperms and the gymnosperm *Ginkgo biloba* (Suárez et al. 2002) indicate that these enzymes may play similar roles to those found in annual herbaceous plants. Thus, the GS2/Fd-GOGAT cycle located within the chloroplast would be involved in the recycling of photorespiratory ammonium in photosynthetic cells.

However, recent findings indicate that in conifers a different situation can be found. In photosynthetic tissues of pine seedlings and other conifers, only cytosolic isoforms of GS have been identified, and various biochemical and molecular approaches have failed to detect a chloroplastic isoform (Cánovas et al. 1991, 1998; Cantón et al. 1993). Although two different isoforms of GS have been characterized in pine seedlings (GS1a and GS1b), both of them are located in the cytosol (Avila et al. 1998; García-Gutiérrez et al. 1998). Nevertheless, the expression of GS1a in conifers resembles the expression pattern of GS2 in angiosperms. This cytosolic isoform is expressed specifically in photosynthetic cells and in a similar way to GS2, GS1a mRNA and polypeptide accumulate preferentially in the light and their levels drop in darkness (Cantón et al. 1999). Moreover, GS1a expression depends on chloroplast integrity as occurs with nuclear genes encoding photosynthetic proteins SSU and LHC2b (Cantón et al. 1999). Comparison with the GS amino-acid sequences from angiosperms showed that GS1a contains amino-acid residues exclusively present in the GS2 polypeptide. On the other hand, another cytosolic GS characterized in pine, GS1b, is more similar to cytosolic GS from angiosperms than to GS1a in the same species (Avila et al. 2000). Moreover, phylogenetic analysis of GS genes suggested that GS1a is unique to gymnosperms

(Suárez et al. 2002). The particular expression pattern together with the specific structural characteristics of the cytosolic GS1a suggest a role for this isoform in reassimilating ammonium liberated in photorespiration (Cantón et al. 1999; Avila et al. 2001). Overexpression of GS1a in poplars conferred enhanced vegetative growth to the transgenic lines (Gallardo et al. 1999) and this was a consequence of transgene expression and assembly of GS1a subunits into a new functional holoenzyme in the cytosol of poplar photosynthetic cells (Fu et al. 2003). The ectopic expression of the enzyme in this particular cell type could improve the capacity of the leaf to recycle ammonium released in secondary processes such as photorespiration (Gallardo et al. 2003).

The location of Fd-GOGAT in the chloroplast of pine photosynthetic cells (García-Gutiérrez et al. 1995) implies a separation of glutamine and glutamate biosynthesis in different subcellular compartments in conifer photosynthetic cells, and suggests the existence of an active glutamate/glutamine exchange between cytosol and chloroplast to maintain a functional GS/GOGAT cycle able to cope with the flux of ammonium from photorespiration. Possible models for the transport of 2-oxoglutarate, glutamate and glutamine into conifer plastids have been proposed (Weber and Flügge 2002), and there is experimental evidence supporting the existence of a translocator in the chloroplast membranes of *Pinus pinaster*, that may be responsible for the import of glutamine into the organelle in antiport with glutamate (Suárez et al. 2002).

Recycling of N released in the phenylpropanoid metabolism

The phenylpropanoid/phenylpropanoid-acetate pathway is responsible for the synthesis of a major group of structural and nonstructural constituents in vascular plants, such as lignins, lignans, flavonoids, suberins and tannins. These plant phenolic compounds play various essential roles for the successful adaptation of vascular plants to land: structural support, pigmentation, defense and signalling. The relevance of this pathway is not only qualitative but also quantitative, as 30 to 45% of plant organic matter is derived from the phenylpropanoid/phenylpropanoid-acetate pathway. Therefore, vascular plants divert large amounts of carbon into the biosynthesis of this class of product. The amino acid phenylalanine, and to a much lower extent tyrosine, are the only donors for the phenylpropane skeleton in this metabolic pathway. The PAL enzyme catalyzes the deamination of phenylalanine and tyrosine to cinnamic and *p*-coumaric acids, respectively. This is a crucial metabolic step connecting primary N metabolism through the shikimate pathway with the allocation of carbon for the biosynthesis of phenylpropanoids. In woody perennials, most metabolic flux through this pathway leads to the biosynthesis of lignin, an important constituent of wood. PAL is encoded by a multigene family in trees (Butland et al. 1998), as previously reported in annual plants, but the specific physiological and biochemical roles of individual gene members remain to be determined. The metabolic reaction catalyzed by PAL has been proposed as a rate-limiting step in the phenylpropanoid pathway. However, recently, it has been shown that the availability of phenylalanine may limit carbon allocation to lignin biosynthesis in *Pinus taeda* (Anterola and Lewis 2002). These results suggest the existence of regulatory steps upstream of the reaction catalyzed by PAL. It is, therefore, possible that primary N assimilation and/or the reassimilation of ammonium represent control points for the regulation of lignin biosynthesis. This assumption is supported by the report of active N recycling in lignifying pine cells via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway (van Heerden et al. 1996). In fact, in cells undergoing active lignification an efficient N recycling mechanism should be operative to avoid severe N deficiency.

