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Assembly of a cytosolic pine glutamine synthetase holoenzyme in leaves of transgenic poplar leads to enhanced vegetative growth in young plants

J. FU[1]*, R. SAMPALO[2]*, F. GALLARDO[2], F. M. CÁNOVAS[2] & E. G. KIRBY[1]

[1]*Department of Biological Sciences, Rutgers University, University Heights, Newark, New Jersey 07102 USA and*
[2]*Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias – Instituto Andaluz de Biotecnología, Unidad Asociada CSIC-UMA, Universidad de Málaga, E-29071 Málaga, Spain*

ABSTRACT

Over-expression of glutamine synthetase (GS, EC 6.3.1.2), a key enzyme in nitrogen assimilation, may be a reasonable approach to enhance plant nitrogen use efficiency. In this work phenotypic and biochemical characterizations of young transgenic poplars showing ectopic expression of a pine cytosolic GS transgene in photosynthetic tissue (Gallardo *et al*., *Planta* 210, 19–26, 1999) are presented. Analysis of 22 independent transgenic lines in a 6 month greenhouse study indicated that expression of the pine GS transgene affects early vegetative growth and leaf morphology. In comparison with non-transgenic controls, transgenic trees exhibited significantly greater numbers of nodes and leaves (12%), and higher average leaf length and width resulting in an increase in leaf area (25%). Leaf shape was not altered. Transgenic poplars also exhibited increased GS activity (66%), chlorophyll content (33%) and protein content (21%). Plant height was correlated with GS content in young leaves, suggesting that GS can be considered a marker for vegetative growth. Molecular and kinetic characterization of GS isoforms in leaves indicated that poplar GS isoforms are similar to their counterparts in herbaceous plants. A new GS isoenzyme that displayed molecular and kinetic characteristics corresponding to the octomeric pine cytosolic GS1 was identified in the photosynthetic tissues of transgenic poplar leaves. These results indicate that enhanced growth and alterations in biochemistry during early growth are the consequence of transgene expression and assembly of pine GS1 subunits into a new functional holoenzyme in the cytosol of photosynthetic cells.

Key-words: metabolic engineering; nitrogen assimilation; plant development; poplar.

Correspondence: Edward G. Kirby.
E-mail: ekirby@andromeda.rutgers.edu

*These authors have equally contributed to this work.

INTRODUCTION

Molecular strategies to increase biomass production of forest trees are now feasible because of the availability of efficient transformation protocols. Clones of *Populus* spp. have been utilized as model systems for genetic engineering of forest trees because of their small genome size, facile vegetative propagation, and ease of transformation (Leplé *et al*. 1992; Merkle & Dean 2000). Key enzymes in primary metabolism were first considered as targets in strategies to increase biomass production in herbaceous plants (Foyer & Ferrario 1994). However, the increase in activity of a particular enzyme using a genetic engineering approach is complex. Introduction of homologous genes into the plant genome can result in down-regulation of the endogenous genes (Napoli, Lemiex & Jorgensen 1990). Thus, introduction of heterologous sequences may be preferred in order to over-produce a protein of interest. Moreover, many key enzymes in metabolic pathways are oligomeric proteins and assembly of their subunits in the heterologous system is required to obtain a functional enzyme. The increase in the activity of an enzyme of interest can also be difficult if the steady-state level of the endogenous enzyme is high in target plant tissues, or if the enzyme is subjected to a post-translational regulation, as is commonly the case in key enzymes in primary metabolic pathways.

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme in nitrogen metabolism and has been considered a target for metabolic engineering. GS represents the main entry point of ammonium into organic nitrogen compounds and is therefore a central step in the biosynthesis of nitrogenous compounds (Miflin & Lea 1980). Functional GS is a holoenzyme composed of eight subunits that are encoded by a small family of nuclear genes. Two main GS isoenzymes are differentially expressed in plant tissues: GS1 in the cytosol and GS2 in chloroplasts. In many plants GS2 is highly abundant in the mesophyll cells of the leaf where it is involved in assimilation of ammonium from the reduction of nitrate, and in recycling the ammonium released during photores-

piration. In contrast, GS1 is expressed in several organs and tissues, including the vascular elements of the leaf, stem, root, flower and fruits (Lea 1997; Cren & Hirel 1999). Post-translational regulation by phosphorylation and interaction with 14-3-3 proteins has recently been described for both GS isoenzymes (Finnemann & Schjoerring 2000; Riedel, Tischner & Mäck 2001).

