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Developing insect resistance with fusion gene transformation of chitinase and scorpion toxin gene in maize (*Zea mays* L)

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Abstract

Transgenic plants with introduced pest-resistant genes provide an efficient alternative insect control. A binary insect-resistant gene combination, containing an insect-specific chitinase gene (*chi*) and a scorpion insect toxin gene (*Bmk*), was introduced into a maize cultivar via pollen-mediated transformation. Thirty-eight putative transgenic plantlets with kanamycin-resistance were obtained. Transgenic statuses of plants were confirmed by Southern blot analysis. Bioassay by inoculation of Asian corn borer (*Ostrinia furnacalis* Guenée; ACB) larvae indicated that the degree of ACB resistance varied among the transgenic plants. The highest average calibrated mortality of larvae was approximately 67%. The genetic analysis of T1 progeny confirmed that the inheritance of introduced genes followed the Mendelian's rules.

Keywords: maize, insect-resistant gene combination, chitinase gene, scorpion insect toxin gene, transgenic plant, calibrated mortality of larvae

Abbreviations: ACB, Asian corn borer (*Ostrinia furnacalis* Guenée); Bt, *Bacillus thuringiensis*; DBM, diamondback moth (*Plutella maculipenis*); GM, genetically modified

Introduction

Maize (*Zea mays* L) is one of the important staple crops in the world. Demand for maize is increasing across the world, predominantly in Asia. However, maize yield and quality are severely compromised by pests. Asian corn borer (*Ostrinia furnacalis* Guenée) is among the most serious pests affecting maize production. ACB causes more than 10% yield loss, with significant negative economic impact each year worldwide (He et al, 2003). ACB belongs to the Pyrralidae family in Lepidoptera order, and its larvae feed on the above-ground tissues of corn plants. They also bore into maize tassel, ear shank, and stalk, thus forming cavities that hinder the translocation of water and nutrients, weaken the strength of the stalk and ear shank, and predispose corn plants to stalk breakage and ear drop.

Traditional maize pest control depends mainly on application of chemical insecticides and breeding of insect resistant varieties. However, high cost, environmental pollution, and health hazards to farmers make the application of chemical insecticides undesirable for effective insect control. Breeding efforts for insect resistance through conventional breeding methods are constrained due to the narrow insect resistance gene resources.

Transgenic plant techniques offer an effective alternative to develop insect resistant crop varieties. The insecticidal properties of the soil bacterium *Bacillus thuringiensis* (Bt) have long been recognized and applied as a biological insecticide for decades. Bt crystal protein (or δ-endotoxin) are proven effective and widely used in controlling insect larvae infestation in many important crops, including maize (Koziel et al, 1993; Du et al, 2013), potato (Perlak et al, 1993), rice (Fujimoto et al, 1993; Cheng et al,1998) and cotton (Perlak et al, 1990). Genetically modified Bt maize, expressing genes encoding the Bt Cry proteins, have been widely produced for controlling pest Lepidoptera. Since the first report of insect-resistant transgenic maize with *cry1Ab* gene (Koziel et al, 1993), GM Bt maize have been grown in many countries (Szabala et al, 2014). In addition, many *Cry* genes have been used in GM Bt maize for pest control (Du et al, 2013). GM Bt maize varieties play an important role in maize production. Nevertheless, GM maize, solely expressing Bt genes, can be narrow in insect-resistant spectrum and at risk of developing insect resistance to the Bt proteins (McGaughey and Whalon, 1992; van den Berg et al, 2013).

Busseola fusca (Fuller; Lepidoptera: Noctuidae) is an important pest of maize. Previous study indicate that *B. fusca* have developed resistance to Bt toxins due to a mutation in the midgut receptors, that leads to the disruption of Bt toxin binding to the receptors, a common mechanism of insect resistance (Ferré, 2002). van Rensburg reports the resistance of *B. fusca* to Bt maize, with field collected larvae in the

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2005/06 crop growing season (van Rensburg, 2007). Laboratory studies showed a considerable number of F1 generation of diapause larvae survived on Bt maize in South Africa. Within one year of the first official reported pest resistance, other cases of control failure were observed by farmers in South Africa (Kruger et al, 2011). Therefore, novel insect resistance genes are of agriculture interest.

