

# Growth, photosynthesis, and herbicide tolerance of genetically modified hybrid poplar

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Hybrid poplar clone NC-5339 (*Populus alba* × *Populus grandidentata* cv. Crandon) was genetically modified for glyphosate (*N*-(phosphonomethyl)glycine) tolerance by *Agrobacterium*-mediated transformation with genetic constructs (pPMG 85/587 and pCGN 1107) that included the mutant *aroA* gene for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) and the neomycin phosphotransferase selectable marker gene. pCGN 1107 also harbored the coding sequence for a chloroplast transit peptide and the CaMV 35S promoter fused to the mutant *aroA* gene. Transformants were selected for kanamycin tolerance, and integration of the *aroA* gene was verified by Southern blot analysis. Cuttings of NC-5339 and the derived transformants were rooted and grown in glasshouses at separate locations, with maximum photosynthetic photon flux density of 1600 and 750  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Productivity was assessed by growth studies and photosynthesis measurements at both locations. Glyphosate tolerance was tested by (i) measurement of chlorophyll concentration in herbicide-treated leaf discs and (ii) whole-plant spray tests. Plants transformed with construct pCGN 1107 were the most herbicide tolerant. Perhaps high-level expression of the *aroA* gene by the CaMV 35S promoter, transport of mutant EPSP synthase into the chloroplasts, or both facilitated glyphosate tolerance. Plants grown at higher photosynthetic photon flux densities (1600 vs. 750  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) had significantly higher maximum net photosynthesis (19.8 vs. 16.2  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and more biomass accumulation (47.6 vs. 33.7 g). However, there were no significant differences between NC-5339 and transformants within location for net photosynthesis or any growth parameter. Genetic modification of hybrid poplar NC-5339 for glyphosate tolerance did not adversely affect plant productivity at either location.

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Le clone de peuplier NC-5339 (*Populus alba* × *Populus grandidentata* cv. Crandon) a été modifié génétiquement pour la tolérance au glyphosate (*N*-phosphonométhyl)glycine) par la méthode de transformation par *Agrobacterium*. Les constructions génétiques (pPMG 85/587 et pCGN 1107) transférées au sein du génome du clone de peuplier incluaient le gène mutant *aroA* codant pour la 5-enolpyruvylshikimate-3-phosphate (EPSP) synthétase (EC 2.5.1.19) et le gène marqueur de sélection néomycine phosphotransférase. La construction pCGN 1107 incluait aussi la séquence codant pour le peptide de transfert au chloroplaste et le promoteur CaMV 35S fusionné au gène mutant *aroA*. Les transformants ont été sélectionnés pour la résistance à la kanamycine et l'intégration du gène *aroA* a été vérifiée par analyse de transfert Southern. Des boutures du clone NC-5339 ainsi que les transformants ont été enracinés et mis en croissance en serre au sein de deux environnements caractérisés par des densités maximales de flux de lumière photosynthétique (PPFD) de 1600 et 750  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Le rendement a été évalué pour chacun des deux environnements à l'aide d'études de croissance et de mesures de la photosynthèse. La tolérance au glyphosate a été évaluée par (i) la mesure de la concentration en chlorophylle au sein de disques foliaires traités à l'herbicide et (ii) des tests de pulvérisation de l'herbicide sur des plantes entières. Les plantes transformées avec la construction pCGN 1107 affichaient la plus grande tolérance à l'herbicide. Cette tolérance au glyphosate pourrait être causée par de

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forts niveaux d'expression du gène *aroA* par le promoteur CaMV 35S, par le transfert de la synthétase mutante EPSP vers les chloroplastes ou encore, une combinaison de ces deux facteurs. Les plantes mises en croissance sous forte PFD ( $1600$  vs  $750 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) affichaient une valeur de photosynthèse nette significativement plus élevée ( $19,8$  vs  $16,2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) et une meilleure accumulation de biomasse ( $47,6$  vs  $33,7$  g). Toutefois, il n'y avait pas de différences significatives entre le clone NC-5339 et les transformants au niveau de chacun des environnements, que ce soit pour la photosynthèse nette ou n'importe quel paramètre de croissance. La modification génétique du clone de peuplier hybride NC-5339 pour la tolérance au glyphosate n'a pas affecté négativement le rendement des plantes et ce, au niveau de chacun des deux environnements.

