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Minireview

Molecular physiology of glutamine and glutamate biosynthesis in developing seedlings of conifers

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Nitrogen is a limiting factor in tree growth and development. The incorporation of ammonium ions in carbon skeletons is catalyzed by the sequential action of the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). Most studies on nitrogen-assimilating enzymes have been reported for annual crop plants. Knowledge of these enzymes in woody plants is much more limited, particularly at the molecular level. Here, we review current available information on glutamine/glutamate biosynthesis and chloroplast development in conifers.

Key words – Amino acid biosynthesis, forest trees, gene expression, glutamate, glutamine, gymnosperms.

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Introduction

The availability of inorganic nitrogen in the soil is frequently the limiting factor in plant growth. Nitrate is the most common form utilized by the plant, except in acidic soils where little nitrification occurs and ammonium is predominant (Berg 1986). After uptake, nitrate is reduced to ammonium by the cell, prior to its incorporation into the organic pool of the plant. However, direct uptake from soil and reduction of nitrate (primary sources) are not the only sources of ammonium ions in a plant cell. Other metabolic processes also release ammonium (secondary sources), such as protein and amino acid catabolism, photorespiration, and biosynthesis of phenylpropanoids. Regardless of their origin, ammonium ions are assimilated into organic nitrogen mainly by the glutamine synthetase/glutamate synthase cycle. Glutamine synthetase (GS, EC 6.3.1.2) catalyses the incorporation of ammonium to glutamate for glutamine biosynthesis whereas glutamate synthase (GOGAT, EC 1.4.7.1; 1.4.1.14) catalyses the transfer of the amide group from glutamine to 2-oxoglutarate, producing two molecules of glutamate and of which one is recycled for glutamine biosynthesis. Both glutamine and glutamate are nitrogen donors for the biosynthesis of major nitrogenous compounds in plants, including amino acids, nucleotides, chlorophylls, polyamines and alkaloids.

In plants, native GS is an octameric enzyme with a molecular mass of about 330–380 kDa. It exists as two isoforms that are easily resolved by anion exchange chromatography: GS1 localized in the cytosol and GS2 confined to the plastid (McNally and Hirel 1983). Two molecular forms of glutamate synthase are also found that differ in their respective source of reductant for enzyme catalysis: NADH-GOGAT and ferredoxin

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(Fd)-GOGAT (Suzuki and Gadal 1984). The biochemistry and molecular biology of GS and GOGAT have been studied extensively during the past decade, no doubt due to their key role in the assimilation of inorganic nitrogen into biological compounds (Lea et al. 1990, Lam et al. 1996). However, most of the studies have been on annual crop plants, and molecular studies of nitrogen metabolism in woody plants, particularly the gymnosperms are scarce. This is in spite of the economic and ecological importance of forests and the fact that nitrogen is a limiting factor in tree growth and development. The study of the enzymes involved in glutamine and glutamate biosynthesis in trees is interesting for several reasons: (1) Limited information is available on the molecular characteristics and regulatory properties of these proteins, their corresponding genes and regulation of their expression. (2) Comparative studies between gymnosperms and angiosperms are informative for phylogenetic analysis and molecular evolution of plants. (3) In the inital stages of conifer growth, developmentally and environmentally regulated processes are better separated than in angiosperms. For example, chloroplast biogenesis may be less dependent on light and it can be accomplished through carbon and nitrogen compounds stored in the seed. (4) In trees, available inorganic nitrogen from the soil is incorporated into amino acids and in many cases accumulated as vegetative storage proteins to be used when the plant initiates periods of active growth and development, i.e. in the next growing season. GS and GOGAT have a key role in nitrogen recycling, storage and remobilization. (5) Conifer trees grow in acidic soils where the predominant inorganic nitrogen form is ammonium instead of nitrate.

In the present paper, the current available information on glutamine/glutamate biosynthesis during early phases of conifer development is reviewed. We will focus especially on considering recent contributions on nitrogen storage in the seeds, its mobilization and metabolic fates during the initial stages of tree development. A short reference on GS and GOGAT in angiosperms is also included, covering molecular characteristics and developmental regulation during greening. More detailed information can be found in major and comprehensive reviews already published (Lea et al. 1990, Lam et al. 1996).