The insect-specific neurotoxin, BmkIT from the venom of the scorpion (*Buthus martensii* Karsch), causes contractive paralysis of insectts. The *BmkIT* gene encods a 69 amino acid protein that selectively and specifically bind to the Na ion channels in the insect cell membranes. The fast inactivation of the Na channels induces rapid paralysis and eventually death of the insects (Liang et al, 1999). BmkIT is thus toxic to many lepidoptera insects.

Chitin is a major component of cuticle and gut epidermis of lepidoptera insects. Chitinases (EC 3.2.1.14) are enzymes with a specific chitin hydrolytic activity. The constitutive expression of a chitinase gene in plants reduces insect damage (Kramer and Muthukrishnan, 1997). In this study, a cDNA encoding the major molting fluid chitinase of the tobacco hornworm, *Manduca sexta*, was used in combination with the *Bmk* gene in transgenic plants to effectively prevent insect damage (Wang et al, 2005). The chitinase (chi) and Bmk binary was introduced into maize by a simple yet effective pollen-mediated transformation method. Bioassays were used to demonstrate that some transgenic plants exhibit high resistance against ACB larvae infestation.

Materials and Methods

Materials

Maize (*Zea mays* L) inbred lines 478 , kindly provided by the Crop Science Research Institute, Shanxi Academy of Agricultural Sciences, China, was used as receptor.

Agribacterium tumefaciens strain LBA4404 harboring binary vector pBI101-Bmk-chi was used in the experiments. The plasmid pBI101-Bmk-chi contains, within T-DNA region, a neomycin phosphotranferase II (NPTII) gene as the kanamycin-resistant selectable marker, the *chi* gene, and the *Bmk* gene. The *NPTII* gene is regulated by the nopaline synthase promoter and terminator, the *chi* gene is regulated by the Cauliflower mosaic virus 35S promoter (CaMV35S) and terminated by the polyadenylation sequence, and the *Bmk* gene is regulated by two tandem-linked CaMV35S promoters and terminated by the nopaline synthase terminator (Figure 1). The binary vector pBI101-Bmk-chi was constructed by Zhang et al (2004).

The plasmid DNA was purified from *E. coli* using the PCR Fragment Recovery Kit purchased from TaKaRa Biotech (Dalian, China).

Genetic transformation method

The genetic transformation was enabled by the pollen mediated transformation method (Wang et al, 2001). Maize were planted in experimental plots in late April in Taiyuan, China, and their florescence emerged in mid July. Maize ears were bagged before silking. Approximately 0.3 g of fresh pollens were collected in the morning, and mixed with 5-10 μg of the plasmid DNA in 20 ml of solution with 0.2 M sucrose. The mixture was treated with ultrasonication using a JY92-II ultrasonicator (from Ningbo Xinzi Scientific Instrument Institute). The parameters for sonication treatment were: 300 W sonic intensity, 5 treatments each with 10 s interval. Subsequently, the treated pollens were used to pollinate clipped maize silks. The maize ears pollinated with DNA-treated pollens were bagged again until they reach maturity.

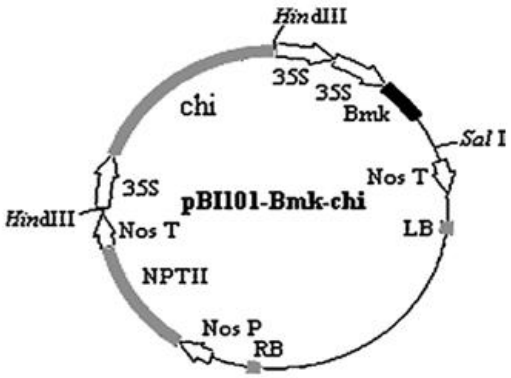

Figure 1 - Schematic map of vector pBI101-Bmk-chi.

Southern blot analysis

Genomic DNA were extracted from fresh leaves of the putative transgenic plants and wild-type plants using the cetyl trimethyl ammonium bromide (CTAB) method (Allen et al, 2006). The genomic DNA were subject to PCR amplification using following primers designed according to the sequences of the *chi* gene:
forward
5'-GAATGGGCCTCGCCGACACACC-3'
reverse
5'-GCCGGTACCTTAGGGTTGTTGACATTC-3'
and *Bmk* genes:
forward
5'-GCCCCCGGGATGAAATTTTTCCTTATATTT-3'
reverse
5'-GCCGTCGACTTAACCAATTATTTGGAC-3'

