ACQUIREMENT OF TRANSGENIC COTTON (*GOSSYPIUM HIRSUTUM* L.) RESISTANT TO HERBICIDE AND INSECT USING GLYPHOSATE-TOLERANT *aroAM*12 GENE AS A SELECTABLE MARKER

XIE LONGXU[1], LI FEN[1], CHEN MINGXIA[1], LU LONGDOU[1]*

Keywords: transgenic cotton, glyphosate, Bt, selectable marker

Abstract: A new binary vector, pAM12-S1m, harboring the *aroAM*12 gene encoding for 5-enolpyruvyl- shikimate-3-phosphate synthase (EPSPS) and a synthetic recombinant *BtS1m* toxin gene consisting of 331 N-terminal amino acids of CryIAc and 284 C-terminal amino acids of CryIAb has been constructed. The truncated *aroAM*12 gene, which was obtained through gene shuffling technology, was ligated with a transit sequence of *Arabidopsis* EPSPS and expressed in cotton plants driven by cauliflower mosaic virus 35S (CaMV35S) promoter. The chimeric *BtS1m* toxin gene was fused with DNA sequence encoding PR1b secretary signal peptide and expressed under the control of 2E-35S promoter and “Ω” translation enhancer sequence derived from tobacco mosaic virus. The mutant EPSPS of *aroAM*12 gene product conferring highly resistant to glyphosate, the active ingredient in herbicide Roundup®, was used as a dominant selectable marker for cotton plant transformation. The genes were introduced into commercial cultivar Zhongmian12 of cotton (*Gossypium hirsutum* L.) by *Agrobacterium*-mediated transformation. The transformants were directly selected on medium supplemented with 80μmol/L glyphosate. In this research, 40 regenerative cotton plantlets were obtained through screening. Integration of *aroAM*12 and *Bts1m* genes was confirmed by PCR and Southern blot, the results indicated that all the 40 plants possessed the *aroAM*12 gene, 28 of which possessed both the *aroAM*12 and *BtS1m* genes. Expression of both the genes was established by Western blots. Insect bioassay and glyphosate resistance assay indicated that the transgenic cotton plants obtained were highly resistant to glyphosate and insect. The results of glyphosate resistance and insect bioassay of T_1 generation showed that the numbers of resistance and sensitive phenotypes showed Mendelian segregation ratio.

INTRODUCTION

Cotton, a global crop, is a very important source of fiber, feed and edible oil. However, due to the weeds and insects damages, the yield and quality of cotton decrease seriously. So, the development of cultivars resistant to herbicide and insect is of tremendously importance to cotton production throughout the world. The tools of gene engineering technology have made it possible to develop cotton that are resistant to glyphosate and insect.

At present, several classes of herbicides are used for broad-spectrum weed control, one such herbicide is glyphosate, the active ingredient in Roundup herbicide. Glyphosate is a broad-spectrum, nonselective herbicide, it is immobilized by the soil and readily degraded by soil microorganisms, so it can’t persist in nature. On the other hand, it is also proved that glyphosate has extremely low toxicity to animals. Glyphosate specifically binds to and blocks the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS), an enzyme of the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate and phosphate. The reaction takes place in the chloroplast (Comai et al, 1985). In the past, the tactics to achieving glyphosate resistant crops were: (I) to introduce the EPSPS gene (*aroA*) which has an overexpression in the plants to compete the inhibition of glyphosate ; (II) to carry out site-directed mutagenesis to obtain none or decreased affinity for glyphosate. The research of glyphosate-tolerant crops has been pursued since the 1980’s. So far, there are variety of glyphosate resistant crops appeared (Della-Cioppa et al, 1986; Padgette, et a l., 1995; Debbi et al.,1996; Marie Mannerlôf, et a l., 1997). In our research, the glyphosate- resistant gene, *aroAM*12, which is obtained through the gene shuffling technology (He et al., 2002), was transferred into cotton.

