

Transgenic cabbage (*Brassica oleracea* var. *capitata*) resistant to Diamondback moth (*Plutella xylostella*)

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A synthetic fusion gene of *Bacillus thuringiensis* encoding a translational fusion product of Cry1B and Cry1Ab δ -endotoxins was transferred to a tropical cabbage breeding line by *Agrobacterium*-mediated transformation. Selection of transformants was carried out on media containing kanamycin. Polymerase chain reaction (PCR) analysis revealed that twelve of the putative transformants contained the transgene. Insect bioassays carried out with the leaves of PCR-positive plants and neonate larvae of Diamondback moth (DBM) showed that one of the transgenic plants was completely resistant to repeated infestation by the larvae. Southern hybridization confirmed gene integration in the DBM-resistant plant. Double-antibody sandwich Enzyme-Linked Immunosorbant Assay (ELISA) analysis revealed accumulation of fusion protein up to 0.16% of total soluble protein in the leaves of the transgenic plants. Progeny (T₁ generation) of the selfed transgenic plants were analyzed for the transgene segregation and insect protection. These studies clearly demonstrated the efficacy of Cry1B-Cry1Ab fusion protein to confer protection to cabbage against DBM infestation. The transgenic cabbage plants will serve as a good system to study the role of gene pyramiding in resistance management strategies intended to prevent evolution of resistance in DBM.

Keywords: *Bacillus thuringiensis*, insecticidal protein genes, *cry1Ab*, *cry1B*, cabbage, Diamondback moth, transgenic plants

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Introduction

The Diamondback moth (DBM; *Plutella xylostella* L.; Lepidoptera: Plutellidae) is an important pest of cruciferous crops and enjoys a worldwide distribution¹. It has become the most destructive pest of cruciferous plants throughout the world². In India, DBM infests important cruciferous crops, such as cabbage, cauliflower, Kohlrabi, radish, turnip, beetroot, mustard, *Brassica campestris* var. *toria* and *B. campestris* var. *sarson*. DBM causes more than 50% loss in marketable yield of cabbage³.

DBM has become resistant to every synthetic chemical used against it and it is one of the insect species that developed resistance to Bt sprays in the open field⁴. Although there are no reports of insects

directly developing resistance to Bt-transgenic plants, survival to maturity has been reported for resistant strains of DBM (broccoli and canola), tobacco budworm and pink bollworm (cotton) on Bt-transgenic plants expressing single toxin⁵. Here, the authors report for the first time introduction and expression of a fusion Bt *cry* gene encoding Cry1B and Cry1Ab as translational fusion protein in a tropical cabbage breeding line which flowers and sets seeds without vernalization in subtropical conditions prevailing in northern plains of India. The resultant transgenic plants were analyzed for resistance to DBM.

Materials and Methods

Construction of Fusion Gene and Plant Transformation Vector

The synthetic *cry1B* gene modified for plant expression was obtained from Dr Roger Frutos

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(CIRAD, Montpellier, France) and synthetic *cryIAb* gene was obtained from Dr. Illimar Altosaar⁶. The fusion gene was constructed by using polymerase chain reaction (PCR) technique. A primer was designed to introduce a *Bam*HI site and to remove the translation stop codon at 3'end of *cryIB* gene sequence as shown below. Such a step preserves the protease-processing site in *cryIB* sequence. The *cryIB* gene was amplified by PCR using *Pfu* DNA polymerase and the PCR product was restricted with *Nco*I and *Bam*HI and cloned in pBluescript KS⁺.

3'end sequence of *cryIB* synthetic gene:

5'-CTTGGAGAGGTAATGAATTCCTGCAG-3'

Primer sequence used to modify *cryIB* synthetic gene (3'end sequence)

Reverse 5'-CTTGGAGAGGGGATCCATTCCTGCAG-3'

The *cryIAb* gene (1.845 kb) from pGEM-4Z vector⁶ was cut with *Bam*HI and *Eco*RI and fused to 3'end of *cryIB* gene (1.95 kb) in pBluescript KS⁺ vector. The *cryIB-cryIAb* fusion gene was cut with *Nco*I and *Sal*I and the fragment was inserted into the binary vector pBinAR⁷, which contains a CaMV 35S promoter and octopine synthase poly A sequence. The T-DNA of recombinant binary vector is shown in Fig.1. The resulting vector was designated as pBinBt 6 and transformed into *A. tumefaciens* strain EHA 105⁸ by freeze-thaw transformation method⁹.

Reagents and Enzymes

Restriction endonucleases, *Pfu* DNA polymerase and T4-DNA ligase were purchased from MBI Fermentas. *Taq* polymerase was purchased from GenTaq, Singapore. Hybond N⁺ membranes were procured from Amersham, UK. All the reagents and plant hormones were of molecular biology grade (Sigma, USA).

