Research Article

Journal of Bioscience & Bioengineering

Obtained And Detection For Transgenic Tobacco With High Gloyphosate-Resistance

Zongying Zhang[a], Xiangru Chen[c], Xiaojun Liu, Wei Lu[b], Ying Wang[a], Chenggui Han[a]*, Dawei Li[a], Jialin Yu[a]

A State Key Laboratory for Agrobiotechnology and Department of Plant Pathology, China Agricultural University Beijing 100193
b Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory of Crop Biotechnology, Ministry of Agriculture, Beijing, 100081
C Key Laboratory of Agricultural Microbiology, College of Agriculture, Guizhou University, Guiyang, Guizhou 550025

***Correspondence to:**
Zongying Zhang*,
A State Key Laboratory for Agrobiotechnology and Department of Plant Pathology, China Agricultural University Beijing 100193
Email: zhangzongying@cau.edu.cn

Received Date: 08 Nov 2023
Accepted Date: 23 Nov 2023
Published Date: 30 Nov 2023

Citation:
Zongying Zhang. Obtained And Detection For Transgenic Tobacco With High Gloyphosate-Resistance. Journal of Bioscience & Bioengineering2023.

1. Abstract

Glyphosate acetylation is an active modified process for glyphosate detoxification. N-acetyltransferase transfers glyphosate to N-Acetylglyphosate before the plant cell death caused by inhibiting the EPSPS synthesis. In this study, a vector containing glyphosate N-acetyltransferase (GAT) gene was constructed and transformed into the Nicotiana tabacum L. cv. Xanthi, the gat gene was integrated into the tobacco genome by PCR and Southern blot detection. The specific antiserum of GAT was prepared successfully, and was diluted to 1:1000 for genetically modified crops specificity testing.

2. Keyword:
Glyphosate N-acetyltransferase gene, glyphosate resistance, transgenic tobacco, antiserium

3. Introduction

The herbicide glyphosate is widely used cause its non-selective broad-spectrum, its efficacy against all plant species, low cost, low mammalian toxicity and benign environmental impact (Franz et al., 1997). It blocks plant growth by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, designated as AroA) (Steinrucken and Amrhein, 1980). At first, some commercial glyphosate-resistance plants were generated by transforming the non-sensitive microbial epsps into plants, including soybean, maize, canola, cotton, sugar beet and alfalfa (James, 2010). However, this mode has some disadvantages, the glyphosate remains and accumulated in the plant [Gougler and Geiger, 1981; Pline et al., 2002]. Castle screened a collection of microbial isolate and obtained a low-activity GAT; then they cloned the gene from Bacillus licheniformis following optimized by gene shuffling and obtained the gat gene which could express GAT with high N-acetyltransferase activity [Castle et al., 2004; Siehl et al., 2005]. This N-acetyltransferase transfers glyphosate to N-acetylglyphosate efficiently before the plant cell death and forms a novel mechanism of glyphosate tolerance in transgenic plants. This mechanism has been used in commercial transgenic crops like OptimumTMGATTM maize (98140) and soybean (356043) (OptimumTMGATTM is a registered trademark of Pioneer Hi-Bred International, Inc.) (Appenzeller et al., 2008, 2009).

In China, Dun constructed a metagenomic library using DNA from glyphosate polluted soils, screened a novel glyphosate tolerance gat gene and cloned it into E. coli expression system to detecte its function [Dun et al., 2006]. This provides the theoretical and practical basis for a new type glyphosate-tolerance transgenic crop with independent intellectual property rights. In this study, a marker-free transgenic tobacco with high glyphosate-tolerance was obtained by transforming the gat gene into it.

4. Meterial and Method

Vector construction

The vector with gat gene, p35s-2301-gat, was provided by Professor Wei Lu (Chinese Academy of Agricultural Sciences). The plasmid, p35s-2301-gat, was an agrobacturium transformation vector which had right border and left border and used npt-II as the marker gene (Figure 1). We used the same enzyme restrict site (Xho I) to delete the marker gene, so that p35s-2301-gat became a non-marker gene vector, p35s-2301-gat- npt-II.