Genomic DNA from the PCR-positive and wild-type plants were analyzed by Southern blot analysis. For each sample, 10 μg genomic DNA was digested with *Hind*III. The digested genomic DNAs were fractioned on 0.8% agarose gels, then transferred onto a Hybond TM N^+ membrane (Hybond N^+, Amersham). The membrane was hybridized with Dig-dUTP labelled Bmk probe at 42°C overnight. To generate the Bmk probe, the 0.28 kb *Bmk* gene fragment from the plasmid pBI101-Bmk-chi was amplified and labeled using the PCR DIG probe synthesis kit following the

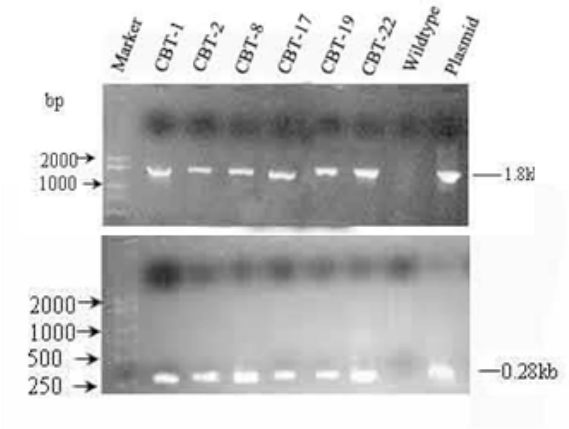

Figure 2 - The result of PCR amplification of transgenic treated plants and wildtype plant for detected alien genes with special primers. Samples CBT-1, CBT-2, CBT-8, CBT-17, CBT-19, and CBT-22 were transgenic treated plants.
Upper pannel: The detected gene was *chi*. Lower pannel: The detected gene was *Bmk*.

manufacturer's instruction (Roche Co, Ltd).

After hybridization the membrane was developed with disodium 3-(5'-chloro-4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo[3.3.1.13,7]decan]-4-yl) phenyl phosphata (CSPD) florescence stain and exposed to X-ray film.

Insect resistance bioassay

All transgenic maize plants were inoculated with ACB larvae in an *in vitro* leaf-feeding assay. The leaves of tested plants were inoculated with five 2nd instar larvae and scored by leaf damage (visual-estimation) and larval mortality after 7days.

The total insect weight was obtained by weighing live larvae at 7th day after inoculation. The test was replicated 3 times for each plant. Live ACB larvae were provided by the Crop Protection Institute of Chinese Academy of Agricultural Sciences, Beijing, China.

Table 1 - Pest resistance analysis of transgenic plants.

Transgenic plants	Calibrated mortality of larvae (%)	Increased insect weight (mg) alive pests	Scale of pest damage in leaves
CBT-1	57.14	12.8 ± 4.651	1
CBT-2	67.29	19.493 ± 4.684	1
CBT-5	42.85	20.697 ± 7.013	3
CBT-8	35.71	21.817 ± 8.399	3
CBT-10	49.99	22.287 ± 3.631	2
CBT-11	49.99	13.297 ± 8.718	2
CBT-13	49.9	13.067 ± 10.81	2
CBT-14	28.56	17.443 ± 5.953	4
CBT-15	49.99	16.24 ± 2.576	2
CBT-16	35.71	17.783 ± 2.67	3
CBT-17	42.86	21.056 ± 2.043	3
CBT-18	49.99	11.917 ± 10.958	2
CBT-19	35.71	21.495 ± 7.177	3
CBT-22	57.14	23.033 ± 2.615	1
CBT-23	49.99	19.12 ± 4.708	2
CBT-24	49.99	14.887 ± 4.812	2
CBT-25	49.99	17.707 ± 7.73	2
CBT-26	49.99	20.91 ± 7.397	3
CBT-27	42.86	15.02 ± 12.844	3
CBT-29	35.71	44.73 ± 24.154	3
Wildtype	0	68.79 ± 2.7	4

The following formulas were used in the bioassay analysis:
mortality = (dead larvae) (total inoculated larvae)$^{-1}$ ×100%; calibrated mortality = (mortality of transgenic plant - mortality of wild type plant) (1–mortality of wild type plant)$^{-1}$ ×100%

Leaf damages were scored using five grades: 0, only a few tiny holes on leaves; 1, unconnected small holes on leaves; 2, connected small holes, but leaves were intact; 3, bigger holes, leaves were almost intact with large amount of leaf residuum; 4, only leaf vein and little leaf residuum left.

