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GENERATION AND GENETIC ANALYSIS OF TRANSGENIC MAIZE (*ZEA MAYS* L.) RESISTANT TO HERBICIDE GLYPHOSATE

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ABSTRACT

Two new glyphosate-resistant genes from different sources were introduced into a maize hybrid, Hi-II by Agrobacterium-mediated transformation, and the transgenic T_0 plants were identified by 2% glyphosate application and PCR detection. Their progeny inheriting stability was determined with Southern-blot hybridization and the transgene segregation ratios were recorded by glyphosate screening.Results showed that the glyphosate-resistant genes were integrated into maize genome which conferred the transgenic plants higher glyphosate tolerence. Transgenic gene in pAX1677 T_1 plants followed Mendelian segregation model while it was not the case in pAX1676 T_1 plants. Development of T_1 plants showed construct pAX1677 excelled to pAX1676.

Keywords: Maize, Herbicide tolerance, Glyphosate, Transformation, Plant growth, Genetic analysis.

Contribution/ Originality

The paper's primary contribution is that we have introduced two new glyphosate-resistant genes with two different transit peptides into maize, which conferred the transgenic plants higher glyphosate tolerance. Furthermore, the transgene of pAX1677 in T1 plants followed Mendelian segregation model while it was not the case for the pAX1676. The growth and development performance of T1 plants showed that the transgene pAX1677 excelled to pAX1676.

1. INTRODUCTION

Since 1996, glyphosate-resistant crop (GRC), either with the GR genes alone or stacked with other traits, have become the most important transgenic crops, covering about 80% of the more than 175 million hectares planted globally with transgenic crops (James, 2014).

Glyphosate [N-(phosphonomethyl) glycine] inhibits 5-enolypyruvyl-shikimate -3-phosphate synthase (EPSPS), which catalyzes a reaction in the aromatic amino acid biosynthetic pathway,

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and thereby exhibits broad spectrum herbicidal activity (Amrhein *et al*., 1980). Glyphosate has been the most widely used herbicide in the world owing to a number of characteristics in the weed management system, including nonselective post emergent weed control, crop safety, low toxicity on nontarget organisms, being rapidly broken down by soil microorganisms, flexibility of application, and relatively low cost (Franz *et al*., 1997). It has helped enable minimum/zero tillage, providing productivity and soil conservation benefits. However evolved glyphosate resistance, first was reported in *Loliumrigidum* (Powles *et al*., 1998); (Pratley *et al*., 1999), presently, there are 222 known instances of HER (evolved herbicide resistance) to glyphosate (Heap, 2014) and the recent research concludes weeds with HER are far from a random sample of weedy plant species in certain families, particularly those species with short life cycles, will rapidly evolve herbicide resistance under high selection pressure (Holt *et al*., 2013).

We know glyphosate usage have dramatically increased with the adoption of genetically modified glyphosate-resistant crops and resistance to glyphosate will evolve where glyphosate-selection pressure is high (Powles and Preston, 2006). Gene flow is another risk to evolve glyphosate-resistant weed, therefore, it is important to find more glyphosate resistant genes from varied resources so as to reduce the potential occurrence of glyphosate resistant weed due to the identical resistant gene stress.At the present study, two new glyphosate-resistant genes derived from *Brevundomonasvesicularis* and *Enterobacteriaceae*sp, respectively were used as target genes and selectable markers. Compared with antibiotic markers, a significant advantage of glyphosate as a selectable marker for maize is simple selection by just spraying the plants with an appropriate concentration of glyphosate (Howe *et al*., 2002); (Norris *et al*., 2009).Several glyphosate tolerant crops have already been successfully commercialized, including Roundup Ready® soybeans, canola, cotton, and corn.

The selection of a promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. The first chloroplast transit peptide sequence was from the acetohydroxy acid synthase, AHAS, (Mazur *et al*., 1987) which should allow import into the chloroplast. In the present study, two newly-isolated glyphosate resistant genes were fused to two chloroplast transit peptide sequences, and gene fusions were cloned behind the Tripro5 promoter in a binary expression vector pSBII constructs, respectively, and were named as pAX1676 and pAX1677. Transgenic maize plants carrying above two constructs were obtained, and their susceptibility to glyphosate was investigated with application of 2% glyphosate solution. Also, glyphosate resistance was compared between pAX1676 and pAX1677 progenies, and the inheritance stability of the transgenes was analyzed as well.