[Traduit par la Rédaction]

## Introduction

Poplar hybrids have high light-saturated photosynthetic rates and potential utility as a renewable biofuel, but they lack tolerance to commercially important herbicides that may be needed for successful plantation management. Tolerance to glyphosate (*N*-(phosphonomethyl)glycine) has been conferred to many plants by *Agrobacterium*-mediated transformation with the mutant *aroA* gene, which encodes glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) (Comai et al. 1985). While foreign DNA has been inserted into the genome of numerous herbaceous species by *Agrobacterium*-plant DNA transfer systems (Davey et al. 1986; Zambryski 1992), genetic transformation of hybrid poplar has only recently been reported (Fillatti et al. 1987b). Owing to long sexual regeneration times of trees and their large size at maturity, genetic engineering is particularly useful for development of herbicide-tolerant poplar varieties (Nelson and Haissig 1986).

To enhance herbicide tolerance by target enzyme protection, hybrid poplar clone NC-5339 (*Populus alba* × *Populus grandidentata* cv. Crandon) (NC represents a permanent plant accession record of the USDA Forest Service North Central Forest Experiment Station) was genetically modified by *Agrobacterium*-mediated transformation with pPMG 85/587, a genetically engineered plasmid containing the mutant *aroA* gene fused to a mannopine synthase promoter (*mas-aroA*) (Fillatti et al. 1987a; Riemenschneider and Haissig 1990; Riemenschneider et al. 1988). Successful transformation with pPMG 85/587 was verified by hybridization using *aroA* as a probe (Fillatti et al. 1987a). The minimal herbicide tolerance of the derived transformants was attributed to the low-level expression of the *aroA* gene as directed by the *mas* promoter and localization of mutant enzyme in the cytoplasm rather than chloroplasts (Riemenschneider and Haissig 1990). Comai et al. (1985) proposed that herbicide tolerance of *Petunia* transformed with pPMG 85/587 was possibly due to transport of metabolites across the chloroplast envelope.

Plasmid pCGN 1107, which also harbors the mutant *aroA* gene, has been used successfully to confer glyphosate tolerance to tomato (Fillatti et al. 1987a). In pCGN 1107 the mutant *aroA* gene was fused to a cauliflower mosaic virus promoter (35S CaMV) and RuBP carboxylase small subunit coding sequences from *Pisum sativum* and *Glycine max*. The 35S promoter was used to direct high-level expression of the mutant *aroA* gene, and the transit peptide coding sequence was included to direct the transport of the mutant enzyme into chloroplasts. The effect of transformation with this genetic construct on glyphosate tolerance and productivity of hybrid poplar is unknown.

While genetic recombination may facilitate production of a herbicide-tolerant mutant enzyme, it may also have unpredicted and detrimental effects on plant growth. A reduction

in plant productivity accompanied the introduction of glyphosate tolerance in *Petunia* (Shah et al. 1986), tomato (Fillatti et al. 1987a), and tobacco (Comai et al. 1985). In contrast, there was no reduction in biomass accumulation of two mutant glyphosate-tolerant strains of the fern *Ceratopteris richardii* (Chun and Hickok 1992), transformed tobacco (Pospisilova et al. 1989), or glufosinate-tolerant transgenic tobacco and tomato (DeGreef et al. 1989).

Our objectives were to (i) genetically modify hybrid poplar clone NC-5339 with pCGN 1107 to introduce glyphosate-tolerant EPSP synthase into chloroplasts and (ii) measure the herbicide tolerance, growth, and net photosynthesis of NC-5339 and derived transformants to determine whether genetic transformation with plasmids pPMG 85/587 or pCGN 1107 conferred glyphosate tolerance or altered biomass production.

## Methods

Leaf discs of hybrid poplar clone NC-5339 were co-cultivated with *Agrobacterium tumefaciens* strain C58 harboring construct pCGN 1107, which included a modified T-DNA with mutant genes for neomycin phosphotransferase (NPT II') and glyphosate-tolerant EPSP synthase (mutant *aroA* gene). NPT II' was spliced to an octopine synthase promoter (*ocs*) and is a selectable marker that confers kanamycin tolerance and facilitates initial screening for transformed plants. Co-cultivation and transformation of NC-5339 was performed as previously reported (Fillatti et al. 1987b), and plasmid constructs have been described elsewhere (Riemenschneider and Haissig 1990).

