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Expression of a conifer glutamine synthetase gene in transgenic poplar

Fernando Gallardo[1,2], Jianming Fu[1], Francisco R. Cantón[2], Angel García-Gutiérrez[2], Francisco M. Cánovas[2], Edward G. Kirby[1]

[1]Department of Biological Sciences, Rutgers University, University Heights, Newark, New Jersey 07102, USA
[2]Departamento de Biología Molecular y Bioquimica, Facultad de Ciencias-Instituto Andaluz de Biotecnología, Universidad de Málaga, E-29071 Málaga, Spain

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Abstract. The assimilation of ammonium into organic nitrogen catalyzed by the enzyme glutamine synthetase (GS; EC 6.3.1.2) has been suggested to be the limiting step for plant nitrogen utilization (H-M. Lam et al. 1995, Plant Cell 7: 887–898). We have developed a molecular approach to increase glutamine production in transgenic poplar by the overexpression of a conifer GS gene. A chimeric construct consisting of the cauliflower mosaic virus 35S promoter fused to pine cytosolic GS cDNA and nopaline synthetase polyadenylation region was transferred into pBin19 for transformation of a hybrid poplar clone (INRA 7171-B4, *Populus tremula* × *P. alba*) via *Agrobacterium tumefaciens*. Transformed poplar lines were selected by their ability to grow on selective medium containing kanamycin. The presence of the introduced gene in the poplar genome was verified by Southern blotting and polymerase chain reaction analysis. Transgene expression was detected in all selected poplar lines at the mRNA level. The detection of the corresponding polypeptide (41 kDa) and increased GS activity in the transgenics suggest that pine transcripts are correctly processed by the angiosperm translational machinery and that GS1 subunits are assembled in functional holoenzymes. Expression of the pine GS1 gene in poplar was associated with an increase in the levels of total soluble protein and an increase in chlorophyll content in leaves of transformed trees. Furthermore, the mean net growth in height of GS-overexpressing clones was significantly greater than that of non-transformed controls, ranging from a 76% increase in height at 2 months to a 21.3% increase at 6 months. Our results suggest that the efficiency of nitrogen utilization may be engineered in trees by genetic manipulation of glutamine biosynthesis.

Key words: Amino acid biosynthesis – Chlorophyll – Glutamine synthetase – Nitrogen assimilation – *Populus* – Transgenic trees

This paper is dedicated to the memory of Dr. Claude Crétim

Abbreviations: CaMV = cauliflower mosaic virus; GS = glutamine synthetase; NPTII = neomycin phosphotransferase II; PCR = polymerase chain reaction

Correspondence to: F.M. Cánovas;
E-mail: canovas@uma.es; Fax: 34 (95) 213 2000

Introduction

In plants, ammonium is assimilated into organic nitrogen mainly through the reaction catalyzed by glutamine synthetase (GS; EC 6.3.1.2). The amide group from the product of the GS reaction, glutamine, is then transferred to glutamate by the action of the glutamate synthase (GOGAT; EC 1.4.7.1 and 1.4.1.14). This metabolic pathway is of crucial importance, since glutamine and glutamate are the donors for the biosynthesis of major nitrogen-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Miflin and Lea 1980). The biochemistry and molecular biology of the GS/GOGAT cycle has been extensively studied due to the key role these enzymes play in plant growth and development (Lam et al. 1996; Lea 1997). Glutamine synthetase is encoded by a small family of homologous nuclear genes and the enzyme is represented by two main isoenzymes: GS1 is localized in the cytosol; GS2 is a chloroplastic enzyme. Octameric GS holoenzymes also differ in their subunit compositions, GS1 consists of polypeptides of 38–41 kDa in most plant species whereas the size of the GS2 polypeptide is about 45 kDa. The physiological roles of GS1 and GS2 are now relatively well established in angiosperms. In leaves, GS2 is expressed in photosynthetic cells and it has been proposed to function in the assimilation of ammonium derived from nitrate assimilation and photorespiration. The GS1 isoenzyme is mainly expressed in vascular elements and could be involved in the generation of glutamine for nitrogen transport within the plant (Lam et al. 1996; Lea 1997).

Few biochemical and molecular studies have addressed nitrogen assimilation in woody perennials, including forest trees (Cánovas et al. 1998). This is in

spite of the economic and ecological significance of forests and the fact that inorganic nitrogen availability in the soil is frequently a limiting factor for tree growth (Cole and Rapp 1981). Recent studies report that conifers exhibit GS expression patterns distinct from angiosperm species in that cytosolic GS is the predominant enzyme in both photosynthetic and non-photosynthetic tissues (Cánovas et al. 1991; García-Gutiérrez et al. 1998). Accordingly, to date only GS1 cDNAs have been isolated from conifers (Cánovas et al. 1998).

In the present report we describe a first attempt to improve nitrogen utilization efficiency in trees by the expression of a gene encoding cytosolic pine GS in transgenic poplar. The increase in the capacity for nitrogen assimilation may be of particular importance in forest trees because of their perennial growth and long life cycle.