5. Plant material and tobacco transformation

Tobacco seeds Nicotiana tabacum L. cv. Xanthi was stored in our lab. The seeds were sterilized with 75% ethanol for 2-3 min followed by one wash with sterile distilled water and with 10% NaClO for 10 min followed by 3-5 washes with sterile distilled water, and then germinated in the glass tubes which had solidified 1/2 MS medium (containing 30 % sucrose, pH 5.8) under 24oC, 16/8 (L/D). After 3-4 weeks, the leaves of

the sterile plants were used for transformation. Tobacco leaves were cut into 2 cm × 2 cm pieces, co-cultured with agrobacterium bacteria solution for 20-30 min and transferred to solid MS media (Murashige and Skoog, 1962), containing 2 mg L-1 Kinetin (KT), 1 mg L-1 Indole-3-acetic acid (IAA) and 100 mg L-1 Acetosyringone (AS), for 2-3 days at 25oC in darkness, the infected leaf pieces were transferred to regeneration medium MSK2I1Z1Carb500 (MS basic media containing 2 mg L-1 KT, 1 mg L-1 IAA, 1 mg L-1 ZT, 500 mg L-1 Carbenicillin, Carb). After regeneration, the shoots were transferred to shoot elongation and root induction media, 1/2 MS. The health regeneration plants were transferred into soil and seeds were harvested.

6. Genomic PCR and Southern blotting

Tobacco genomic DNA was extracted from young leaves of transgenic and non-transgenic plants by using the cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980)or the Axygen Nucleic Acid Purification Kit. Genomoic DNA PCR was performed using the primers gat-F (TCT AGA ATG GCG CAA GTT AGC AGA) / gat-330R (GAA CAT GCA AGC CTC GAT TGG T), resulting in 330 bp amplified DNA product. The PCR products were analyzed by agarose gel electrophoresis. For Southern blotting analysis, 10 µg of tobacco genomic DNA was digested with EcoR I (New England Biolabs), separated on agarose gels, transferred to a hybond-XL nylon membrane (GE, Healthcare), and subjected to Southern Blot analysis (Sambrook and Russell, 2001).

7. Preparation of GAT antiserum and Western blot analysis

In this part, purified GAT was injected to the rabbit to produce the antiserum. Total protein was extracted from leaves which collected from transgenic and non-transgenic tobacco plants. 100 mg leaf samples were ground in a Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) and homogenized with 100 µl 2 × SDS protein extraction buffer (0.25 M Tris-HCl, pH 6.8, 8% 2-mercaptoethanol, 20% glycerol and 8% SDS) and 100 µl ddH2O. After denaturation and centrifuge, the total protein were separated in 16% SDS polyacrylamide gel, transferred to nitrocellulose filter membrane and immunoblotted with 1:1000 dilution of GAT rabbit poly-clonal antibody.

8. Seed germination selection and glyphosate tolerance spray test

The herbiciede RoundupTM (active ingredient isopropylamine salt of glyphosate, 41%) was used in these tests. The seeds of transformation lines were harvest in the greenhouse and were sterile by NaClO method to detect the glyphosate-resistance on MSG2000 media which contains herbicide RoundupTM [1:2000, RoundupTM : distilled water] for two weeks and then transfer to MSG4000 for two weeks following on MS without glyphosate. The seeds which have resistant will be uniformly green and grow normal, while the sensitive seeds will be bleached and dead [Yan et al., 2011]. The seeds of transformation lines were planted in the greenhouse and the plants were sprayed with herbicide RoundupTM dilution by distilled water at 4-6 leaf stage using pressure watering can[Ye et al., 2001]. Ten days after spraying glyphosate, the symptoms will show up, and two weeks after spraying, the glyphosate-resistance lines will distinctly differ from the sensitive lines. The plants which have resistant will be uniformly growth and the sensitive plants will be dry death.