Phenylalanine deamination

Recent studies carried out in lignifying cells have provided new data on the biochemistry of this important process. The administration of [^{15}N]-L-phenylalanine to potato discs in the light has been used to trace the metabolic fate of ammonium liberated in the PAL reaction (Razal et al. 1996). Using nuclear magnetic resonance (NMR) spectroscopic analyses it was shown that the α-amino N released from phenylalanine was first incorporated into the amide group of glutamine and latter into glutamate. The generation of labelled

glutamine and glutamate was prevented when the incubation was performed in the presence of methionine-S-sulphoximine, a specific inhibitor of GS activity. In *P. taeda* cell cultures the incubation with high levels of sucrose induces an active synthesis of lignin (van Heerden et al. 1996). This experimental system was used to clarify the mechanism for recycling of ammonium during phenylpropanoid metabolism in lignifying cells of a woody plant. The metabolic fate of externally supplied N in different forms was traced by combining high-performance liquid chromatography and ^{15}N NMR and gas chromatography-mass spectroscopy. When cell cultures were incubated with ^{15}N-phenylalanine an increase in labelled glutamine and glutamate was detected in the first 24 h while additional increases in serine and alanine were detectable later, after a 96-h incubation period. After the addition of L-α-aminooxy-β-phenylpropionic acid (L-AOPP), a specific PAL inhibitor, the amount of ^{15}N-phenylalanine remained essentially constant in the cells indicating that phenylalanine was mainly committed to phenylpropanoid biosynthesis via the PAL reaction. The addition of GS or GOGAT specific inhibitors provoked the accumulation of ^{15}N either in the form of released NH_4^+ or in glutamine respectively, beside the α-amino N of phenylalanine, indicating that ammonium recycling was inhibited (van Heerden et al. 1996). Similar experiments were carried out by feeding the pine cell cultures with either $^{15}NH_4Cl$, $^{15}N(\delta)$-glutamine or ^{15}N-glutamate in the presence of the PAL inhibitor L-AOPP, to clearly show that a major amount of the provided N was destined to phenylalanine synthesis. Therefore, the authors concluded that in active lignifying cells a phenylpropanoid-N cycle must exist involving the enzymes PAL, GS and possibly GOGAT with two major characteristics: (i) ammonium liberated by PAL reaction during active lignin biosynthesis is rapidly recycled by sequential GS and GOGAT activities. The synthesized glutamate is conscripted to the synthesis of the phenylalanine precursor arogenate; (ii) when exogenous N is supplied in the form of NH_4Cl, glutamine or glutamate, this is made available not only for phenylalanine synthesis, but also for general amino acid/protein synthesis. However, when phenylalanine was provided as the unique N source, it was transferred mainly, if not exclusively, to glutamine and glutamate at first and later also to serine and alanine. Therefore, N released during phenylalanine deamination is not made available for general amino acid/protein synthesis, but instead appears to be strictly destined to synthesize arogenate for phenylalanine regeneration (Figure 1). These results suggest that the phenylpropanoid-N cycle is a tightly compartmentalized process and separated from the general N metabolism in actively lignifying cells, so that the cells can maintain high rates of lignification without causing a collapse of N content in the plant.

C1 metabolism

During the conversion of cinnamic acid to monolignols, *O*-methylation reactions introduce methyl groups in specific positions. Therefore cells undergoing lignification must consume vast amounts of methyl groups. Two *O*-methylases have been identified as specifically involved in methylation of lignin precursors: caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT) and caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (Zhong et al. 1998; Anterola and Lewis 2002). In both reactions S-adenosylmethionine (SAM) is consumed as methyl group donor. The enzyme catalyzing SAM synthesis, S-adenosylmethionine synthetase, is considered a housekeeping protein owing to the multiple cellular processes consuming SAM as methyl group donor (Hanson and Roje 2001). Nevertheless, enzymatic activity measurements in poplar (Vander Mijnsbrugge et al. 1996), and the relatively high abundance of