Nitrogen reserves in seeds of gymnosperms

In dicotyledonous seeds, the two principal types of storage proteins are the 11S and 7S globulins which vary in their relative proportions in different species (Shotwell and Larkins 1989). Although a limited number of studies are available on storage proteins in gymnosperms, the occurrence of proteins with similar characteristics to those of the angiosperm 11S and 7S has been demonstrated in the seeds of several gymnospermous species such as spruce (Hakman et al. 1990, Newton et al. 1992), pine (Allona et al. 1992, 1994a), and Douglas fir (Leal and Misra 1993) by different experimental approaches including electrophoretic analysis, protein sequencing and cDNA primary structure. Proteins with similar structure to 11S globulins have also been described in the non-conifer gymnosperm *Ginkgo biloba* (Arahira and Fukazawa 1994). Recently, Aragoncillo and coworkers (Allona et al. 1994b) characterized a fraction of low molecular mass globulins in maritime pine seeds with high homology to the 2S proteins in angiosperms.

Noteworthy of reserve proteins in gymnosperms is their high proportion of arginine, the highest nitrogen-rich amino acid. Thus, in *Pinus pinaster* the 2S proteins contain about 25% arginine (Allona et al. 1994b), whereas in the glutelins, the main storage proteins, arginine residues represent about 15% of total amino acids (Allona et al. 1992). When storage proteins are degraded following seed germination, the most abundant amino acids in pine seedlings have been reported to be arginine and the amides, glutamine and asparagine (Guitton 1964). The abundance of arginine in reserve protein appears to be a general characteristic of other gymnosperm species (Feirer 1995; C. Aragoncillo, personal communication) and is consistent with a nitrogen storage function. These findings suggest the possibility that gymnosperms, and particularly conifers, have a great capacity for nitrogen storage in their seeds.

Chloroplast development and gene expression

During germination, nitrogenous and lipid reserves in the seed are mobilized, supplying material for the growing embryo until the photosynthetic machinery of the developing seedling permits autotrophic growth. Storage proteins are degraded to their respective amino acids, which are used in protein synthesis or are subsequently deaminated. Ammonium released in this process is reassimilated into organic nitrogen by the GS/GOGAT metabolic pathway. Therefore, the mobilization of seed nitrogen reserves and their conversion to glutamine and glutamate for the biosynthesis of nitrogen compounds are essential events in the development of photosynthetic organelles and early plant growth. Glutamine is an important means of nitrogen translocation in angiosperms (Lea et al. 1990) and gymnosperms (Barnes 1963).

In angiosperms, when seedlings are maintained in the dark, the proplastid develops into an etioplast, a plastid lacking photosynthetic pigments and polypeptides. Etiolated seedlings exposed to light accumulate chlorophylls and photosynthetic proteins, such as the apoprotein of light-harvesting complex of photosystem II (LHCII b) and the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are synthesized in a light-dependent fashion.

This is the consequence of light activation of the chloroplast and nuclear gene expression (Tobin and Silverthorne 1985).

The induction of GS/GOGAT enzyme activities, polypeptides, and mRNAs has also been demonstrated when etiolated plants are transferred to the light. The accumulation of chloroplastidic GS stimulated by light has been described in a variety of plant species and in most cases is due to de novo protein synthesis (McNally and Hirel 1983). A substantial increase in GS2 mRNA occurs after irradiation of etiolated leaves (Tingey et al. 1988, Edwards and Coruzzi 1989), and the response appears to be mediated by phytochrome. In mature leaves, GS2 mRNA abundance is affected by environmental and developmental factors associated with mature chloroplasts (Edwards and Coruzzi 1989). Similarly, Wallsgrove et al. (1982) have described in maize seedlings the induction by light of Fd-GOGAT activity, which is accounted for by de novo synthesis of the corresponding polypeptide (Suzuki et al. 1987). It has also been reported that stimulation by light of Fd-GOGAT activity and Fd-GOGAT message is mediated by the phytochrome receptor (Elmlinger and Mohr 1991, Becker et al. 1993). These data indicate that the GS/GOGAT cycle is active in developing chloroplasts.