In addition, another important trait concerning cotton, the insect –resistance, has been extensively carried out. Transgenic cotton producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used for insect control. (Firozabady et al., 1989; EL-Hiatemy et a l ., 1990; Debbie et al., 1996) .The *Bt* gene we introduced is *BtS1m*, which is a chimeric gene with its production consisting of 331 N-terminal amino acids of CryIAc and 284 C-terminal amino acids of CryIAb.

The objective of this research was to reconstruct a high efficient binary plant expression vector harboring the *aroAM*12 and *BtS1m* genes that confer resistance to glyphosate and insect, respectively. The genes were transferred to

commercial cultivar Zhongmian12 utilizing *Agrobacterium*-mediated transformation method, and as we know, it is the first report to use *aroAM*12 as a selective marker gene to screen off the transformants of cotton plants.

MATERIALS AND METHODS

Plasmid constructs

A 1.3 kb DNA fragment carrying the *aroAM*12 gene was obtained by gene shuffling technology. Another 0.22 kb DNA fragment, ASP (*Arabidopsis* signal peptide), was attached to *aroAM*12. This recombinant fragment was cloned into a plant expression vector, pCAMBIA1300, that included a cauliflower mosaic viral 35S promoter (CaMV35S), Nos (polyA) addition signal. The coding region of *aroAM*12 gene is flanked by a MCS (multiclone site) at the 5′end and a *Xho*| site at the 3′end. The above cloning resulted in the plasmid pGAT1300 .The plasmid pBtS1m harboring the *BtS1m* gene was received from Tian Y.C, Institute of Microbiology, CASB, China. The chimeric *BtS1m* gene were inserted between promoter of the 2E-35S(CaMV35S promoter supplemented with four repeats of the enhancer domain to stimulate the transcription of *BtS1m* gene in plants and nopaline synthase (Nos) terminator sequences. The 5′end of *Bts1m* gene was fused to the untransformed Ω leader sequence of tobacco mosaic virus to enhance the translation of mRNAs. The coding region of *BtS1m* cassette is flanked by a *Hind*III site at the 5′end and a *Xba*I site at the 3′end. Both the pGAT1300 and *pBts1m* plasmid DNA were digested with *Hind*III and *Xba*I. A plant expression vector, pCM12-S1m, was constructed by assembling the *aroAM*12 and *BtS1m* cassette (Fig.1).

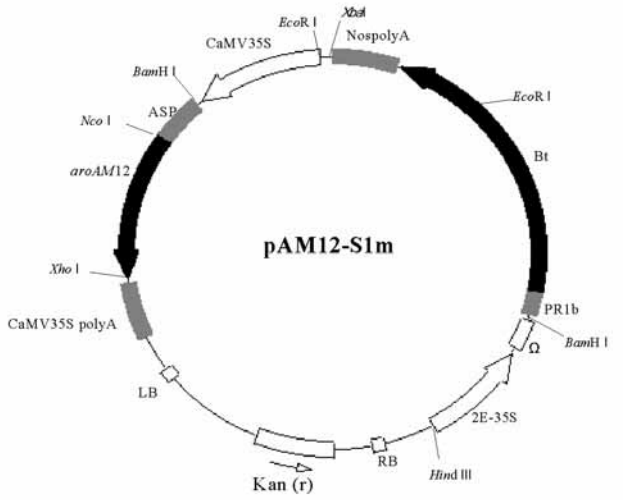

Fig.1. Construction of a high efficient expression binary vector pCM12-S1m with *aroAM*12 and *BtS1m* genes

Bacterial strain and vector

Agrobacterium strain LBA4404 carrying the binary plasmid pCM12-S1m, with *aroAM*12 as a selective marker, was used as the vector system for cotton transformation and regeneration experiments.

Plant materials

Seeds of *Gossypium hirsutum* L. cv. Zhongmian12 were obtained from the Cotton Research Institute, CAAS, and Anyang, Henan Province.

Seed germination

Seeds were sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 5 minutes, rinsed subsequently with sterilized deionized water at least 4 times. The seeds were germinated aseptically on MS medium at 28°C.The pH of the medium was adjusted to 5.8~6.2 prior to autoclaving at 121°C for 20 min.

