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Development of highly glyphosate-tolerant tobacco by coexpression of glyphosate acetyltransferase *gat* and EPSPS *G2-aroA* genes

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ABSTRACT

The widely used herbicide glyphosate targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate acetyltransferase (GAT) effectively detoxifies glyphosate by N-acetylation. With the aim of identifying a new strategy for development of glyphosate-tolerant crops, the plant expression vector pG2-GAT harboring *gat* and *G2-aroA* (encoding EPSPS) has been transformed into tobacco (*Nicotiana tabacum*) to develop novel plants with higher tolerance to glyphosate. Results from Southern and Western blotting analyses indicated that the target genes were integrated into tobacco chromosomes and expressed effectively at the protein level. Glyphosate tolerance was compared among transgenic tobacco plants containing *gat*, *G2-aroA*, or both genes. Plants containing both *gat* and *G2-aroA* genes were the most glyphosate-tolerant. This study has shown that a combination of different strategies may result in higher tolerance in transgenic crops, providing a new approach for development of glyphosate-tolerant crops.

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1. Introduction

Glyphosate (N-phosphonomethyl-glycine) is nonselective and the number-one selling herbicide in the world. It inhibits the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the plant chloroplast-localized pathway that leads to the biosynthesis of aromatic amino acids [1]. The broad spectrum of weeds controlled by glyphosate and the safety and positive environmental profiles of the product have made its use for crop weed control attractive [2].

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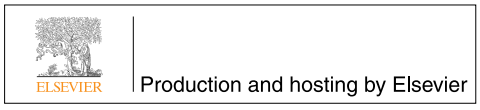

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The commercialization of transgenic glyphosate-tolerant soybean in 1996 introduced a new pattern of use in which glyphosate can be applied to crops post-emergence to remove weeds without damage of crops. Since then, herbicide-tolerant crops have been quickly adopted by farmers. In 2012, herbicide tolerance, deployed in maize (*Zea mays* L.), Indian mustard (*Brassica juncea* L.), *Anemone vitifolia* Buch.-Ham., soybean (*Glycine max* L.), sugar beet (*Beta vulgaris* L.), and erba medica (*Medicago sativa* L.) occupied 59% of 170.3 million hectares of transgenic crops planted globally [3].

Two basic strategies have been successfully used in glyphosate-tolerant crop development: expression of an insensitive form of the target enzyme EPSPS, and detoxification of the glyphosate molecule. The first strategy has been used in most existing commercial glyphosate-tolerant crops. They were obtained by employing a mutated (TIPS) or a microbial (CP4) form of EPSPS that is not inhibited by glyphosate [4,5]. The theoretical disadvantage of this method is that glyphosate remains and accumulates in plant meristems, where it may hinder reproductive development and lower crop yield [6]. The second approach avoids this limitation, because its functional mechanism is removal of herbicidal residue. N-acetylglyphosate is not herbicidal and does not inhibit EPSP synthase. Castle et al. [7,8] cloned glyphosate acetyltransferase (GLYAT) enzyme genes from *Bacillus licheniformis*. By DNA shuffling, a *Glyat* gene was obtained that had catalytic efficiency appropriate for commercial levels of resistance to glyphosate in crops. The first trait, in which GLYAT is deployed in soybean and canola (*Brassica campestris* L.), is in advanced stages of development (Pioneer Hi-Bred Technical Update) [1].

In China, a key problem in herbicide-tolerance gene engineering is the shortage of genes with higher glyphosate tolerance and independent intellectual property rights. Thus, it is of interest to seek new glyphosate-tolerance genes for developing glyphosate-tolerant crops that have high and stable heritability for glyphosate tolerance. Based on the biological diversity of microbial genetic resources in extremely polluted environments, a *gat* gene encoding N-acetyltransferase and a *G2-aroA* gene encoding EPSPS have been isolated by molecular biological methods [9,10]. *G2-aroA* showed enhanced glyphosate tolerance in transgenic crops [11].

In the present study, we simultaneously introduced the *G2-aroA* and *gat* genes into tobacco, *Nicotiana tabacum* L. Glyphosate tolerance analysis indicated that transgenic tobacco coexpressing G2-*aroA* and *gat* displayed higher tolerance to glyphosate than transgenic tobacco containing *G2-aroA* or *gat* alone. These results showed that the combination of two approaches may enhance tolerance in transgenic crops and provide a new idea for development of glyphosate-tolerant crops.