In contrast to angiosperms in which chlorophyll biosynthesis and accumulation of gene products associated with photosynthesis are regulated by light, it is well documented that seedlings of pine and other gymnospermous species are able to accumulate photosynthetic pigments and develop functional chloroplasts in darkness (Bogdanovic 1973, Mariani et al. 1990). In several pine species, the light-independent synthesis of LHCII b and other chloroplast polypeptides such as LSU and SSU has been reported (Yamamoto et al. 1991, Cánovas et al. 1993, García-Gutiérrez et al. 1993). The extreme abundance of nitrogen-rich amino acids in conifer storage proteins would support the high demand of nitrogen for chloroplast development during early stages of plant growth, particularly in dark-grown seedlings. On the other hand, since these plants are able to develop chloroplasts in the dark, it was postulated that the nitrogen assimilating enzymes GS and Fd-GOGAT also accumulated in darkness, maintaining the appropriate glutamine and glutamate levels required for the induction of the photosynthetic apparatus under these conditions.

Molecular characterization of GS and GOGAT

Chloroplastidic and cytosolic glutamine synthetase isoenzymes have been purified and characterized from many plant species (reviewed by Lea et al. 1990). Progress in the last few years demonstrated that the genetic basis of GS isoforms is the existence of a small family of nuclear genes that are differentially expressed during development and in response to different external stimuli (Cullimore et al. 1984, Tingey et al. 1988). The complete nucleotide sequence of cytosolic and plastidic GS subunits has been deduced from the corresponding cDNAs isolated from a variety of angiosperm species (Forde and Cullimore 1989).

In gymnosperms, GS enzyme has been purified from needles and roots of jack pine (*Pinus banksiana*) and Douglas fir roots (*Pseudotsuga menziesii*), and its physico-chemical and kinetic properties determined (Vézina and Margolis 1990, Bedell et al. 1995). It appears that native coniferous GS is constituted by two polypeptides different in size and relative abundance (Vézina and Margolis 1990, Bedell et al. 1995). Similar findings have been reported in recombinantly-expressed pine GS in *Escherichia coli* (Cantón et al. 1996), where the more abundant polypeptide and that of largest molecular mass corresponded to the expected cDNA product. It remains to be determined whether the less-well represented polypeptide is an alternatively expressed product of the gene or a degradation product of the intact GS protein.

A number of cDNA clones encoding glutamine synthetase and ferredoxin-dependent glutamate synthase have been isolated from pine seedlings by using: (1) synthesis of oligonucleotides matching conserved regions in the sequence of angiosperm genes, (2) purification of enzyme protein and raising of polyclonal antibodies and immunoscreening of expression libraries, (3) screening with heterologous cDNA probes, (4) random sequencing of isolated cDNA clones.

Cantón et al. (1993) were the first to report the GS cDNA sequence from a woody plant, which was isolated by screening a cDNA library of *Pinus sylvestris* seedlings with a synthetic oligonucleotide. A full-length clone, pGSP114, contained an open reading frame encoding a polypeptide of 357 amino acids, with a molecular mass of 39.5 kDa and pI of 6.6. The derived amino acid sequence was closer in homology to cytosolic GS (GS1) (78–82%) than to the chloroplastidic GS (GS2) (71–75%) of angiosperms. An alignment with the GS amino acid sequences available in the data bank showed that the pine sequence lacked the 60 amino acid N-terminal presequence as well as the 16 amino acid C-terminal extension that are characteristic of chloroplastidic GS primary structure (Forde and Cullimore 1989). Although the function of the C-terminal extension is unknown, the N-terminal presequence is involved in the targeting of polypeptide into the chloroplast compartment (Forde and Cullimore 1989). The Scots pine (*P. sylvestris*) GS subunit has been overexpressed in *E. coli* cells, the recombinant protein has been purified and used to raise a high titer antiserum in rabbits (Cantón et al. 1996). The antibody has proved to be specific and it will be a useful molecular tool in the biochemical analysis of nitrogen metabolism in coniferous trees and other woody plant species.

In addition to the full-length cDNA described above, other clones have been characterized in *P. sylvestris*, but all encoded the cytosolic polypeptide (Cantón et al. 1993). Elmlinger et al. (1994) also reported the isolation of a GS1 cDNA sequence from *P. sylvestris*, though in this case a GS2 specific probe was used for screening of the library. Similar findings have been reported by random sequencing of several partial cDNA clones from loblolly pine (Kinlaw et al. 1996). Sequence comparison revealed that all isolated clones were homologous to Scots pine GS1 (C. S. Kinlaw, personal communication).