For shoot regeneration after co-cultivation, leaf discs were (i) cultured on Murashige and Skoog (MS) nutrient medium containing  $5.0 \text{ mg}\cdot\text{L}^{-1}$  IAA and  $0.01 \text{ mg}\cdot\text{L}^{-1}$  benzyladenine (BA) (pH 5.8) in darkness for 72 h, (ii) plated on MS medium containing  $0.1 \text{ mg}\cdot\text{L}^{-1}$  BA (pH 5.8), and (iii) cultured in cool-white fluorescent light ( $25^\circ\text{C}$ ,  $25\text{--}30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 16-h photoperiod). Regeneration and propagation media also contained  $50 \text{ mg}\cdot\text{L}^{-1}$  kanamycin and  $300 \text{ mg}\cdot\text{L}^{-1}$  cefotaxime. Regenerated shoots morphologically indistinguishable from NC-5339 were identified as putative transformants of NC-5339 and propagated in vitro for three or four passages of 30 days each and rooted to establish stock plants, hereafter referred to as plant transformants (PT).

Integration of the mutant *aroA* gene into the nuclear genome was tested by hybridization of a radiolabelled probe with poplar DNA fragments separated by gel electrophoresis. Nuclear DNA was extracted from leaves of transformants (PT1A, PT1B, PT3, PT7, and PT11), according to methods of Fillatti et al. (1987b). DNA from each transformant was digested with *SalI* restriction endonucleases (Promega Biotec, Madison, Wis.), separated electrophoretically on 0.7% agarose, and transferred to a 0.45- $\mu\text{m}$  nylon membrane (Managraph, MSI, Westboro, Mass.). A 1.3-kb *BamHI-SalI* fragment was isolated from plasmid pARO9 (Stalker et al. 1985), labelled, and hybridized to membrane-bound DNA according to Ausubel et al. (1987). Membranes were hybridized for 18 h at  $37^\circ\text{C}$  and washed at  $42^\circ\text{C}$  according to the protocol of Ausubel et al. (1987) for nylon membranes. The washed membranes were exposed to X-ray film for 15 h at  $-50^\circ\text{C}$ .