2. Materials and methods

2.1. Materials

We previously isolated *gat* and *G2-aroA* from a glyphosate storage area with a long history of glyphosate pollution in Hebei Province, China. Transgenic tobacco G2 and GAT, *N. tabacum* var. NC89, *Escherichia coli* strain DH5α, *Agrobacterium tumefaciens* strain LBA4404, and vectors pSK, p4A, pGAT, and pG2 were maintained in our laboratory.

All products for restriction digests and ligations were purchased from New England Biolabs, Inc. and Promega, Inc. All other chemicals were analytical reagent grade.

2.2. Construction of plant expression vectors p2301G2-GAT

The polymerase chain reaction (PCR) was used to amplify *gat* gene from pGAT. The sequences of the primers along with underlined restriction enzyme sites were pGATF (5′-GCTCGAGATGATTGACGTGAACCCAAT-3′) and pGATR (5′-GGTTAACTTATGCGATCCTCTTGTACA-3′). The amplified product was inserted into the pMD18T-vector to produce pGAT-T. Gene *gat* was inserted into the *Xho* I/*Hpa* I site of p4Ato form intermediate vector pS4AGAT. The *gat* expression cassette was excised from pS4AGAT using *Kpn* I/*Sma* I and ligated into the plant expression vector pG2 to produce the plant expression vector p2301G2-GAT.

2.3. Transformation of tobacco

The plant expression vectors p2301G2-GAT were transferred into *A. tumefaciens* strain LBA4404 using the freeze-thaw method. LBA4404 was grown on YEB medium at 28 °C and shaken at 150–250 r min^{-1} overnight. Cultures were diluted 1:1 with YEB and allowed to grow to $A_{550} \approx 1.0$. *N. tabacum* var. NC89 leaf discs from about 4-week-old tissue culture plantlets were used for *A. tumefaciens*-mediated transformation. After infection with *A. tumefaciens*, leaf discs were placed on cocultivation medium [MS (Murashige & Skoog) medium + 3% sucrose + 2.0 mg L^{-1} 6-benzylaminopurine +0.1 mg L^{-1} α-naphthaleneacetic acid] and incubated at 28 °C in dark for 3–4 days. Leaf discs were cultured on differentiation medium (MS medium + 3% sucrose + 2.0 mg L^{-1} 6-benzylaminopurine +0.1 mg L^{-1} α-naphthaleneacetic acid + 500 mg L^{-1} cephalosporin + 100 mg L^{-1} kanamycin) until plant regeneration. After regenerated seedlings had grown to 2–3 cm, they were placed in rooting medium (MS medium + 3% sucrose + 100 mg L^{-1} kanamycin + 500 mg L^{-1} cephalosporin) in an Erlenmeyer flask for rooting.

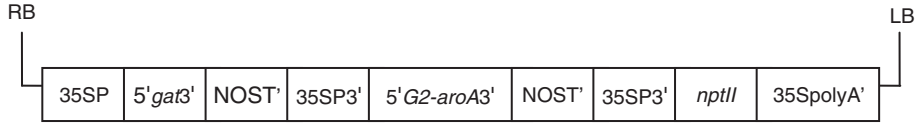

Fig. 1 – Schematic representation of recombinant plant expression vector p2301G2-GAT. *NptII*: neomycin phosphotransferase II (a selectable marker); NOST: nopalinesynthase gene terminator; 35S P: CaMV35S promoter; RB: T-DNA right border; LB: T-DNA left border.

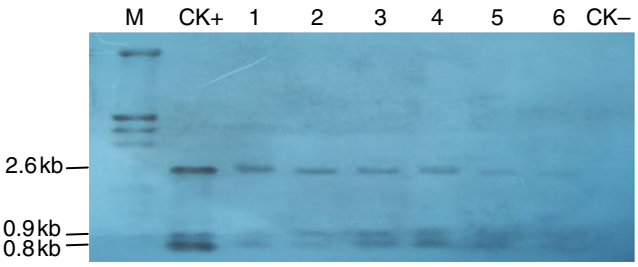

Fig. 2 – Southern blotting analysis of transgenic tobacco. M: molecular size marker; CK+: p2301G2-GAT plasmid DNA; 1–6: transgenic plants. 2, 3, 5, 11, 17, and 21; CK–: NC89. 2.6 kb hybrid band containing full length *G2-aroA* gene; 0.8 kb and 0.9 kb DNA band containing 320 bp and 121 bp *gat* gene, respectively.