Materials and methods

Plant materials. Hybrid poplar (*Populus tremula* × *P. alba*, clone INRA 717 1-B4) was maintained in vitro, as described by Leplé et al. (1992).

Gene construction. A chimeric gene composed of the cauliflower mosaic virus (CaMV) 35S promoter fused to the pine cytosolic GS cDNA (Cantón et al. 1993) and nopaline synthetase polyadenylation region (NOS) was used to transform hybrid poplar (Fig. 1). The 1.4-kb *Eco*RI insert containing the full-length cytosolic GS cDNA from pGS114 (Cantón et al. 1993) was isolated and blunt-ended using the Klenow fragment of DNA polymerase I. In parallel, the 1.0-kb *Bam*HI fragment containing the neomycin phosphotransferase II (NPTII) gene from pCaMVNEO (Fromm et al. 1986) was excised and the digested plasmid was blunt-ended. The 1.4-kb GS cDNA was then cloned into the digested pCaMVNEO to yield p35SGSp. The new plasmid has a 2.1-kb *Hin*dIII fragment containing the CaMV 35S-GS-NOS construct (Fig. 1). The orientation of the GS cDNA was verified by sequencing the junctions. This 2.1-kb *Hin*dIII construct was then cloned into the *Hin*dIII site of the *Ti*-derived disarmed binary vector pBin19 (Bevan 1984). The new vector, pBin35SGSp, was transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Holsters et al. 1978).

Preparation of Agrobacterium. A single colony of *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid vector, pBin35SGSp, as described above, was cultured in 2YT (Ausubel et al. 1987) liquid medium containing antibiotics: streptomycin (200 mg L^{-1}) and kanamycin (50 mg L^{-1}). After 48 h at 28 °C (300 rpm), the bacterial suspension was centrifuged and bacteria resuspended in liquid M1 plant cell culture medium (see below) to an OD_{660} of 0.3.

Inoculation, co-cultivation, decontamination, selection, and regeneration. When in vitro grown plantlets reached a height of 5–10 cm, leaves were removed and pre-cultured in darkness for 48 h on solidified M1 medium consisting of MS salts (Murashige and Skoog 1962), MW vitamins (Morel and Wetmore 1951), 3% (w/v) sucrose, L-cystein (1 mg L^{-1}) and Bacto-agar (8 g L^{-1}). Pretreated leaves were cut into segments of 1 cm × 1 cm. Leaf segments were placed directly in bacterial suspension at room temperature for 2 h, then blotted onto sterile filter paper to remove excess bacteria. Explants were co-cultivated in darkness for 48 h on solidified M1 medium. For decontamination and selection for antibiotic resistance, explants were transferred to M2 medium consisting of M1 medium containing timentin (200 mg L^{-1}), kanamycin (50 mg L^{-1}), and 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg L^{-1}) in darkness. After 4 weeks, calli were separated from leaf segments and transferred to M3 medium [consisting of M1 medium, kanamycin (50 mg L^{-1}), and thidiazuron (0.1 μM)] in the light for regeneration of shoots. After shoots reached a height of 2–3 cm, they were separated and cultured on M4 medium (consisting of M1 with half-strength MS macronutrients) for root induction.

Plant culture conditions. Unless otherwise noted, in-vitro cultures were maintained in a constant-temperature facility at 24 °C and provided with low light (30 μmol m^{-2} s^{-1}; 16 h photoperiod; General Electric Cool-White fluorescent bulbs). After roots were induced, rooted shoots of transformed and control plants were transferred to a potting mix consisting of MetroMix 200 (Scotts