Results

Seed setting of transgenic treatmented ears

According to the method of genetic transformation method above, the 69 out of 231 pollinated pollinated maize ears produced seeds. A total of 221 seedlings were recovered from 266 seeds, a germination rate of 83%.

Nucleic acid analysis of the transgenic treatmented ears

Total DNA, extracted from leaves of the 221 transgenic treatmented seedlings and wild-type seedlings, were subjected to PCR amplification using primers specific for the Bmk and chi gene sequences. A total of 47 PCR-positive seedlings were detected to have both *Bmk* and *chi* genes (Figure 2).

Ten PCR-positive samples were randomly selected and further analyzed by Southern blot hybridization. Nine out of the ten seedlings showed strong and clear positive *Bmk* gene signal (data not shown), indicating the integration of Bmk gene into the maize genome. Among the positive samples, CBT-29 revealed three discrete bands; CBT-22, CBT-17, and CBT-8 revealed two discrete bands; and CBT-19, CBT-13, CBT-4, CBT-2, CBT-1 generated only one band. CBT-3 and wild type showed no distinctbands.

Insect resistance bioassay of the transgenic plants

Thirty ears were harvested from T0 transgenic lines, and seeds were germinated to produce T1 plants. Young leaves from 20 randomly selected T1 and wild-type plants were subjected to insect resistance bioassay by placing the 2nd instar ACB larvae onto the detached leaves. We observed that all of the transgenic plants showed ACB resistance to various degrees. The insect resistance of tested plants was scored according to leaf damage and larvae mortality after 7th day. Three out of the 20 tested transgenic plants were grade 1 with high resistance; eight plants showed moderate grade 2 resistance; eight plants showed grade 3 resistance; and one plant was as susceptible as the wild-type. The insecticidal activity assay results were shown in Table 1 and Figure 3. The transgenic plants with high resistance suffered