9. Results

Generation of tobacco primary transformant

The vector that used in this study hadn't the marker gene, so the health regeneration plants were transferred into soil directly without selection by any antibiotics. Twenty-two transgenic lines were obtained and used for detecting and seeds harvest.

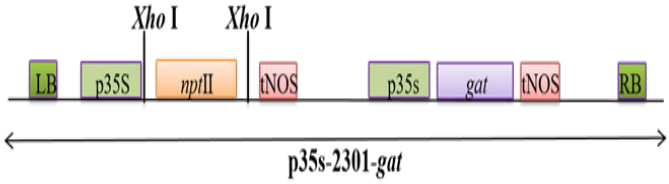

Figure 1. Schematic maps of the gene cassette used for production of the transgenic tobacco line.

Diagrams of the constructs are not to scale.

p35S: CaMV 35S promoter, npt-II: neomycin phosphotransferase II coding sequence, tNOS: nopaline synthase terminator, gat: GAT gene encoding sequence, LB: left board; RB: right board.

Genomic PCR and Southern blotting

Selecting the glyphosate-resistance transgenic plants

The genomic DNA PCR of T0 was carried out and the agarose analysis showed that eight of 22 regeneration tobaccos were positive [Fig. 2 showed the positive lines].

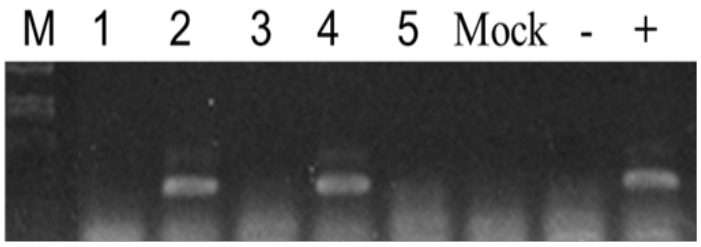

Fig. 2 Genomic PCR of transgenic tobacco lines and non-transgenic tobacco

M: λ DNA marker (Hind III / EcoRI); Lane 1-5: transgenic lines; Mock: non-transgenic lines; -:negative control (water as template); +: positive control (plasmid as template). The arrow shows the interest band.

Journal of Bioscience & Bioengineering

One of the positive lines [p35s-2301-gat- npt-II Line 1, 5, 6, 7, 8, 9, 10, 11] , line 5, was choose to carry out the following experiments. The transgenic tobacco genomic PCR of T1 and T2 for line 5 showed the interest band [Fig. 3].

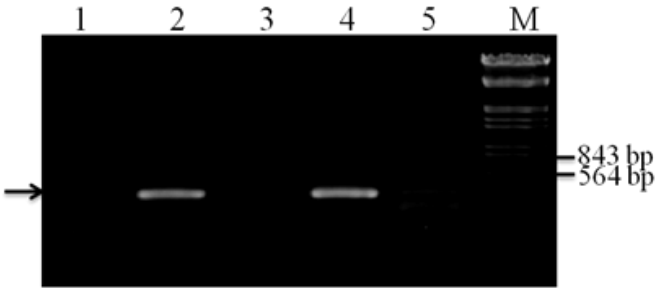

Fig. 3 Transgenic tobacco genomic PCR of T1 and T2 for g-5

Lane1: Non-transgenic tobacco (the receptor SR as template); Lane 2: T1 transgenic tobacco line (g-5); Lane 3, 5: negative transgenic line; Lane 4:T2 transgenic tobacco line (g-5);
M: λ DNA marker (Hind III / EcoRI). The arrow shows the interest band.

The genomic DNA of T1, Line 5, and non-transgenic line were extracted to do Southern blot. They were hybridized with a 600-bp gene fragment from 35S promoter that had been PCR amplified with 35S-F [GCT CCT ACA AAT GCC ATC ATT GC) and 35S-R (GAT AGT GGG ATT GTG CGT CAT CCC] primers and labeled with α-32P according to instructions provided in the Ready-To-Go dCTP labeling kit (GE, Healthcare). The results proved that the transgenic line had one positive band [Fig. 4].

