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The *CP4* transgene provides high levels of tolerance to Roundup® herbicide in field-grown hybrid poplars

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Abstract: We tested two genes together in hybrid poplars (genus *Populus*), *CP4* and *GOX*, for imparting tolerance to glyphosate (the active ingredient in Roundup® herbicide). Using *Agrobacterium*-based transformation, 80 independent transgenic lines (i.e., products of asexual gene transfer) were produced in a variety of hybrid poplar clones (40 lines in *Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr., 35 lines in *Populus tremula* L. × *Populus alba* L., and five lines in *P. tremula* × *Populus tremuloides* Michx.). We evaluated glyphosate tolerance over 2 years in field studies conducted in eastern and western Oregon. Ten percent of our transgenic lines showed no foliar damage or reduced growth after being sprayed with Roundup® at concentrations above normal commercial rates. Lack of damage was associated with expression of the *CP4* gene but not of the *GOX* gene. It was suspected that *GOX* caused undesirable side effects, so we produced 12 lines into which only the *CP4* gene was inserted. The performance of these newly regenerated lines was compared with an identical number of lines, produced in the same genotype, that had previously been engineered to contain both *CP4* and *GOX*. Growth of the lines transformed with just *CP4* was significantly better than those containing both genes and exhibited less damage in response to glyphosate treatment. This is the first report of transgenic poplars exhibiting high levels of glyphosate tolerance when grown under field conditions. With a modest transformation effort, it is possible to produce lines with commercially useful levels of glyphosate tolerance and little apparent collateral genetic damage.

Résumé : Les auteurs ont étudié l'effet combiné de deux gènes chez des peupliers hybrides (genre *Populus*), *CP4* et *GOX*, qui confèrent la tolérance au glyphosate (l'ingrédient actif de l'herbicide Roundup©). À l'aide de la transformation par *Agrobacterium*, 80 lignées transgéniques indépendantes (i.e., les produits du transfert génétique asexué) ont été produites à partir d'une variété de clones de peupliers hybrides (40 lignées de *Populus trichocarpa* Torr. & Gray × *Populus deltoides*, 35 lignées de *Populus tremula* L. × *Populus alba* L. et cinq lignées de *P. tremula* × *Populus tremuloides* Michx). La tolérance au glyphosate a été évaluée pendant 2 ans dans des dispositifs au champ établis dans l'est et l'ouest de l'Oregon. Dix pour cent des lignées transgéniques n'ont pas subi de dommages foliaires ou de réduction de croissance après avoir été arrosées avec du Roundup© à des concentrations supérieures à celles normalement utilisées commercialement. L'absence de dommages était associée à l'expression du gène *CP4* mais pas à celle du gène *GOX*. Soupçonnant que le gène *GOX* pouvait causer des effets secondaires indésirables, les auteurs ont produit 12 lignées comportant uniquement le gène *CP4*. La performance de ces nouvelles lignées régénérées a été comparée à celle d'un nombre identique de lignées produites à partir du même génotype mais comportant les deux gènes *CP4* et *GOX*. La croissance des lignées comportant uniquement le gène *CP4* était significativement supérieure à celle des lignées comportant les deux gènes, en plus de subir moins de dommages en réponse au traitement au glyphosate. Cette étude

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est la première à rapporter que des peupliers transgéniques démontrent un degré élevé de tolérance au glyphosate en conditions de croissance au champ. On peut, avec un modeste effort de transformation, produire des lignées dotées d'un degré commercialement utile de tolérance au glyphosate sans grands dommages secondaires apparents au plan génétique.

[Traduit par la Rédaction]

Introduction

Rigorous weed control is highly desirable for establishing poplar plantations that exhibit high levels of survival and rapid growth (Hansen and Netzer 1985). Because poplars are sensitive to most pre- and post-emergent herbicides, weeds are usually eliminated through cultivation and specialized chemical treatments. A variety of herbicides are applied either before buds elongate in the spring or as directed sprays during the growing season to minimize leaf contact. Although weed-control measures only need to be used until canopy closure is reached, they are labor and (or) equipment intensive and, therefore, costly. It may be possible to control weeds in poplar plantations more economically and more completely if herbicide-tolerant lines are available.

Glyphosate, the active ingredient in the herbicide Roundup®, is frequently used for weed control in poplar plantations. It is a broad-spectrum, post-emergent herbicide that has a number of desirable attributes. These include immediate inactivation by absorption to soil colloids (Bronstad and Friestad 1985), rapid degradation, little or no toxicity to living organisms other than plants (Kishore et al. 1992), minimal potential for bioaccumulation (Tooby 1985), and low volatility (Rueppel et al. 1977). Glyphosate-tolerant poplars should allow preferential and more effective use of Roundup®, resulting in fewer or more environmentally benign chemical inputs, and possibly reduced tillage (Strauss et al. 1997), during poplar culture.

Transformation of hybrid poplar was first described by Fillatti et al. (1987) and involved the insertion of an herbicide tolerance gene, *aroA*, from *Salmonella typhimurium*. A point mutation in this gene results in the formation of a 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS, E.C. 2.5.1.19) that is resistant to inhibition by glyphosate (Comai et al. 1983). Expression of this gene under the control of the mannopine synthase promoter (*mas*) produced unexpectedly low levels of herbicide tolerance (Riemenschneider et al. 1988). This was thought to be the result of low-level and cytosolic transgene expression. When the same poplar clone (NC-5339, cv. Crandon, *Populus alba* L. × *Populus grandidentata* Michx.) was transformed with *aroA* under the control of the cauliflower mosaic virus 35S promoter and a chloroplast transit peptide, the resulting transgenic plants exhibited markedly higher tolerance for glyphosate (Riemenschneider and Haissig 1991; Donahue et al. 1994).