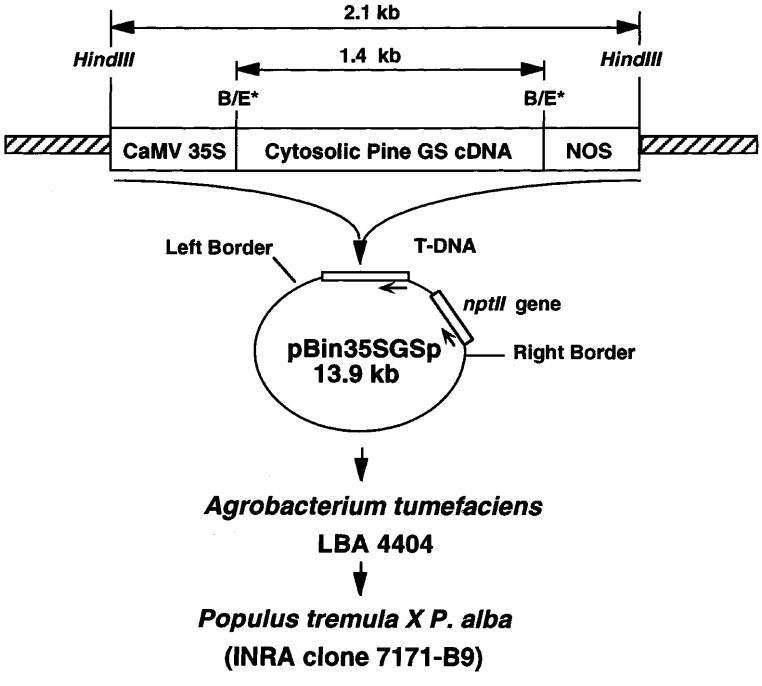

Fig. 1. Strategy for transformation of hybrid poplar [*Populus tremula* × *P. alba* (INRA 717 1-B)] with *Agrobacterium tumefaciens* (LBA 4404) containing the binary vector pBin35SGSp. *B*/*E**, blunt-ended *Bam*HI/*Eco*RI site. The *arrows* in pBin35SGSp indicate the sense of transcription of CaMV 35S-GS-NOS and *nptII* genes

Co., Marysville, Ohio, USA). Throughout the greenhouse study, plants were not supplied with any supplementary nutrients.

Protein extraction. Measuring from the apex, the fourth youngest leaf of 3- to 4-month-old greenhouse-grown plants were ground in a mortar with a pestle using glass beads (Sigma) in 50 mM Tris-HCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol (v/v), pH 8.0. The ratio of extraction buffer to glass beads to plant material was 3 ml:0.5 g:1 g. Extracts were centrifuged at 22000 *g* and the supernatant was used for GS activity and protein determinations.

Analysis of GS activity and protein determination. The GS activity was determined by the synthetase assay as described previously (Cánovas et al. 1991). The protein content was estimated by the method described by Bradford (1976) using bovine serum albumin as standard.

Electrophoresis and immunoblot detection of GS polypeptides. Total soluble proteins were separated by SDS-PAGE (10% acrylamide) using the discontinuous buffer system of Laemmli (1970). Polypeptides were visualized by Coomassie-blue staining to confirm that equal amounts of protein were loaded in each lane. Proteins were electrotransferred to nylon filters and GS polypeptides were detected using polyclonal antibodies raised against the recombinant pine GS (Cantón et al. 1996). Protein blotting, saturation of blot, and subsequent incubations with the antiserum and washing steps were performed as described elsewhere (Gallardo et al. 1995). Immunocomplexes were detected with peroxidase-conjugated immunoglobulin with a molar ratio of peroxidase to immunoglobulin of 3.3 (Vector Laboratories, Burlingame, Calif., USA).

Chlorophyll determination. Total chlorophyll was extracted from the fourth youngest leaf of transgenic lines and controls using 80% (v/v) acetone. Values were determined spectrophotometrically (Graan and Ort 1984).

Nucleic acid isolation and analysis. Genomic DNA was isolated from poplar leaves according to Dellaporta et al. (1993). Total RNA was extracted from 5 g of leaves using the guanidine isothiocyanate method as described elsewhere (Ausubel et al. 1987). Southern and northern blotting were carried out following standard procedures (Ausubel et al. 1987) and hybridized with ^{32}P-labeled pine GS cDNA (1.4 kb; Cantón et al. 1993) using the random-primer method. A mitochondrial β-ATP synthase probe from *Nicotiana plumbaginifolia* of 1.25 kb (Boutry and Chua 1985) was used as control in northern blot experiments. Filters were washed at 42 °C in 2×, 1× and 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) containing 0.2% SDS, and at 55 °C in 0.1× SSC, 0.2% SDS before exposure.

Height growth measurements. After transfer of regenerated transgenic and control plants to the greenhouse, height growth measurements were taken at monthly intervals for 6 months. A total of 78 transgenic plants was measured, representing 22 independent transformations, and 5 non-transgenic controls.

Results

Glutamine synthetase polypeptides in leaves of hybrid poplar. In order to determine the pattern of GS polypeptides in poplar leaves, total soluble proteins were extracted from areas enriched in photosynthetic cells (leaf tip) and areas enriched in vascular tissue (petioles). Proteins were then separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised against the pine GS (Cantón et al. 1996; Fig. 2). Photosynthetic tissues

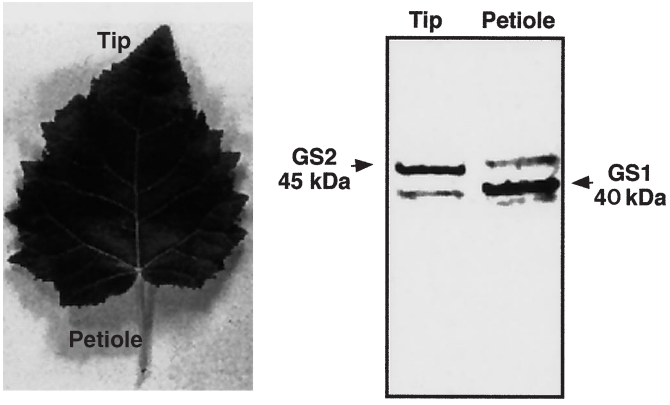

Fig. 2. Immunoblot analysis of GS polypeptides in poplar leaves. Total soluble proteins were extracted from leaf tips (blades) and petioles, subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed with antibodies raised against pine GS1. Polypeptides corresponding to 40-kDa cytosolic GS (GS1) and the 45-kDa chloroplastic GS polypeptide (GS2) are indicated by *arrows*. A minor GS polypeptide of 38 kDa was also detected and its relative abundance was higher in vascular tissue. This cross-reacting band may represent either an additional polypeptide of the poplar GS isoprotein family, or a degradation product of the intact GS protein. Twenty micrograms of protein was loaded per lane