Although the role of chloroplastic GS is widely accepted, the biological function of the cytosolic GS is still a matter for discussion. The cytosolic holoenzyme is involved in the recycling of ammonium produced from secondary processes, and in primary nitrogen assimilation in the root. However, over the past decade a number of reports have proposed that GS1 plays a role in the development of photosynthetic tissues in angiosperms. Variation in the relative proportions of GS isoenzymes, or up-regulation of cytosolic GS expression, have been observed during plastid differentiation (Gallardo *et al*. 1988; Gálvez, Gallardo & Cánovas 1990) and have been associated with leaf senescence (Kawakami & Watanabe 1988). Furthermore, GS1 has been shown to be regulated in response to biotic or abiotic stresses (Pérez-García *et al*. 1995; Bauer *et al*. 1997). In conifers, cytosolic GS is the only GS enzyme detected in seedlings and its expression is associated with the development of the seedling and the chloroplast (García-Gutiérrez *et al*. 1998; Cánovas *et al*. 1998; Suárez *et al*. 2002). Based on accumulated information, over-production of GS1 in photosynthetic tissues was considered a reasonable strategy to improve nitrogen use efficiency, and to study the biological role of this GS isoenzyme. We have previously reported genetic transformation of poplar plants with a gene encoding the pine cytosolic GS1. Expression of the transgene was detected at mRNA and protein levels and preliminary characterization of transformed plants indicated that transgenic lines exhibit enhanced vegetative growth (Gallardo *et al*. 1999). In this paper we show that differences in vegetative growth of poplar expressing the pine transgene are correlated with their GS contents. To our knowledge, this is the first report describing the production and characterization of a new functional GS holoenzyme in transformed plants. This new GS holoenzyme is exclusively composed of pine subunits.

MATERIALS AND METHODS

Plant materials and culture conditions

Untransformed (control) hybrid poplar (*Populus tremula* × *Populus alba*, clone INRA 7171-B4) and transgenic lines expressing a cytosolic pine GS gene under the control of cauliflower mosaic virus 35S promoter were produced and maintained *in vitro*, as described (Leplé *et al*. 1992; Gallardo *et al*. 1999). Rooted shoots of transformed and control plants were transferred to the greenhouse in 4 inch (10 cm) plastic pots containing a potting mix (MetroMix 200; Scotts Co., Marysville, OH, USA) with no mineral fertilizer. The greenhouse was maintained at approximately 21 °C.

Protein and chlorophyll extraction and determination

Leaves corresponding to the fourth youngest leaf of 3- to 4-month-old-plants were selected for analysis. Preliminary studies indicated that GS activity recovered from the fourth youngest leaves remained constant and was independent of plant age. In order to obtain sufficient material for ion-exchange separations, the fourth to tenth leaves were used. Protein extracts were prepared as described (Gallardo *et al*. 1999). In leaf extracts prepared for subsequent fractionation by ion-exchange chromatography, midribs were removed. Quantification of soluble proteins in the extracts was performed using the procedure described by Bradford (1976) with bovine serum albumin as standard. Total chlorophyll was extracted using 80% (v/v) acetone and the chlorophyll content was determined spectrophotometrically (Graan & Ort 1984).

Fractionation of proteins by ion-exchange chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Proteins from crude extracts were precipitated with 80% (w/v) saturated ammonium sulphate and dialysed overnight at 4 °C against 200 volumes of running buffer (Tris-HCl 50 mM, 2 mM ethylenediaminetetraacetic acid, 10 mM magnesium sulphate, 10 mM 2-mercaptoethanol, pH 8·0). Concentrated protein extracts were loaded onto a 40 mL DEAE-Sephacel column Sigma Chemical, St Louis, MO, USA equilibrated with running buffer. After washing the column, elution of bound proteins was carried out with a linear gradient of 0–0·6 M KCl. Chromatography was performed at 4 °C with a flux of 40 mL h^{-1}. Elution of GS was monitored in 4 mL fractions by determination of enzyme activity (transferase assay). Separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out using 10% (w/v) acrylamide gels using the discontinuous buffer system of Laemmli (1970). Detection of GS polypeptides was performed by Western blot analysis.