Transformation and cotton regeneration

Hypocotyl sections (0.3-0.5cm) taken from 5-day-old seedlings were directly used to be infected with LBA4404 culture (A600 0.4-0.6). The hypocotyls were immersed in the bacterial suspension for approximately 7 min and

subsequently blotted dry on the sterile filter paper. Then they were co-cultivated on MS_0 media supplemented with 0.1mg/L 2,4-D, 0.1mg/L KT and 200mg/L Acetosyringone (pH 5.0 ,solidified with 0.2% Gelrite) under dark conditions for 48 hours at 28±2°C. After co-culture, the hypocotyls were rinsed thoroughly, blotted dry, then, transferred to MS_1 medium (same as MS_0, except that acetosyringone was taken out, pH 5.8-6.2) containing 400mg/L cefotaxime and 0.91g/L $MgCl_2$ and incubated at 28±2°C, under a 16 hours photoperiod. After 7 days cultivation, the explants were transferred to MS_2 medium (same as MS_1, except that glyphosate was added) containing 80μM glyphosate to induce the callus formation. After two subcultures on MS_2, the glyphosate-resistant calli were transferred to the proliferation medium (MS_0 +0.91g/L $MgCl_2$). Again after several subcultures, the calli were transferred to MSB medium (MS saults,B5-organics, 1g/L asparagines, 2g/L glutamine, 3% glucose, pH 5.8-6.2,solidified with 0.25% Gelrite), cultured on this medium under a 16 hours photoperiod at 28±2°C, with monthly subculture until somatic embryos appeared, germinated, rooted. , then the individual plantlets were transferred to soil and kept under high humidity for two weeks. At last, they were transferred to large pot with fertile soil and grown to maturity in the greenhouse.

PCR Analysis

Total DNA was extracted from the leaves of cotton plants utilizing cetyl-trimethyl ammonium bromide (CTAB) method as described in ref [9]. Transgenic plantlets were checked by PCR with *aroAM*12 specific primers (5′-CATGCCATGG AATCCCTG ACG TTA CA A-3′forward, and 5′-CGCGGATCCTTAGCAGGCTACTCATTC-3′,reverse) and *BtS1m* specific primers (5′-TATCCCATTGTTCGCAGTCC-3′forward, and 5′-CAT CACGACTCAAGTTGTTA-3′,reverse). PCR reaction was performed in 20μL volume under the following conditions: 31 cycles of 94°C for 1 min (denaturation), 54°C for 1.5 min (annealing), and 72°C for 1 min (extension). Cycles were preceded by denaturation for 5 min at 94°C and a final extension at 72°C for 10 min. PCR products were electrophoresed on 0.8% agarose gel.

Southern blot Analysis

The procedures for Southern blot hybridization analysis were according to Sambrook et al (1989). Total genomic DNA from transgenic and untransformed plants were used for Southern blot, 15μL of DNA per sample was thoroughly digested with EcoRI,separated on 0.8% agarose gel, then transferred to a nylon membrane (HybondTM-N+,Amersham) by capillary action. The blot was prehybridized and subsequently hybridized with *aroAM*12 gene probe and *BtS1m* gene probe, respectively. Labeling of the probe with ^{32}P was done by Hexal Label PlusTM DNA Labeling kit (MBI Fermentas, USA) and purified with CENTRI.SPIN Columns (PRINCETON). Following hybridization , the membranes were washed properly, then exposed 4 days to Kodak X-film at -70°C for autoradiography.

Western bot Analysis

Approximately 200 mg fresh leaves of cotton plants were quickly ground to a fine powder in liquid nitrogen. The powder was homogenized in 200μL extraction buffer (100m mol/L Tris-HCl, pH 8.0 ,5mmol/L MgCl2, 2% SDS,10% glycerol, 5% β-mercaptoethanol), boiled for 10min. The sample was centrifuged and the supernatant collected. Protein concentrations were determined by using the Bradford assay, with BSA as the protein standard. 10% SDS-PAGE was performed to separate the proteins, subsequently transferred to HybondTM-P PVDF membrane (Amershan) by using the Seminphor transphor unit (Amershan). After transferring, the membrane was incubated with antibodies against the 48 kDa EPSPS (1: 250) and the polyclonal antibodies against the 68 kDa CryAc protein (1: 300), respectively. The western blot was performed following the Western Blot Kit BCIP/NBT System (Kirkegaard & Perry Laboratories, USA).