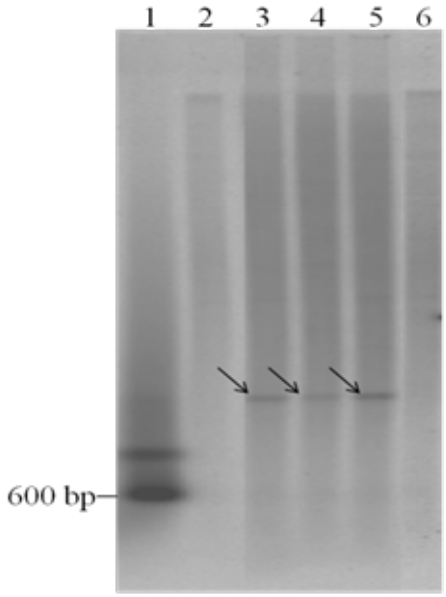

Fig. 4 Southern blotting analysis of T1 transgenic progeny for the 35s promoter gene

Lane 1: CK+ (PCR fragment of 35s); Lane 2: Negative control (the receptor SR); Lane 3-6: Transgenic tobacco lines. The arrows presented the interest bands.

GAT antiserum and Western blot analysis

The GAT gene was cloned into the expression vector, successfully, the interest protein was purified and made the antiserum by Chinese Academy of Sciences genetics. The antiserum was successful to use and detect the GAT protein. Western blot was carried out using this antiserum for T1 and T2 of Line 5, the result was shown in Figure 7. The interest protein which was 17 kDa was detected in T1 and T2 [Fig. 5].

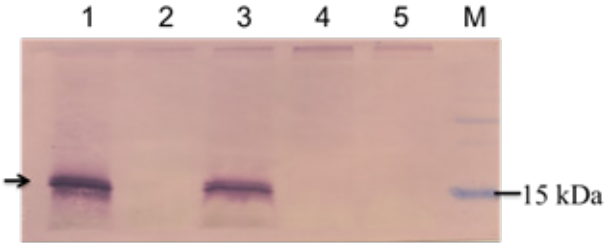

Fig. 5 Western blot analysis of transgenic tobacco plants carrying GAT gene.
Total protein extracted from leaves of transgenic lines was subjected
1: T2 progeny of line (g-5); 2: SR; 3:T1 progeny of line (g-5); 4,5: negative transgenic line; M: Protein Marker (SM0671, NEB). The interest band is 17 kDa.

Analysis of T1 and T2 transgenic tobaccos with herbicide glyphosate Seed germination selection and glyphosate tolerance spray test For seed germination selection, the T1 seeds of PCR positive lines [p35s-2301-gat- npt-II Line 1, 5, 6, 7, 8, 9, 10, 11] were sterilized and seeded on MS containing glyphosate, the images was shown in Figure 6 [a-c]. The line 5 showed high glyphosate-resistant line while the other transgenic lines and non-transgenic line were light yellow and stopped growth after germination. T2 seeds of resistant line 5 were harvest and used for germination detection, the images were shown in Figure 6 [d-f]. The glyphosate-resistant phenotype is stably inherited in T2 plant.

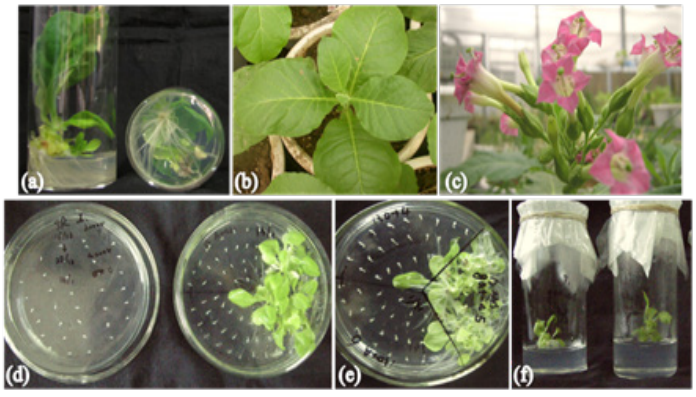

Fig. 6 Obtainment of T0 transgenic tobacco and analysis of T1 transgenic tobacco with glyphosate tolerance