Isolated cDNA clones have been used to investigate the genomic organization of GS genes in *Pinus*. Southern analysis of genomic DNA from pine embryos suggested that, as in angiosperms, GS is encoded by a gene family in pine (Cantón et al. 1993, Kinlaw et al. 1996).

The availability of a full-length cDNA sequence from a gymnosperm allowed the analysis of the molecular evolution of GS genes in plants. Data derived from this analysis show that pine GS shares a common evolutive origin with cytosolic GS in angiosperms and that GS1/GS2 split occurred before the angiosperm/gymnosperm separation (F. R. Cantón. 1993. Thesis, Univ. Málaga, Spain).

Ferredoxin and NADH-dependent glutamate synthases have also been characterized in many angiosperm species (Lea et al. 1990). Based on the different physico-chemical, immunological and regulatory properties it was assumed for a long time that they were distinct proteins (Suzuki and Gadal 1984, Lea et al. 1990). This assumption has been confirmed by the isolation and characterization of different cDNA clones encoding Fd- (Sakakibara et al. 1991, Zehnacker et al. 1992, Avila et al. 1993, Nalbantoglu et al. 1994) and NADH-dependent (Gregerson et al. 1993) glutamate synthases. In angiosperms, Fd-GOGAT is localized in the chloroplast (Botella et al. 1988) and represents the predominant molecular form in green tissues, where it is involved in the assimilation of ammonia derived from nitrate reduction and the reassimilation of ammonia released in photorespiration and protein catabolism (Lea et al. 1990). The structure of the alfalfa NADH-GOGAT gene was reported recently. Its expression is restricted to functional root nodules where it appears to play a significant role in the assimilation of ammonium derived from symbiotic N_2 fixation (Vance et al. 1995).

Molecular studies on the Fd-GOGAT enzyme in gymnosperms have also been reported recently. This protein was purified from pine cotyledons by ammonium sulphate precipitation, ion-exchange and hydrophobic chromatography (García-Gutiérrez et al. 1995). As in angiosperms, pine Fd-GOGAT is a monomeric enzyme with a native molecular mass of about 165 kDa. Antibodies were raised against the purified protein and used to isolate several cDNA clones by immunoscreening of a cDNA library from *P. sylvestris* seedlings. The longest cDNA insert (2.1 kb) contained an open reading frame coding for 550 amino acid residues of the C-terminal region of pine Fd-GOGAT enzyme and a noncoding region of 449 nucleotides including a poly(A) tail (García-Gutiérrez et al. 1995). This region includes three conserved cysteines involved in an iron-sulphur cluster and a flavin mononucleotide-binding site, two putative domains conserved in other plant Fd-GOGATs (Sakakibara et al. 1991, Zenacker et al. 1992). Comparison of the pine amino acid sequence with those of mono- and dicotyledonous sequences available in the data bank revealed an 83–85% identity, indicating that Fd-GOGAT primary structure is well conserved in spite of the evolutionary distance between angiosperms and gymnosperms. From a recent report, it is concluded that this may also be extended to photosynthetic organisms other than higher plants (Navarro et al. 1995).

NADH-GOGAT activity has also been detected in young pine seedlings but the enzyme has not been characterized at the molecular level (Elmlinger and Mohr 1991, García-Gutiérrez et al. 1995).

Cellular distribution and subcellular compartmentation of GS and GOGAT

It is currently accepted that in angiosperms ammonium released in nitrate reduction and photorespiration is assimilated in the chloroplast by the sequential action of chloroplastidic GS (GS2) and Fd-GOGAT. Although nitrate reductase is localized in the cytosol, the chloroplastidic localization of nitrite reductase supports a direct role of GS2 in assimilation of ammonium derived from nitrate reduction. GS2 is also responsible for the maintenance of nitrogen economy under photorespiratory conditions: barley mutants lacking chloroplastidic GS normally grow in a CO_2-enriched atmosphere, but are unable to survive when exposed to normal O_2 levels (Wallsgrove et al. 1987). In concordance with its role in photosynthetic nitrogen metabolism, the GS2 isoenzyme is the predominant molecular form in the leaves of many plants. Cytosolic GS is a minor enzyme in green tissues, with the exception of plants exhibiting low levels of photorespiratory activity (C_4 type plants) (Hirel et al. 1984) and under particular physiological conditions such as senescence or pathogen infection (Kawakami and Watanabe 1988, Pérez-García et al. 1995).