Although Karnosky et al. (1997) showed that the 35S promoter resulted in higher expression levels than *mas* and that the transit peptide directed transgene product to the chloroplast, the performance of lines containing *aroA* driven by 35S still fell short of commercially adequate levels. After greenhouse spray tests, chlorophyll content in all transgenic lines was negatively correlated with glyphosate concentration; height growth was arrested following herbicide treatment, even though terminal buds and immature leaves appeared unaffected; and only one line retained live leaves 6 weeks following treatment at 1.12 $kg \cdot ha^{-1}$ (Donahue et al. 1994).

We tested a new construct for its ability to impart glyphosate tolerance to transgenic poplar. Using an *Agrobacterium*-mediated transformation protocol (Han et al. 2000; Leple et al. 1992), we generated transgenic plants in six clones of hybrid poplar (four clones of *Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr. and one clone each of *Populus tremula* L. × *Populus alba* L. and *P. tremula* × *Populus tremuloides* Michx.). All lines were transformed with two glyphosate tolerance genes, *CP4* and *GOX*. The former is an alternative form of EPSPS, cloned from *Agrobacterium* strain CP4, for which glyphosate has a low affinity. *GOX* is a gene isolated from *Achromobacter* strain LBAA that encodes the enzyme glyphosate oxidoreductase, which metabolically inactivates glyphosate. The initial breakdown product of the reaction catalyzed by the GOX enzyme is aminomethylphosphonic acid (AMPA; Barry et al. 1992).

Several agronomic crops transformed with both *CP4* and *GOX*, including corn, cotton, potato, rapeseed, soybean, sugar beet, and tomato, have been field tested and deregulated (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm). In many crops containing both genes, a chlorotic phenotype has been observed in response to glyphosate treatment, possibly resulting from the conversion of glyphosate to AMPA. Therefore, we produced a separate population of hybrid cottonwood that contained only the *CP4* gene to determine if it alone could confer high levels of glyphosate tolerance. Our transgenic plants were field tested 2 years in trials planted west and east sides of the Cascade Mountains in Oregon, which represent maritime and high desert environments, respectively.

Materials and methods

Plant material

Leaf discs from four triploid clones of hybrid cottonwood (19-53, 24-305, 184-402, and 189-434; all *P. trichocarpa* × *P. deltoides*) and two diploid clones of hybrid "aspen" (Institut national de la recherche agronomique (INRA) 717-1B4, *P. tremula* × *P. alba*; INRA 353-38, *P. tremula* × *P. tremuloides*) were used to generate the transgenic lines. (For the purposes of this study, "clone" refers to a poplar genotype, "line" denotes a plant derived from an independent transformation event, and "ramet" is a vegetative propagule

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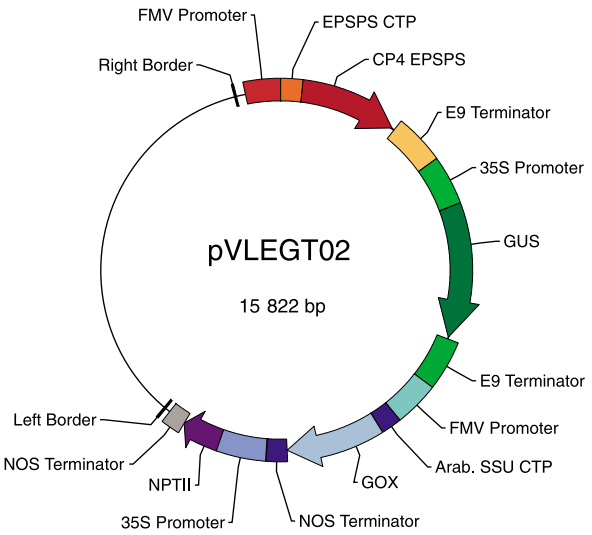
of a line.) Leaf discs for cocultivation were derived from both in vitro and growth room grown plants.

Constructs

Nearly all transgenic lines were produced with the plant transformation vector, pV-LEGT02 (provided by Monsanto Co.), which contains four transcriptional units within its T-DNA (Fig. 1). At the right border, the *Agrobacterium* strain CP4 *EPSPS* gene (Barry et al. 1992) is expressed as a fusion with the chloroplast transit peptide (CTP) from *Arabidopsis thaliana* EPSPS (Klee et al. 1987) under the control of the caulimovirus figwort mosaic virus (FMV) promoter (Gowda et al. 1989; Richins et al. 1987; Sanger et al. 1990) and the polyadenylation signal from the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase–oxygenase gene of pea (E9; Coruzzi et al. 1984; Morelli et al. 1986). Next is the GUS gene (Jefferson et al. 1986), which is controlled by an enhanced version of the cauliflower mosaic virus (CaMV) 35S promoter (Kay et al. 1987) and the E9 terminator. The *GOX* gene follows and is expressed as a fusion with the CTP from the *A. thaliana* SSU gene (Stark et al. 1992) under the control of the FMV promoter and the nopaline synthase (NOS) terminator (Bevan et al. 1983). Finally, nearest the left-hand border is the neomycin phosphotransferase gene (*NPT II*) driven by the 35S promoter and terminated by NOS 3′ sequences.

Twelve additional transgenic lines were produced in clone 189-434 using a second binary vector (pMRR2). This construct contained only the *CP4* operon from pV-LEGT02 between its left- and right-hand borders. In this case, the transgene also served as the selectable marker for transformation.

