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Review

Genetic modification of amino acid metabolism in woody plants

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Abstract

Forest trees comprise a large group of angiosperm and gymnosperm species of economic importance that play a crucial role in the ecosystems. Nitrogen is frequently a limiting factor for growth of forest trees, thus development of a fundamental understanding of nitrogen assimilation and metabolism is particularly important in broadening our understanding of fundamental tree biology. There are a number of fundamental ways in which woody plants differ from herbaceous species, including seed dormancy and germination, growth habit and enhanced secondary development, management of reduced nitrogen during dormancy, and the metabolic requirements for secondary growth, a major sink for both reduced nitrogen and carbon. Poplar species (*Populus* spp.) have emerged as model systems for research in woody angiosperms. Modification of metabolism using genetic engineering approaches has recently focussed on altering the biosynthesis of glutamine, polyamines, glutathione, and lignin. These approaches potentially affect plant development and stress tolerance. The aim of this minireview is to integrate the experimental genetic engineering approaches in the context of developing an increased understanding of overall nitrogen and amino acid metabolism in trees.

Keywords: Nitrogen assimilation; Plant metabolism; Trees; Transgenic plants

1. Introduction

Progress in nitrogen assimilation and amino acid biosynthesis is of special interest for plant scientists and breeders, since plant development and biomass production depend on the availability of inorganic nitrogen in soil, and nitrogen fertilizers are widely used to get better yields. In addition, key enzymes in amino acid biosynthesis are the target of a number of relevant herbicides that are used for weed control, and the synthesis of amino acids is also associated to resistance to stress in plants [37]. Therefore, an increased knowledge on nitrogen metabolism and the generation of plants with higher efficiency in nitrogen assimilation and metabolism are of broad interest for plant breeders, especially to decrease the cost/production ratio, that includes relevant issues such as (a) increase in yield, (b) decrease in the pollution associated to the use of fertilizers, (c) improving the resistance of cultures to different stresses and (d) the control of competitive and undesirable weeds.

Most of the studies in nitrogen metabolism have been performed in annual herbaceous plants, and much less is known on this subject in woody perennial species. The aim of this minireview is to provide an overview of the recent works on genetic manipulation of amino acid metabolism in woody plants, including ammonium assimilation and glutamine biosynthesis, polyamine metabolism, and glutathione biosynthesis. Special attention will focus on the integration of the experimental approaches in the context of overall nitrogen and amino acid metabolism in trees (Fig. 1). A related metabolic pathway is the biosynthesis of the aromatic amino acids

Abbreviations: ADC, arginine decarboxylase; γ-ECS, γ-glutamylcysteine synthetase; GOGAT, glutamate synthase; GS, glutamine synthetase; GSHs, glutathione synthetase; QTLs, quantitative trait loci; ODC, ornithine decarboxylase; Put, putrescine; VSPs, vegetative storage proteins.

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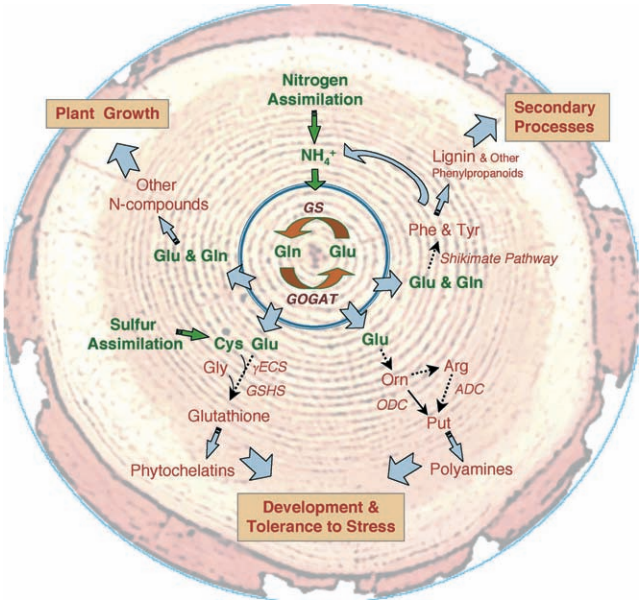

Fig. 1. An overview of nitrogen metabolism depicting the major pathways studies in woody plants. ADC, arginine decarboxylase; γ-ECS, γ-glutamylcysteine synthetase; GOGAT, glutamate synthase; GS, glutamine synthetase; GSHs, glutathione synthetase; ODC, ornithine decarboxylase; Put, putrescine.

phenylalanine and tyrosine (through the shikimate pathway), which leads to the biosynthesis of lignin. Reviews addressing lignin manipulation and wood formation have recently appeared [1,3,26] and, therefore, this topic will not be considered in this paper.

2. Trees as experimental plant models, relevance and difficulties

Most of our knowledge on plant metabolism and physiology has been gained using cell cultures, seedlings or whole plants of herbaceous species, including model systems and other species of agronomic importance. Recently, it has been shown that a number of trees may be suitable for both basic and applied research [32,63]. Progress from molecular and physiological studies in trees has been constrained by various difficulties that trees present for experimental work, including their large physical size, the usually large genome in many species of economic interest, long life cycle of perennial species, difficulties for genetic transformation and regeneration in vitro, and difficulties for molecular and biochemical analyses. However, there are important reasons to study woody plants. They provide a range of products of commercial interest, including wood, pulp, wood products, and important secondary metabolites. Trees are of great environmental interest for reforestation programs, soil retention, as essential components of the natural landscape, and for recreation. Moreover, forest ecosystems play a crucial role in global carbon budgeting, responses to global climate change, and maintenance of biodiversity.