Determination of GS activity, kinetic studies and detection of GS polypeptides by Western blot analysis

GS assays were carried out as described previously (Cánovas *et al*. 1991). The GS activity in crude extracts was determined using the synthetase assay. For monitoring the elution of GS activity in the ion-exchange separations, the more sensitive transferase assay was used. Kinetic studies were performed with enzyme preparations desalted by gel filtration. Values of kinetic constants for ATP, glutamate and hydroxylamine were determined using the synthetase assay at different concentrations of one substrate and at the saturation concentrations of the other substrates. Because GS isoenzymes exhibit similar affinities for hydroxylamine and ammonium, as observed for the recombinant pine

holoenzyme expressed in poplar (this work) and in *Escherichia coli* (de la Torre *et al*. 2002), only the K_m value for hydroxylamine was determined. For Western blot analysis, GS polypeptides were detected using polyclonal antibodies raised against recombinant pine GS (Cantón *et al*. 1996). Protein transfers were conducted with a Multiphor II Novablot apparatus (Pharmacia Biotech, Uppsala, Sweden) by the application of 0·8 mA cm^{-2} and constant current for 90 min. Immunocomplexes were identified with peroxidase-conjugated immunoglobulin with a peroxidase : immunoglobulin molar ratio of 3·3 (Vector Laboratories, Burlingame, CA, USA).

Gel filtration chromatography

Preparations of GS holoenzymes partially purified by ion-exchange chromatography were fractionated through a Sephacryl S-300 column (89·5 cm × 1·6 cm; Pharmacia Biotech) equilibrated with running buffer as described (Gallardo & Cánovas 1992). Molecular mass was estimated by calibration of Sephacryl S-300 with ferritin (450 kDa) catalase (240 kDa), yeast alcohol dehydrogenase (141 kDa), bovine serum albumin (66 kDa), and cytochrome *c* (12·4 kDa).

Height growth and leaf size measurements

After regenerated plants were transferred to the greenhouse, height measurements were made weekly. A total of 78 transgenic plants were measured, representing 22 independent transformation events and five non-transgenic controls. Measurements of leaf length and width were made on fully expanded mature leaves of 22 transgenic lines and controls 4 months after the plants were transferred to the greenhouse. A total of 390 leaves derived from 22 transgenic lines and 25 leaves from control plants were measured. The numbers of leaves of 78 transgenic plants representing 22 transgenic lines and five control plants were counted after 2, 3 and 6 months in the greenhouse. Individual leaf areas were estimated using the equation: leaf area = 0·5(leaf length × leaf width). Total leaf area per plant was calculated by multiplying the average area per leaf by the average number of leaves per plant.

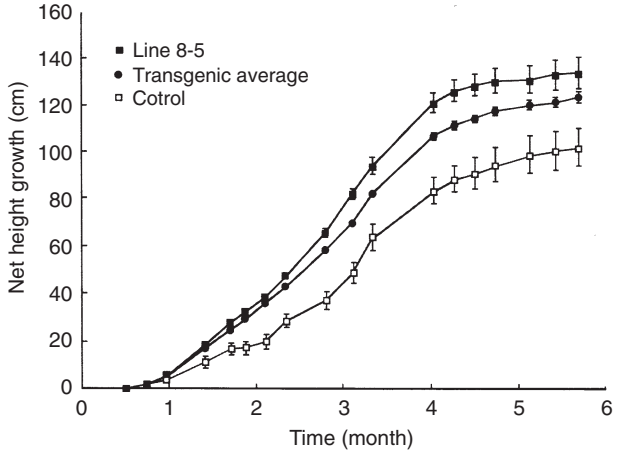

Figure 1. Growth of control and transgenic poplar lines. Height data from 78 transgenic plants representing 22 primary independently transformed lines and five non-transformed plants (controls) were included in this study. Line 8–5 displayed the highest growth of all transgenic lines included in the present study.

Table 1. Average leaf number per plant of transgenic plants and controls

		Leaf number	*P*	Transgenics > controls
2 months	Transgenics	25·3	***	7·3 (41%)
	Controls	18·0		
3 months	Transgenics	35·6	**	8·1 (30%)
	Controls	27·5		
6 months	Transgenics	48·0	**	5·0 (12%)
	Controls	43·0		

The leaf numbers were determined 2, 3 and 6 months after plants were transferred into the greenhouse, respectively. The leaf numbers of 78 transgenic plants representing 22 primary independent transgenic lines and five non-transformed plants (controls) were counted. ***$P < 0{\cdot}001$; **$P < 0{\cdot}01$.