Fig. 1. Schematic map of binary vector used to produce the transgenic lines containing both glyphosate tolerance genes. Abbreviations are as follows: Arab., *Arabidopsis thaliana*; *CP4 EPSPS*, 5-enolpyruvyl-3-phosphoshikimate synthase gene from *Agrobacterium* sp. strain CP4; *CTP*, chloroplast transit peptide gene; *E9*, polyadenylation signal from the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase–oxygenase gene from pea; FMV, figwort mosaic virus; *GOX*, glyphosate oxidoreductase gene from *Achromobacter* sp.; *GUS*, β-glucuronidase gene from *Escherichia coli* Tn5; *NOS*, nopaline synthase gene from *Agrobacterium tumefaciens*; *NPT II*, neomycin phosphotransferase gene from *Escherichia coli*; 35S, promoter from 35S gene of cauliflower mosaic virus.



Tissue culture

Cottonwood transformation and regeneration were performed as described by Han et al. (2000). Sterile leaf discs were cocultivated with *Agrobacterium* strain ABI containing the binary vector pV-LEGT02. Hybrid aspens were transformed using disarmed *Agrobacterium* strain C58pMP90 as described by Leple et al. (1992). Forty independent lines were generated in cottonwood (7 in 19-53, 6 in 24-305, 8 in 184-402, and 19 in 189-434) and 40 in aspen (35 in 717-1B4, 5 in 353-38). Each line was propagated in vitro by rooting excised nodes from mother plants on kanamycin-containing basal media. After roots were established in the presence of kanamycin, plants were transferred to antibiotic-free basal media (half-strength Murashige and Skoog (MS) media; Han et al. 2000).

Conditioning

In vitro grown plants were rinsed with tap water and transplanted into 5.7 × 8.3 cm Rose Pots (Anderson Die and Manufactuing Co., Portland, Oreg.) containing 2:1 Sunshine Mix : perlite (McConkey Co.). Pots were sealed in Zip-Lok® sandwich bags (3 pots/bag) containing approximately 100 mL of water and placed in a growth room under constant light (cool white fluorescent lamps) and temperature (25°C). The Zip-Lok® bags were opened for progressively longer intervals each day to allow acclimation to ambient conditions. After 2 weeks, the plants were removed from the bags. Following about 4 weeks in the growth room, plants were transferred to a greenhouse. Trees were further acclimated in a lath house for 2 weeks prior to being planted in the field.

Verification of transgenics

Selection

All transformants produced using pV-LEGT02 were rooted in kanamycin (25 mg/L); those with just the *CP4* gene were rooted in the presence of 2 mg/L glyphosate. Leaf tissue from each line was histochemically stained for GUS activity in 1 g/L X-Gluc (Jefferson et al. 1987) and cleared in 95% ethanol. Approximately half of the transgenic lines containing both herbicide tolerance genes were pretested by rooting in glyphosate-containing basal media (2 mg/L).

Polymerase chain reaction

GOX- and *CP4*-specific primers were used to confirm the presence of glyphosate tolerance genes using the polymerase chain reaction (PCR). The forward and reverse *GOX* primer sequences (5′ to 3′) were GCT GAG AAC CAC AAG AAG GTT GGT ATC and GGA TGC AGG ACC AGT TTG CTT GG, respectively; the *CP4* primer sequences were GCA ACT GCT CGT AAG TCC TCT G and CTT AGC TCC AAG ACC AGC CAT C. For resistant lines in which a product could not be amplified (perhaps because of PCR inhibition or mutations–rearrangements at a priming site), Southern blots were used to confirm transgene insertion.

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Southern analysis

Genomic DNA was extracted from nontransgenic controls and from lines transformed with pV-LEGT02 for which no amplification product was detected via PCR. Ten micrograms of genomic DNA was digested with *Eco*RI to generate border fragments, separated by gel electrophoresis, and blotted to Nytran nylon membrane (Schleicher and Schuell) under alkaline conditions (Sambrook et al. 1989). PCR-generated fragments for the coding regions of *CP4*, *GOX*, and *NPT II*, radiolabelled (^{32}P-dCTP) via random-hexamer priming, were used sequentially as probes. Prehybridizations, hybridizations, and washes were conducted under high-stringency conditions (0.1 × SSPE, 0.1% SDS, 65°C final wash; Sambrook et al. 1989). Before reprobing, the blots were stripped according to the membrane manufacturer's protocol.

Protein analysis

Sample processing

Leaf tissue was taken from all transgenic lines and their corresponding nontransgenic controls. All plants were greenhouse grown and had never been treated with glyphosate. After removal, samples were immediately frozen in liquid nitrogen, transported on dry ice, and maintained at –80°C until processing. GOX protein was extracted from this tissue using a 1:100 tissue to buffer ratio. The GOX extraction buffer contained 100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.05% Tween-20, 6.5 mM CHAPS (pH 7.8), and 0.2% L-ascorbic acid (added just prior to use). CP4 protein was extracted from leaf tissue using extraction buffer containing 137 mM sodium chloride, 8.1 mM sodium phosphate dibasic, 15 mM potassium phosphate monobasic, 4.2 mM potassium chloride, and 0.05% Tween 20 (pH 7.4), using a 1:20 tissue to buffer ratio. Tissue was ground for 30 s using a Polytron (Brinkmann Instruments) set at 17 500 rpm. Extracts were kept on ice throughout the extraction process. Insoluble debris was pelleted by centrifugation, and the supernatant was stored at –80°C until enzyme-linked immunosorbent assays (ELISAs) were conducted using antibodies generated in house by Monsanto Company.

ELISA analyses

CP4 ELISA

Tissue matrix effects were evaluated by adding various amounts of extract to purified CP4 during standard curve determinations. Minor inhibition in antibody binding was observed when extract was added to the standards, suggesting that CP4 levels may be slightly underestimated by the ELISA. The minimum detectable level of CP4 protein was 0.02 μg/g fresh mass (FM) of leaf tissue.