(a) T0 transgenic tobacco in glass tube with root media; (b): T0 transgenic tobacco were planted in the soil; (c): flowering of T0; (d), (e): tolerance analysis; SR, the non-transgenic tobacco was left of (d); three transgenic lines were in the right of (d) and in the (e); (f): the glyphosate-resistance plants of T1 in the glass tubes.

The glyphosate spray test was carried out with seed germination at the same time. The T1 seeds of eight PCR positive lines were planted in the greenhouse to do the glyphosate spray test. Images of T1 glyphosate-tolerance were taken before spraying glyphosate and at 10 days after sprayed RoundupTM [1:250, RoundupTM: distilled water] and the transgenic line, Line 5, was uniformly growth while the non-transgenic line and other seven transgenic lines were dry to death [Figure 4 a-c]. The T2 seeds of Line 5 were harvest and planted in the greenhouse to do the spray test. Images of this line were taken before spraying glyphosate and at 30 days after sprayed RoundupTM (1:100, RoundupTM: distilled water), the glyphosate-resistant tobacco was green and the non-transgenic control was totally death [Fig. 7 d-e]. This experiment had the same results with seed germination test.

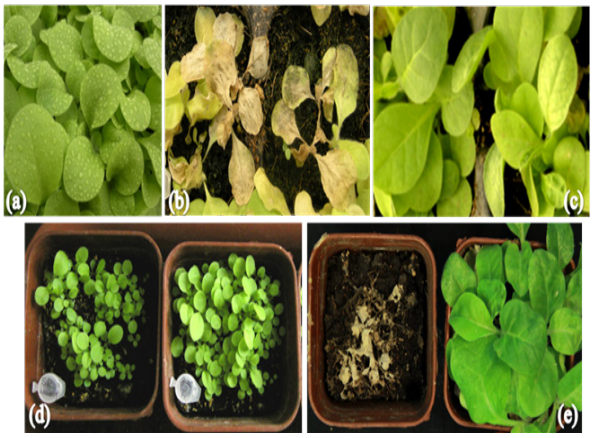

Fig. 7 Analysis of T1 and T2 transgenic tobaccos with herbicide glyphosate (a) T1 plants sprayed by glyphosate;(b) and (c) were non-transgenic and transgenic tobaccos 10 days later after sprayed the herbicide; (d) T2 plants before spraying; (e): non-transgenic tobacco (left) and transgenic tobacco (right) over one month after sprayed.

10. Discussion

Transgenic tobacco was obtained with high glyphosate tolerance in this study,The vector with interest gene, which was a selection marker gene at the same time, was carried out the Agrobacterium-mediated transformation. And the specific antiserum was made successfully and the interest protein was detected by western blot. Over the past several years, consumer and environmental groups have expressed concern about the use of marker genes from an ecological and food safety perspective. Although no scientific basis has been determined for these concerns, generating marker-free plants would certainly contribute to the public acceptance of transgenic crops. Considering that crops containing these genes with products elicit little if any harmful biological activities, particularly when compared to the environmental damage occurring due to widely used pesticides for insect and fungal control. Nevertheless there is a possibility that marker genes could be transferred to weeds or pathogenic micro-organisms in the gastrointestinal tract or soil, and endow these organisms with resistance [Nap et al., 1992; Smalla et al., 2000]. Hence it is incumbent on the research community to generate selectable marker-free transgenic plants or the the interest gene as the marker that are acceptable for use in breeding programs or commercial deployment. Considering the consumers and the environment, the marker-free transgenic crops are the necessary need under the biotechnological crops grown more and more. Recently, the effect of gat was realized gradually and was applied in transgenic crops rapidly after the high efficient gat reported [Appenzeller et al., 2008, 2009]. It was used as a selection marker too [Vikram and Koiwa, 2009], and the findings provide that Glyphosate N-acetyltransferase (GAT) can detoxify the herbicide glyphosate by N-acetylation of glyphosate [Guo,et al,2021].