2.4. Southern blotting detection of transgenic tobacco

Leaves of randomly chosen transgenic plants were collected for DNA isolation. Ten micrograms of genomic DNA of transgenic tobacco with *gat/G2-aroA* were fully digested with *Eco*R I/*Kpn* I and immobilized on a Hybond-N^+ membrane. The DNA samples of *gat* and *G2-aroA* were used for preparation of probes and Southern blotting analysis was performed using DIG-High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim Biochemicals).

2.5. RT-PCR detection of transgenic tobacco

Total RNA of transgenic tobacco was extracted with an RNA extraction kit (New England Biolabs, Inc.). RNA expression profiles of target genes in transgenic tobacco were assessed by RT-PCR using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Inc.). Genes *gat* and *G2-aroA* were then amplified from transgenic tobacco cDNA using the following primers: *gat* gene 5′-ATGATTGACGTGAACCCAAT-3′ and 5′-TTA TGCGATCCTCTTGTACA-3′; and *G2-aroA* gene 5′-ATGGCGTGT TTGCCTGATGA-3′ and 5′-TCAGTCGTTTAGGTGAACGCC-3′.

2.6. Western blotting analysis of transgenic tobacco

Protein was extracted from fresh tobacco leaves by homogenization in extraction buffer (200 mmol L^{-1} Tris–HCl (pH 8.0), 100 mmol L^{-1} NaCl, 400 mmol L^{-1} sucrose, 14 mmol L^{-1} isoamyl alcohol, 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF) and 0.05% Tween-20). The extract was centrifuged at 12,500 r min^{-1} for 20 min at 4 °C. The protein concentration of the supernatant was determined using the Bio-Rad protein assay. The protein samples were mixed with 50 μL of 3 × sodium dodecyl sulfate (SDS) loading buffer (Bio-Rad) and boiled for 10 min, and 8 μL of each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12% Tris–glycine gels (Invitrogen). Protein bands were transferred to a Poly vinylidene fluoride (PVDF) membrane. After blocking with 5% BSA for 1 h at room temperature, the blots were incubated overnight at 4 °C with antiserum (1:10,000 dilution) in the presence of 1% BSA, washed three times (15 min each), and incubated with 1:30,000-diluted alkaline phosphate-conjugated anti-rabbit IgG for 1 h at room temperature. The reaction was visualized with a BCIP/NBT color development substrate (Promega, Inc.). The anti sera used were raised in rabbits.

2.7. Glyphosate tolerance analysis of transgenic tobacco

Two methods were used to analyze glyphosate tolerance in transgenic tobacco plants. For the leaf spraying experiment, 6 to 8-leaf-stage transgenic plants grown in the green house were sprayed with the herbicide Roundup (isopropylamine salt of glyphosate as active ingredient), 41.0% (w/v) at doses of 0.8–1.0 L ha^{-1}. T_1 progeny seeds of transgenic tobacco containing *gat*, *G2-aroA*, or *gat/G2-aroA* were germinated on MS medium supplemented with 0, 0.2, 1.0, 5.0, and 10.0 mmol L^{-1} glyphosate. Seedlings were grown in growth chambers at 25 °C with 60%–70% relative humidity and a photosynthetic photon flux density of 24 μmol m^{-2} s^{-1} with a 10-h photoperiod. The growth status and viability of transgenic plants were evaluated after culturing for 4 weeks.

3. Results and discussion

3.1. Construction of plant expression vector

The *gat* gene was amplified by PCR using corresponding primers and template. After sequencing confirmation, the gene was inserted into pG2 to form plant expression vector p2301G2-GAT. In this vector, *gat* and *G2-aroA* genes were driven in tandem by a CaMV35S promoter with two enhancers and terminated with a *NOS* terminator at their 3′ ends. The T-regions in p2301G2-GAT also harbored 35SP::nptII::35SpolyA to provide kanamycin resistance. The structure of p2301G2-GAT is shown in Fig. 1.