Significant research efforts have focussed on the development of protocols for mass clonal propagation of forest species of economic importance, including a number of conifers (such as pines, spruces and Douglas fir) and angiosperm species of the genera *Eucalyptus* and *Populus* [40]. Genetic transformation of conifers, the most important group of gymnosperms, is still a difficult task, although protocols for *Agrobacterium*-mediated transformation have been reported for embryogenic cultures of spruce and loblolly pine [61]. Furthermore, the transformation of shoots of loblolly pine using *Agrobacterium* infiltration has also been recently reported [27]. Somatic embryogenesis represents a useful model to study the developmental regulation of gene expression [17]. Such recent technological advances are opening the possibility of modifying the metabolism in gymnosperms.

In angiosperm forest trees, *Populus* has emerged as the model for experimental research [9,56]. The poplar species have small genomes and are characterized by an easy vegetative propagation. Furthermore, some genotypes are amenable to transformation via *A. tumefaciens*. In addition, the availability of fast growing poplar clones allow for the transfer of molecular studies to field trials in a relatively short period of time [35]. Moreover, because of their distinct juvenile and mature phases of growth, woody plants offer opportunities to study specific metabolic processes, thus, complementing our knowledge acquired from herbaceous plants.

3. Modification of nitrogen metabolism in trees

3.1. Genetic manipulation of glutamine biosynthesis

Availability of inorganic nitrogen in the soil is frequently a limiting factor for plant growth. Thus, the uptake of inorganic nitrogen and its incorporation to amino acids have been the main area of interest in plant biochemistry and physiology. Knowledge on nitrogen assimilation has been recently revised, and research efforts have centered on elucidation of key steps of the process, including nitrate uptake and reduction, incorporation of ammonium into glutamine, and biosynthesis of glutamate [37]. Although the physiology of nitrogen uptake is well characterized, the modification of nitrate uptake using transgenic approaches is complicated, because of the existence of multiple genes involved in nitrogen transport systems in plants [25,45]. However, the reduction of nitrate to ammonium and the assimilation of ammonium into glutamine are well understood. Thus, by the introduction of specific transgenes, transgenic plants could provide a means to study the role of various factors that affect and regulate ammonium assimilation.

Early studies in the 1990s indicated that an increase in the level of key enzymes, including nitrate and nitrite reductases, glutamine synthetase (GS) and asparagine synthetase, in transgenic plants resulted in very limited or no effect on the phenotype of the modified plant [18,57]. Therefore, co-transformation with other enzyme(s) to avoid the generation of new limiting steps in the metabolic pathways was suggested [18]. Nearly one decade of research has been neces-

sary to demonstrate that the modification of GS alone may affect the biosynthesis of nitrogen compounds and plant development. GS exists as two different isoenzymes in most plant tissues: a chloroplastic enzyme (GS2), which is involved in the assimilation of ammonium from nitrate reduction and photorespiration in photosynthetic cells; a cytosolic enzyme (GS1), which is expressed mainly in roots and non-photosynthetic tissues. The biological role of GS1 is not well defined, but it seems to be involved in the primary assimilation of ammonium from the soil and in the assimilation of ammonium released through other secondary metabolic processes other than photorespiration. Since GS1 in the leaf is expressed specifically in the vascular bundles, a role for GS1 in the transport of glutamine to other plant organs has been considered [13,47]. However, recent work from our group on GS expression in pine seedlings has demonstrated the presence of GS1 in photosynthetic cells growing vegetatively [24], and in the mesophyll cells of tomato leaves infected by *Pseudomonas syringae* [49].

Using the transformation approaches, modification of the expressions of GS1 and GS2 has been achieved in a number of species including tobacco, *Lotus*, rice, and wheat, and in the woody perennial poplar. Results confirm the relevance of GS isoenzymes in plant development, biomass production, and yield (reviewed in [41]). In angiosperm and gymnosperm trees, the study and the modification of nitrogen assimilation hold special interest. During the early stages of development, the GS expression is regulated somewhat differently in conifers than in angiosperms, specifically affecting the biogenesis of chloroplasts. In conifers, the regulation of development of chloroplasts and the expression of chloroplast-specific enzymes are less dependent on light than in angiosperm species. This includes the expression of genes implicated in ammonium assimilation [12,55]. The economy of reduced nitrogen is of special importance in trees, since inorganic nitrogen is incorporated into amino acids, and to a great extent excess nitrogen is accumulated in the bark of the stem as vegetative storage proteins (VSPs). Moreover, woody tissues of the trunk of the trees are important sinks for the carbon and nitrogen assimilated during the tree life cycle. The demand for human use of products derived from forest trees, for example wood, is increasing. Knowledge on metabolic and developmental processes relating to wood and biomass production to efficiency of nitrogen utilization will lead to applications in increasing overall forest productivity.

The mechanisms whereby trees manage the reduced nitrogen, during the onset of dormancy and resumption of active growth, are of importance in developing a complete understanding of nitrogen homeostasis. VSPs serve as sinks for re-absorption of nitrogen from senescing leaves; thus, they act as a reservoir of reduced nitrogen to support growth during the start of each growing season [62]. Recent research has centered on the regulation of expression of VSPs and the effects of nitrogen status, photoperiod, and abiotic stress [14]. Changes in growth in response to environmental factors may alter carbon and nitrogen partitioning and, thus, provoke VSP production [64]. Accumulation of VSPs appears to be a component of the overall nutrient use efficiency of the plant. Thus, manipulation of VSP production could offer an important avenue for enhancing biomass production in trees.