RESULTS

Phenotypic and biochemical characteristics of transgenic poplar expressing the pine cytosolic GS

The vegetative growth of 22 transgenic poplar lines and controls was analysed over a period of 6 months in the greenhouse (Fig. 1). Differences in height growth between transgenic and control lines were significant throughout the study period with the greatest differences observed during the first 3 months. The transgenic plants reached average heights that were 51 and 76% greater than control plants after the first and second months, respectively. Transgenic plants reached an average height of greater than 1 m after 4 months, whereas control plants required 5 months to reach the same height. At the end of the experiment (6 months) the transgenic plants achieved an average height of 123·9 cm, which is 21% greater than control plants (101·9 cm).

Transgenic and control lines exhibited linear increases in growth rate during the first 3 months of the study, reaching peak rates (>1 cm d^{-1}) during the second and third months (data not shown). The growth rate of all lines declined after the third month, possibly indicating nutrient depletion from the soil. No nutrient supplements were provided during the period of the study.

After 2 months, transgenic plants produced 41% more leaves than controls (Table 1). At the end of the study, the transgenic plants maintained on average a leaf number that was 12% greater than control plants (Table 1). Average leaf length and width were 17 and 9% greater, respectively, in transgenic plants than in controls (Table 2), resulting in the

Table 2. Average leaf length, width and area per leaf of transgenic and control plants

		Average	*P*	Transgenics > controls
Length	Transgenics	12·2 cm	*	1·7 cm (17%)
	Controls	10·5 cm		
Width	Transgenics	8·6 cm	*	0·7 cm (9%)
	Controls	7·9 cm		
Areas	Transgenics	51·9 cm^2	*	10·0 cm^2 (25%)
	Controls	41·9 cm^2		

The measurements of leaf length and width were made on the fully expanded mature leaves of 22 independently transformed transgenic lines and non-transgenic controls 5 months after plants were transferred to the greenhouse. Measurements were made from a total of 380 leaves derived from 22 transgenic lines and 20 leaves from control plants. *$P < 0{\cdot}05$.

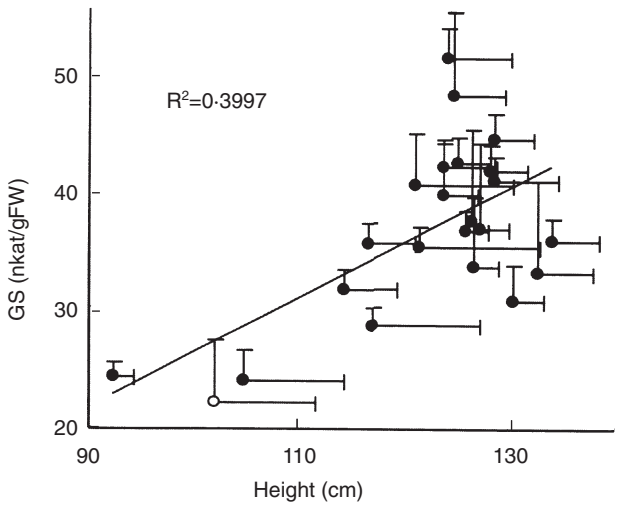

Figure 2. Relationship between GS activity and height growth for control and transgenic poplar lines. Values are averages of primary independent transgenic lines (closed circles) and the controls (open circle). Bars represent the average standard error values for each variable. The R^2 value was calculated from the average data. **$P < 0{\cdot}01$ ($R = 0{\cdot}6322 > R_{0{\cdot}01} = 0{\cdot}526$).

transgenic plants having an average leaf area 25% greater than controls. Although leaf area varied between transgenic plants and controls, the leaf shape did not appear to be affected by the transformation.

In our preliminary studies (Gallardo *et al*. 1999), analysis of only four transgenic lines showed that leaves of transgenic plants had enhanced glutamine synthetase activity and greater protein and chlorophyll contents than controls. These observations were confirmed in the extensive present study. The GS activity, protein and chlorophyll contents were 66, 33 and 21% greater in the transgenic plants than in control plants (fresh weight basis) (Table 3).

A linear correlation between GS activity and height growth was observed and is statistically significant ($P < 0{\cdot}01$) (Fig. 2). These results suggest that GS activity in young leaves can be used as a marker for vegetative development in the poplar lines. Weak correlations were observed between protein and chlorophyll contents and height growth (data not shown).