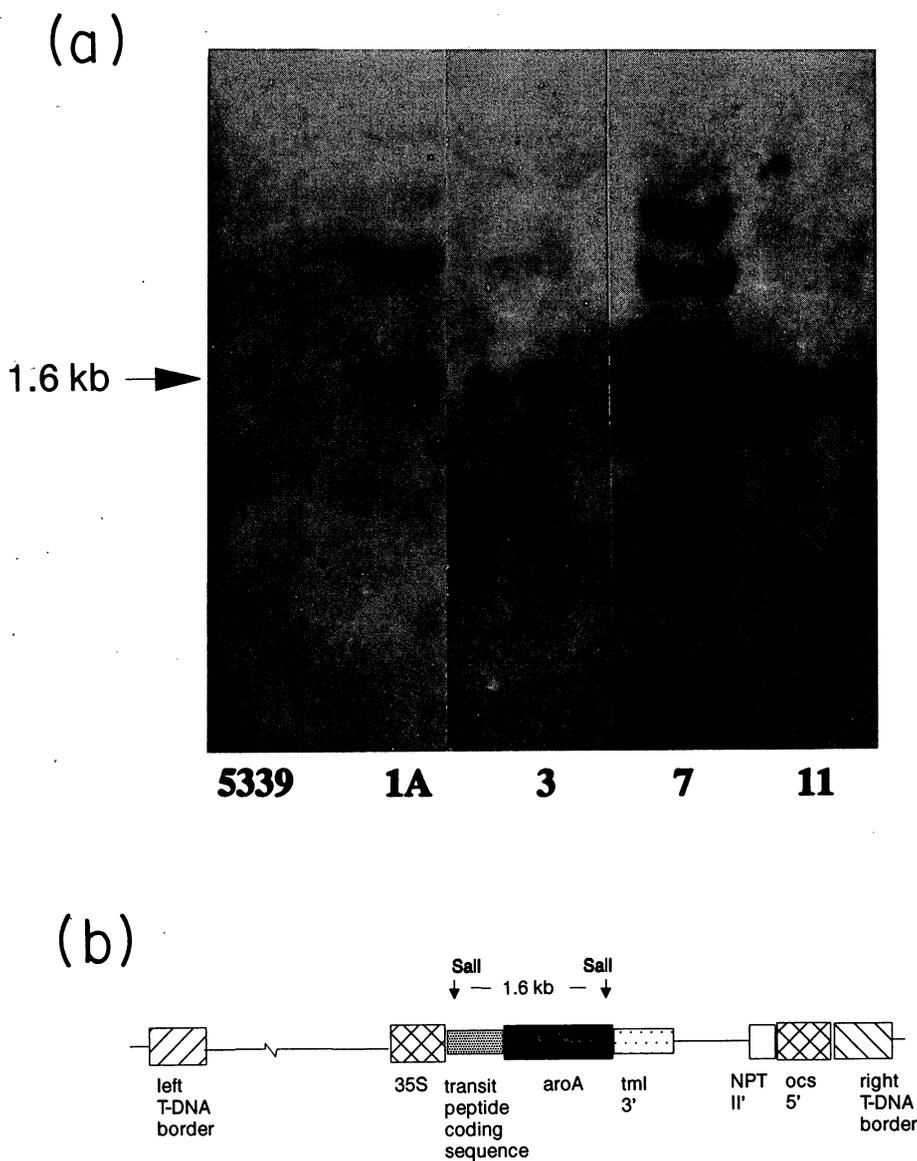


FIG. 1. (a) Southern blot analysis of 10  $\mu\text{g}$  of DNA per lane from NC-5339 and transformants. The 1.3-kb probe hybridized to a 1.6-kb *SalI* nuclear DNA fragment expected to contain the mutant *aroA* structural gene plus the transit peptide coding sequence. (b) Physical map of the T-DNA showing the location of the *SalI* endonuclease cleavage sites, the transit peptide coding sequence, and the mutant *aroA* gene.

We measured the herbicide tolerance and productivity of NC-5339 and transformants derived from transformation with genetically engineered plasmids pPMG 85/587 (12804 and 12812) and pCGN 1107 (PT1A, PT1B, PT3, PT7, and PT11), hereafter referred to as constructs 1 and 2, respectively. Construct 1 and 2 transformants with the most herbicide tolerance were selected from earlier screening of numerous samples (Riemenschneider and Haissig 1990). Clonal populations of each transformant were established from rooted cuttings (10 cm) grown in soil fertilized with 17:9:13 N-P<sub>2</sub>O<sub>5</sub>-K<sub>2</sub>O Osmocote (Redi Earth plus Osmocote, Grace Sierra, Milpitas, Calif.) in 15 cm diameter pots. NC-5339 and derived transformants were grown in glasshouses at two locations: (i) FSL (Forestry Sciences Laboratory, North Central Experiment Station, USDA Forest Service, Rhinelander, Wis.), maximum PPFD = 750  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  sunlight supplemented with high-pressure sodium lamps, and (ii) A&M (Research and Extension Center, Texas A & M University, Dallas, Tex.), maximum PPFD = 1600  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  sunlight.

Transformants 12804, 12812, PT1A, PT3, PT7, and PT11 were sprayed with 0, 0.56, 0.84, and 1.12  $\text{kg}\cdot\text{ha}^{-1}$  Roundup<sup>®</sup> in whole-

plant spray tests at FSL. The number of live leaves and height of each plant were recorded 1 week before spraying and weekly thereafter for 4 weeks. Leaves with more than 10% of the lamina turgid and green were defined as living.

Herbicide tolerance of NC-5339 and transformants 12812, PT1A, PT1B, PT3, PT7, and PT11 was also tested at A&M by measurement of chlorophyll concentration in glyphosate-treated leaf discs. Samples were removed from fully expanded mature leaves, LPI 4 (LPI defined by Larson and Isebrands 1971), with a number 5 cork borer and placed on filter paper saturated with a glyphosate solution (0, 20, 40, 60, 80, and 100  $\text{mg}\cdot\text{L}^{-1}$  active ingredient) in Petri dishes irradiated continuously (50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , Sylvania 40-W daylight fluorescent lamps) at 28–30°C. Chlorophyll was extracted from the leaf discs with 80% acetone, and optical density was measured at 665 nm at 5, 10, and 15 days.