Herbicide resistance assays

Transgenic and untransformed plantlets were treated with 0.5% Roundup® (370g/L), once every 7 days, when the plants were approximately 40cm tall, and scored for phytotoxic effects after three weeks.

Insect Bioassays

The leaves with leafstalk of young seedling plants of transgenic lines and non-transgenic cultivars (negative control) were picked at the same seedling ages. They were placed on moist paper in Petri dishes with tight-fitting lids. Five second-instar larvae of *H.assulta* were distributed in each leaf. After three days in culture, the leaf damage index and the number of surviving larvae were recorded. Treatment was replicated 4 times.

Herbicide resistance and Insect Bioassays in T_1 generation

Selfed seeds collected from T0 transgenic plants were germinated, and T_1 progenies were subjected to the Roundup® test and insect bioassays. Untransformed Zhongmian12 were used as comparable controls in both assays. The plants which have lateral root growth (growing on MS media containing 25 μmol/L glyphosate) were scored as resistant, while the plants, which have no appearance of lateral root, were recorded as susceptible.

RESULTS AND DISCUSSIONS

Transformation of cotton

Cotton hypocotyls explants were transformed with *Agrobacterium* strain LBA4404 harboring the binary plasmid pCM12-S1m containing the *aroAM*12 and *BtS1m* genes. The regenerated putative plantlets were screened by PCR with primers against the *aroAM*12 and *BtS1m* genes, respectively, for the presence of the T-DNA inserts. The results were described as in Table 1.

Table1 Transformation frequency calculated as obtained transgenic plantlet/total number of explants

Cultivar	Explants	Transgenic plantlets*	Transformation frequency (%)
Zhongmian12	1500	40	2.67

*Plant number of PCR positive with *aroAM*12 primers.

In the 40 plantlets examined, all have the predicted 1.2 kb fragment by using the *aroAM*12 specific primers, untransformed plant DNA, as expected, showed no products. Only 28 plantlets generated a predicted 0.96 kb PCR product by using the *BtS1m* specific primers, the left and untransformed ones had no PCR products. The results indicated that during the T-DNA integration to plant genome, approximately 25% *BtS1m* gene were lost. Fig.2 was the part PCR results of regenerated plantlets.

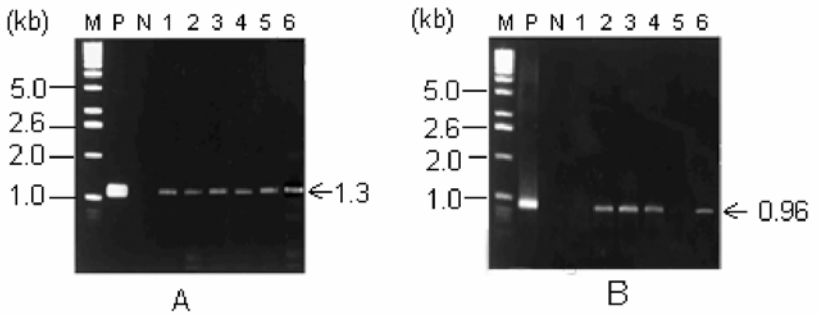

Fig. 2. PCR analysis of putative transformants and untransformed plant using two primer sets:

(A): *aroAM*12 gene specific and (B) *BtS1m* gene specific. Lane M, 1kb ladder ; lane P, pCM12-BtS1m plasmid DNA ; lane N, untransformed plant DNA ; lanes 1-6, putative transformants: M12-Bt/a、M12-Bt/ b 、M12-Bt/c M12-Bt/d 、M12-Bt/e and M12-Bt/f.