2. MATERIALS AND METHODS

2.1. Plants Material, *Agrobacterium* Strain and Plasmid Construct

Maize (*Zea mays* L.) hybrid High-II and vectors named as pAX1677 and pAX1677 were provided by Athenix Corporation, Durham, NC, USA. The plasmid pSBII contains, within the T-

DNA (transfer DNA) region, an EPSPS gene, which was derived from *Brevundomonasvesicularis* for pAX1676, the sequence named as GRG8 (SEQ ID NO:2), and from*Enterobacteriaceae* sp. for pAX1677, the sequence was named as GRGl (SEQ ID NO:6), respectively (Hammer and Hinson, 2005). They were used as both target genes and selectable markers and they were driven by different leading sequences. The EPSPS genes conferred glyphosate resistance for plant selection and were regulated by the Tripro5 promoter and terminated by the cauliflower mosaic virus35S terminator (CaMV 35S). The leading sequence derived from *Chlamydomonas*acetolactate synthase (AHAS) for pAX1676 and maize AHAS for pAX1677 encoded transit peptide that targeted the interested protein into the chloroplasts (Fig. 1).

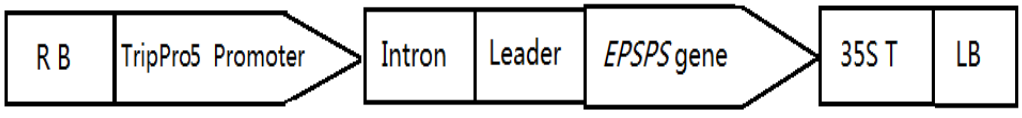

Fig-1. T-DNA region of vector pSBII. Within the T-DNA (transfer DNA) region, an EPSPS gene was both the target gene and a selectable marker. The EPSPS gene confer glyphosate resistance to plant and was regulated by Tripro5 promoter and terminated by the cauliflower mosaic virus 35S terminator (CaMV 35S) sequence

2.2. *Agrobacterium*-Mediated Transformation

Embryo preparation. Maize ears were bagged as soon as they appeared, and pollinated when their silks were in full blossom. Then the pollinated ears were collected at 10-12 days after pollination and sterilized with 20% clorox for 20 min on a rotary shaker, rinsed for 3-4 times with sterile water. Immature embryos with the size between 1.0 mm to 1.5 mm were used as explants in the transformation.

***Agrobacterium*-mediated transformationand plant regeneration**. Immature embryos were suspended in liquid inoculation medium M1 (Table 1), then *Agrobacterium* suspension were added to the medium, which was gently vortexed for 30 S and then let the embryos settled down. Five minutes later, the liquid was decanted and the embryos were inoculated into Petri dishes containing solid co-cultivation medium M2 with flat side down. The Petri dishes were placed at 22°C in dark for 2-3 days, and the embryos were transferred onto callus induction medium M3, and incubated at 28°C for 10-12 days in dark. Then the calli were transferred onto selection medium M4 and incubated for 2 weeks at 28°C in dark. Surviving embryo genic calli were transferred onto the regeneration medium M5 and incubated at 28°C in dark. Ten days later, the calli were transferred onto fresh medium and incubated at 26°C in light till the seedlings differentiated. Fully developed plantlets were transferred into rooting medium M6 and incubated at 26°C in light. Seedlings with well-developed roots were transplanted into pots and removed into the greenhouse. Ears were bagged before silking, and were either self-pollinated or backcrossed with pollen from High-II plants. Multiple seedlings generated from same callus were

considered to be clones of the same transgenic events, and were labeled with the same event designation.