Untreated NC-5339 and derived transformants were grown at each location (A&M, June 1991; FSL, December 1991) for 5 weeks for growth analysis and measurement of net photosynthesis ( $P_{\text{net}}$ ). Plant height, leaf number, leaf area (leaf area meter

TABLE 1. Analysis of variance for optical density of chlorophyll extracted from leaf discs of all clones exposed to glyphosate

Source	df	Mean square	F
Clone	6	0.250 96	26.98**
Day	2	0.879 42	210.27**
Dose	5	0.187 65	20.80**
Clone × day	12	0.003 24	1.61ns
Clone × dose	30	0.008 07	4.01**
Day × dose	10	0.002 96	1.47ns
Clone × day × dose	60	0.002 01	7.27**
Error	252	0.000 28	

NOTE: \*\*, significant at  $p < 0.01$ . ns, not significant.

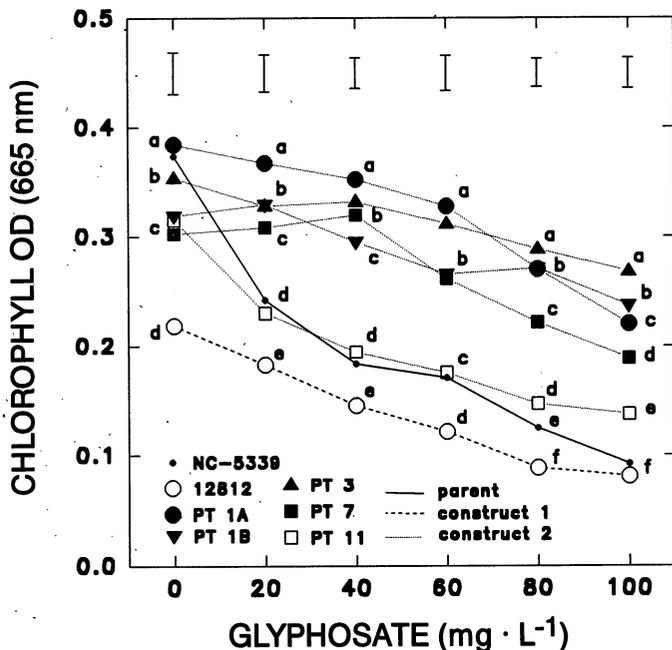


FIG. 2. Loss of chlorophyll from poplar clone leaf punches associated with increasing concentration of glyphosate. Chlorophyll concentration was assayed by optical density (OD) at 665 nm of 80% acetone solution extracted from leaf discs of NC-5339 and derived transformants treated with 0, 20, 40, 60, 80, and 100  $\text{mg}\cdot\text{L}^{-1}$  of glyphosate. Mean values were pooled for measurements taken at 5, 10, and 15 days. Standard error was pooled, and letters indicate significant difference between clones at each treatment level.

LI-3000, LI-COR Inc., Lincoln, Nebr.), root/shoot ratio, and leaf, stem, root, and total biomass were compared for each clone.  $P_{\text{net}}$  was measured on mature leaves (LPI 7 to 10) with a closed gas-exchange photosynthesis system (LI-6250, LI-COR Inc., Lincoln, Nebr.) (FSL) and an open ADC-LCA3 carbon dioxide analysis system with a Parkinson PLC leaf chamber (Analytical Development Company, Hoddesdon, England) (A&M). Measurement conditions were  $[\text{CO}_2] = 350$  and  $390$  ppm; leaf temperature = 25–30 and 29°C; PPFD = 800 and 1500  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at FSL and A&M, respectively. Some transformants were not included in every experiment owing to the small populations of clonally propagated plants available at each location.