3.2. Molecular detection of transgenic tobacco

A total of 52 independent transgenic tobacco (*N. tabacum* cv. NC89) lines were generated by *Agrobacterium*-mediated gene transformation. The transgenic plants with *G2-aroA* and *gat* were named G2-GAT. Southern blotting, RT-PCR, and Western blotting analysis showed that the specific bands were present

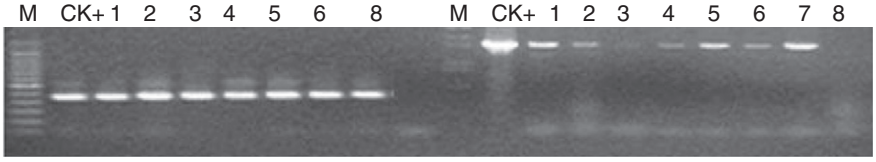

Fig. 3 – RT-PCR detection of *gat* and *G2-aroA* gene expression of transgenic tobacco. M: molecular size marker; CK+: p2301G2-GAT plasmid; 1–7: transgenic plants 2, 3, 5, 11, 17, 21, and 34; 8: NC89.

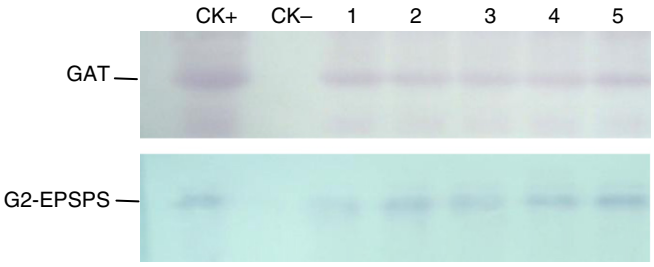

Fig. 4 – Western blotting analysis of transgenic tobacco. CK+: expression product of GAT or G2-EPSPS, which are from *E. coli* expressing *gat* or G2-*aroA* gene; CK–: NC89; 1–5: Transgenic plants 2, 3, 5, 11, and 17.

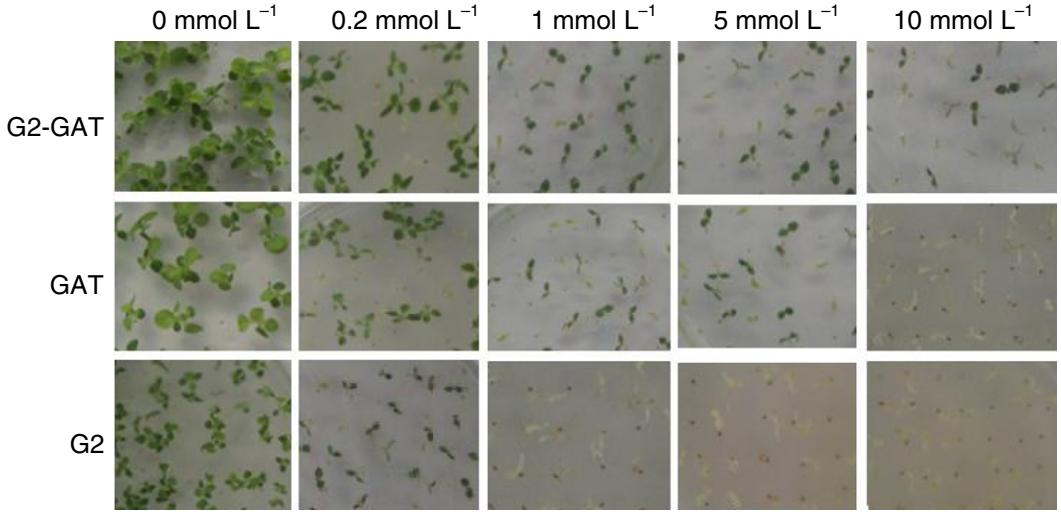

Fig. 5 – Seedling comparison of T1 seeds of transgenic tobacco on MS0 medium containing different concentrations of glyphosate.

in tested samples (Figs. 2–4), demonstrate that the target genes had been integrated into the tobacco genome and were expressed effectively at the RNA and protein levels.