Southern blot analysis

The *aroAM*12 gene PCR positive plantlets were further analyzed with Southern hybridization to confirm the site-specific integration of T-DNA and to establish inserts number. Total DNA from each random transgenic and untransformed cottons were digested with *Eco*RI , and two blots were made from these samples. The blot shown in Fig.3B was probed with ^{32}P primer-labeled 1.3kb size *aroAM*12 DNA fragment. Single band was present in lanes 1, 2,5,6; two bands were detected in lanes 3,4. No hybridization signal could be detected for the untransformed plant DNA, as expected. According to the distribution of *Eco*RI sites on the T-DNA region and the hybridization pattern, we can confirmed that the copy number of inserts were equal to the number of bands. The number of inserts in the transformants varied from one to two copies. The blot shown in Fig.3C was probed with ^{32}P primer-labeled 0.96kb DNA fragment; two, three, three and

two bands were detected in lane 2,3,4,6, respectively. Single band of approximately 1.0 kb size fragment were detected in all the 4 lanes. As can be seen in Fig.3A, there were two *Eco*RI sites in the T-DNA region, one flanks at the 5’ of *aroAM*12 gene, the other is located inside the *Bts1m* gene. The putative transformant in lane 1, 5 and the control plant showed no hybridizing fragment.

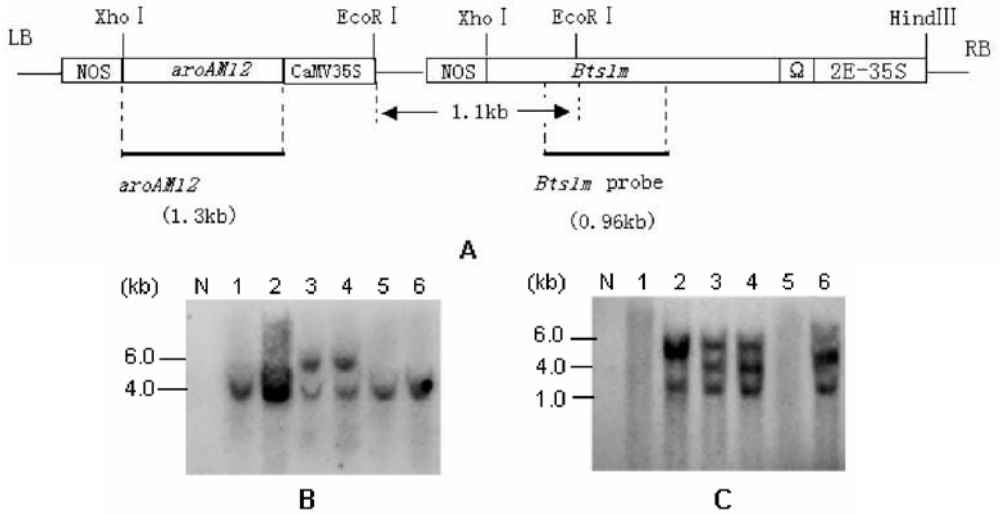

Fig.3. Southern blot analysis of DNA isolated from leaves of independent transformants and untransformed control.

A, map of the T-DNA region of the pCM12-S1m; B, Total genomic DNA of transformants was digested with *Eco*RI and hybridized with *aroAM*12 DNA fragment. LaneN: untransformed plant; Lanes1-6: M12-Bt/a、M12-Bt/b 、M12-Bt/c 、M12-Bt/d 、M12-Bt/e 、M12-Bt/f; C, Total genomic DNA of transformants was digested with *Eco*RI and hybridized with *BtS1m* DNA fragment .Lanes1-6: M12-Bt/a、M12-Bt/b 、M12-Bt/c 、M12-Bt/d 、M12-Bt/e 、M12-Bt/f; Lane7: untransformed plant.

Immunoblot Analysis

To test whether the *aroAM*12 mRNA and *BtS1m* mRNA were properly translated; total soluble protein obtained from transformed and untransformed leaves was subjected to Western blot analysis. The blot shown in Fig. 4A was probed with anti-EPSP synthase serum. In the six random selected transgenic plants, as well as the positive control (EPSP synthase protein, purified from an *E.coli* overproducing strain, in preparation), a band of 48 kDa size was detected with different band intensity. The blot shown in Fig. 4B was probed with anti-CryIAc serum. In lanes 2,3,4,6, bands of approximately 68 kDa size were detected, co-migrated with the positive control (CryIAc protein, in preparation), while the putative plantlet in lanes 1,5, as well as the untransformed plant, no visible signals were detected. This result coincided with the southern blot mentioned above.