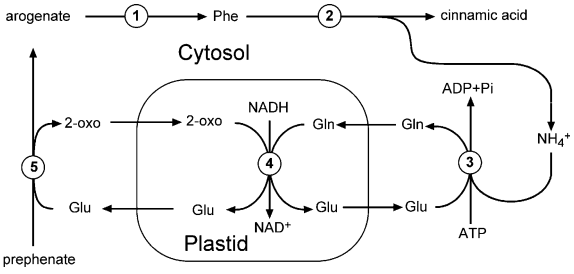

Figure 1. Nitrogen recycling in *Pinus taeda* during active phenylpropanoid metabolism Enzymes are as follows: (1) arogenate dehydratase; (2) PAL, phenylalanine ammonia-lyase; (3) GS, glutamine synthetase; (4) NADH-GOGAT, NADH-glutamate synthase; (5) prephenate aminotransferase. Redrawn from Van Heerden et al. (1996) with modifications. 2-oxo, 2-oxoglutarate.

S-adenosylmethionine synthetase ESTs in developing-xylem libraries from poplar (Sterky et al. 1998) and pine (Whetten et al. 2001) indicate that high levels of this enzyme must be required in xylem to provide methyl groups for monolignol synthesis. It has been estimated that in woody plants, lignin biosynthesis demands 10-fold more one-carbon units than all of primary metabolism combined (Hanson and Roje 2001). SAM synthesis requires 5-methyl-tetrahydrofolate to produce the SAM precursor methionine in the reaction catalyzed by methionine synthase (Hanson and Roje 2001). This 5-methyl-tetrahydrofolate can be produced by conversion from 5,10 methylene-tetrahydrofolate in the reaction catalyzed by 5,10 methylene-tetrahydrofolate reductase (MTHFR) (Roje et al. 1999).

The amino-acid serine is at the basis of C1 metabolism in many biological systems (Mouillon et al. 1999). In plants, it has been proposed that the cytosolic or plastidial conversion of serine into glycine, catalyzed by serine hydroxy-methyl transferases (SHMT), could be an important (if not the main) one-carbon source in plant C1 metabolism. Therefore serine catabolism would mainly be driven by the need for CH2-THF synthesis as methyl donor (Figure 2). As a result of this metabolic process, glycine is formed which would be recycled to serine in mitochondria by the glycine decarboxylase (GDC)-SHMT system. Serine synthesized in plastids from 3-P-glycerate could also contribute to C1 metabolism (Ho and Saito 2001). Therefore, a rapid regeneration of THF from CH2-THF by SAM synthesis together with serine accumulation by rapid glycine oxidation in the mitochondria and synthesis in the plastid will push the equilibrium of the reaction catalyzed by cytosolic SHMT to glycine and CH2-THF synthesis, providing therefore C1 units for lignin synthesis among other metabolic needs (Figure 2).

In consequence, the levels of ammonium released during active lignin synthesis by the high levels of mitochondrial GDC-SHMT activity required for glycine recycling to serine must also compromise the N economy in a woody perennial, specially in trees where trunks are organs of massive wood production (Figure 2). Therefore it is expected that an active reassimilation mechanism would be operative, also avoiding the severe N losses that otherwise would occur.

Several lines of evidence support this hypothesis:

(1) Studies of non-photosynthetic sycamore cambial cells support a role of serine and glycine as sources of C1 units in plants, via glycine decarboxylation in mitochondria by GDC and serine synthesis by mitochondrial SHMT and serine-to-glycine conversion by most probably cytosolic SHMT to provide the cytosol with the required levels of CH2-THF for SAM synthesis (Mouillon et al. 1999).
(2) Enzymes involved in SAM synthesis, together with GDC, SHMT and GS are among the most abundant protein/transcript in developing xylem in poplar and pine, as deduced from proteomic and EST data (Whetten et al. 2001; Vander Mijnsbrugge et al. 2000)
(3) In differentiating secondary xylem of pine and poplar all the genes involved in the C1/N cycle are expressed, as shown by the presence of ESTs in the databases.
(4) Expression levels of some of these genes are higher in developing xylem with high lignin content (compression wood) than in xylem with lower amount of lignin (normal wood). This conclusion comes from the analysis of DNA arrays and EST relative abundances (Table 1).

The enzymes for ammonium reassimilation

All the above mentioned findings suggest the occurrence of molecular mechanisms for a tightly coordinated expression of genes involved in lignin synthesis, SAM production and N recycling.