The use of transgenic plants to analyse the expression of GS genes has contributed notably to a better understanding of the physiological role of GS isoenzymes. Genetically-engineered tobacco plants have been produced that express the β-glucuronidase (GUS) gene under the control of pea GS1 and GS2 promoters. Analysis of GUS expression in different tissues of transformed plants revealed that GS2 was mainly expressed in mesophyll cells (containing chloroplasts),

whereas GS1 expression was restricted to phloem cells (Edwards et al. 1990). The presence of the protein in leaf veins has been determined by immunostaining using light and electron microscopy (Carvalho et al. 1990). These findings suggest that GS1 is involved in the translocation of nitrogen compounds to sink tissues and strongly support non-overlapping roles for GS1 and GS2 in photosynthetic organs. Recently, Hayakawa et al. (1994) reported experimental evidence suggesting that NADH-GOGAT is responsible for the biosynthesis of glutamate in developing sink leaves.

Current available data indicate that seedlings of pine and other conifers mainly express cytosolic GS in photosynthetic tissues (Cánovas et al. 1991, Cantón et al. 1993, 1996, García-Gutiérrez et al. 1998). In previous studies on glutamine synthetase in pine, GS2 isoenzyme and its corresponding polypeptide were not detected in maritime (Cánovas et al. 1991, Cantón, 1993) and Scots pines (Cantón et al. 1993) even though a number of biochemical and molecular approaches were used. Molecular data derived from the characterization of GS cDNA clones in several laboratories show that the GS1 gene is actively expressed in developing pine seedlings; GS2 gene expression has not yet been reliably demonstrated. The above data indicate that glutamine biosynthesis occurs in the cytosol of pine cells, at least during early phases of plant growth. This molecular-based assumption has been recently confirmed by the immunocytochemical detection of GS1 in mesophyll and phloem cells of pine seedlings (García-Gutiérrez et al. 1998) and suggests a key role for cytosolic GS in photosynthetic cells of conifers. However, it remains to be determined whether the same or different gene products (GS1) are present in both cell types. As occurs in other plants, pine Fd-GOGAT is a soluble protein located in the chloroplast's stroma and therefore glutamate synthesis is confined to the plastid (García-Gutiérrez et al. 1995). The localization of GS in the cytosol implies that synthesized glutamine should be transported into the chloroplast for amino acid and chlorophyll biosynthesis. Consequently, a glutamine translocator should be operative in green pine tissues in order to provide the required glutamine and glutamate levels for chloroplast biogenesis and early plant growth. The metabolic role of this transporter in the control of nitrogen economy of conifers might be of significant importance.

Developmental and environmental expression of GS and GOGAT genes in conifers

Primary seedling development starts by the activation of a set of genes involved in the utilization of nitrogen and carbon reserves stored in the dry seed. The expression of some proteolytic enzymes has been reported during germination of several coniferous species (Transbarger and Misra 1995). The activity of the corresponding gene products is potentially responsible for protein breakdown and amino acid production for protein biosynthesis in the developing plant. Recent advances have been reported on expression of GS and GOGAT genes, which are involved in the recycling of nitrogen derived from mobilization of nitrogen reserves. The expression of these genes has been studied during early development of conifer seedlings at the levels of enzyme activity, protein and mRNA.