11. AUTHORS' CONTRIBUTION

ZZ carried out most of the experiments and wrote the manuscript. XC prepared the antiserum, XL anticipated transplantation and detection of the regeneration tobacco, WL sponsored the vector containing the interest gene and the YW, DL, JY and CH conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Reference:

1. Appenzeller, L., Munley, S., Hoban, D., et al., 2008. Subchronic feeding study of herbicide-tolerant soybean DP-356Ø43-5 in Sprague-Dawley rats. Food and Chemical Toxicology 46, 2201-2213.,
2. Appenzeller, L., Munley, S., Hoban, D., et al., 2009. Subchronic feeding study of grain from herbicide-tolerant maize DP-Ø9814Ø-6 in Sprague-Dawley rats. Food and Chemical Toxicology 47, 2269-2280.
3. Castle, L., Siehl, D., Gorton, R., et al., 2004. Discovery and directed evolution of a glyphosate tolerance gene. Science 304, 1151-1154.
4. Dun, B., Lu, W., Ping, S., et al., 2006. Isolation of a novel glyphosate tolerance N-acetyltransferase gene and expression in E. coli. High technology letter 16, 943-947.
5. Franz, J., Sikorski, J.and Mao, M., 1997. Glyphosate: a unique global herbicide. American Chemical Society.
6. Gougler, J.and Geiger, D., 1981. Uptake and distribution of N-phosphonomethylglycine in sugar beet plants. Plant physiology 68, 668.
7. James, C., 2010. Global Status of Commercialized Biotech/GM Crops: 2010. ISAAA. Metro Manila, Philippines. ISBN 978-1-892456-49-4.
8. Murashige, T.and Skoog, F., 1962. A revised medium for rapid

Journal of Bioscience & Bioengineering

growth and bio assays with tobacco tissue cultures. Physiologia plantarum 15, 473-497.

9. Murray, M.and Thompson, W., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8, 4321-4326.
10. Nap, J.P., Bijvoet, J.and Stiekema, W.J., 1992. Biosafety of kanamycin-resistant transgenic plants. Transgenic research 1, 239-249.
11. Pline, W., Wilcut, J., Duke, S., et al., 2002. Tolerance and accumulation of shikimic acid in response to glyphosate applications in glyphosate-resistant and nonglyphosate-resistant cotton (Gossypium hirsutum L.). Journal of agricultural and food chemistry 50, 506-512.
12. Sambrook, J.and Russell, D., 2001. Molecular cloning: a laboratory manual. Cold spring harbor laboratory press.
13. Siehl, D., Castle, L., Gorton, R., et al., 2005. Evolution of a microbial acetyltransferase for modification of glyphosate: a novel tolerance strategy. Pest management science 61, 235-240.
14. Smalla, K., Heuer, H., Gotz, A., et al., 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Applied and environmental microbiology 66, 4854.
15. Steinrucken, H.and Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochemical and Biophysical Research Communications 94, 1207-1212.
16. Vikram, M.and Koiwa, H., 2009. Glyphosate resistance as a versatile selection marker for Arabidopsis transformation. Plant Molecular Biology Reporter 27, 132-138.
17. Yan, H., Chang, S., Tian, Z., et al., 2011. Novel AroA from pseudomonas putida confers tobacco plant with high tolerance to gyphosate. PloS one 6, e19732.
18. Ye, G., Hajdukiewicz, P., Broyles, D., et al., 2001. Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. The Plant Journal 25, 261-270.
19. Guo, BF., Hong, HL., Sun, LP. et al.,2021. Transcriptome analysis reveals differing response and tolerance mechanism of EPSPS and GAT genes among transgenic soybeans. Mol Biol Rep 48, 7351–7360.