displayed a GS polypeptide pattern enriched in the 45-kDa polypeptide characteristic of the chloroplastic GS2 (Fig. 2, leaf tip), whereas vascular tissues (Fig. 2, petiole) showed a GS pattern characterized by the abundance of a smaller, 40-kDa polypeptide subunit of the cytosolic GS1. Since these tissues are composed of distinct populations of cell types, the chloroplastic and cytosolic GS polypeptides were also detected as secondary bands in the protein extracts prepared from petioles and leaf tip, respectively. This pattern is typical of those reported for other angiosperm species (Lam et al. 1996).

Gene fusions and transformation of hybrid poplar with the pine GS1. A recombinant plasmid (pBin35SGSp) containing a chimeric GS gene construct was transferred via *Agrobacterium* to leaf segments of receptor hybrid poplar plants (Fig. 1). The gene construct consisted of the 1.4-kb pine cytosolic GS cDNA (Cantón et al. 1993) under control of the CaMV 35S promoter and the *nos* termination sequence defining a *Hin*dIII DNA-casette of 2.1 kb (see *Materials and methods* section). The T-DNA in the plasmid binary system also contained *nptII* as a selectable marker. Kanamycin-resistant cultures were selected and plants regenerated using standard protocols (data not shown).

Pine GS1 sequences in the genome of transformed poplar plants. To determine the presence of the GS1 transgene in the genome of kanamycin-resistant plants, total genomic DNA was isolated from leaves of independently transformed poplar lines, digested with *Hin*dIII, restriction fragments separated on an agarose gel, and probed with radiolabeled 1.4-kb GS1 cDNA in a Southern blot (Fig. 3). A hybridization signal corresponding to the 2.1-kb *Hin*dIII-fragment of the chimeric construct (Fig. 1) was detected in all the kanamycin-resistant plants

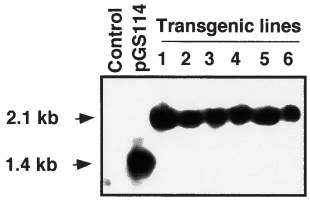

Fig. 3. Southern blot analysis of control and transformed hybrid poplar lines. Genomic DNA was isolated from leaves of control (non-transgenic) and six independently transformed poplar lines (lanes 1–6), digested with *Hin*dIII and separated on an agarose gel. Blots were hybridized with ^{32}P-labeled pine GS cDNA. *pGS114,* positive hybridization control of the pine GS1 cDNA (1.4 kb). Ten micrograms of total DNA was loaded per lane. The absence of hybridization with genomic DNA from control plants can be explained by the low identity of pine GS sequence with GS cDNAs from angiosperms (66–75% at the nucleotide level)

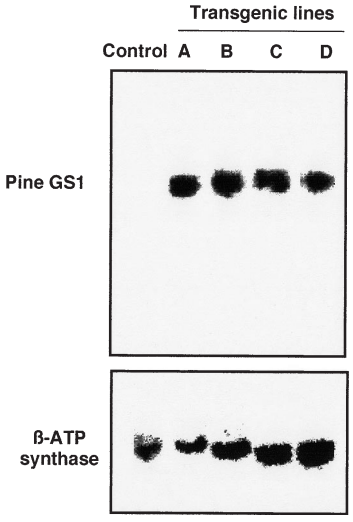

Fig. 4. Northern blot analysis of pine GS transcripts expressed in transgenic poplar. *Upper panel*, total RNA was isolated from leaves of control (non-transgenic) plants and transformed poplar lines and probed with radiolabeled pine GS1 cDNA, as described. *Lower panel*, the same blot hybridized with the mitochondrial β-ATP synthase probe from *Nicotiana plumbaginifolia.* Ten micrograms of total RNA was loaded per lane

analyzed (Fig. 3). No hybridization with labeled pine GS cDNA was detected in digests of genomic DNA of control, non-transgenic plants (Fig. 3, control). The above data clearly indicate the presence of the introduced GS sequences in the selected transgenic clones and were confirmed by polymerase chain reaction (PCR) analysis using pine GS-specific primers. Furthermore, the copy number of the introduced gene was estimated by Southern blot analysis (data not shown); four transgenic lines containing a single copy of the transgene per genome were selected for molecular and biochemical characterization.