Enzyme activities, which are very low in the embryo, increase dramatically during seed germination and this increase is supported by a parallel accumulation of GS and Fd-GOGAT polypeptides (Cánovas et al. 1991, García-Gutiérrez et al. 1995). The isolation of cDNA sequences encoding these nitrogen-assimilating enzymes allowed their use as molecular probes for detection of the corresponding mRNAs (Cantón et al. 1993, García-Gutiérrez et al. 1995). These results suggest that the increase in the activity of the enzymes is to a great extent due to mRNA acccumulation and de novo GS and Fd-GOGAT protein synthesis. Therefore, the expression of GS and GOGAT genes seems to be regulated, at least partially, at the transcriptional level, although other mechanisms of gene regulation cannot be ruled out. Studies on regulation of GS and GOGAT in other plant species revealed that the relative abundance of these enzymes is regulated by transcriptional activation (Forde and Cullimore 1989, Sakakibara et al. 1991). In angiosperms, light is a regulatory factor that stimulates the accumulation of GS and Fd-GOGAT during seed germination and greening of etiolated leaves (Winter et al. 1982, Suzuki et al. 1987). Nitrogen nutrition also affects the expression of nitrogen-assimilating enzymes in angiosperms (Hirel et al. 1987, Redinbaugh and Campbell 1993). However, the feeding of maritime pine seedlings with nitrate or ammonium has little or no effect on gene expression (Cánovas et al. 1991, García-Gutiérrez et al. 1995). Similar results were reported for GS genes in *Phaseolus vulgaris* cotyledons (Swarup et al. 1990), suggesting that enzyme levels were sufficient to support an eventually increased inorganic nitrogen uptake. One can speculate that the high nitrogen content of the seed may supply the required material for metabolic activity and early growth of the seedling, and consequently, the uptake of external nitrogen is not initiated until the reserves are exhausted. Thus, Guitton (1964) reported that primary development in pine does not imply changes in the total nitrogen of the seedling.

In conclusion, the expression of GS and Fd-GOGAT genes in conifers appears to be controlled by a developmental programme, at least in the initial phases of seedling growth, and environmental factors such as light and/or exogenously supplied nitrogen have a limited role on this programme. In later stages of tree seedling development, when the germination stage is

finishing up, carbon and nitrogen reserves have been mobilized, and then the effect of environmental factors is much more pronounced (Margolis et al. 1988, Elmlinger and Mohr 1992). The observed expression pattern of genes involved in nitrogen metabolism is closely associated with the light-independent chloroplast development observed in conifers (Mariani et al. 1990), and light-independent expression of photosynthesis genes (Yamamoto et al. 1991). All these data strongly suggest the operation of an active GS/GOGAT cycle for the biosynthesis of nitrogen compounds required for plastid biogenesis.

Concluding remarks

Knowledge of molecular characteristics and regulation of nitrogen-assimilating enzymes in forest trees has increased notably in the last few years. The enzymic proteins have been purified from woody plants and their physico-chemical, kinetic and molecular properties determined. GS and Fd-GOGAT show similar characteristics to those described for the corresponding counterparts in angiosperms. Purified preparations of these enzymes have been used to generate polyclonal antibodies that have subsequently been used to determine subcellular, tissue-specific distribution and developmental regulation in developing seedlings, particularly with regard to chloroplast development. Complementary DNA clones have also been isolated and expression of the corresponding genes was studied during early stages of development. Relevant differences found are: (1) Chloroplast development and subsequent expression of genes encoding chloroplast proteins and accumulation of chlorophylls in conifers is far less regulated by light than in angiosperms. (2) Genes involved in glutamine biosynthesis are not so strongly regulated by light and their encoded enzymes accumulate in a light-independent fashion during early growth of conifers. After germination, environmental factors (light and nitrogen nutrition) stimulate gene expression. (3) Glutamine biosynthesis is located mainly in the cytosol in a number of species examined. Accordingly, to date only GS1 cDNA has been isolated from a gymnosperm species. Recent data show that pine GS1 is located in mesophyll and phloem cells, suggesting different metabolic roles for these cytosolic enzymes in developing trees. It remains to be determined whether these proteins are encoded by the same or separate genes as reported in tobacco (Dubois et al. 1996). The characterization of genomic clones encoding GS and Fd-GOGAT will be very helpul in determining the structure of these genes and the analysis of the 5′ regulatory region may elucidate the sequences involved in both developmental (endogenous) and environmental (exogenous) regulation. The isolation and characterization of genes involved in nitrogen metabolism and the continuous improvement in gene transfer to forest trees will lead in the near future to the genetic manipulation of glutamine biosynthesis by the application of molecular biology techniques such as chimeric gene construction, transformation and differential expression analysis in transgenic trees. These new experimental approaches will open the possibility of modifying endogenous glutamine levels and studying the effect of this modification on amino acid metabolism, biomass production and tree growth rate.

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