The enzymes involved in the reassimilation of ammonium in vascular tissues have been characterized in conifers. The isoform of glutamine synthetase, GS1b, is highly abundant in pine hypocotyls and roots, although is also present at a low level in the cotyledons. In all these tissues, GS1b expression is associated with vascular bundles (Avila et al. 2001; Suárez et al. 2002); the pattern is quite similar to that found for GS1 in angiosperms (Dubois et al. 1996) and suggests that GS1b plays an important role in N transport and translocation. However, other functional roles for this isoform in tree biology cannot be ruled out. For example, in pine seedlings the abundant and precise localization of gene expression in the vascular bundles supports a role for the *GS1b* gene product in N recycling associated with lignin

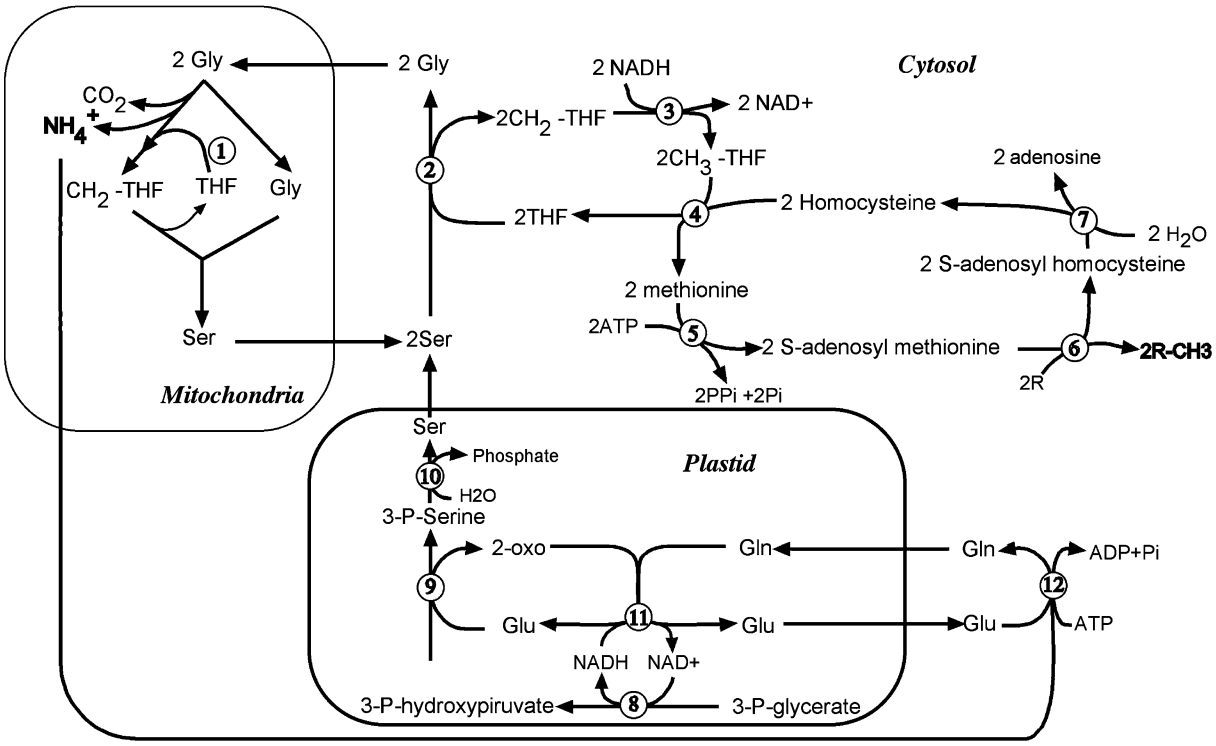

Figure 2. Proposed scheme for Nitrogen/C1 metabolism. The mitochondrial catabolism of glycine together with the plastidial metabolism of 3-P-glycerate provide the cytosol with serine, the donor of C1 subunits. Ammonium released by the activity of the mitochondrial glycine decarboxylase complex will be recycled by glutamine syntetase/NADH-glutamate synthase to generate glutamate which in turn would contribute to Ser biosynthesis in the plastidial compartment. THF, tetrahydrofolate. Enzymes are as follows: (1) glycine decarboxylase/serine hydroximethyltransferase; (2) glycine hydroximethyltransferase; (3) methylene tetrahydrofolate reductase; (4) methionine synthase; (5) S-adenosylmethionine synthetase; (6) methyl transferase; (7) S-adenosylhomocysteine hydrolase; (8) 3-phosphoglycerate dehydrogenase; (9) 3-phosphoserine aminotransferase; (10) 3-phosphoserine phosphatase; (11) NADH-glutamate synthase; (12) glutamine synthetase.