GOX ELISA

As with CP4, matrix effects were evaluated for different amounts of GOX extract to the standard curve determinations. Considerable matrix effects were seen for the GOX ELISA, particularly at low concentrations of GOX extract (1.5 ng/well), where an increase in binding was observed. This would result in an overestimation of actual GOX expression levels. These matrix effects were compensated for by spiking standard curve measurements with extract (matrix) from untransformed plants. The minimum detectable level of GOX protein was 0.54 μg/g FM of leaf tissue.

Field study

Establishment

Transgenic aspens were planted in Benton County, Oregon (central Willamette Valley; mean annual rainfall (MAR), 117.4 cm; and mean annual temperature (MAT), 11.3°C), in July 1995. Transgenic cottonwoods were planted in Morrow County (east of the Cascade Mountains; MAR, 8.6 cm; and MAT, 11.9°C) in May 1996 and in Clatsop County (west of the Cascade Mountains; MAR, 146.9 cm; and MAT, 10.6°C) in June 1996. Rooted plants were grown at a 0.6 × 0.6 m spacing at the Benton County site, 3.0 m (between rows) × 2.3 m (between trees within rows) in Morrow County, and 1.8 × 1.8 m in Clatsop County.

One ramet of each transgenic cottonwood line and the corresponding nontransgenic controls were randomly planted in each of six plots at both the Morrow and Clatsop sites. An unmeasured demonstration plot was also planted along the edge of the trial at Morrow. Here, transgenics and nontransgenics were planted in alternate rows (Fig. 2*g*). One ramet of each transgenic aspen line and corresponding controls were randomly assigned to three separate rows within each of three plots in Benton County.

In a separate 1-year study, established in April 2000, 25 lines (all in clone 189-434) were planted out at Morrow County site using dormant hardwood cuttings. These had been taken from trees first planted in 1998 and propagated in 1999. Twelve of these lines contained *CP4* and *GOX*, an equal number of lines contained only *CP4*, and one line was a nontransgenic control. Two ramets of every line were planted in each of 12 rows. Unrooted cuttings were planted at the same spacing as used in the previous study in Morrow County. The plant rows were evenly divided into three replicate blocks; the four treatment options described below were each randomly assigned to a single row within each block.

Different irrigation schemes were used in each area to accommodate the differences in rainfall between regions. In Morrow County, plants were fertigated daily; in Benton County, they were irrigated as needed throughout the growing season; and in Clatsop County, trees were only irrigated until they were established. Thirty-centimetre wind screens were installed at the Morrow site immediately following establishment to shelter plants from the sun and wind (Fig. 2*g*); screens were removed after the plants were acclimated.

Treatments

Roundup Pro™ (41% glyphosate, the active ingredient (a.i.)) was applied twice each year during periods of active tree growth (generally in early July and late August) at a rate of either 4.7 L/ha (2.0 qt/ac, 2.0 kg a.i./ha; 1×) or 9.3 L/ha (4.0 qt/ac, 3.9 kg a.i./ha; 2×). At the Clatsop and Morrow sites, each treatment rate was applied to two randomly selected replicate blocks; two blocks were left unsprayed as controls. At the Benton site, each treatment level was applied to one plot (i.e., spray levels were not replicated in this small trial). For the *CP4* versus *CP4–GOX* study, a third treatment level was added (2.3 L/ha, 1.0 qt/ac, 1.0 kg a.i./ha).

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Fig. 2. Morphology of transgenic lines after glyphosate application. (*a*) Example of a transgenic hybrid cottonwood (clone 184-402) at the Clatsop site receiving a damage rating of 0 (no damage; Table 1). Spray rate: 4.7 L Roundup Pro™/ha (1×). (*b*) Example of a transgenic hybrid cottonwood (clone 189-434) at Clatsop receiving a damage rating of 1. Spray rate: 9.3 L Roundup Pro™/ha (2×). (*c*) Example of a nontransgenic hybrid cottonwood (clone No. 24-305) at Clatsop receiving a damage rating of 5 (dead). (*d*) A typical transgenic hybrid aspen (clone 717-1B4) in Benton County receiving a damage rating of 0, 1× spray rate. (*e*) Example of a transgenic hybrid aspen (clone 717-1B4) receiving a damage rating of 1, 1× spray rate. (*f*) Highly tolerant transgenic hybrid cottonwood (clone 189-434) in Morrow County after the second 2× treatment in the second year of the study (cumulative total of four treatments), with a damage rating of 0. (*g*) Demonstration plot at the Morrow site, 1× spray rate. The small nontransgenic trees, shown in the right foreground (next to the shade cards and flagging) died, whereas all of the transgenics survived and grew (left foreground). (*h*) Transgenic hybrid cottonwood at the Clatsop site after the first 1× treatment in the second year of the study (cumulative total of three treatments) showing typical *GOX*-associated chlorosis. All pictures were taken 4 weeks after treatment.