### Results

DNA was isolated from NC-5339 and the derived transformants, and Southern blot analysis verified insertion of the mutant *aroA* gene for construct 2 transformants (Fig. 1a). Hybridization with the mutant *aroA* gene probe confirmed

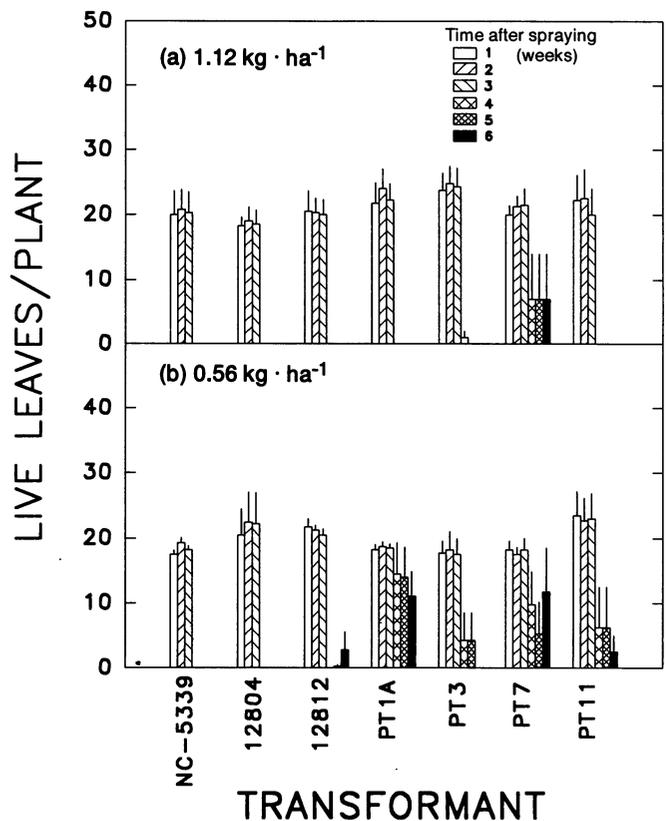


FIG. 3. Number of live leaves remaining on plants after glyphosate treatment. The number of live leaves was plotted for each clone at time zero, when spray was applied, and weekly thereafter for 5 weeks for plants treated with 1.12 and 0.56  $\text{kg}\cdot\text{ha}^{-1}$  Roundup® ( $n = 4$ ).

integration of the mutant enzyme gene sequence in construct 2 transformants (PT1A, PT3, PT7, and PT11) and its absence in NC-5339 (Figs. 1a, 1b). By the density of the hybridized bands we estimate the order of the copy number of integrated genes for the transformants to be PT7 > PT11 > PT3 > PT1A.

Chlorophyll content in untreated leaf discs declined significantly over time for both transformed and untransformed trees (Table 1). However, there was no significant difference between transformants for the effect of time on chlorophyll concentration, and the values measured at 5, 10, and 15 days were pooled to assess glyphosate tolerance (Fig. 2). Chlorophyll concentration was negatively correlated with glyphosate treatment in each case. The greatest effect of glyphosate was noted for NC-5339, which had one of the highest chlorophyll concentrations at 0  $\text{mg}\cdot\text{L}^{-1}$  glyphosate and the lowest chlorophyll concentration at 100  $\text{mg}\cdot\text{L}^{-1}$  glyphosate (Fig. 2). With the exception of PT11, all construct 2 transformants tested retained more chlorophyll than NC-5339 when treated with any concentration of glyphosate (Fig. 2).

For glyphosate-sensitive plants, damage was observed first in growing points in terminal buds and immature leaves, followed by death of mature leaves and stems. Two weeks after spraying, the apical meristems of NC-5339, 12804, and 12812 were dead, growth had ceased, and immature leaves were chlorotic. For construct 2 transformants, increase in plant height also stopped even though the terminal buds and immature leaves appeared unaffected. Five weeks after

TABLE 2. Biomass analysis of NC-5339 and derived transformants from growth studies at two locations

