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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.

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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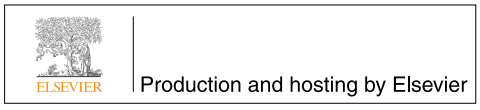

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The commercialization of transgenic glyphosate-tolerant soybean in 1996 introduced a new pattern of use in which glyphosate can be applied to crops post-emergence to remove weeds without damage of crops. Since then, herbicide-tolerant crops have been 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.

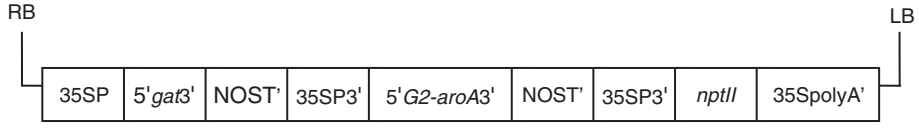

Fig. 1 – Schematic representation of recombinant plant expression vector p2301G2-GAT. *NptII*: neomycin phosphotransferase II (a selectable marker); NOST: nopalinesynthase gene terminator; 35S P: CaMV35S promoter; RB: T-DNA right border; LB: T-DNA left border.