Molecular analysis of pine GS1 expression in transgenic poplar. We next examined whether or not the introduced pine GS1 gene is expressed in transgenic poplar. Total RNA was isolated from the selected transgenic poplar and controls, separated on formaldehyde gels and blotted onto Nytran filters. Northern blots probed with radiolabeled pine GS1 cDNA revealed expression of the pine message in all transgenic lines (Fig. 4). No message was detected among total RNA isolated from non-transformed controls (Fig. 4). As an internal control, the highly conserved, constitutively expressed mitochondrial β-ATP synthase (1.25 kb; Boutry and Chua 1985) was used (Fig. 4).

To further characterize the expression of the transgene we decided to study protein GS expression (Fig. 5). Extracts of total proteins were prepared from whole leaves of control poplar and leaves of transgenic lines, separated by SDS-PAGE, and immunoblots developed using polyclonal antibodies raised against the recombinant pine GS1 (Cantón et al. 1996). In control, non-transformed leaves (Fig. 5A), the major GS polypeptide detected corresponds to the 45-kDa chloroplastic GS2. However, in leaves of four independent transformed

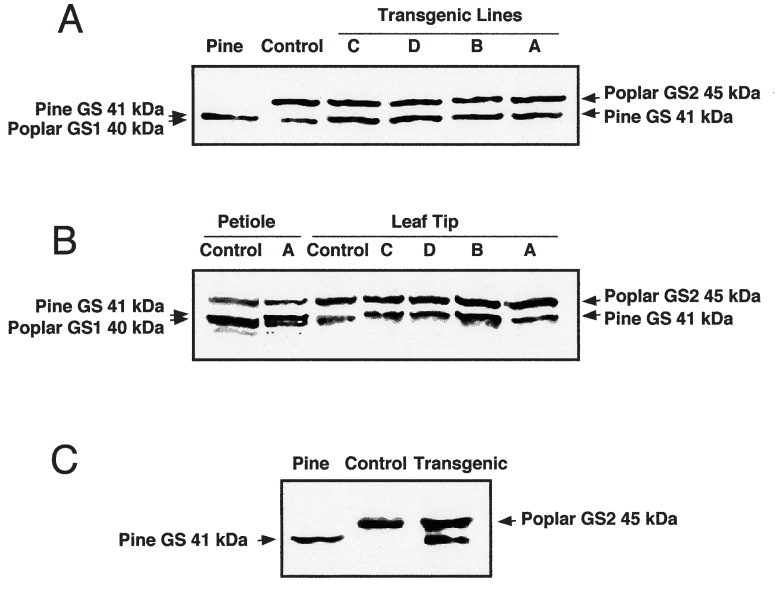

Fig. 5A–C. Immunoblots of GS polypeptide expression in transgenic poplar leaves. Total proteins from crude extracts were separated by SDS-PAGE, transferred onto a nylon filter and probed with specific GS antibodies as described in *Materials and methods*. **A** Total soluble proteins of whole leaves from control plants and transgenic lines, and from pine cotyledons. **B** Total soluble proteins extracted from leaf tips and petioles. **C** Total soluble proteins were prepared from control and transgenic poplar leaves from which midribs had previously been removed. Twenty micrograms of protein was loaded per lane

lines (Fig. 5A), a second major GS polypeptide was detected at 41 kDa, which corresponds in size to the introduced pine GS1. As an additional control, total protein extracts from pine cotyledons were also assessed and showed a single GS polypeptide corresponding to the 41-kDa GS1 (Fig. 5A, pine).

To confirm these data, protein extracts from tissues enriched in either photosynthetic (leaf tip) or vascular tissues (petioles) of control and transgenic poplar were subjected to western blotting analysis (Fig. 5B). It is clear that the major GS polypeptide in vascular bundles of non-transformed poplar is the 40-kDa GS1, whereas in the same tissue transgenic poplars express the endogenous 40-kDa GS1 and an additional polypeptide corresponding to the 41-kDa pine cytosolic GS1 polypeptide (Fig. 5B, compare petiole control and transgenic line A); the same pattern was found in petioles of all transgenics analyzed including lines B, C and D. Moreover, in photosynthetic tissues of non-transgenic controls (Fig. 5B, control leaf tip) the most abundant protein was the 45-kDa polypeptide corresponding to the poplar GS2, while in photosynthetic tissues of transgenic poplar lines, an additional major GS polypeptide of 41 kDa corresponding to the pine cytosolic GS1 was detected (Fig. 5B, leaf tip). These data suggest that cytosolic pine GS is expressed in both non-photosynthetic and photosynthetic poplar cells. To confirm the ectopic expression of the cytosolic GS polypeptide in transgenic photosynthetic cells, protein was extracted from control and transgenic leaves from which midribs had been removed to minimize the presence of vascular elements expressing endogenous cytosolic GS. The western blot analysis of these extracts showed that only the GS2 polypeptide could be detected in control plants (Fig. 5C), whereas both chloroplastic GS2 and pine cytosolic GS polypeptides were present in the extract prepared from transgenic poplar leaves (Fig. 5C).