Nitrogen assimilation and mobilization are crucial processes for the growth and development of perennial species. Modification of the expression of key enzymes in nitrogen metabolism is a reasonable strategy for enhancing growth of forest trees. Increased levels of GS have been achieved in transgenic poplar by the ectopic constitutive expression of a cytosolic pine GS1, under the direction of a CaMV 35S promoter [22] (Table 1). The introduced pine GS is a cytosolic enzyme, and its ectopic expression in poplar has resulted in the production of a pine holoenzyme in photosynthetic cells [20]. Although the role of cytosolic GS is not well defined in the leaves of angiosperms, its expression in photosynthetic tissues has been associated with responses to biotic and abiotic stresses, fruit ripening, and leaf senescence [5,48,10,23]. Therefore, the modification of GS1 levels may have an important effect not only on nitrogen partitioning, but also on stress tolerance. Although there are several reports of transgenic herbaceous plants with altered chloroplastic GS contents [41], the discussion here will focus on an alteration of cytosolic GS expression.

The analysis of transgenic poplar lines expressing the pine cytosolic GS has revealed that the ectopic expression of GS1 in the leaf leads not only to increased GS activity, but also to enhanced chlorophyll and protein contents [20,22]. Moreover, the enhancement of vegetative growth with respect to untransformed plants (Fig. 2) has been reported. Similar results have been obtained in herbaceous plants [21,41], indicating that the modification of cytosolic GS levels may be an appropriate approach for improving growth of crop species. Interestingly, the overexpression of GS1 in *Lotus corniculatus*, a legume plant, resulted in premature flowering and early senescence [59]. This apparent acceleration of development is an interesting observation and may have application in accelerating flowering in plants with long juvenile periods, as in the case of woody perennials.

Greenhouse studies of transgenic poplars overexpressing pine GS1 showed that enhanced GS activity in young leaves was correlated with increases in height growth [20,22]. Fu-

Table 1
Genetic manipulation of glutamine, polyamine and glutathione biosynthesis in woody plants

Host species	Transgene	Gene source	Promoter	Gene transfer	Reference
P. tremula × *P. alba*	Glutamine synthetase	*Pinus sylvestris*	35S (CaMV)	*Agrobacterium tumefaciens*	[20,22]
P. nigra × *P. maximowiczii*	Ornithine decarboxylase	*Mus musculus*	35S (CaMV)	Biolistic bombardement	[6,7]
P. tremula × *P. alba*	γ-Glutamylcysteine synthetase	*Escherichia coli*	35S (CaMV)	*Agrobacterium tumefaciens*	[42,43]
P. tremula × *P. alba*	Glutathione synthetase	*Escherichia coli*	35S (CaMV)	*Agrobacterium tumefaciens*	[19,42]

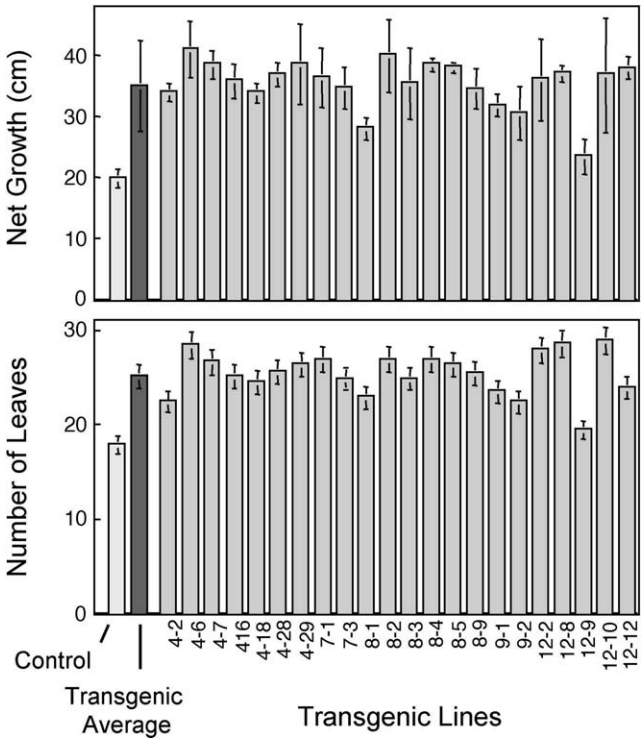

Fig. 2. Analysis of growth in 22 independent transgenic lines of poplar (*P. tremula* × *P. alba*) expressing cytosolic GS1a from *Pinus sylvestris*. Data correspond to 2-month-old plants grown in greenhouse. Average data of net growth and number of leaves of each line are represented with the standard error of the mean.

entes et al. [21] reported that a higher performance of transgenic tobacco plants overexpressing GS1 was observed, specifically when inorganic nitrogen availability was low. Similar results have been observed for GS-overexpressing poplars grown under low (0.3 mM) and high (10 mM) nitrate regimes (Man and Kirby, unpublished data). Similar findings have also been reported by Oliveira et al. [44] using transgenic tobacco plants. Recent work using ^{15}N enrichment has shown that transgenic poplars have enhanced nitrogen assimilation efficiencies, particularly under conditions of low nitrate availability (Man and Kirby, unpublished data). These results suggest that GS-overexpressing poplars may be better able to exploit nitrogen resources in the soil and, thus, require lower nitrogen fertilization regimes. This could result in reduced risk of pollution, as posed by current agricultural practices.