biosynthesis in xylem cells. In fact, *GS1b* transcripts and polypeptide accumulate in developing xylem cells where the activities of PAL and the enzymes involved in C1 metabolism are high, and therefore ammonium is liberated. In keeping with this role of GS1b isoform, the proximal region of the *GS1b* promoter contains AC elements (Gómez-Maldonado et al. 2003), which are frequently found in the promoters of genes encoding enzymes involved in vascular development and lignin biosynthesis, particularly PAL (Gray-Mitsumune et al. 1999). The functional significance, if any, of such putative regulatory elements is currently being examined.

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 should be the enzyme involved in the reassimilation of ammonium in xylem cells. Although the enzyme has not been characterized in woody plants, ESTs for NADH-GOGAT are present in the ESTs data base from poplar and pine xylem (http://www.biochem.kth.se/PopulusDB; http://pinetree.ccgb.umn.edu; http://cbi.labri.fr/outils/SPAM/index.php). The participation in the recycling process of other auxiliary enzymes cannot be excluded. For example, glutamate dehydrogenase has recently been reported to be present in vascular cells (Dubois et al. 2003) and the expression of asparagine synthetase and aspartate aminotransferase is supported by the existence of ESTs in the data banks.

Mobilization and recycling of N contained in seed storage proteins

Again most detailed studies on N mobilization and recycling in woody plants have been performed in

Table 1. Genes encoding enzymes of serine metabolism, C1 metabolism and nitrogen assimilation expressed in developing xylem of trees (Whetten et al. 2001)

Gene	Xylem EST	Differentially expressed (compression > normal wood)	Evidence
Glycine decarboxylase	Yes	Yes	EA, M
Serine hydroxymethyltransferase	Yes	Yes	EA, M
Methylene-tetrahydrofolate reductase	Yes	?	–
Methionine synthase	Yes	Yes	EA
S-Adenosylmethionine synthetase	Yes	Yes	EA, M
Methyl transferase	Yes	?	–
S-Adenosylhomocysteine hydrolase	Yes	Yes	EA
Phosphoglycerate dehydrogenase	Yes	?	–
Phosphoserine aminotransferase	Yes	?	–
3-phosphoserine phosphatase	Yes	?	–
NADH-glutamate synthase	Yes	–	–
Glutamine synthetase	Yes	Yes	EA

EA: differential expression detected by ESTs relative abundance. M: differential expression detected by microarray analysis.

conifers. Mature gymnosperm seeds enclose the embryo within the megagametophyte, a maternally derived tissue that synthesizes metabolic reserves during seed maturation. Although reserve proteins are present in both embryo and megagametophyte, most of them are contained in the latter representing a major N reserve of the seed (Groome and Axler 1991; Stone and Gifford 1997). After initiation of seed germination, amino acids resulting from hydrolysis of storage-proteins do not accumulate in the megagametophyte, but are rapidly and efficiently mobilized to the embryo (King and Gifford 1997). Mobilization of storage proteins by the megagametophyte and uptake by the developing seedling are possibly owing to a close physical relationship between them throughout stratification, germination and early growth (Stone and Gifford 1997). Although the germinative period as well as the seedling growth rate vary notably among gymnosperm species, the mobilization of reserve proteins in the megagametophyte is synchronized with radicle emergency from the seed, in a process that is independent of the embryonic storage protein break-down (King and Gifford 1997; Todd and Gifford 2002). As the embryonic protein reserves, a minor component, are completely exhausted following germination (Groome and Axler 1991; Stone and Gifford 1997), the amino acids imported from the megagametophyte constitute the primary N source during early seedling development, supporting its rapid growth once embryo reserves are depleted and before photosynthetic machinery is functional (Lammer and Gifford 1989).

Protein mobilization during seed germination depends upon both activation and synthesis of enzymes. The depletion of protein reserves during development has been shown to be accompanied by changes in enzyme activities in a number of gymnosperm species such as *Araucaria* (Cardemil and Reinero 1882), *Larix* (Pitel and Cheliak 1986), *Picea* (Gifford and Tolley 1989) and *Pinus* (Gifford et al. 1989; Groome and Axler 1991). Protein synthesis appears to be also obligatory for seed germination in most, if not all species (Bewley and Makus 1990). New proteins are synthesized coinciding with the loss of seed dormancy by the megagametophyte and following germination by the embryo (Schneider and Gifford 1994). Changes in both mRNA and protein profiles are developmentally regulated in the megagametophyte and the embryo during germination and early seedling growth and reflect that the rate of reserve protein hydrolysis is driven by the level of proteolytic enzymes involved in storage protein break-down (Groome and Axler 1991; Mullen et al. 1996).