Table-1. Media used in *Agrobacterium*-mediated maize transformation

Media name	Media component
M1	MS + Casamino + Dicamba
M2	MS +1mg /ml Dicamber +500 mg / l Morpholineethanesulfonic acid (MES) + 40 mg / ml Acetosyringone
M3	MS＋1mg / ml Dicamber + KH_2PO_4 +100 mg / ml Timentin +5 mg / ml $AgNO_3$＋100 mg / ml N-Z-Amine
M4	MS + KH_2PO_4＋1 mg / ml Dicamber + 100 mg / ml Timentin +5 mg / ml $AgNO_3$＋100 mg /ml N-Z-Amine ＋0.5M Glyphosate
M5	MS ＋ KH_2PO_4＋Ancymidol + Kinetin + 100 mg / ml Timentin
M6	MS＋100mg/ml Timentin

2.3. Polymerase Chain Reaction Analysis

Total DNA was extracted from leaf samples of each individual plant using the QIAGEN "Dneasy Plant Mini Kit" according to the manufacturer instructions. Two pairs of primers were designed for the EPSPS gene, which were R 5'-AGACAGCACATTCCAGTAGG-3', F 5'-CACCACGTTCATCGTCAAAG-3' for pAX1676 and R 5'-AGCTGGCGCGCCCTAAGAGAAGTTGAACTGAT-3', F 5'-AGCTGGATCCGGCATGAAGGTGACAATCCAGC-3' for pAX1677, and were synthesized by Sangon Biotechnology Company (Shanghai, China). The fragments sizes between the two primers were 1000 bp and 1200 bp, respectively. Amplification procedure was as following, 95 °C for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2m; and a final extension at 72 °C for 10 min. Reactions were carried out in a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA.). The amplified products were electrophoresed on 1% agarose gel and visualized with ethidium bromide and photographed under ultraviolet light.

2.4. Glyphosate Resistance Screening of T_1Plants

T_0 PCR positive plants were self-pollinated or backcrossed. T_1 seeds were harvested and sown in experimental plots next year, and then the putative transformed plants were preliminarily selected by spaying the plants with 2% glyphosate solution. Two weeks post-application, mortality was visually recorded, and the resistance ratio was calculated for each transgenic event.

2.5. PCR-Southern Dot Analysis

PCR-Southern dot analysis was performed on pAX1677 T_1 plants to confirm that the

introduced transgene was integrated and stably transmitted to the progenies. Total DNA from selected glyphosate tolerant plants and untransformed control were used as template for PCR amplification as previously-described, then amplification products was dripped onto a nylon membrane by a pipetter. The probe (1200 bp fragment of plasmid pSBII) was labeled by Digoxigenin (DIG) -dUTP. PCR-Southern blot was conducted using the DIG DNA Labeling and Detection Kit, following the manufacturer's instruction, and detected with disodium 3- (4-methoxyspiro (1,2-dioxetane-3,2'- (5'- chloro) tricyclo [3.3.1.1] decan) -4-yl) phenyl phosphate (CSPD) and exposed to an X-ray film. The membrane was hybridized with the labeled probe at 68℃over night. The Dig DNA Labeling and Detection Kit and CSPD were from Boehringer Mannheim Co. Ltd, Germany.

2.6. Data Analysis

X^2-test was used to analyze the difference significance of progeny segregation ratio.

3. RESULTS

3.1. Transformation Efficiency and Plant Regeneration

A total of 979 and 625 immature embryos were co-incubated with *Agrobacterium* strain harboring pAX1676 and pAX1677, respectively. The summary of transformation results was showed as Table 2.

Table-2. Summary of transformation results

construct	No. of embryos	No. of calli	No. of resistant calli (%)	No. of transgenic events (%)	No. of seedlings (%)
pAX1676	979	979 (100)	161 (16.4)	15 (1.53)	25 (2.55)
pAX1677	625	625 (100)	122 (19.5)	7 (1.12)	20 (3.20)

*Numbers in parentheses indicate percentages relative to total embryos.

3.2. PCR Detection of T_0 Plant

PCR analysis showed amplifications products of 1000bp and 1200bp bands corresponding to the constructs pAX1676 and pAX1677, respectively. The results demonstrated all plants obtained were positive, implying the glyphosate resistant gene was integrated in T_0 maize plant genome. Some representatives of the PCR-positive transgenic plants were showed in Fig. 2.