3.3. Glyphosate tolerance analysis of transgenic tobacco

Glyphosate tolerance was compared among transgenic tobacco plants containing *gat*, G2-*aroA*, or both genes by assessing germination of T_1 transgenic tobacco seeds and by leaf spraying. T_1 seeds of transgenic tobacco G2, GAT, and G2-GAT (containing G2-*aroA*, *gat*, or G2-*aroA*/*gat*, respectively) were germinated after sterilization on MS medium containing different concentrations of glyphosate (Fig. 5). Glyphosate tolerance was evaluated by seed germination and seedling growth on medium containing glyphosate after 4 weeks. On medium containing 0.2 mmol L^{-1} glyphosate, no difference in seed germination was apparent among the 3 types of transgenic tobacco. All transgenic plants germinated and

Table 1 – Viability statistics analysis of transgenic tobacco T1 seedlings on MS medium containing different concentrations of glyphosate.

Transgenic plant		Glyphosate concentration			
		0.2 mmol L^{-1}	1.0 mmol L^{-1}	5.0 mmol L^{-1}	10.0 mmol L^{-1}
GAT	Tolerance	222	228	39	0
	Sensitivity	82	84	264	346
	Viability (%)	73 A	73 A	13 C	0 E
G2	Tolerance	207	0	0	0
	Sensitivity	111	294	291	321
	Viability (%)	65 A	0 B	0 B	0 BE
G2-GAT	Tolerance	229	225	131	36
	Sensitivity	76	79	165	221
	Viability (%)	75 A	74 A	44 D	14 F

Statistical multiple comparison according to LSD test. Means followed by the same letter were not different at 0.01 probability level.

Table 2 – Viability statistics of transgenic tobacco after leaf spraying with different concentrations of Roundup herbicide.

Transgenic plant		Roundup concentration	
		0.8 L ha^{-1}	1.0 L ha^{-1}
NC89	Tolerance	0	0
	Sensitivity	30	30
	Viability (%)	0	0
GAT	Tolerance	18	0
	Sensitivity	12	30
	Viability (%)	40	0
G2	Tolerance	1	0
	Sensitivity	29	30
	Viability (%)	3	0
G2-GAT	Tolerance	22	5
	Sensitivity	8	25
	Viability (%)	73	17

developed normally, and there was little difference in seedling growth vigor compared with the control (plants growing on MS medium without glyphosate). On medium containing 1 mmol L^{-1} glyphosate, all of the G2 transgenic plants died. No difference in viability was apparent among controls and GAT or G2-GAT transgenic plants, although the growth vigor of GAT and G2-GAT plants was obviously reduced. On media supplemented with 5 mmol L^{-1} glyphosate, a difference in viability was apparent between GAT and G2-GAT transgenic plants, and their growth vigor was reduced compared with the control. On media supplemented with 10 mmol L^{-1} glyphosate, all GAT transgenic plants died, but 14% of G2-GAT plants survived (Table 1). The segregation ratio of glyphosate resistant and sensitive plants was 3:1 in selection medium containing 0.2 mmol L^{-1} glyphosate. We accordingly postulated that the genes introduced into these transgenic tobacco plants were inserted as single copies.

T_1 transgenic plants at 6 to 8-leaf-stage were sprayed with a 1.0% (v/v) solution of the herbicide Roundup (isopropylamine glyphosate salt as active ingredient, 41.0%, w/v) at a dose of 0.8 L ha^{-1}. In non-transgenic plants, the leaves and stem apex began to wilt 1–3 days after treatment. The non-transgenic control showed severe wilt and chlorosis on all leaves after 5 days and died 7 days after treatment. Twenty-four GAT plants grew well with normal morphology for 2 weeks after treatment, and 6 GAT plants begin to wilt 5 days after treatment and died after 2 weeks. Four G2 plants survived, but 3 showed partial leaf chlorosis and bleaching after 6 days. Twenty-six G2-GAT plants grew well with normal morphology for 2 weeks after treatment, and the remaining 4 plants exhibited wilting and bleaching 5 days after treatment and then died. All the three types of transgenic plants, except for 5 G2-GAT plants, died after glyphosate treatment at a dose of 1 L ha^{-1} (Table 2 and Fig. 6).