In conifers, seed reserve proteins are rich in amino acids with a low C/N ratio (Allona et al. 1994; Feirer 1995), making them particularly suited for storage and transport of N (Bray 1983). Arginine, the amino acid with the highest content

in N (four atoms per molecule), constitutes a large portion of the amino acid pool in the seed reserve proteins, particularly in conifer seeds. Arginine represents more than 23% (accounting for over 46% of the total N) in the megagametophyte reserves of loblolly pine (King and Gifford 1997). Similar arginine content has been found in the reserves of other conifer seeds, including maritime pine (Allona 1994), eastern white pine (Feirer 1995) and Douglas fir (Feirer 1995). Following germination, arginine accumulates in the seedling (King and Gifford 1997), suggesting that this amino acid likely represents the major source of N for the biosynthesis of nitrogenous compounds.

Enzymatic activity and expression of enzymes related to N metabolism have been analyzed in detail during conifer seed germination and post-germinative growth and used as an approach to identify functional roles of individual enzymes (Figure 3). The accumulation of N within arginine in the germinating embryo is accompanied by a marked increase in both arginase activity (King and Gifford 1997) and arginase transcripts (Todd et al. 2001) in loblolly pine. Arginase transcript, protein and activity are localized in the expanding cotyledons, structures closely in contact with the megagametophyte during early seedling development. The further hydrolysis of urea by the enzyme urease has been reported in loblolly pine (Todd et al. 2001), supporting that arginine catabolism is an important source of ammonium throughout early seedling development. GS1b has been reported as the enzyme incorporating the ammonium into glutamine at early stages of germination of Scots pine (Suárez et al. 2002), converting two of the four N atoms in the arginine molecule to a form that is easily used for the biosynthesis of nitrogenous compounds. High levels of *GS1b* expression in the embryo hypocotyl precedes vascular element formation, suggesting the function of GS1b in N translocation from the central and basal area of the embryo to the radicle, where a high input of N is required for radicle expansion and differentiation. In the embryo, the expression level of GS1a (Garcia-Gutiérrez et al. 1995; Avila et al. 2001), Fd-GOGAT (Garcia-Gutiérrez et al. 1995) and NADH-GOGAT (A. García-Gutiérrez and F.M. Cánovas, unpublished data) is very low and presumably none of these genes is involved in glutamate synthesis at early embryo germination stage. Glutamate dehydrogenase, an important enzyme during seed germination (Melo-Oliveira et al. 1996), has been proposed as an alternative enzyme providing the