Transgenic plants overexpressing GS also exhibit enhanced photosynthetic and photorespiration capacities [21,44], enhanced tolerance to water stress (El-Khatib et al., unpublished), and enhanced resistance to phosphinothricine, a broad-spectrum herbicide that inactivates GS [46]. These reports provide evidence that enhanced GS expression is correlated with resistance to different types of stress. Moreover, a higher capacity to accumulate bark storage proteins has been observed in the GS-overexpressing poplars, which could be a consequence of their altered nitrogen partitioning, and contribute to enhanced vegetative growth following dormancy (Jing et al., unpublished data). These results suggest that enhanced vegetative growth and development of agriculturally important species can be achieved by establishing lines with enhanced cytosolic GS expression in photosynthetic tissues. The coincidence of GS1 genes with QTLs for yield [41] supports this strategy.

This evidence supports a strong role for GS1 in plant development and crop yield. However, if we consider that the ectopic GS1 expression in transgenic angiosperms may accelerate the plant life cycle by enhancing vegetative growth resulting in premature flowering and senescence, the question on why angiosperms species evolved without GS1 expression in mature photosynthetic cells remains unsolved. It may be speculated that high levels of expression of GS could result in poor adaptation of plants to their environments. It is interesting to note that, based on nucleotide sequence analysis, plant GS2 appears to have evolved from a duplicated GS1 gene around the time of land plant evolution [2]; furthermore, the first multicellular plants evolved with atmospheric oxygen levels similar to present day levels. Transgenics overproducing GS1 have enhanced photosynthetic and photorespiratory capacities [21,44], as also reported for enhanced expression of GS2 in transgenic tobacco [36]. Taking all this into consideration, it is tempting to speculate that the suppression of GS1 expression in photosynthetic cells and its substitution by GS2 occurred when land plants were already exposed to the present oxygen levels, perhaps as an adaptive mechanism to overcome the high levels of ammonium released during photorespiration. Therefore, the role of GS1 in the development and biomass production could be considered from a human perspective of plant productivity and could have a minor relevance, if we take into consideration the available data for plant land evolution. More data from other species transformed with GS genes will be necessary to get a better understanding of the biological relevance of GS1.

3.2. Modification of phenylalanine metabolism

Phenylalanine (tyrosine) ammonia-lyase (PAL) catalyzes the deamination of phenylalanine and tyrosine to cinnamic and *p*-coumaric acids, respectively. This is a crucial metabolic step connecting primary nitrogen metabolism through the shikimate pathway, with the allocation of carbon for the biosynthesis of phenylpropanoids. Most metabolic flux through this pathway leads to the biosynthesis of lignin, an important constituent of wood. As previously reported in annual plants, PAL in trees is encoded by a multigene family [11], but the specific physiological and biochemical roles of individual gene members still remain unknown. To our knowledge, no attempts have been made to manipulate PAL in trees using genetic engineering approaches. However, transgenic tobacco plants with altered lignin contents have been obtained using the bean PAL2 gene in either the sense or antisense orientation [16,33]. These data reinforce a proposed role of PAL 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* [1]. These results sug-

gest the existence of upstream control points in the regulation of reaction catalyzed by PAL. It is, therefore, possible that primary nitrogen assimilation, or reassimilation of ammonium, is involved in providing phenylalanine for lignin biosynthesis. This assumption is supported by the report of active nitrogen recycling in lignifiyng pine cells via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway [58]. It would be interesting to determine if increased growth observed in transgenic poplar overexpressing GS [22] is related to a higher capacity for recycling of ammonium released during lignin biosynthesis.

3.3. Genetic manipulation of the metabolism of polyamines

Alteration of glutamine/glutamate biosynthesis may have a direct effect on polyamine metabolism. Polyamines are nitrogen-rich polycations of low molecular weight. They are involved in a variety of cellular and developmental processes in plants and have been directly associated with plant stress responses [8,60]. The first step in polyamine metabolism is the biosynthesis of putrescine, which can be accomplished by two alternative pathways: either from ornithine via the reaction catalyzed by ornithine decarboxylase (ODC), or from arginine via the reaction catalyzed by arginine decarboxylase (ADC). Since arginine is produced from ornithine, and ornithine can be released from the catabolic breakdown of arginine, the relative significance of these two pathways, which leads to the production of putrescine, is unclear.

Modification of polyamine levels in plants has been achieved in transgenic plants by an overproduction of heterologous ADC or ODC enzymes [4,6,7,39,52]. Transformed tobacco overexpressing ADC showed increased levels of putrescine, leading to significant changes in phenotype, including altered patterns of plant development [39]. In rice, enhanced growth under saline stress conditions in transgenic lines overexpressing ADC was observed [52]. These results indicate that enhanced polyamine production as a result of overexpression of ADC may enhance plant development and stress tolerance.