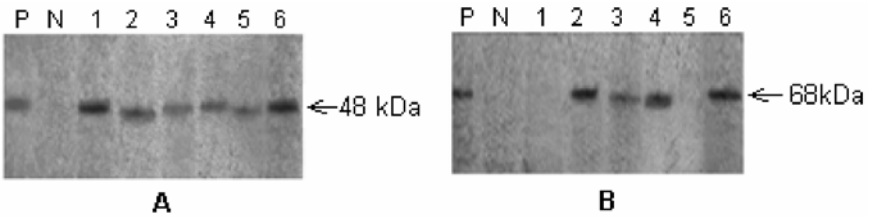

Fig.4. Western blot analysis of total proteins extracted from transformants and untransformed cotton plant.

(a) The EPSPS protein of *aroAM*12 gene product was detected using a polyclonal antibody serum, LaneP: EPSPS protein, positive control; Lane N, untransformed cotton plant; Lanes1-6: M12-Bt/a、M12-Bt/b 、M12-Bt/c 、M12-Bt/d 、M12-Bt/e 、M12-Bt/f. (b) The CryIAc protein of *BtS1m* gene product was detected using a polyclonal antibody serum. Lane P: CryIAc protein, positive control; Lane N, untransformed cotton plant; Lanes1-6: M12-Bt/a、M12-Bt/b 、M12-Bt/c 、M12-Bt/d 、M12-Bt/e 、M12-Bt/f .

Insect Bioassay of T_0 transgenic cotton

Transgenic cotton plants obtained by transformation with the pCM12-Bts1m constructs and untransformed cotton were tested for insect tolerance by feeding leaves to neonate larvae of the bollworm (*H.assult*). Larvae fed on transgenic plant leaves suffered from severe deleterious effects; surviving insects suffered reduction in body weight. Larvae fed on untransformed leaves, however, grew well. The detail results were shown in Fig.5 and Table2

Fig.5. Insect bioassays.

The leaves of transgenic and untransformed plantlets were infested with H. assulta of second instar larvae at three days. TG, transgenic plant; CK, untransformed control.

Table 2 Effects of transgenic cotton on survival and development of H. assulta of second instar larvae at three-day infestation.

Number of plantlet	Mortality (%) 3days	Survival weight (mg) 3days	Leaf damage
1	0	5.50±0.40	++++
2	82.5	0.70±0.25	+
3	73.0	0.85±0.20	++
4	86.0	0.67±0.28	+
5	0	5.52±0.30	++++
6	82.5	0.71±0.25	+
CK	0	5.58±0.40	++++

Herbicide resistance of T_0 transgenic cotton

To discern the extent of protection afforded by the *aroAM*12 gene, resistance of plantlets to different dosage of glyphosate were tested. In greenhouse assay, all regenerated cotton plantlets exhibited tolerance to glyphosate. A typical cotton is shown in Fig.6, from which we could observe that after three times spraying, the transgenic cotton plant grew well, while the control one were completely shriveled and dry.

Fig.6. Glyphosate resistance in transgenic and untransformed cotton plant.

The plantlets were sprayed with 0.5% Roundup®. TG, transgenic cotton of M12-Bt/b line; CK, untransformed cotton plant.

Herbicide resistance and Insect Bioassays in T_1 generation

Resistance to Roundup® were assessed 12 days after growing in media containing 25 μmol/L glyphosate. Some T_1 progenies having lateral root growth showed significant resistant against glyphosate, while others have no lateral root appearance (Fig.7). In insect bioassay, some

progenies showed little damage caused by *H. assulta* of second instar larvae, while others showed severe damage. Both the herbicide resistance and insect assays on segregating progenies of T_1 showed good fit to the Mendelian ratio of three resistant: one susceptible (one inserts) or fifteen resistant: one susceptible to the glyphosate and insect (two inserts), respectively (Table3, 4).