	Height (cm)	Leaf dry wt. (g)	Shoot dry wt. (g)	Root dry wt. (g)	Total dry wt. (g)	Root/shoot ratio <sup>a</sup>	Leaf area (cm <sup>2</sup> ) <sup>b</sup>	No. of leaves	$P_{net}$ ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )
<b>Biomass parameter data (mean <math>\pm</math> SE)</b>									
FSL	102.87 $\pm 1.69$	16.75 $\pm 0.77$	10.36 $\pm 0.59$	6.58 $\pm 0.51$	33.69 $\pm 1.80$	0.238 $\pm 0.011$	3873.69 $\pm 151.08$	29.04 $\pm 0.52$	16.21 $\pm 0.24$
A&M	111.14 $\pm 0.88$	23.98 $\pm 0.45$	13.88 $\pm 0.28$	9.69 $\pm 0.32$	47.55 $\pm 0.95$	0.255 $\pm 0.006$	4119.54 $\pm 60.10$	28.25 $\pm 0.18$	19.79 $\pm 0.28$
Combined	107.60 $\pm 1.02$	20.88 $\pm 0.62$	12.37 $\pm 0.37$	8.36 $\pm 0.34$	41.61 $\pm 1.28$	0.248 $\pm 0.006$	4014.18 $\pm 74.20$	28.59 $\pm 0.25$	18.32 $\pm 0.30$
<b>Analysis of variance mean squares</b>									
Clones (df = 7)	99.84ns	7.95ns	4.54ns	4.39ns	43.25ns	21.17ns	41.81ns	2.94ns	1.02ns
Location (df = 1)	837.91*	720.6**	171.78*	121.63*	2598.52**	13.63ns	205.51ns	4.88ns	154.15**
Location $\times$ clone (df = 4)	33.52ns	23.32ns	12.24ns	9.00ns	112.86ns	53.22*	46.00ns	2.29ns	1.29ns
Error	48.32	10.66	5.32	4.94	53.66	18.06	32.42	4.06	2.55
Among-clone LSD	5.8	14.06	16.76	23.9	15.82	15.4	12.75	6.34	7.84

NOTE: \*, significant at  $p < 0.05$ ; \*\*, significant at  $p < 0.01$ ; ns, not significant ( $p > 0.05$ ).

<sup>a</sup>To obtain actual mean square values, multiply by  $10^{-4}$ .

<sup>b</sup>To obtain actual mean square values, multiply by  $10^4$ .

treatment the terminal bud of every cutting was dead, even though the lateral buds were still viable.

Based on leaf retention throughout the study, NC-5339 and the construct 1 transformants with the mas promoter, 12804 and 12812, were the most sensitive to glyphosate treatment, while construct 2 transformants with the 35S promoter, particularly PT1A, PT3, and PT7, had some herbicide tolerance (Fig. 3). Some mature leaves survived for each construct 2 transformant sprayed at  $0.56 \text{ kg}\cdot\text{ha}^{-1}$  glyphosate and for PT7 sprayed at  $1.12 \text{ kg}\cdot\text{ha}^{-1}$ . Although lateral bud break briefly produced some leaves for one specimen of 12812 (Fig. 3), all of the leaves ultimately died on glyphosate-treated NC-5339 and construct 1 transformants.

There were no significant differences between NC-5339 and derived transformants for biomass parameters,  $P_{net}$ , or growth patterns within either location (Table 2). However, plants grown at higher light intensity (A&M) had significantly higher rates of  $P_{net}$  ( $19.8 \pm 0.28$  vs.  $16.2 \pm 0.24 \mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), had more total biomass accumulated ( $47.6 \pm 0.95$  vs.  $33.7 \pm 1.80 \text{ g}$ ), and were taller than plants grown at lower light intensity (FSL). In contrast, growth patterns (i.e., root/shoot ratio and leaf area) did not vary significantly with location.

### Discussion

Genetic modification of hybrid poplar for herbicide tolerance and its effect on productivity were successfully determined in this investigation. Construct 2 transformants retained the highest concentration of chlorophyll in glyphosate-treated leaf discs (Fig. 2) and were the only plants that survived spray tests and had living leaves and viable lateral buds at the end of the test period (Fig. 3). In addition, transformation for glyphosate tolerance with either construct did not adversely affect photosynthetic capacity or alter growth patterns (Table 2). *Agrobacterium*-plant mediated transformation modified the hybrid poplar genome for herbicide tolerance without decreasing growth.

Glyphosate is easily translocated within plants and is a competitive inhibitor of EPSP synthase, an enzyme in the shikimic acid pathway for biosynthesis of aromatic amino acids in chloroplasts (Cole 1985). Therefore, the effects of glyphosate are usually observed first in growing regions and include the inhibition of chlorophyll accumulation in growing leaves (Cole 1985; Mazur and Falco 1989). For woody plants, enhanced translocation of glyphosate to the roots was measured in glyphosate-sensitive white oak (*Quercus alba*), while it was not translocated in herbicide-tolerant red maple (*Acer rubrum*) (Green et al. 1992). Effective translocation and mode of action of glyphosate explain the damage we observed for NC-5339, 12804, and 12812: (i) chlorosis of immature leaves and terminal buds was likely caused by blocking of the shikimic acid pathway, shortage of aromatic amino acids, and lack of chlorophyll biosynthesis and (ii) translocation to the roots followed by death of root meristems may have caused the death of mature leaves from drought stress. Tests are being expanded on NC-5339, 12812, PT1A, and PT7 to more closely evaluate the developmental and physiological progression of damage in sensitive versus tolerant transformants.