Modification of polyamine metabolism has recently been reported in poplar cell cultures by the expression of a mouse ODC cDNA [6,7] (Table 1). Transformed cells showed elevated ODC enzyme activities and accumulated higher levels of putrescine [6]. The activity of endogenous ADC was unaffected. Interestingly, the inhibition of GS by methionine sulfoximine led to a substantial reduction of polyamine biosynthesis, both in transgenic and control cells suggesting that ornithine biosynthesis occurs mainly via ammonium assimilation into glutamine and not from catabolic utilization of arginine. In grapevine cell suspensions, a similar experimental model, an increased ammonium availability in the culture medium resulted in increased levels of putrescine. It has been proposed that polyamine biosynthesis could be a mechanism for ammonium detoxification under stress conditions [51]. Furthermore, increased putrescine catabolism has also been demonstrated in ODC transformed poplar cells [7]. In addition to resulting in enhanced stress resistance, this may also result in metabolic intermediates with important functions in plant growth and development. Interestingly, putrescine is reported to play a role in the rooting of poplar shoots [30], and in enhancing the frequency of somatic embryogenesis in cell lines of carrot that overexpress ODC [4].

Taking into consideration the varied roles ascribed to polyamines [8], metabolic studies of transgenic poplar cells are extremely valuable for a better understanding of overall nitrogen metabolism. The generation of transgenic trees with altered expression of key enzymes of polyamine biosynthesis will complement these studies and provide new insights into relevant issues in forest biotechnology, including rooting, somatic embryogenesis, and tolerance to abiotic stress.

3.4. Genetic manipulation of glutathione biosynthesis

Sulfur is an essential constituent of all living organisms. In plants, sulfur is contained in amino acids, in the redox iron-sulfur centers of some proteins, in sulfolipids, and in a variety of secondary metabolites. The mechanisms for sulfate uptake and reduction to sulfide are similar to nitrogen uptake and reduction to ammonium. Once sulfur is reduced to sulfide, it is incorporated into cysteine, the precursor of all other sulfur-containing molecules in plants, including the tripeptide glutathione [38]. Glutathione (GSH) is a stable thiol compound involved in redox regulation. In glutathione, cysteine is linked by peptide bonds to the γ-carboxyl group of glutamate and to the α-amino group of glycine. In plants, glutathione is present in all subcellular compartments, where it is essential in protection against oxidative stress and the detoxification of toxins, xenobiotics and heavy metals [38].

The biosynthesis of GSH has been engineered in trees by an overexpressing of bacterial genes coding for enzymes involved in GSH biosynthesis (Table 1) [19,42]. In the first step of this metabolic pathway, the enzyme γ-glutamylcysteine synthetase (γ-ECS) catalyzes the biosynthesis of γ-glutamylcysteine from cysteine and glutamate. In the second step, the formation of the tripeptide is catalyzed by the enzyme glutathione synthetase (GSHs). The overexpression of GSHs in transgenic trees had no effect in GSH metabolism, however, the overexpression of γ-ECS in hybrid poplar (*P. tremula* × *P. alba*) increased GSH levels in leaves and roots, when compared to untransformed controls [31,43,54]. These results suggest that the control of GSH biosynthesis is exerted at the level of the first enzyme committed to the pathway, representing a key point of connection between nitrogen and sulfur metabolism.

Recently, there has been an increased interest in the use of forest trees, particularly *Populus* for phytoremediation of environmentally troublesome compounds [15]. Trees are considered more suitable for phytoremediation than herbaceous plants, because of their significant biomass and long life cycles. Thus, transgenic poplars overexpressing γ-ECS have been proposed as tools for phytoremediation of heavy metals and herbicides. Increased levels of GSH in transgenic lines of poplar support stimulation of the biosynthesis of phytochelatins, small polypeptides synthesized from glu-

tathione that show enhanced capacity for heavy metal detoxification [28]. Herbicide resistance is due to the enhanced capacity of γ-ECS transgenics to inactivate pesticides by conjugation and by transfer of toxin to the vacuole [29].

4. Potential benefits in forest biotechnology and future prospects

As discussed above, the manipulation of nitrogen metabolism in trees may have a potential impact on forestry production. However, most of the studies have been performed in controlled laboratory conditions or in greenhouses. Therefore, field trials are required to confirm that the laboratory and greenhouse performance of transgenic plants is maintained under field conditions.

In the case of genetically engineered trees, field tests are essential to improve our knowledge on the stability of expression of inserted genes in long-lived species. For commercial applications, it is critical that transgenes continue to be expressed throughout the rotation period, which can range from several years to decades. Field studies that use transgenic poplar trees overexpressing GS have been initiated at locations in Spain and in the United States (Fig. 3). The field sites have been intensively prepared, including mechanical tilling, fertilizer application, and periodic elimination of weeds. No major incidents of insect, pest or disease problems were noted in either transgenics or controls. No unusual growth characteristics, such as high incidents of branching, were observed. Significantly, expression of the transgene appeared to be stable during the two-first growing seasons, and increased growth in plant height and stem diameter of transgenics trees have been recorded, confirming results of the greenhouse studies (Jing et al., unpublished).

Very recently, agronomic and pulping performance of transgenic trees with altered lignin metabolism was reported from a field study carried out in two sites, in France and England [50]. The expression of the transgenes was found to be stable for the duration of the 4 years study. Plants remained healthy throughout the study, and growth parameters did not differ from untransformed trees. Interactions with insects were normal, and no changes in soil microbial communities were detected beneath the transgenic trees. These results agreed quite well with the data reported by Kaldorf et al. [34], which indicated no significant differences in the degree of mycorrhizal colonization between transgenic aspen trees (*P. tremula* × *P. tremuloides*) expressing the *rol C* gene and non-transgenic controls. These studies were of special relevance for genetically engineered forest plants, which have to develop over decades without external fertilization.