Biochemical analysis of transgenic poplar. Independent immunoblots for GS polypeptides from the fourth youngest leaf of control and transgenic plants were quantified by densitometry, the total content of GS polypeptide and the relative content of GS1 were expressed relative to the control plants and compared with specific GS activity determined in the same leaf (Fig. 6). In both control and transgenic leaves, the total GS polypeptide pattern (GS1 + GS2 signals) matched well the specific activity of the organ, with an average increase in the transgenic leaves of 42.5% in the specific activity, and 49.8% in total GS polypeptide with respect to the control. These differences are, in part, due to the

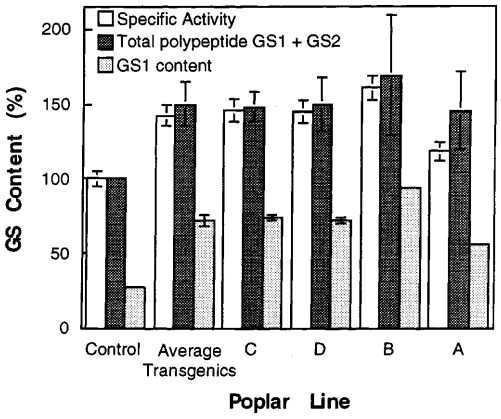

Fig. 6. Glutamine synthetase content in the fourth youngest leaf of various poplar lines. Specific activity was expressed as the percentage difference over control values; 100% of activity correspond to 2.83 nkat (mg protein)$^{-1}$. The total GS (GS1 + GS2) and GS1 polypeptide content was estimated by densitometric analysis of immunoblots and it is also expressed as percentage with respect to control values. The GS1 content is expressed with respect to the total GS polypeptide value in each poplar line. The average GS1 content in the leaf of control and transgenic lines was 27.5 ± 0.5 and 48.1 ± 2.3% of total GS content, respectively. Data are means of three independent determinations ± SE

different GS1 polypeptide contents which represents 27.5% of total GS polypeptide in control leaves and an average of 48.1% of total GS polypeptide in the transgenic leaves.

Total soluble protein content and chlorophyll levels were also determined in the fourth youngest leaf and compared with GS activity values (Fig. 7). The GS activity in transformed poplars expressed on a fresh-weight basis was higher than in controls, with levels 2- to 3-fold higher in some transgenics. Furthermore, in some transgenic lines, soluble protein levels were significantly higher than in controls (poplar lines A and B), whereas in other transgenic lines protein levels did not appear to be altered (poplar lines C and D). In addition to these changes, chlorophyll levels appeared to be significantly higher in some transgenic lines (Fig. 7, poplar line A) in correspondence with the observed increase in GS activity per gram fresh weight.

Assessment of growth in height of transgenic poplar. Average heights of transgenic poplar containing the pine cytosolic GS1 and controls are presented in Table 1. In order to have representative data for the impact of GS1 overexpression a large number of independent primary transformants (n = 22) was considered. Data are

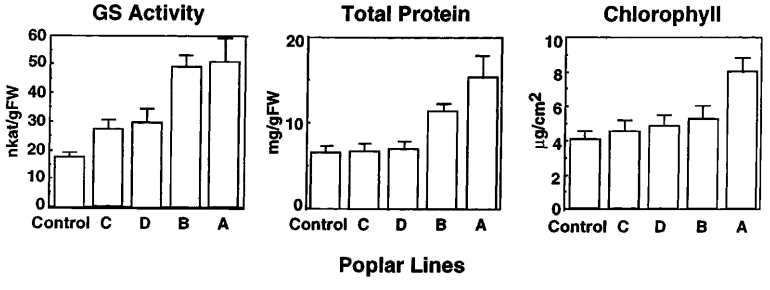

Fig. 7. Biochemical characteristics of the fourth youngest leaf of transgenic poplar lines. Total GS activity, soluble protein content and chlorophyll levels were determined in the leaves of control (non-transgenic) plants and in transgenic lines (A–D). The GS activity was determined by the synthetase assay. The protein content was estimated by the method described by Bradford (1976) using bovine serum albumin as standard. Total chlorophyll was extracted from leaves using 80% (v/v) acetone

Table 1. Growth of transgenic and control poplar. Control and transgenic poplars containing the pine cytosolic GS1 construct were grown in the greenhouse and height was measured after 2, 4, and 6 months. A total of 5 controls and 78 transgenic plants was measured representing 22 independent transformation events

Age		Average height (cm)	*P*	Transgenics > control Δ height (cm)
2 months	Transgenics	35.2	***	15.2 (76.0%)
	Controls	20.0		
4 months	Transgenics	111.2	***	22.4 (25.2%)
	Controls	88.8		
6 months	Transgenics	123.6	***	21.7 (21.3%)
	Controls	101.9		

***$P < 0.001$

reported for 2, 4, and 6 months growth in the greenhouse. At all time points, transgenics showed significantly greater growth in height than controls, ranging from 76.0% increases over controls at 2 months, to 21.3% increases over controls at 6 months. All differences were significant at the 0.001 level (Student *t*-test). Phenotypic differences for representative transgenics and control (untransformed) poplars are compared in Fig. 8.