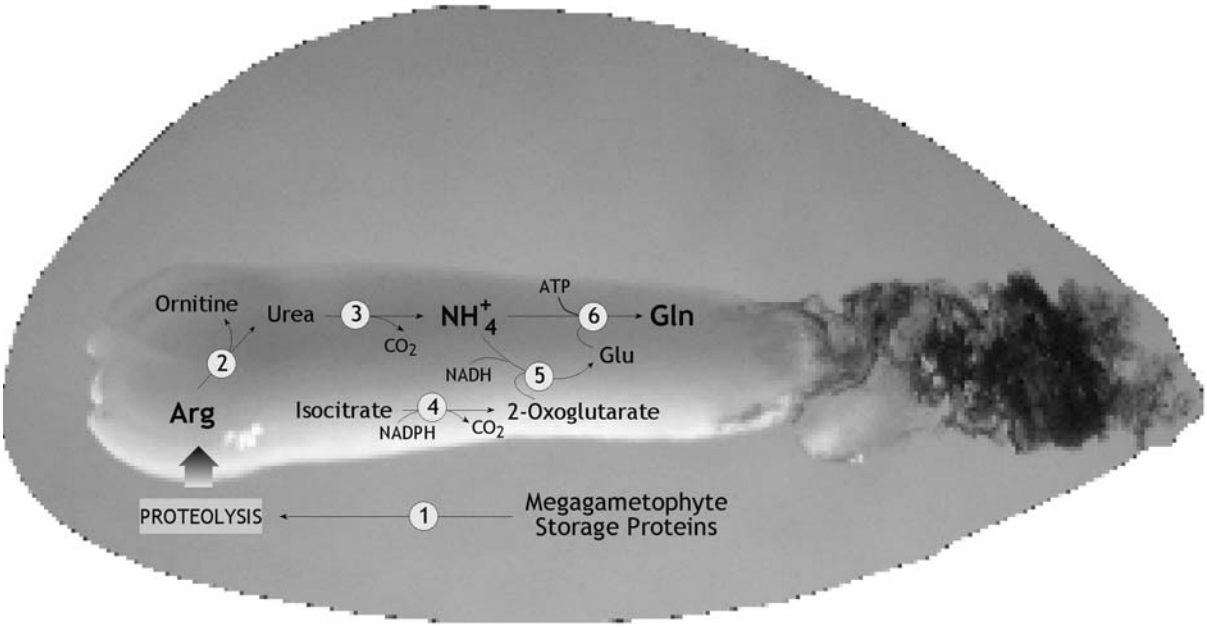

Figure 3. Enzymes of nitrogen metabolism in germinating seeds of conifers. (1) proteases; (2) arginase; (3) urease; (4) isocitrate dehydrogenase; (5) glutamate dehydrogenase; (6) glutamine synthetase.

glutamate necessary for glutamine synthesis. NADP isocitrate dehydrogenase, active in germinating embryos (Palomo et al. 1998), could supply the carbon skeleton in the glutamate dehydrogenase reaction (Avila et al. 2001).

Both arginase and glutamine synthetase (reviewed in Suárez et al. 2002) enzymes are under strong developmental control in conifers. In order to study the mechanisms triggering these enzymatic activities, model systems for *in vitro* analysis have been developed. The regulation of loblolly pine arginase has been studied using zygotic embryos in an *in vitro* culture system (Todd and Gifford 2002). The zygotic embryo itself is able to initiate arginase gene expression, but the presence of the megagametophyte is necessary for maintaining or up-regulating arginase expression (Todd and Gifford 2002) probably because arginine as such regulates arginase transcripts levels (Todd and Gifford 2003). To study the mechanisms involved in the transcriptional regulation of these two enzymes, the molecular and functional characterization of their gene promoters has been initiated (D. Brownfield and D.J. Gifford, personal communication; Gómez-Maldonado et al. 2004). *GS1b* gene expression is enhanced by exogenously supplied gibberelic acid (GA) in germinating pine embryos and pine seedlings. Deletion analysis of the *GS1b* promoter revealed that sequences containing the GA-responsive element, located between −1005 and −724 bp were essential for the increased promoter activity observed in response to GA (Gómez-Maldonado et al. 2004).

Mobilization and recycling of N contained in vegetative storage proteins

In many trees the N and C present in leaves are mobilized during autumn and stored in perennial tissues to be remobilized at the beginning of the next growing season. Vegetative storage proteins (VSPs) play a key role in this process; they are synthesized and accumulated to recycle carbon and N that would otherwise be lost as litter (Stepien et al. 1994). Two genes for VSPs were first analyzed in soybean by Mason and Mullet (1990). In this species the expression of genes encoding VSPs is induced by source/sink relationships, high N supply, water stress, wounding and treatment with jasmonic acid. There have also been recent advances in the knowledge of VSPs in woody plants, mainly in poplar (Coleman and Chen 1993; Lawrence et al. 1997). Most of the work concerning VSP in woody perennials has been carried out in broadleaved temperate deciduous trees, where overwintering VSPs accumulated in the stem play a critical role in recycling the N from senescing leaves in autumn in order to support spring growth (Stepien et al. 1994) However, the occurrence of overwintering VSPs in conifers suggests that N storage and recycling in vegetative organs is not restricted to broadleaved trees (Roberts et al. 1991; Harms and Sauter 1992).

Therefore, it seems that a general strategy for trees is the use of VSPs to obtain readily accessible N for initial stages of budbreak. However, the high variability among species and dormant tissues concerning the contribution of identified VSPs to the total N remobilized and the fact that the decline in the VSP content is insufficient to explain the spring fall in total protein content, has led to the suggestion that other type of proteins contribute to the general N mobilization (Gomez and Faurobert 2002). Moreover, it has been suggested that the VSP role in growth initiation could be more qualitative than quantitative, because in the perennial plant model soybean and in the woody perennial peach trees VSP are not the major N storage form (Gomez and Faurobert 2002). However, owing to their storage yield in winter and strong and early remobilization during spring regrowth, these proteins must play an important role in spring growth initiation.