In principle, the use of transgenic trees in plantation forestry may present fewer concerns for consumers than transgenic food crops plants, because the final consumer products are not ingested and, therefore, no effect on the human health is expected. As occurs with transgenic herbaceous plants, another important issue to be considered for the implementation of transgenic forest crops is the assessment of environmental risks associated with the spread of transgenes to native populations. These concerns could be addressed by co-engineering reproductive sterility in transgenic lines [53].

Fig. 3. Perspectives on transgenic tree research. The picture at the top corresponds to in vitro-cultivated transgenic poplar (*P. tremula* × *P. alba*) overexpressing GS1 [22]. Higher growth of transgenic poplars, with respect to control (bottle on the right) plants, was observed in vitro and in greenhouse studies [20,22]. Approved field trials are necessary to confirm the higher growth and development of transgenic trees in natural conditions. The picture at the bottom corresponds to transgenics overexpressing cytosolic GS1 in a field test in the province of Granada (Spain). The test was started in 2001 with rooted plants of 50 cm in height. The picture was taken on October 2002 and the height of the trees was approximately 5 m.

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References

[1] A.M. Anterola, N.G. Lewis, Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity, Phytochemistry 61 (2002) 221–294.

[2] C. Avila, R. Muñoz-Chapuli, C. Plomion, J.-M. Frigerio, F.M. Cánovas, Two genes encoding distinct cytosolic glutamine synthetases are closely linked in the pine genome, FEBS Lett. 477 (2000) 237–243.

[3] M. Baucher, B. Monties, M. Van Montagu, W. Boerjan, Biosynthesis and genetic engineering of lignin, Crit. Rev. Plant Sci. 17 (1998) 125–197.

[4] D. Bastola, S. Minocha, Increased putrescine biosynthesis through transfer of mouse ornithine decarboxylase cDNA in carrot promotes somatic embryogenesis, Plant Physiol. 109 (1995) 63–71.

[5] D. Bauer, K. Biehler, H. Fock, E. Carrayol, B. Hirel, A. Migge, T.W. Becker, A role for cytosolic glutamine synthetase in the remobilization of leaf nitrogen during water stress in tomato, Physiol. Plant. 99 (1997) 241–248.

[6] P. Bhatnagar, B.M. Glasheen, S.K. Bains, S.L. Lomg, R. Minocha, C. Walter, S.C. Minocha, Transgenic manipulation of the metabolism of polyamines in poplar cells, Plant Physiol. 125 (2001) 2139–2153.

[7] P. Bhatnagar, R. Minocha, S.C. Minocha, Genetic manipulation of the metabolism of polyamines in poplar cells. The regulation of putrescine catabolism, Plant Physiol. 128 (2002) 1455–1469.

[8] A. Bouchereau, A. Aziz, F. Larher, J. Martin-Tanguy, Polyamines and environmental challenges: recent development, Plant Sci. 140 (1999) 103–125.

[9] H.D. Bradshaw, R. Ceulemans, J. Davis, Emerging model systems in plant biology: poplar (populus) as a model forest tree, J. Plant Growth Regul. 19 (2000) 306–313.

[10] N. Brugière, F. Dubois, C. Masclaux, R.S. Sangwan, B. Hirel, Immunolocalization of glutamine synthetase in senescing tobacco (*Nicotiana tabacum* L.) leaves suggests that ammonia assimilation is progressively shifted to the mesophyll cytosol, Planta 211 (2000) 519–527.

[11] S.L. Butland, M.L. Chow, B.E. Ellis, A diverse family of phenylalanine ammonia-lyase genes expressed in pine trees and cell cultures, Plant Mol. Biol. 37 (1998) 15–24.

[12] F.M. Cánovas, F.R. Cantón, A. García-Gutiérrez, F. Gallardo, R. Crespillo, Molecular physiology of glutamine and glutamate biosynthesis in developing conifer seedlings, Physiol. Plant. 103 (1998) 287–294.

[13] H. Carvalho, S. Pereira, C. Sunkel, R. Salema, Detection of cytosolic glutamine synthetase in leaves of *Nicotiana tabacum* L. by immunocytochemical methods, Plant Physiol. 100 (1992) 1591–1594.

[14] G. Coleman, Seasonal vegetative storage proteins of poplar, in: N. Kloptenstein, Y. Chun, M. Kim, M. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*, Gen. Tech. Rep. RM-GTR-297, U.S. Department of Agriculture, Rocky Mountain Forest and Range Experiment. Station, Fort Collins, CO, 1997, pp. 124–130.

[15] M.E. Dix, N.B. Klopfenstein, J.-W. Zhang, S.W. Workman, M.-S. Kim, The potential use of *Populus* for phytoremediation of environmental pollution in riparian zones, in: N. Kloptenstein, Y. Chun, M. Kim, M. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*, Gen. Tech. Rep. RM-GTR-297, U.S. Department of Agriculture, Rocky Mountain Forest and Range Experiment. Station, Fort Collins, CO, 1997, pp. 206–211.

[16] Y. Elkind, R. Edwards, M. Mavandad, S.A. Hedrick, O. Ribak, R.A. Dixon, C.J. Lamb, Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene, Proc. Natl. Acad. Sci. USA 87 (1990) 9057–9061.

[17] L.H. Filonova, P.V. Bozhkov, S. von Arnold, Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking, J. Exp. Bot. 51 (2000) 249–264.