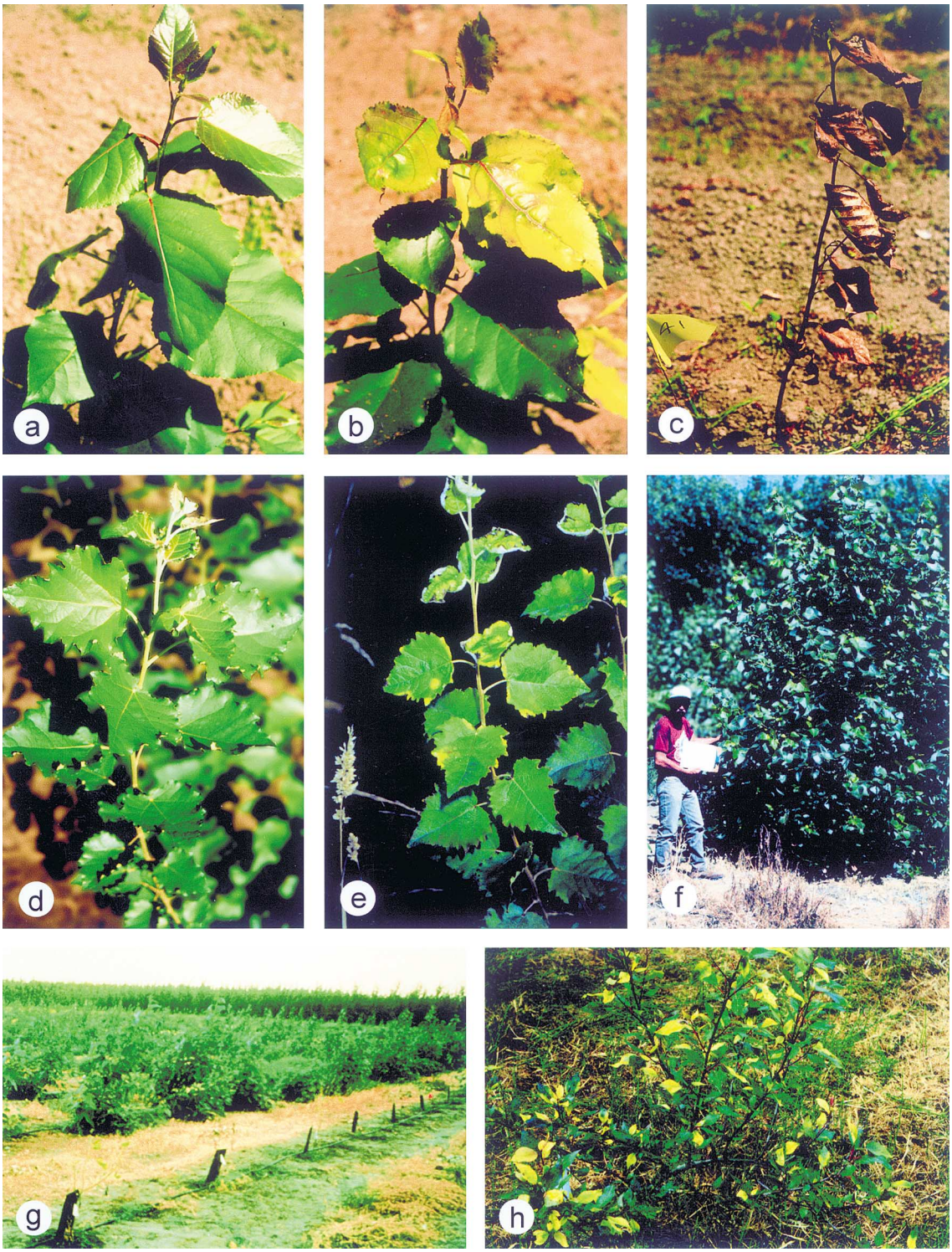

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Table 1. Rating system used to score glyphosate damage in transgenic plants containing both herbicide tolerance genes.

Score	Description
0	No damage
1	Some visual color change, no apical damage
2	Necrosis or discoloring in apical area
3	Entire tree is moderately affected, slight top dieback, leaf discoloring
4	Entire tree is substantially damaged, some green foliage present
5	Death; no green foliage present

Table 2. Rating system used to evaluate chlorosis due to glyphosate treatment in the one- versus two-gene study.

Score	Description
0	No apparent damage
1	Less than 5% of the leaf area affected
2	5–25% of the leaf area affected
3	26–50% of the leaf area affected
4	51–75% of the leaf area affected
5	76–100% of the leaf area affected
6	Death; no green foliage present

During the first growing season, all treatments were applied over the top of the trees using a boom sprayer that was tractor mounted at the cottonwood sites and hand held at the aspen site. During the second growing season, a tractor equipped with three nozzles was driven between planting rows to apply herbicide to the cottonwoods. Two nozzles were angled toward the base of trees in the rows on either side of the tractor, and the third nozzle was directed downward between the rows. This spray pattern resulted in foliar coverage of up to 1 m off the ground on both sides of each tree. During the second year of the study, aspens were treated manually with a backpack sprayer using a "waving-wand" technique (Newton et al. 1998) to maximize coverage of the canopy with herbicide.

The tractor-mounted spray equipment was calibrated immediately before each use. A stopwatch was used to determine the time required for the tractor to traverse 15 m in first gear at the lowest throttle setting. A graduated cylinder was used to measure volume delivered by each nozzle in 15 s at a constant rate with an electric pump. A tape measure was used to measure the width of the spray pattern for each nozzle. Taken together, these measurements were used to calculate the volume delivered on a per-hectare basis. The appropriate volume of herbicide was then added to each 150-L batch to achieve the desired delivery rate.

Weeds in the unsprayed plots were controlled exclusively through cultivation to provide comparable levels of weed control in sprayed and unsprayed plots. At the Clatsop site, a rototiller was driven between and within rows, whereas at the Morrow site, tilling was only done between plant rows (including the *CP4* vs. *GOX* experiment). Hand hoeing was also done to remove persistent weeds near the base of each tree at both sites. Hand hoeing alone was used to remove weeds in the unsprayed plots at the Benton site.

Measurements

Heights and basal diameters were taken on all trees immediately after planting. Trees were evaluated for damage 4 weeks after treatment using the damage rating system shown in Table 1. Height and basal diameters were also measured (both in cm) when damage ratings were taken and at the end of each growing season. In the one- versus two-gene study, heights and diameters were only taken at the end of the growing season, and herbicide damage was assessed visually according to the rating system listed in Table 2.