Characterization of GS isoenzymes in transgenic and control plants

We previously reported the presence of pine GS polypeptides in protein extracts from young transgenic leaves (Gallardo *et al*. 1999). GS is an oligomeric enzyme. As such, in transgenic plants, pine GS polypeptides could be assembled in combination with poplar GS polypeptides, producing a heteromeric recombinant enzyme composed of a mixture of pine and poplar subunits. Alternatively, the pine GS polypeptides could be assembled into a pure pine GS1 holoenzyme producing an entirely new isoenzyme in transgenic poplars. In order to investigate the GS isoenzyme patterns in leaves of transgenic and control poplar lines, it is important to know if the higher level of GS activity shown in transgenic leaves is associated with the alteration of the GS isoenzyme pattern. To examine the pattern of GS expression in transgenic and control lines, we fractionated leaf proteins using a DEAE-Sephacel column (Gallardo *et al*. 1988; Gálvez *et al*. 1990). To avoid the presence of endogenous poplar cytosolic GS in the extracts, midrib tissues were carefully removed from leaves prior to extraction. The profile of GS activity from control leaves indicated the presence of a single GS peak that eluted at 0·4 M KCl (Fig. 3, top). When a similar experiment was performed with proteins prepared from transgenic leaves, two peaks of GS activity were detected: a major peak at 0·4 M KCl (Fig. 3, top, peak II), and a minor peak at 0·27 M

Table 3. GS activity, protein content, and chlorophyll content for transgenic and control poplars

	GS activity (nkat g^{-1}FW)	Protein content (μg g^{-1}FW)	Chlorophyll content (μg g^{-1}FW)
Control	22·2	3846·7	484·2
Average of transgenics	36·8	5132·5	586·1
Transgenics > control	14·6 (66%)	1285·8 (33%)	101·9 (21%)
P	***	***	***

Twenty-two transgenic lines and control plants were assayed with three replicates each. ***$P < 0{\cdot}001$.

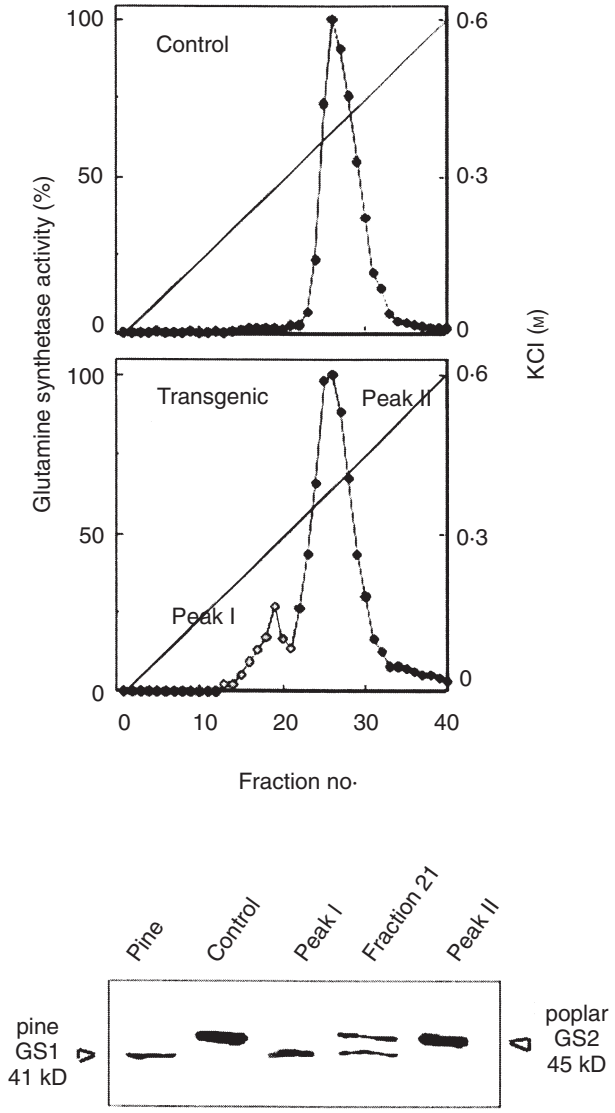

Figure 3. GS isoenzyme content in transgenic and control poplar leaf tissues. (Top) Proteins from control and transgenic leaf extracts enriched in photosynthetic cells were fractionated by ion-exchange chromatography. Elution of GS was monitored by enzyme assay after application of a linear KCl gradient. (Bottom) Western blot analysis of polypeptides taken from the peak fractions using polyclonal GS antibodies (Cantón *et al.* 1996). A pine cotyledonary extract (10 μg protein) was also introduced for analysis.