Although successful transformation with construct 1 for 12804 and 12812 was previously reported (Riemenschneider et al. 1988), the minimal herbicide tolerance conferred by construct 1 was for a brief period (2 weeks) and treatment with a low concentration of glyphosate ( $0.17 \text{ kg}\cdot\text{ha}^{-1}$ ). It is not surprising that construct 1 did not increase herbicide tolerance markedly, since there was no coding sequence for the transit peptide to facilitate transport of mutant EPSP synthase into chloroplasts, where the shikimic acid pathway is localized. In addition, the mas promoter used in construct 1 is less effective in higher plants than other promoters such as the CaMV 35S used in construct 2 (Odell et al. 1985; Riemenschneider and Haissig 1990). This difference may be due to subcellular targeting, differential expression with cell type, and (or) a difference in overall

level of expression. Although we verified insertion of construct 2 by Southern blot analysis, we can only infer that glyphosate tolerance in PT1A and PT7 was caused by a high concentration of mutant EPSP synthase in the chloroplasts. Western analysis of subcellular distribution of the mutant enzyme is currently being conducted.

With the exception of PT11, construct 2 transformants were the most tolerant in whole-plant spray tests and also retained the highest concentrations of chlorophyll. This finding implies a potential role of products of the shikimic acid pathway in chlorophyll biosynthesis or retention. Glyphosate inhibited chlorophyll biosynthesis and decreased carotenoids in *Chlorella pyrenoidosa* (Hernando et al. 1989). Muñoz-Rueda et al. (1986) also measured a decrease in chlorophyll concentration in glyphosate-treated alfalfa and clover and proposed that glyphosate interferes with electron transport pathways by destruction of chlorophyll. In our investigation, chlorophyll concentration decreased at the same rate in unsprayed NC-5339 and transformants (Table 1), while mutant EPSP synthase restricted glyphosate damage in construct 2 transformants. Therefore, our results support the contention that glyphosate decreased chlorophyll concentration by inhibition of chlorophyll biosynthesis rather than by a direct effect on chlorophyll. An investigation of the interaction of glyphosate concentration and irradiance on chlorophyll concentration in glyphosate-treated leaf discs could help elucidate the mechanism by which glyphosate affects chlorophyll.

The difference in  $P_{\text{net}}$  at the two locations is not surprising for hybrid poplar grown at different light levels.  $P_{\text{net}}$  ( $19.79 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) measured for transformants grown at  $1600 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  is similar to maximum rates measured for various hybrid poplars (Nelson 1984). The greater height and amount of biomass accumulated by the trees grown at high light (A&M) could thus be attributed to higher photosynthetic rates at the higher light level. More importantly, there was no significant difference in root/shoot ratio between locations, indicating that differences in environmental conditions between the two locations did not alter growth patterns nor confound any potential differences in productivity between the transformants and NC-5339. The significant location  $\times$  clone interaction for root/shoot ratio (Table 2) was probably due to the larger range of clone means at FSL (range 0.101) than at A&M (range 0.019). In addition, we attribute the interaction to location-related differences in experimental error, since differences among clones in root/shoot ratio were not significant at either location when locations were analyzed separately.

The lack of any significant difference between transformants at separate locations for biomass accumulation and photosynthetic capacity is noteworthy because both constructs induce the production of mutant EPSP synthase, which could divert biochemical resources and alter resource allocation. In addition, construct 2 includes the sequence for a transit peptide also used for transport of the small Rubisco subunit into chloroplasts. Expression of this sequence could potentially affect Rubisco availability in chloroplasts and ultimately decrease photosynthetic capacity and productivity. Apparently, the use of the transit peptide in these transformants did not interfere with Rubisco availability nor limit carbon assimilation. Thus, genetic modification for glyphosate tolerance in hybrid poplar did not adversely affect net photosynthesis or biomass production of cuttings within a 5-week period.

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