Quantitative analyses

For each tree, volume index was calculated by multiplying the square of basal diameter by height ((diameter)^2(height)). To correct for differences in initial tree sizes, growth was determined by subtracting the natural log of the volume index 1 month following the final treatment from the natural log of the volume index at the time of establishment (ln(ending volume index) – ln(starting volume index)). Relative growth was used as a measure of herbicide tolerance and was calculated as the difference in mean growth between untreated ramets and treated ramets of the same line.

Pearson's correlation coefficient was used to determine relationships between levels of protein per transgenic line and herbicide tolerance. We determined the relationship between protein levels, growth, and damage at each field site by first calculating the least-square means (LSMs) per line to remove the effects of blocks and then performing stepwise multiple regression with the LSMs, using damage or growth as the response variables and CP4 and GOX protein levels as the explanatory variables.

Using only the top-performing line for each clone, ANOVA was used to test the effects of spray level (0, 1×, 2×), transgenic line, and site on growth. We also compared the growth of those lines that were sprayed but showed no signs of damage to controls in the unsprayed rows. For these tests, an ANOVA was used with line and block as explanatory variables.

Results

Molecular and protein characterizations

All field-tested lines had rooted in kanamycin and stained positive for GUS activity; those that exhibited glyphosate tolerance in the field had at least one intact copy of either *GOX* or *CP4* (data not shown). CP4 protein (mean 0–26 μg/g FM) was present at lower levels than GOX protein (mean 0–1122 μg/g FM), and in some cases, there were detectable levels of only a single gene product. Mean GOX protein levels were lower in the aspen than in the cottonwood, and a greater percentage of transgenic aspen lines had low (below threshold of detectability) or no detectable levels of CP4 (7.7 vs. 11.4%, respectively) and GOX protein (10.3 vs. 15.9%, respectively).

Levels of CP4 and GOX protein were correlated in cottonwood (Pearson's $r = 0.50$, $p = 0.0006$) but not in aspen ($p = 0.55$). Levels of CP4 protein were negatively associated with damage at both the Morrow and Clatsop County sites for both low and high spray levels (Table 3). Also, CP4 protein levels were positively associated with cottonwood growth at Clatsop and Morrow County sites. GOX protein levels were

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Table 3. Results of stepwise multiple regression analyses of damage and natural log of growth as a function of the levels of GOX and CP4 proteins detected by ELISA.

		Damage				Growth			
Site	Spray	*N*	β	Adjusted R^2	*P*	*N*	β	Adjusted R^2	*P*
Clatsop	Low	44	–0.37	0.18	0.004	44	1.44	0.23	0.001
	High	44	–0.45	0.26	0.0004	44	1.56	0.25	0.001
Morrow	Low	44	–0.32	0.13	0.02	44	1.21	0.15	0.001
	High	44	–0.47	0.25	0.0005	44	1.63	0.22	0.001
Benton	Low	35	–0.06	0.15	0.02				
	High	35	–0.05	0.11	0.05				

Note: For growth, the response variable was the least-squares mean for each line for the log-transformed difference in volume index, calculated from an ANOVA that included block and row as main effects. The field-plot design at the Benton County site precluded an analysis of growth. Levels of GOX protein were not significantly associated with damage or growth and did not enter the model. *N*, number of lines (i.e., independent transformation events); β, slope for CP4 protein; R^2, coefficient of determination; *P*, probability that slope differs from zero.

not significantly (α = 0.05) correlated with damage or growth at either spray level or any of the sites.

Tolerance

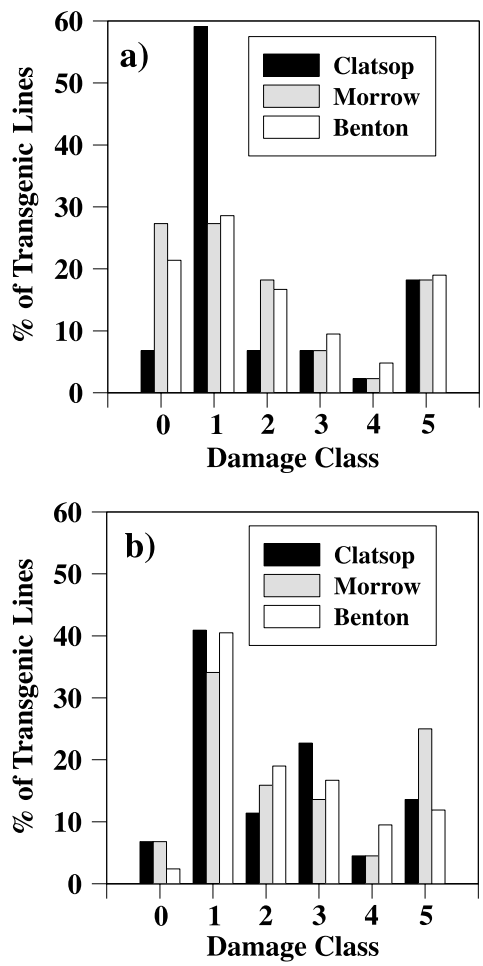
Figure 3 is a frequency distribution of mean damage (across ramets and lines) by site for the first 1× spray in both years. Nearly two-thirds of all lines had either no visible damage or only minor chlorosis (damage classes 0 and 1, respectively; Figs. 2*a*, 2*b*, 2*d*, 2*e*, and 3). Three cottonwood lines in Clatsop and Morrow Counties and one aspen line planted in Benton County showed no damage in response to the 2× treatment (Fig. 3). The corresponding number of lines falling into damage class 1 were 18, 15, and 16, respectively. Note that a disproportionately large number of lines fell into damage class 1 at the Clatsop site. Following the first treatment in the second year of the study, chlorotic spots appeared on leaves that were within the spray zone; no other evidence of chlorosis was observed on the affected plants (Fig. 2*h*). These spots occurred only on plants at the Clatsop site; they eventually disappeared and did not occur following the second application. In general, transgenic lines showed slightly more damage in the second year of the study than in the first. All lines that succumbed to glyphosate treatment (damage class 5) were ones that had not been rooted in glyphosate prior to field testing (Fig. 2*c*). Trees that had only the *CP4* gene exhibited few damage symptoms, whereas those with both *CP4* and *GOX* were severely chlorotic in response to treatment (Figs. 4 and 5).