KCl representing approximately 18% of the total GS activity (Fig. 3, top, peak I). Polypeptide characterization of the peak fractions was conducted by Western blot analysis (Fig. 3, bottom). One GS subunit isoform corresponding to the chloroplastic GS2 (45 kDa) was detected in preparations from control leaves. This GS2 polypeptide was also detected in the peak II of transgenic leaves. In extracts of transgenic leaves an additional polypeptide was present in the peak I. This polypeptide corresponded in size to the pine GS1 subunits (41 kDa). Both the endogenous poplar GS2 and the pine transgene product GS1 co-eluted in the fraction 21. Similar results were obtained for three different transgenic lines, indicating the presence of a new GS enzyme (pine GS1) in photosynthetic tissues of transgenic lines.

In order to determine whether the new GS enzyme ectopically expressed in poplar leaves exhibits characteristics similar to endogenous poplar GS isoforms, molecular characterization of the GS enzymes was undertaken using gel filtration (Fig. 4). For these experiments we used partially purified proteins prepared by ion-exchange chromatography, as described above. Molecular masses of native enzymes were estimated using a Sephacryl S-300 column previously calibrated with protein markers of known size. Estimated values for molecular masses of endogenous poplar chloroplastic and cytosolic GS isoenzymes were 418 and 320 kDa, respectively (Fig. 4). These data suggest that poplar GS isoenzymes are octomeric enzymes, as are their counterparts in herbaceous and other woody plants. The molecular mass of the new GS isoform from transgenic leaves was estimated to be approximately 340 kDa (Fig. 4). This determination is consistent with an octomeric pine GS1 holoenzyme composed of 41 kDa subunits.

In order to gain a better understanding of biochemical characteristics conferred to transgenic poplars because of the presence of the new GS holoenzyme, further characterization of GS isoforms focused on their catalytic properties. The velocity kinetics of the new GS isoenzyme in transgenic poplar composed of pine subunits were examined using ATP, glutamate and hydroxylamine as variable substrates (Table 4). In all cases, graphical representations of enzyme kinetic data according to Lineweaver–Burk, Eadie–Hofstee and Hanes equations (Price & Stevens 1989) showed non-cooperativity of the enzyme for the substrates. The K_m values for the pine holoenzyme for ATP, glutamate and hydroxylamine were 0·5, 4·2 and 0·27 mM, respectively. These kinetic data were similar to those obtained for the native pine GS enzyme produced in *E. coli* (de la Torre

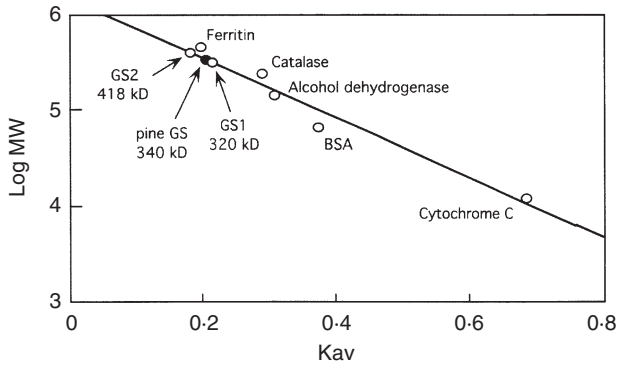

Figure 4. Molecular weight estimation of GS holoenzymes detected in transgenic and control poplar leaves. The log of the molecular weight was plotted versus the partition coefficient ($K_{av} = V_e - V_o/V_t - V_o$) of the proteins eluted through a Sephacryl S-300 column.

Table 4. Kinetic properties of GS holoenzymes

GS holoenzyme	Substrate		
	ATP	Glutamate	Hydroxylamine
Pine GS	K_m = 0·5 mM	K_m = 4·2 mM	K_m = 0·27 mM
Poplar GS1	K_m = 0·5 mM	K_m = 3 mM	$S_{0·5}$ = 0·49 mM nH = 0·84
Poplar GS2	K_m = 0·6 mM	$S_{0·5}$ = 19 mM nH = 0·75	K_m = 0·82 mM
Higher plant GS[a]	0·1–1·5 mM	1–13 mM	0·04–0·7 mM

[a]Stewart *et al*. (1980).

et al. 2002), and are in the range of values reported for GS in higher plants (Stewart, Mann & Fentem 1980).