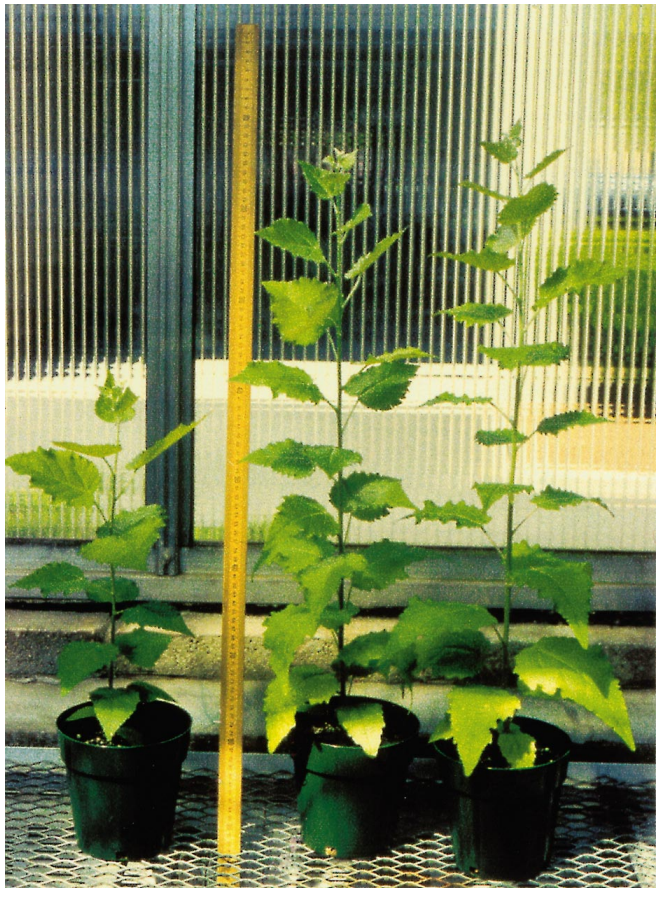

Fig. 8. Phenotype of hybrid poplars (*Populus tremula* × *P. alba*) expressing pine GS. The two plants on the *right* are representatives of two 3-month-old transgenic lines; the plant on the *left* is an untransformed control

Discussion

Plant growth and biomass accumulation are dependent on the availability, absorption, and assimilation of nutrients from the environment. Approaches to modify levels of key enzymes involved in steps in carbon and nitrogen assimilation and primary metabolism have been considered as a means to improve vegetative growth and biomass production (Foyer and Ferrario 1994, and references therein). However, overproduction of a key enzyme usually results in alteration of the content of specific metabolites with very limited (if any) observable effect on vegetative growth (Hudspeth et al. 1992; Brears et al. 1993; Foyer et al. 1994; Gallardo et al. 1995). For this reason, it has been suggested that other limiting steps are in place and that co-transformation with genes encoding other enzymes should be considered in order to overcome these limitations (Foyer and Ferrario 1994; Gallardo et al. 1995). However, most work on metabolic engineering using transgenic plants has been done in herbaceous species (e.g. *Arabidopsis* and tobacco), making it difficult to evaluate differences in long-term growth between transgenics and untransformed plants. Because of their comparatively slow growth rate, woody perennials present an alternative model for assessment of the significance of a particular enzyme or metabolic pathway in influencing plant growth and development, especially when targets for metabolic engineering are enzymes involved in assimilatory and primary metabolism, therefore having widespread effects on plant development.

Poplar (*Populus* sp.) is now considered a model in molecular investigations of forest trees because of its small genome size, facile vegetative propagation, and its amenability to *Agrobacterium*-mediated gene transfer (Ahuja 1987; Leplé et al. 1992). Analysis of GS isoenzymes in poplar leaves showed a similar pattern to that found in herbaceous angiosperm plants (Fig. 2). The majority of GS polypeptides accumulate in photosynthetic tissues and corresponds to chloroplastic GS2 (45 kDa), while the cytosolic GS1 polypeptide (40 kDa) is mainly detected in tissues enriched in leaf vascular elements (petioles and midribs, Fig. 2). Because of the similarity in their polypeptide patterns, we assume that poplar GS isoenzymes are involved in metabolic processes analogous to their corresponding counterparts in herbaceous plants. Although chloroplastic GS is the main isoenzyme in the leaves of many angiosperms, and the unique GS isoform expressed in mesophyll cells, a number of reports indicate a role for cytosolic GS in the development of photosynthetic tissues in response to changes in physiological conditions during development (Gallardo et al. 1988; Gálvez et al. 1990), senescence (Kawakami and Watanabe 1988), in response to pathogen attack (Pérez-García et al. 1995) or water stress (Bauer et al. 1997). Furthermore, cytosolic GS has an important physiological role in conifer photosynthetic metabolism (Cánovas et al. 1998 and references therein). Therefore, the modification of cytosolic GS expression seems to be a reasonable approach to establish the roles and relevance of this isoenzyme in plant development. To address this project