Growth

Figure 6 is a frequency distribution of relative growth (across ramets and lines) at each site for trees receiving glyphosate treatment. At the 1× spray rate, about 75% of the transgenic lines were unaffected or appeared to grow somewhat better when treated with glyphosate (Fig. 6*a*). At the 2× spray rate, 39–83% of the lines (depending on site) were unaffected or grew slightly better in response to treatment (Fig. 6*b*).

Analysis of variance revealed no significant (α = 0.05) difference in growth of the best-performing line within each clone whether weeds were controlled through cultivation or by spraying, at either spray level. In addition, for any given clone in the unsprayed rows, mean growth of the transgenics

Fig. 3. Damage to transgenic poplars following the first glyphosate treatment in 1996 and 1997 at the 1× (*a*) and 2× (*b*) rates of Roundup Pro™. Damage ratings were taken 4 weeks after the first application in both years. Damage values are averaged across ramets, lines, and years for each site.



was not significantly (α = 0.05) different than the nontransgenic controls, at either the Clatsop or Morrow sites (data not shown). There were also no obvious differences in

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Fig. 4. Damage to transgenic poplars (clone 189-434) containing either one or both glyphosate tolerance genes. "Low", "medium", and "high" correspond to 2.3, 4.7, and 9.3 L/ha Roundup Pro™. Damage ratings (Table 2) were taken 4 weeks after each of two treatments. Damage values are averaged across ramets, lines, and treatments dates. Error bars are SEs.

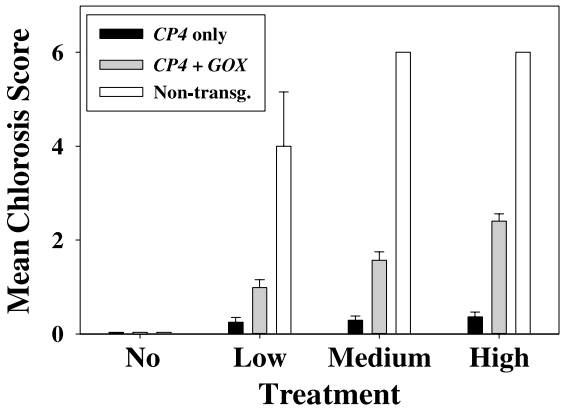


Fig. 5. Typical damage symptoms exhibited in the one- versus two-gene study following a single glyphosate treatment at the 4.7 L/ha level. The tree on the left contains both *CP4* and *GOX*, the one on the right contains just the *CP4* gene.

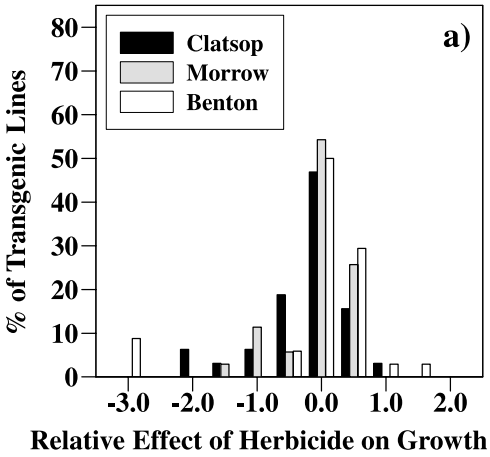
leaf morphology between tolerant transgenic and nontransgenic lines, whether sprayed or not.

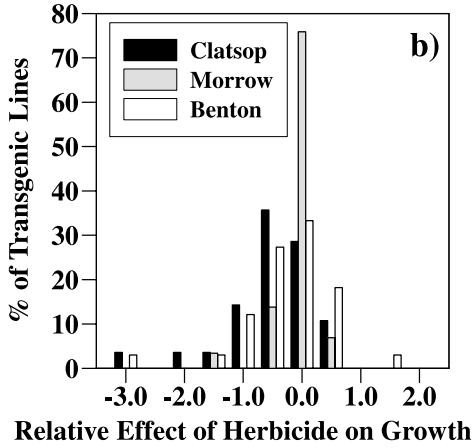
However, on average, the transgenic lines containing both the *CP4* and *GOX* genes grew significantly (α = 0.05) less when treated with glyphosate than the corresponding unsprayed lines (Fig. 7), whereas those lines containing just *CP4* were not significantly affected by treatment. Growth of the transgenic plants, containing either one or both glyphosate tolerance genes, was not significantly different than that of the nontransgenic plants in the unsprayed control rows (Fig. 7).

Discussion

When poplar was transformed with both *CP4* and *GOX*, treatment with glyphosate resulted in the appearance of chlorosis that, in our experience, was not typical of glyphosate damage. When poplar was engineered with just the *CP4* gene, no chlorosis developed in response to herbicide treatment. The association of the chlorotic symptoms with the presence of the *GOX* gene suggests that the enzyme may be responsible for metabolic changes that result in chlorosis. One known activity of GOX enzyme is to convert glyphosate

Fig. 6. Effects of Roundup Pro™ on overall relative growth (defined in Materials and methods) of all transgenic lines by site at the 1× (*a*) and 2× (*b*) rates. Positive values indicate superior growth after spraying compared with unsprayed trees.





into AMPA. Further experimentation should be undertaken to determine whether this conversion is related to the appearance of chlorotic symptoms in transgenic poplar.