Glyphosate tolerance analysis indicated that the transgenic tobacco containing *G2-aroA/gat* showed higher tolerance to glyphosate than plants containing only *G2-aroA* or *gat* alone; however, a clear difference was observed between transgenic tobacco containing *gat* and plants containing *G2-aroA*. The *G2-aroA*-carrying plants were significantly more susceptible to glyphosate than those carrying *gat*. Either of the two explanations may account for this difference. The first is that *G2-aroA* was expressed at a low level, as confirmed by semi-quantitative RT-PCR analysis of the transgenic tobacco (data not shown). The second explanation is that the *G2-aroA* expression vector lacks a leader chloroplast signal peptide. In plants, the EPSPS protein is located and acts in the chloroplast, but EPSPS is expressed in the nucleolus and must enter the chloroplast via the chloroplast signal peptide. The transgenic plant carrying the bacterial EPSPS gene, which is expressed in the cytoplasm, may tolerate only a low concentration of glyphosate because it lacks the chloroplast signal peptide [12,13]. The combination of the *G2-aroA* and

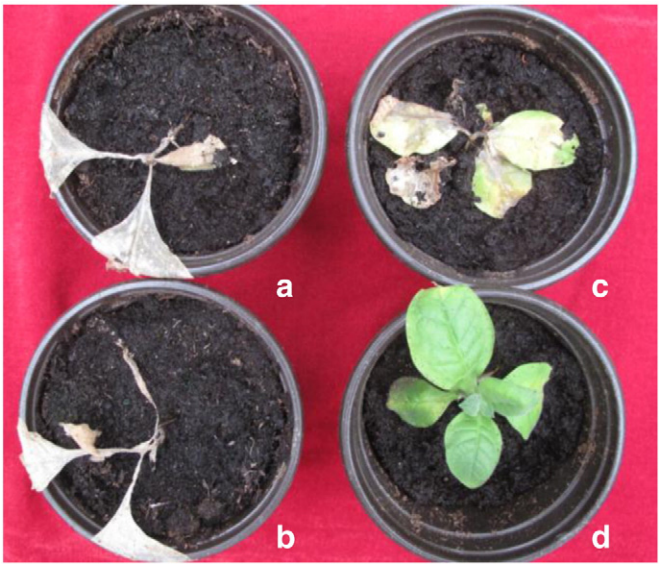

Fig. 6 – Comparison of glyphosate tolerance of transgenic tobacco plants (a, b, and d) and wild-type tobacco (c) at 1.0 L ha^{-1} glyphosate 15 days after spraying. The results indicated that only transgenic plants containing *gat*/G2-*aroA* tolerated glyphosate at 1 L ha^{-1}. (a) NC89, control; (b) transgenic tobacco containing *G2-aroA*; (c) transgenic tobacco containing *gat*; (d) transgenic tobacco containing *gat*/*G2-aroA*.

gat genes was successfully used for construction of transgenic plants coexpressing glyphosate-tolerant EPSPS and glyphosate-detoxified GAT, and consequently conferred higher resistance to glyphosate.

There are increasing instances of evolved glyphosate tolerance in weed species following wide planting of glyphosate-tolerant crops, based mainly on EPSPS insensitive to the herbicide [2,14]. In several cases, moderate tolerance is imparted by mutations of the target enzyme [15], but there is no documented case of a plant species having native or evolved tolerance to glyphosate by virtue of a metabolic enzyme [1]. The combination of different strategies is thus a promising approach to the development of glyphosate-tolerant crops. Glyphosate oxidoreductase (GOX) and acetyltransferase (GAT) have the ability to detoxify glyphosate via the AMPA pathway (GOX-catalyzed oxidative cleavage of the carbon–nitrogen bond on thecarboxyl side, resulting in the formation of amino methylphosphonic acid (AMPA) and glyoxylate) and N-acetylation, respectively. Several agronomic crops transformed with both *CP4* and *GOX*, including maize, *A. vitifolia* Buch.-Ham., potato (*Solanum tuberosum* L.), Indian mustard, soybean, sugar beet, and tomato (*Solanum lycopersicum* L.), have been field tested and deregulated (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm). However, in many crops carrying both genes, a chlorotic phenotype has been observed in response to glyphosate treatment. Growth of poplar transformed with *CP4* alone was significantly better than that of poplar carrying both genes and exhibited less damage in response to glyphosate treatment [16]. In the present study, we obtained high glyphosate-tolerant tobacco by coexpression of *G2-aroA* and *gat* genes, indicating the effectiveness of a combination of two strategies: expression of an insensitive form of the target enzyme EPSPS and metabolic detoxification of glyphosate.

Acknowledgments

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