in forest trees, hybrid poplar was transformed via *Agrobacterium* with a chimeric gene composed of the pine cytosolic GS under the direction of the CaMV 35S promoter, and a gene conferring antibiotic resistance (NPTII) as selectable marker (Fig. 1). All plants regenerated in the presence of the antibiotic were shown to contain the pine GS gene by Southern blotting (Fig. 3) and PCR analysis. Pine GS transgene expression was also detected in all selected poplar lines and high levels of pine GS mRNA were shown in leaf tissues of transgenics (Fig. 4). The 41-kDa pine GS polypeptide was also detected both in leaf regions enriched in photosynthetic cells (leaf blades) and in vascular elements (petioles), indicating that pine GS transcripts are correctly processed by the angiosperm translational machinery (Fig. 5). Since cytosolic GS expression in angiosperm leaves is confined to vascular elements, the presence of the pine GS1 polypeptide in transgenic leaf devoid of midribs suggests that the pine GS1 protein is stable in mesophyll cells (Fig. 5C). It is interesting to note that detection of cytosolic GS in the whole leaf and the leaf blade of control plants was due to the presence of midribs in the material used for protein extracts; however, in the leaves of transgenic plants, the predominant cytosolic polypeptide corresponded in size to the transgene product (Fig. 5A,B). These results strongly suggest that down-regulation of endogenous cytosolic GS could occur as result of the transgene expression in vascular elements of the leaf blade. The analysis of the polypeptides in petioles clearly shows the presence of both the endogenous and the pine cytosolic GS in the transgenic plants (Fig. 5B). The coexistence of these products could be explained by the extreme abundance of endogenous cytosolic GS in the tissue, but could also reflect the more complex content of cell types in the petiole. In fact, photosynthetic cells are also present in the petiole, as denoted by detection of the chloroplastic GS polypeptide in extracts from control and transgenic plants (Figs. 2 and 5B).

The densitometric analysis of immunoblots from leaves indicated that the content in GS polypeptide (GS1 + GS2) was 49.8% higher in transgenic than in control leaves. Similar relative values of total GS polypeptide and GS specific activity in control and transgenic leaves were also observed (Fig. 6), suggesting that most of the pine GS polypeptides are assembled into functional holoenzymes. In addition, these data also suggest that no additional post-translational regulation controlling GS holoenzyme assembly is acting in the transgenic leaves at this physiological state; nevertheless, we cannot rule out such a possibility in other tissues or physiological conditions, as suggested in transgenic tobacco by Temple et al. (1993). When the GS1 content was quantified, the cytosolic subunit accounted for 27.5% of total GS polypeptide in the control leaf, while an average of 48.1% was estimated in the leaf transgenics. This increase in cytosolic GS as result of the transgene expression cannot explain differences found in total GS polypeptide (49.8% increase respect to control) or in specific activity (42.5% increase respect to control), suggesting that expression of pine GS in poplar provokes more than a simple additive effect on the content of total GS polypeptide or specific activity in the leaf. Interestingly, when GS activity was expressed on a fresh-weight basis an increase of 2- to 3-fold was observed in the leaves of several primary transformants (Fig. 7A); furthermore, the content of GS activity per g fresh weight was positively correlated with increased protein and chlorophyll accumulation in the leaf of transgenic lines (Fig. 7B,C). Hence, these results suggest that up-regulation of cytosolic GS in poplar leaves may lead to a global effect on the synthesis of nitrogenous molecules. Although the presented studies were carried out with the same leaf, the observed values of GS activity, protein and chlorophyll content may reflect differences in transgene expression during the ontogeny of the leaf due to a positional effect on the poplar genome. The balanced increase in GS activity in the transgenics when expressed on a protein-content basis (42.5%) may, therefore, be explained by the increase in total protein content which can also affect the abundance of GS2. Similar results describing an increase in nitrogenous compounds, including total protein and/or total chlorophyll or biomass, have been reported in herbaceous non-legume and legume plants overexpressing GS (Temple et al. 1993; Hirel et al. 1997). However, in other reports, no phenotypic effects were apparent in transgenic plants overproducing GS1 (Eckes et al. 1989; Hirel et al. 1992). These discrepancies may indicate instability of the holoenzyme in the different heterologous systems, or may reflect differences attributable to the different plant models (species) used in the transformation studies.

In our forest tree model, the expression of the transgene is also accompanied by alteration of the phenotype with increased growth in height especially noticeable during the first months of growth in the greenhouse (Table 1, Fig. 8). In this context, a recent report has postulated that the enhancement of cytosolic GS expression in transgenic *Lotus corniculatus* can activate physiological processes leading to early flowering and plant senescence (Vincent et al. 1997). Our results in a tree model also support the hypothesis that up-regulation of cytosolic GS can produce a global effect on plant growth and development. The present work is, to our knowledge, the first study addressing the increase in nitrogen-use efficiency in transgenic trees by overproducing a key enzyme involved in amino-acid biosynthesis. Work is in progress to evaluate growth characteristics of engineered trees in open-field trials and to characterize the GS holoenzyme produced as result of the transgene expression.

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