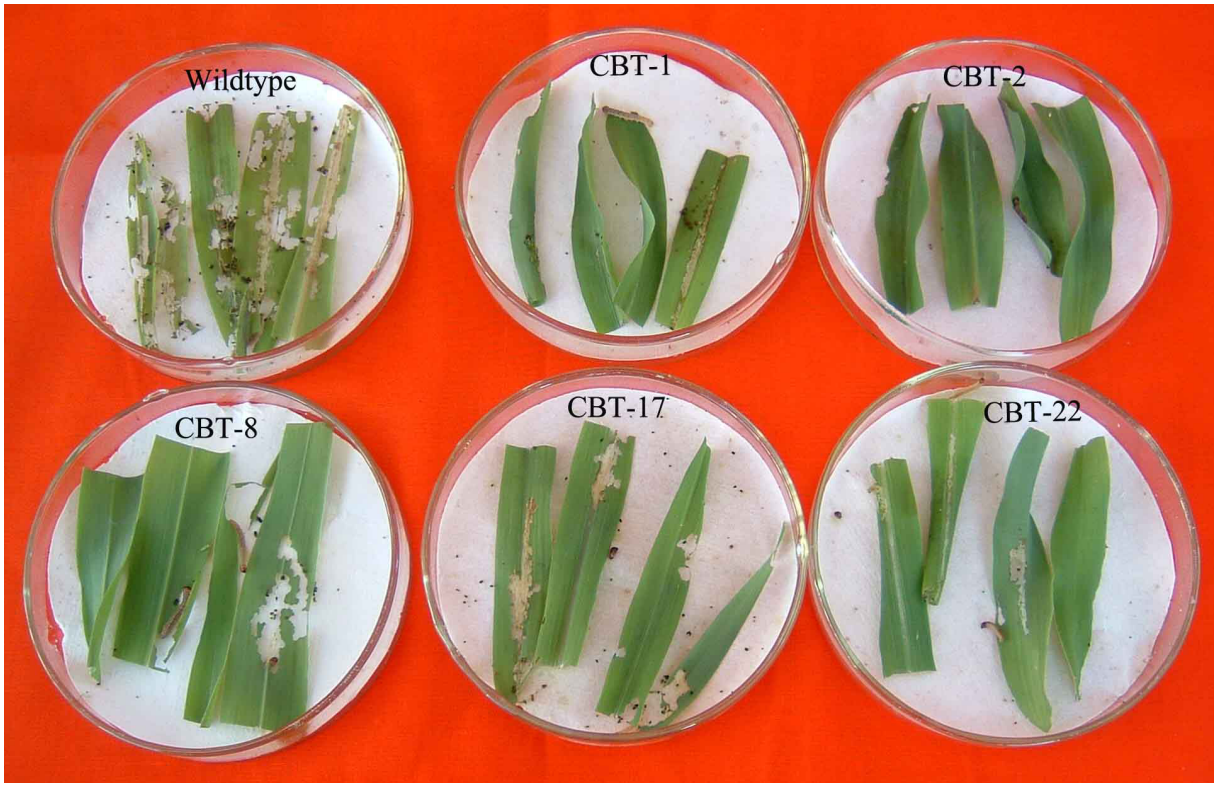

Figure 3 - The result of insecticidal activity bioassay of transgenic T1 plants against *Ostrinia furnacalis* Guenée larvae infestation. The picture was taken at the 5th day after inoculation.

very little feeding damage and resulted in high larvae mortality, and those with moderate resistance suffered a little feeding damage and resulted in a moderate larvae mortality. Most of the tested plants showed moderate to low ACB resistance. The highest average calibrated mortality of larvae was 67.3%. Taken together, above results indicate that the binary insect resistant genes were efficiently transformed into maize by the pollen-mediated approach, and the expression of the transgenes conferred the ACB resistance.

The heredity assay of the T1 progeny

The heredity analysis of T1 progeny were performed by PCR amplification with *Bmk* gene specific primers using total DNA from young leaves. The segregation ratio of PCR-positive and PCR-negative plants agreed with the Mendelian's rules (Table 2).

Discussion

Bt genes encode insecticidal-endotoxins that are widely-used for the development of insect-resistant crops including maize. Bt maize containing the *Cry1* transgene, most effective against lepidoptera larvae, was initially released for commercial production in the USA in 1996 (Carpenter et al, 2010). Since then, various Bt maize hybrids have been developed that contain transgenes of either the *Cry1* or *Cry2* family, all targeting lepidopterous pests. Bt maize expressing *Cry* genes are currently planted in many countries. Evolution of resistance is a primary threat to the continuing success of Bt maize. Field resistance is a genetically-based decrease in susceptibility of a population to a toxin caused by field exposure (Tabashnik, 1994). Continuous use of transgenic maize producing the same insecticidal Bt toxin increases selection pressure and consequently the risk of evolved resistance to Bt proteins (van Rensburg, 2007). Simultaneously introducing more than one insect-resistant gene with different modes of action into plants could slow down or minimize the risk of developing resistance by insects.

Ding et al (1998) introduced the *chi* gene from *Manduca sexta* into tobacco, and showed resistance against *Heliothis virescens* larvae infestation (Ding et al, 1998). In a previous study, we introduced the combination of *Bmk* and *chi* genes into a rapeseed cultivar using Agrobacterium-mediated transformation. The results showed that high-level expression of both Bmk and chi genes enhanced the resistance of transgenic plants against diamondback moth (*Plutella maculipenis*) larvae infestation (Wang et al, 2005). In this study, we introduced the binary insect-resistant gene combination into maize using pollen-mediated transformation, and obtained transgenic plants that are highly resistant to ACB larvae infestation. To the best of our knowledge, this is the first report of generation of highly ACB larvae resistant maize through

Table 2 - Segregation of the Bmk gene in T1 transgenic plants.

Plants	amplification results		N° of plants		χ^2	p value
	P$	N	obtained	expected		
CBT-1	24	7	3.43 : 1	3 : 1	0.0110	< 0.01
CBT-2	21	9	2.33 : 1	3 : 1	0.2111	< 0.01
CBT-4	25	7	3.57 : 1	3 : 1	0.0417	< 0.01
CBT-8	35	2	17.5 : 1	15 : 1	0.0162	< 0.01
CBT-22	33	2	16.5 : 1	15 : 1	0.0476	< 0.01

$P specific amplication product present, N specific amplification product absent

introduction of such gene combination. Our results provide a new approach to create insect resistance maize without the use of *Bt* genes. This approach broadens the insecticidal spectrum of GM crops and reduces the risk of insects developing resistance resulted from the use of single insect toxin gene.

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