Kinetic properties for the endogenous poplar cytosolic and chloroplastic GS isoenzymes were also determined in partially purified preparations from leaves of control plants (Table 4). Petioles of control leaves were used for preparation of the poplar cytosolic GS because of their high content in cytosolic GS polypeptide (Gallardo *et al*. 1999). The K_m values for ATP were similar for poplar GS1 and GS2 (0·5 and 0·6 mM, respectively), but the isoenzymes differed in their kinetic behavior for glutamate and hydroxylamine. Poplar GS1 exhibited negative cooperativity for hydroxylamine (nH = 0·84), whereas the GS2 holoenzyme exhibited negative cooperativity for glutamate (nH = 0·75). The K_m or $S_{0·5}$-values were similar to values previously reported for GS isoenzymes from herbaceous plants (Stewart *et al*. 1980).

DISCUSSION

Because glutamine and glutamate are the starting points in metabolic pathways leading to the biosynthesis of nitrogen-containing molecules in plants, and as glutamine is the nitrogen donor for the biosynthesis of glutamate, it is clear that glutamine synthetase plays a fundamental role in plant growth and development. In addition, metabolic processes, including photorespiration and the synthesis of phenylpropanoids, regulate the recycling of ammonium through glutamine. Thus, engineering enhanced activity and expression of functional GS holoenzymes could lead to enhanced growth.

In this work we confirm that vegetative growth in poplar can be enhanced by ectopic expression of a pine GS transgene. Furthermore, we demonstrate that this enhanced growth is linearly correlated with increases in total GS activity and is accompanied by the assembly of heterologous pine GS1 subunits into a new cytosolic GS holoenzyme in transgenic leaf cells. Transgenic lines exhibited a higher growth rate that was especially noticeable during the first 3 months of growth. These enhancements in vegetative growth correspond with an increase in total photosynthetic surface area. Similar results have recently been reported in transgenic tobacco expressing a cytosolic alfalfa GS under conditions of nitrogen starvation (Fuentes *et al*. 2001). Although preliminary analysis of our data indicates that enhanced growth of transgenic poplar may result from higher number of leaves, it is difficult to assess the molecular changes associated with their higher vegetative growth because GS expression is subject to a developmental control (Lam *et al*. 1996).

Biochemical analysis of young leaves of transformed poplar lines confirming that GS activity is significantly enhanced in transgenic lines. Greater chlorophyll and protein levels in young leaves of transgenic plants indicate that transgene expression has a significant effect on biosynthesis of nitrogen-containing compounds. These biochemical changes could be responsible for improved growth of transgenic lines. It is intriguing that GS activity in young leaves correlates better with height growth than with protein and/or chlorophyll contents, suggesting that ectopic GS expression could affect pathways that contribute to vegetative growth other than through enhanced biosynthesis of nitrogen compounds. This has been shown by Kozaki & Takeba (1996) and Fuentes *et al*. (2001), who report that overexpression of chloroplastic and cytosolic GS in transgenic tobacco causes changes in the photosynthetic and photorespiratory capacities of the leaf, thus affecting vegetative growth.

The linear correlation between GS activity in young leaves and plant height suggests that GS activity can serve as a marker for early vegetative growth. This conclusion is consistent with the role of GS in primary development (Cánovas *et al*. 1998; Cren & Hirel 1999; Suárez *et al*. 2002). Furthermore, in mapping experiments, cytosolic GS genes are reported to co-locate with quantitative trait loci for various agronomic and physiological traits, including grain yield in maize (Hirel *et al*. 2001) and vegetative growth in pine (C. Plomion and J-M. Friggerio, personal comm.).