[18] C.H. Foyer, S. Ferrario, Modulation of carbon and nitrogen metabolism in transgenic plants with a view to improved biomass production, Biochem. Soc. Trans. 22 (1994) 909–915.

[19] C.H. Foyer, N. Souriau, S. Perret, M. Lelandais, K.-J. Kunert, C. Pruvost, L. Jouanin, Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees, Plant Physiol. 106 (1995) 1047–1057.

[20] J. Fu, R. Sampalo, F. Gallardo, F.M. Cánovas, E.G. Kirby, Assembly of a cytosolic pine glutamine synthetase holoenzyme in the leaf of transgenic poplar leads to an enhanced vegetative growth of young plants, Plant Cell Environ. 26 (2003) 411–418.

[21] S.I. Fuentes, D.J. Allen, A. Ortiz-López, G. Hernández, Overexpression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations, J. Exp. Bot. 52 (2001) 1071–1081.

[22] F. Gallardo, J. Fu, F.R. Cantón, A. García-Gutiérrez, F.M. Cánovas, E.G. Kirby, Expression of a conifer glutamine synthetase gene in transgenic poplar, Planta 210 (1999) 19–26.

[23] F. Gallardo, S. Gálvez, M.A. Quesada, F.M. Cánovas, I. Núñez de Castro, Glutamine synthetase activity during the ripening of tomato fruit, Plant Physiol. Biochem. 26 (1988) 747–752.

[24] A. García-Gutiérrez, F. Dubois, F.R. Cantón, F. Gallardo, R.S. Sangwan, F.M. Cánovas, Two different modes of early development and nitrogen assimilation in gymnosperm seedlings, Plant J. 13 (1998) 187–199.

[25] A.D.M. Glass, D.T. Brito, B.N. Kaiser, J.R. Kinghorn, H.J. Kronzucker, A. Kumar, M. Okamoto, S. Rawat, M.Y. Siddiqi, S.E. Unkles, J.J. Vidmar, The regulation of nitrate and ammonium transport systems in plants, Inorganic Nitrogen Assimilation Special issue, J. Exp. Bot. 53 (2002) 855–864.

[26] J. Grima-Pettenati, D. Goffner, Lignin genetic engineering revisited, Plant Sci. 145 (1999) 51–65.

[27] J.H. Gould, Y. Zhou, V. Padmanabhan, M. Magallanes-Cedeno, R.J. Newton, Transformation and regeneration of loblolly pine: shoot apex inoculation with *Agrobacterium*, Mol. Breed. 10 (2002) 131–141.

[28] E. Grill, S. Löffler, E.-L. Winnacker, M.H. Zenk, Phytochelatins, the heavy-metal binding peptides of plants, are synthesised from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatine synthase), Proc. Natl. Acad. Sci. USA 86 (1989) 6838–6842.

[29] G. Gullner, T. Kömives, H. Rennenberg, Enhanced tolerance of transgenic poplar plants overexpressing γ-glutamylcysteine synthetase towards chloroacetanilide herbicides, J. Exp. Bot. 52 (2001) 971–979.

[30] J.F. Hausman, C. Kevers, T. Gaspar, Involvement of putrescine in the inductive rooting phase of poplar shoots raised in vitro, Physiol. Plant. 92 (1997) 201–206.

[31] C. Herschbach, L. Jouanin, H. Rennenberg, Overexpression of γ-glutamylcysteine synthetase, but not of glutathione synthetase elevates glutathione allocation in the phloem of transgenic poplar (*Populus tremula* × *Populus alba*) trees, Plant Cell Physiol. 39 (1998) 447–451.

[32] C. Herschbach, S. Kopriva, Transgenic trees as tools in tree and plant physiology, Trees 16 (2002) 250–261.

[33] P.A. Howles, V.J.H. Sewalt, N.L. Paiva, Y. Elkind, N.J. Bate, C. Lamb, R.A. Dixon, Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis, Plant Physiol. 112 (1996) 1617–1624.

[34] M. Kaldorf, M. Fladung, H.-J. Muhs, F. Buscot, Mycorrhizal colonization of transgenic aspen in a field trial, Planta 214 (2002) 653–660.

[35] N. Kloptenstein, Y. Chun, M. Kim, M. Ahuja, Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*, Gen. Tech. Rep. RM-GTR-297, U.S. Department of Agriculture, Rocky Mountain Forest and Range Experiment. Station, Fort Collins, CO, 1997.

[36] A. Kozaki, G. Takeba, Photorespiration protects C3 plants from photooxidation, Nature 384 (1996) 557–560.

[37] P. Lea, J.-F. Morot-Gaudry, B. Hirel, Inorganic Nitrogen Assimilation, J. Exp. Bot. 53 (2002) 773–979 Special Issue.

[38] T. Leustek, K. Saito, Sulfate transport and assimilation in plants, Plant Physiol. 120 (1999) 637–644.

[39] C. Masgrau, C.T. Altabella, R. Farras, D. Flores, A.J. Thompson, R.T. Besford, A.F. Tiburcio, Inducible overexpression of oat arginine decarboxylase in transgenic tobacco plants, Plant J. 11 (1997) 465–473.

[40] S.A. Merkle, J.F.D. Dean, Forest tree biotechnology, Curr. Opin. Biotech. 11 (2000) 298–302.

[41] B. Miflin, D.Z. Habash, The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops, J. Exp. Bot. 53 (2002) 979–987.

[42] G. Noctor, A.-C.M. Arisi, L. Jouanin, C.H. Foyer, Manipulation of glutathione and amino acid biosynthesis in the chloroplast, Plant Physiol. 118 (1998) 471–482.