Whatever the specific role of VSP, it seems clear that a large amount of N is remobilized from storage to sink after break dormancy (Millard et al. 1998; Malaguti et al. 2001), and this is an essential process in the annual dormancy/growth cycle in woody perennials. It has been estimated that N remobilized from storage provides up to 87% of the required N during the initial steps of growth of spur leaves in apple trees, just until net uptake becomes the major contribution of N for tree growth (Malaguti et al. 2001). After breakdown of the reserves, the released N must be transported from sources to sink in the form of soluble N-compounds during spring foliation. It has been shown that during bud burst and initial leaf growth, N concentration in the xylem sap

reaches a peak, which was attributed to remobilization (Glavac and Jockheim 1993; Schneider et al. 1994). The most common form of N transport reported in the xylem sap of different tree species are the amides asparagine and glutamine, citrulline and arginine (Malaguti et al. 2001). The relative importance of each of these amino acids varies among species. For example, in apple trees asparagine accounts for more than half the total amino acid N in the sap during remobilization, followed by glutamine and aspartate (Malaguti et al. 2001). On the contrary, glutamine is the major form in which N is translocated in xylem sap of *Prunus* (Youssefi et al. 2000), *Populus* (Schneider et al. 1994), and the conifers *Picea abies* (Stoerner et al. 1997) and *Pinus* spp (Barnes 1963). Citrulline has also been described as the predominant N compound in xylem sap from various tree species (Barnes 1963; Millard et al. 1998) whereas arginine and glutamine alternate as the main N compound in xylem saps from a broad range of species in Australia between the dry and wet season (Schmidt and Stewart 1998).

Whatever the form of N transport, glutamine is required for its synthesis and, therefore, glutamine biosynthesis should be an essential process for stored N remobilization during spring growth in trees. The specific pathways and enzymes involved in mobilization of N in adult woody perennials are still largely unknown, but stored N contained in protein must be mobilized through proteolytic and hydrolytic activities to yield ammonium. GS, NADH-GOGAT and possibly GDH activities are key players in the seasonal N cycling in apple trees (Titus and Kang 1982). Although no molecular analyses of the GS isoforms were undertaken in this study, cytosolic GS was probably involved. The conservation of N mobilized during autumnal senescence in woody tissues of the tree and reutilization in the spring for new growth are critical events for overall N economy. The application of the now available major breakthrough technologies for global analysis of gene expression could provide new and valuable knowledge on these important processes.

Concluding remarks and future prospects

Considerable progress has been made in recent years on different molecular aspects of N mobilization and recycling in woody plants and this new knowledge is of crucial importance for a better understanding of fundamental tree biology. Some of the key genes involved in these processes have now been characterized and studies of gene expression and functional analysis in transgenic trees provide new insights on their specific roles. However, additional research is needed to elucidate the molecular nature of other genes/proteins involved in N mobilization and recycling and how they are regulated during tree development and by metabolites. For example, very little is known on the spatial and temporal regulation of genes involved in lignin biosynthesis and N metabolism during secondary xylem development. Molecular studies are also needed on the localization and role of key enzymes in protein mobilization during the onset of dormancy and resumption of active growth in long lived woody perennials.

Another important area of research in the near future will deal with C/N balance. In order to maintain a balance between C and N metabolism, plant cells should be able to sense changes in the C/N ratio and convert this information into molecular signals that are able to alter specific cellular activities, such as enzymatic activities or gene expression. Recent findings indicate that 2-oxoglutarate is a possible candidate as a metabolic signal to regulate the co-ordination of C and N metabolism (Hodges et al. 2002). The role of the 2-oxoglutarate binding protein PII in this regulation is well established in bacterial systems (Ninfa and Atkinson 2000) and functional homologues have recently been characterized in plants, including conifers (Moorhead and Smith 2003; F.M. Cánovas, C. Avila and F.R. Cantón, unpublished) suggesting this protein may also play a similar role. Plant PII proteins contain a plastid transit peptide and its presence inside the plastids has been reported in *Arabidopsis* (Hsieh et al. 1998). The occurrence of PII in the chloroplasts, where the GS/GOGAT cycle is located in angiosperms, may be evidence of a global co-ordination among N metabolism, C assimilation and photorespiration in photosynthetic cells. This co-ordination is important not only for photosynthesis but also for the phenylpropanoid pathway and other metabolic processes where C and N are balanced. In summary, woody plants require molecular mechanisms to sense changes in C/N ratio and co-ordinate the particular needs of

source and sink tissues in order to maintain a continuous overall balance by the allocation of resources. A network of sensing and signaling molecules must be integrated to allow the plant a distribution of the resources in an efficient manner. PII may be part of these mechanisms and research efforts are needed to clarify the role of this regulatory protein.

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