The observed increase in GS activity in transgenic poplar could result from the assembly of hybrid GS holoenzymes composed of subunits encoded by both endogenous GS genes and the pine GS transgene. Such hybrid GS holoenzymes could exhibit altered kinetic properties resulting in the higher GS activity detected in transgenic leaves. Alternatively, transgene expression could result in the formation of a pure pine GS holoenzyme, whose presence could have an additive effect, thus resulting in higher GS activity. Our data support this second hypothesis. Only a limited number

of reports (Hirel *et al*. 1992, 1997; Vincent *et al*. 1997) have utilized ion-exchange chromatography to investigate the GS isoenzyme pattern in transgenic plants. The present work is to our knowledge the first report describing molecular and kinetic properties of a transgenic GS holoenzyme produced as the result of the transgene expression. Characterization of endogenous poplar GS isoenzymes in photosynthetic cells of non-transgenic controls revealed that GS2 is the only GS isoenzyme present. This enzyme exhibited molecular and kinetic characteristics similar to other chloroplastic GS isoenzymes, indicating that poplar GS2 is similar to its counterparts in herbaceous plants. However, in transgenic poplar, a new GS holoenzyme, representing 18% of the total GS activity, is also present in photosynthetic cells. Molecular characterization of this new transgenic enzyme, including determination of subunit size and native molecular weight, indicates that the new enzyme corresponds to the pine GS holoenzyme. This conclusion is also based on the absence of other (poplar) GS polypeptides in the preparation of the holoenzyme. Furthermore, this shows that no hybrid holoenzymes, that is those consisting of endogenous poplar and transgenic pine subunits, are produced in photosynthetic cells of transgenic poplar. We conclude therefore that changes in photosynthetic tissues of transgenic plants, which affect vegetative growth, can be attributed to the formation of this new cytosolic pine GS holoenzyme. Interestingly, the percentage of GS activity corresponding to the pine enzyme (18%) is similar to the increase in GS1 polypeptide observed in young leaves of the transgenic plants (21%) (Gallardo *et al*. 1999). However, the increase in total GS activity observed in the 22-independent transgenic lines was higher (65%). As GS2 expression is associated with chloroplast development, the increase in total GS activity may result from enhanced chloroplast development in the leaves of transgenic plants, as evidenced by their higher protein and chlorophyll contents (Table 3). These data indicate the possible role of cytosolic pine GS holoenzyme in enhancing the development of photosynthetic tissues of transgenic plants. However, the production of hybrid GS holoenzymes in non-photosynthetic tissues cannot be ruled out as a contributing factor to enhanced growth because poplar GS1 and pine GS elute from ion exchange columns at a similar ionic strength (data not shown).

Recently, we have used stable ^{15}N-enrichment to investigate the efficiency of nitrogen assimilation in poplar GS transgenic plants. Levels of free ammonium and nitrate in leaves of transgenic plants were significantly less than in non-transgenic controls. However, greater levels of assimilated nitrogen (total free amino acid levels and structural nitrogen compounds) were produced in transgenic plants than in controls. This indicates a higher efficiency of nitrogen assimilation in GS transgenic plants (Man and Kirby, in preparation).

Because GS1 isoenzymes from poplar, pine and other plants exhibit similar kinetic properties (Table 3), enhancement of vegetative growth and synthesis of nitrogen-containing compounds could be expected as a result of ectopic expression of a cytosolic GS holoenzyme, regardless of origin. Enhancement of vegetative growth has been observed in herbaceous species expressing a cytosolic GS transgene, including *Lotus corniculatus* (Vincent *et al*. 1997) and tobacco (Fuentes *et al*. 2001); however, no molecular or kinetic evidence of a new GS holoenzyme was described in those works.

In conclusion, our data indicate that early growth in poplar can be enhanced by ectopic expression of a cytosolic GS holoenzyme in photosynthetic tissues. In the current work, and in a previous report on tobacco (Fuentes *et al*. 2000), plants were maintained under low nitrogen conditions. The ectopic expression of a GS holoenzyme in the cytosol of photosynthetic cells could improve the capacity of the leaf to recycle the ammonium released via secondary processes. The importance of re-assimilation of ammonium lost in photosynthetic cells is clear. *Arabidopsis* and barley mutants with reduced capacity to re-assimilate ammonium are lethal under photorespiratory conditions (Somerville & Ogren 1980; Wallsgrove *et al*. 1987). The improved efficiency of photosynthetic cells of transgenic poplar to recycle ammonium could allow better dissipation of the excess reducing power generated in photosynthetic cells as indicated by Kozaki & Takeba (1996). This enhanced efficiency for nitrogen recycling could result in better exploitation of nutrient resources. Investigation of the possible alteration of GS isoenzyme patterns in other organs is currently underway to evaluate the contribution of transgene expression in non-photosynthetic tissues to vegetative growth.

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