Fig .7. Effect of glyphosate on lateral root development of T_1 seedlings
A, Seedlings with well-developed lateral roots; B, Seedlings with no lateral roots.

Table3 Segregation of glyphosate tolerance in T1 progenies

Progenies of Tranformants	No. of plants Resistant	No. of plants Sensitive	χ^{2*}	P
1	50	30	5.33 (3:1)	0.010-0.025
2	53	27	2.74 (3:1)	0.050-0.100
3	72	8	1.41 (15:1)	0.100-0.250
4	71	9	2.00 (15:1)	0.100-0.250
5	54	26	2.05 (3:1)	0.100-0.250
6	52	28	3.51 (3:1)	0.050-0.100

* Uncorrected chi-square goodness-of-it test for hypothesis of 3:1 or 15:1 segregation. None of the chi-square values are significant at the 95% confidence level.

Table4 Segregation of insect- resistance in T_1 progenies

Progenies of Tranformants	No. of plants Resistant	No. of plants Sensitive	X^2	P
1	0	12	-	-
2	28	16	2.45 (3:1)	0.100-0.250
3	27	5	2.13 (15:1)	0.100-0.250
4	24	3	1.80 (15:1)	0.100-0.250
5	0	10	-	-
6	18	10	1.40 (3:1)	0.100-0.250

CONCLUSIONS

Analysis of cotton plants regenerated from *Agrobacterium*-inoculated hypocotyls confirmed the feasibility of transferring the *aroAM*12 gene and *BtS1m* gene, conferring resistance to the broad-spectrum glyphosate and insects, respectively. We also demonstrated the use of *aroAM*12 gene as a convenient selectable marker gene in plant transformation experiments. To the best of our knowledge, in previous studies, the commonly used selectable marker genes for cotton are antibiotic resistant gene such as *NPT*IIand *HPT* genes (Satyavathi et a l.,2002; Yue et al.,2002). Glyphosate-resistant cotton was also obtained (Debbie et al.,1996), but the CP4 EPSPS gene transferred was only as a commercial interest, not as a selective marker gene. Glyphosate selection has many advantages, compared to the antibiotic genes. First, glyphosate is readily degraded in soil; the EPSPS is present merely in plants and bacteria, not in animals. In terms of safety, there exists the possibility that the antibiotic resistant genes migrate to the microorganisms to lead to the invalidation of medical antibiotics. Second, a very low frequency of non-transgenic escapes happened during the whole transformation process. In our research, all the 40 plantlets selected were proved to be transgenic. The *aroAM*12 gene we introduced was through gene shuffling technology; several amino acids were changed compared to the original EPSPS gene (*Salmonella typhimurium* and *Escherichia coli*), which caused the EPSP enzyme with an extremely high ki for glyphosate and low km for the substrate PEP (He et al., 2002). The levels of tolerance depend on the expression level of *aroAM*12 –encoded EPSP synthase. As to the *BtS1m* gene, the feeding assay revealed that the CryIAc protein was toxic to the larvae of *H.assulta*. It had been observed that the insect resistance varied among different plants, this is perhaps due to the activity of the transgene’s promoter or the translation enhancer “Ω” fragment. It was also observed that in the whole transformation process, 25% *BtS1m* gene was lost during the integration of T-DNA. According to Tinland (Tinlind,1991), the T-DNA integrates into the plant genome from the right to the left border, so the DNA adjacent to the left border might be lost. While in this study, the *BtS1m* gene adjacent to the right border of T-DNA was lost, what caused the disagreement need further research. Based on the southern blot, we can make a conclusion that the T-DNA inserted into the genome at random by illegitimate recombination, so that the number of the inserted copies, their chromosomal location varies among the transformants. Together with the immunnoblot, it seems that there is no direct correlation

between copy number and the level of gene expression, and sometimes there exist the inverse correlation. Gene silencing might cause this phenomenon (Vaucheret and Fagard,2001).

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1) Life of Science, Henan Normal University, Xinxiang, 453007, China