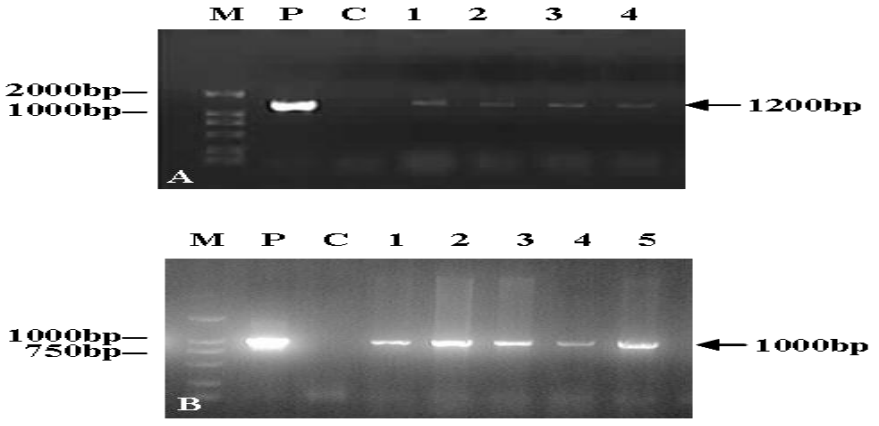

Figure-2.

Fig-2.PCR amplification of EPSPS genes.Samples from putative transgenic T_0 plants of pAX1677 (A) and pAX1676 (B), respectively.Lane M, DNA molecular weight marker (DL2000); Lane P, positive control (pSBII);Lane C, DNA from untransformed plant (control); Lanes 1– 4, DNA from transgenic plants showing amplification of EPSPS fragments

3.3. Glyphosate Resistance Screening of T_1Generation

All T_1 plants of pAX1676 and pAX1677 were spayed with 2% glyphosate solution in the next generation. It was observed that 62.38% of pAX1676 backcrossed T_1 plants were tolerant to glyphosate injury, and for the pAX1677 transgenic population, 53.42% of backcrossed T_1 plants and 65.52% of self-pollinated T_1 plants showed glyphosate resistant (Table 3). And all plants survived from glyphosate application were PCR positive, indicating that glyphosate application was reliable for putative transgenic plants selection.

Table-3. Glyphosate resistance screening of T_1 generation

Population	Pollinate model	No. of plants treated with glyphosate	No. of survived plants	No. of PCR positive	Expected segregation ratio	*X2*	Accordance to Mendelian segregation model.
pAX1676 T1	backcross	101	63 (62.38)	63 (62.38)	1:1	5.7	N
pAX1677 T1	backcross	73	39 (53.42)	39 (53.42)	1:1	0.22	Y
	self- pollinated	29	19 (65.52)	19 (65.52)	3:1	0.29	Y

a X^2 test (1df, p = 0.05) was performed on the basis of observed and expected phenotypes. $X^2_{0.05}$, =3.84, $X^2 < X^2_{0.05}$, means the target gene in the progeny population followed Mendelian segregation model, vice verse.

bNumbers in parentheses indicate percentages of positive relative to total plants

The pAX1677 T_1 population derived from self-pollination of T_0 plants segregated independently for glyphosate resistance following the 3:1 segregation ratio (Table 3). And the segregation ratio was 1:1 in its backcross population. As for backcrossed pAX1676 T_1 population, the segregation ratio did not consist with the expected 1:1 Mendelian segregation ratio.

Symptoms (mainly mild bleaching leaves and slightly retardation in plant development) of glyphosate were observed about one week after the application. Overall seedlings of pAX1677 T_1

plants were much less affected than those of pAX1676 in that less leaves were injured and their plants were taller than those of pAX1676 T_1 (Figure 3). Average plant height of pAX1676 T_1 was 128.04 cm, which was significantly shorter than 158.10 cm of pAX1677 T_1 and 163.33 cm of the WT High-II ($p< 0.05$). The difference of plant heights between pAX1677 T_1 and WT High-II was not significant ($p> 0.05$).