Our results suggest that both glyphosate-tolerance transgenes are not needed to impart high levels of tolerance; expression of a single gene appears to be sufficient. There are three independent lines of evidence to substantiate this claim. Firstly, relatively low levels of GOX protein resulted in highly tolerant plants (i.e., low damage score). For example, among the three most highly tolerant lines selected, one had a GOX protein concentration of only 7.1 μg/g FM (the mean level was 60.98 μg/g FM; maximum level detected was 1122 μg/g FM). Secondly, lines that contained only *CP4* exhibited less herbicide damage and grew better than lines with both *CP4* and *GOX*. Finally, levels of CP4 protein were negatively correlated with herbicide damage and positively correlated with growth, but GOX protein levels were not correlated with damage or growth.

The differences in effects of *GOX* and *CP4* were apparent in part because of the relatively weak correlation between GOX and CP4 protein levels in plants containing both genes. It is widely known that *Agrobacterium* is capable of inserting T-DNA in a variety of ways (see references in Ülker et

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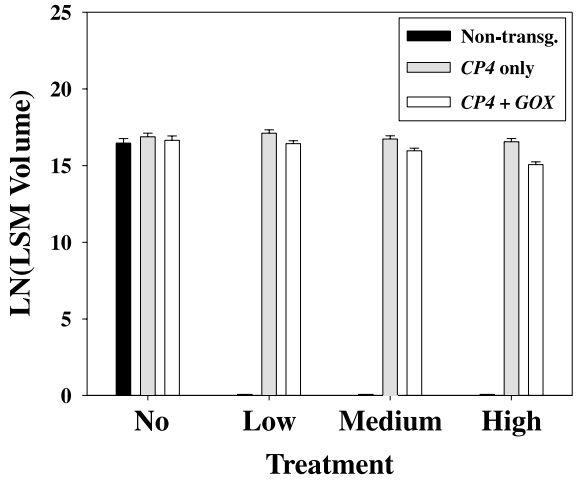
Fig. 7. Mean growth (volume index) for transgenics containing a single herbicide tolerance gene (*CP4*) and those containing both genes (*CP4* and *GOX*) following two treatments with glyphosate at the Morrow site. Error bars are SEs.



al. 1999). Options can include partial (truncated), single, and multiple insertions. The latter can result from many copies at a single locus (direct or inverted repeats) or single and multiple inserts at several loci. We have observed differences in the complexity of T-DNA inserts among poplar genotypes, with aspen typically showing much more complex insertion profiles than cottonwood. These differences, combined with the fact that different promoters were used to drive the expression of *CP4* and *GOX*, could explain the lack of correlation between CP4 and GOX protein levels in hybrid aspen.

In 2 lines of the 80 tested, we observed an abrupt increase in mean damage for a line from one year to the next (in one case, the mean damage rating went from 0.5 to 4.5; in the other case, the change was from 1.5 to 4.5). One possible explanation for loss of tolerance is transgene silencing, which has been reviewed extensively (e.g., Finnegan and McElroy 1994; Stam et al. 1997; Matzke and Matzke 1998). One form of silencing occurs at the level of transcription and usually involves methylation of promoter regions but may also involve chromatin modification (van Blokland et al. 1997; Finnegan et al. 1998). A recent report described how cold-induced dormancy led to elevated methylation, which in turn, led to partial transgene silencing (Callahan et al. 2000). The possibility of year-to-year variation in transgene expression highlights the importance of conducting multiyear trials. We have vegetatively propagated all lines used in this study, planted them on another site, and plan to monitor their growth and glyphosate tolerance for several years.

There was little evidence for somaclonal variation during our 2-year study. The transgenic plants showed no obvious morphological variation, and transgenics and controls grew equally well when unsprayed. However, one of the highly tolerant lines, a derivative of triploid clone 24-305, did show altered morphology and slower growth after it was propagated in a stool bed and planted in a subsequent field trial. Although it retained its herbicide tolerance, the propagation seemed to release latent somaclonal variation. We have also observed delayed manifestation of morphological variation following propagation in two other transgenic cottonwood lines, and both lines retained their transgene expression. Although the occurrence of somaclonal variation is infrequent, it seems prudent to study transgenic trees for several years and multiple propagation cycles before their commercial use to ensure that no significant collateral genetic damage has been introduced.

These results are promising, and we are now evaluating whether glyphosate tolerance will result in more effective and less expensive weed control. Vegetative propagules from previously screened, herbicide-tolerant transgenic trees have been used to establish a large-scale trial in which transgenics and nontransgenics are being grown under operational conditions. To determine the utility of herbicide tolerance, we are using this study to evaluate weed control and tree growth under a conventional management regime and one that relies exclusively on the use of Roundup®.

Although additional field trials are needed to verify yield and the absence of morphological abnormalities in successive years and other environments, it appears possible to produce poplars that are highly tolerant of glyphosate and exhibit normal growth. The next step in commercial development could be to combine herbicide tolerance with genes that prevent flowering (Skinner et al. 2000). This would greatly reduce the chance of herbicide tolerance spreading outside of plantations. Flowering control may also provide other benefits, such as improving stem growth, restricting movement of exotic poplar genes (from hybrids) into wild stands, reducing plantation in-growth, eliminating nuisance floral structures, and reducing production of airborne allergens (pollen).

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