[43] G. Noctor, M. Strohm, L. Jouanin, K.-J. Kunert, C.H. Foyer, H. Rennenberg, Synthesis of glutathione in leaves of transgenic poplar overexpressing γ-glutamylcysteine synthetase, Plant Physiol. 112 (1996) 1071–1078.

[44] I.C. Oliveira, T. Brears, T.J. Knight, A. Clark, G.M. Coruzzi, Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light and photorespiration, Plant Physiol. 129 (2002) 1170–1180.

[45] M. Orsel, S. Filleur, V. Fraisier, F. Daniel-Vedele, Nitrate transport in plants: which gene and which control? Inorganic Nitrogen Assimilation Special issue, J. Exp. Bot. 53 (2002) 825–834.

[46] M.B. Pascual, Efecto del herbicida fosfinotricina sobre chopos transgénicos que sobreexpresan glutamina sintetasa, Tesis de Licenciatura Universidad de Málaga, 2002.

[47] S. Pereira, H. Carvalho, C. Sunkel, R. Salema, Immunocytolocalization of GS in mesophyll and phloem of leaves of *Solanum tuberosum* L, Protoplasma 167 (1992) 66–73.

[48] A. Pérez-García, F.M. Cánovas, F. Gallardo, B. Hirel, A. de Vicente, Differential expression of glutamine synthetase isoforms in tomato detached leaflets infected with *Pseudomonas syringae* pv. Tomato, Mol. Plant-Microbe Interact. 8 (1995) 96–103.

[49] A. Pérez-García, S. Pereira, J. Pisarra, A. García-Gutiérrez, F. Cazorla, R. Salema, A. de Vicente, F.M. Cánovas, Cytosolic localization in tomato mesophyll cells of a novel glutamine synthetase induced in response to bacterial infection or phosphinothricin treatment, Planta 206 (1998) 426–434.

[50] G. Pilate, E. Guiney, K. Holt, M. Petit-Conil, C. Lapierre, J.C. Leple, B. Pollet, I. Mila, E.A. Webster, H.G. Marstorp, D.W. Hopkins, L. Jouanin, W. Boerjan, W. Schuch, D. Cornu, C. Halpin, Field and pulping performances of transgenic trees with altered lignification, Nat. Biotechnol. 20 (2002) 607–612.

[51] N.I. Primikirios, K.A. Roubelakis-Angelakis, Cloning and expression of an arginine decarboxylase cDNA from *Vitis vinifera* L.-cell-suspension cultures, Planta 208 (1999) 574–582.

[52] M. Roy, R. Wu, Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice, Plant Sci. 160 (2001) 869–875.

[53] S.H. Strauss, W.H. Rottmann, A.M. Brunner, L. Sheppard, Genetic engineering of reproductive sterility in forest trees, Mol. Breed. 1 (1995) 5–26.

[54] M. Strohm, M. Eiblmeier, C. Langebartels, L. Jouanin, A. Polle, H. Sandermann, H. Rennenberg, Responses of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing glutathione synthetase or glutathione reductase to acute ozone stress: visible injury and leaf gas exchange, J. Exp. Bot. 50 (1999) 365–374.

[55] M.F. Suárez, C. Avila, F. Gallardo, F.R. Cantón, A. García-Gutiérrez, M.G. Claros, F.M. Cánovas, Molecular and enzymatic analysis of ammonium assimilation in woody plants, Inorganic Nitrogen Assimilation Special issue, J. Exp. Bot. 53 (2002) 891–904.

[56] G. Taylor, *Populus*: arabidopsis for forestry. Do we need a model tree?, Ann. Bot. 90 (2002) 687–766.

[57] S.J. Temple, S. Bagga, C. Sengupta-Gopalan, Can glutamine synthetase activity levels be modulated in transgenic plants by the use of recombinant DNA technology?, Biochem. Soc. Trans. 22 (1994) 915–920.

[58] P.S. van Heerden, G.H. Towers, N.G. Lewis, Nitrogen metabolism in lignifying *Pinus taeda* cell cultures, J. Biol. Chem. 271 (1996) 12350–12355.

[59] R. Vincent, V. Fraisier, S. Chaillou, A. Limami, E. Deleens, B. Phillipson, C. Douat, J.-P. Boutin, B. Hirel, Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium assimilation and plant development, Planta 201 (1997) 424–433.

[60] R. Walden, A. Cordeiro, A.F. Tiburcio, Polyamines: small molecules triggering pathways in plant growth and development, Plant Physiol. 113 (1997) 1009–1013.

[61] A.R. Wenck, M. Quinn, R.W. Whetten, G. Pullman, R. Sederoff, High efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*), Plant Mol. Biol. 39 (1999) 407–416.

[62] S. Wetzel, C. Demmers, J. Greenwood, Seasonally fluctuating bark proteins are a potential form of nitrogen storage in three temperate hardwoods, Planta 178 (2001) 275–281.

[63] S.D. Wullschleger, S. Jansson, G. Taylor, Genomics and forest biology: *Populus* emerges as the perennial favorite, Plant Cell 14 (2002) 2651–2655.

[64] B. Zhu, G. Coleman, Phytochrome-mediated photoperiod perception, shoot growth, glutamine, calcium and protein phosphorylation influence the activity of the poplar bask storage protein gene promoter (bspA), Plant Physiol. 126 (2001) 342–351.