Figure-3.

Fig-3. T_1 plants development. A: pAX1677 T_1 Plants; B: pAX1676 T_1 plants

3.4. PCR-Southern Analysis of T_1 Plants

PCR-Southern hybridization of pAX1676 and pAX1677 T_1 lines survived from glyphosate application revealed the integration of glyphosate resistant gene into their genome. Some representatives of the PCR-Southern hybridization results of transgenic plants were showed in Fig. 4.

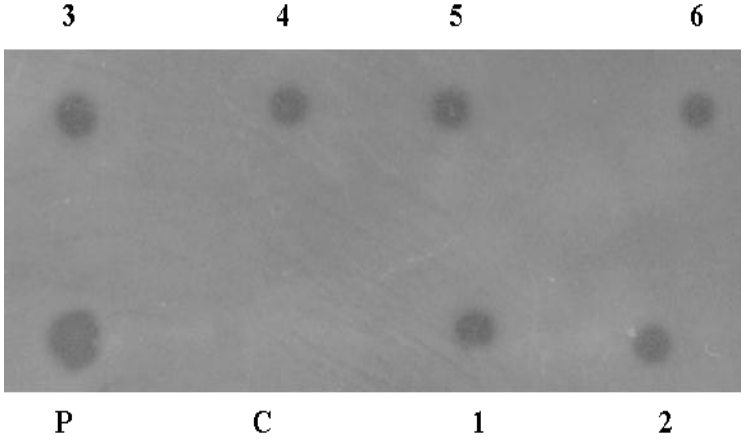

Figure-4.

Fig-4. PCR-Southern blot analysis of total DNA of pAX1677 T_1 plants. Lanes 1, 2, 3, 4, 5, 6: DNA from transgenic plants; C: DNA from untransformed control. P: DNA from plasmid pSBII

4. DISCUSSION

EPSPS is the target enzyme of nonselective herbicide glyphosate. This enzyme is located in the chloroplasts of the higher plants. Two different sequences encode maize or chalmy AHAS

transit peptide were fused to the EPSPS gene for directing the gene expression in chloroplast. In our research, untransformed control plants turned brown and permanently wilted after 0.2% glyphosate application, whereas a portion of transgenic seedlings recovered from 2% glyphosate injury within a week and those of pAX1677 T_1 showed higher tolerance. The does was expected to inhibit growth of resistant weeds as waterhemp (*Amaranthustuberculatus*) (Nandula *et al*., 2013) whose resistance presented globally (Heap, 2014).

We observed some symptoms from glyphosate application on transgenic plants. Yellow stripe appeared in both pAX1676 and pAX1677 T_0 generations, which might be caused the by insertion gene that hindered the chlorophyll synthesis. The development of both transgenic lines was inhibited where the average plant height was 138cm and 152cm, respectively, which was significantly lower than untransformed control of 180cm. And their generative growth delayed as well as their pollen amount was reduced, that we had to use the wild type High-II plants as pollen donators to get seeds for some T_0 plants. Besides, abnormal development was observed, e.g., multi-ears appeared in same husks of 3.5% pAX1676 T_0 plants. The above responses might be contributed to insertion occurred into a functional gene. Compared with pAX1676 T_0 and T_1 generations, less negative effects showed in pAX1677 progenies. We know that each transgenic event randomly generated is a unique one, regardless what vector is used. At the present study, the two target genes were from different sources, so their sequences differed, and, they also have different leading sequences that might or might not affect their expression and performance. Observing and analyzing transgenic progenies of pAX1676 and pAX1677, we found that the performance of pAX1677 progenies generally excelled to those of pAX1676, e.g., in plant height. Besides, the EPSPS gene in pAX1677 T_1 plants followed Mendelian segregation model as well. We conclude that the construct pAX1677 was better than pAX1676 and may be used as a new glyghosate-resistance source and some of progenies harbouringpAX1677 might have commercial value after undergoing biosafety evaluation.

5. ACKNOWLEDGEMENTS

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