Plant Transformation

Tropical cabbage (*B. oleracea* var. *capitata*) breeding line, DTC 507 was chosen as the plant material. Cabbage seedlings were raised aseptically on half-strength Murashige and Skoog (MS)¹⁰ medium in Magenta boxes. Hypocotyl segments (0.5 cm length) proximal to cotyledonary node of 5-6 day old seedlings were excised and cultured on preculturing medium (PCM) containing MS salts, B5 vitamins, 2% sucrose, 2 mg/l BAP, 0.2 mg/l NAA or IAA, 0.7% Phytigel (pH 6.0) and incubated at

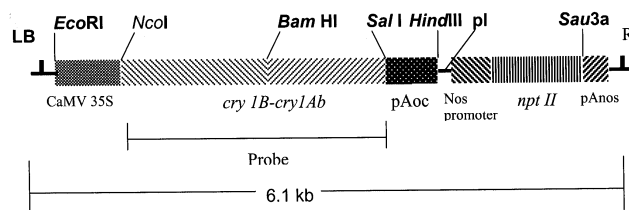


Fig. 1—T- DNA of plant transformation vector pBinBt6 carrying *cryIB-cryIAb* fusion gene; *npt II*, neomycin phosphotransferase II; LB, left border; RB, right border; pAocs, poly (A) sequence of octopine synthase gene; pAnos, poly (A) sequence of nopaline synthase gene.

25±2°C, 16 hrs photophase with light intensity of 40 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Agrobacterium tumefaciens was grown in yeast extract-mannitol (YEM) medium for 22 hrs at 28°C and diluted 100-fold before use. Hypocotyls precultured for 2 days were infected in *Agrobacterium* suspension for 2-5 min, blotted dry with sterile Whatman 3 MM filter paper and air-dried for 10 min. Infected explants were placed back on the same plates containing PCM and cocultivated at 22 °C for 2 days in the dark.

The cocultivated hypocotyl segments were transferred to *Agrobacterium* elimination medium (AEM) consisting of PCM plus 500 mg/l cefotaxime. After three days, explants were transferred to selection medium containing PCM plus 500 mg/l cefotaxime, 20 mg/l kanamycin and 10 μM AgNO₃. Small green shoots appeared on pale green callus after four to six weeks and were allowed to attain 1-2 cm length in the selection medium. These green shoots selected on the selection medium were carefully excised and transferred to shoot growth and development medium (SGM) containing MS salts supplemented with B5 vitamins, 0.2 mg/l BAP, 500 mg/l Cefotaxime, 0.8% agar, 2% sucrose and kanamycin was excluded. When putative transformants shoot attained a length of 3-5 cm, they were transferred to rooting medium consisting of half-strength MS salts, B5 vitamins, 0.8% agar and 2% sucrose. Rooted plantlets were transferred to small pots containing vermiculite and peat moss in 3:1 ratio. After establishment, the plants were shifted to large earthen pots in the greenhouse.

Southern Analysis

Genomic DNAs were isolated according to Doyle and Doyle¹¹ from the leaves of one T₀ transgenic plant and eight plants of T₁ generation, digested with *Hind*III, electrophoresed on 0.8% agarose gel and

transferred to positively charged nylon membrane. Decalabel™ DNA labelling kit (MBI Fermentas, Lithuania) was used to prepare the radiolabelled probe according to the manufacturer's instructions. Southern hybridization was carried out with a ³²P-dCTP-labelled *NcoI-SalI* insert (3.8 kb) separated from pBluescript KS+ carrying *cryIB-cryIAb*¹².

Enzyme Linked Immunosorbant Assay (ELISA)

Rapidly growing leaves (500 mg) were frozen and ground in liquid nitrogen. The leaf powder was extracted with phosphate-buffered saline/Tween (PBST)¹². The leaf extracts were centrifuged and the supernatants were immediately used for protein estimation¹³. The extracts were tested by a double antibody sandwich quantitative ELISA, as described previously¹⁴. Antibodies raised in rabbits against pure CryIB and CryIAb proteins were used.

Insect Bioassays

A field population of *P. xylostella* was obtained from Indian Agricultural Research Institute farm and was reared on organically grown cabbage leaves at 27±2°C and 60% relative humidity under a 16 hrs photophase. Insect bioassays were carried out using leaf discs (3.5 cm²), detached leaves (25 cm²) and whole plants (50 d old). Ten neonate larvae were placed on leaf discs; 15 larvae of second, third and fourth instar stages were used for detached leaf bioassays and 90-120 second and third instar larvae were used for whole plant bioassays. In addition, eggs (40-50) laid on parafilm strips that were smeared with cabbage leaf tissues were used for bioassays. Two strips each were clipped to transgenic and wild type cabbage plants.

Progeny Analysis

The seeds obtained from the T₀ transgenic plants by bud pollination were germinated in small pots. The T₁ generation plants (30 plants) were analyzed by PCR using *npt II* primers.

Forward primer: 5'- CAATCGGCTGCTCTGATGCCG-3'

Reverse primer: 5'- AGGCGATAGAAGGCAATGCGC-3'

Results and Discussion

Regeneration and Transformation

Regeneration of cabbage shoots from hypocotyl segments was tested using PCM containing either NAA or IAA. Both the media were found efficient for regeneration of viable green shoots from hypocotyl

segments. On PCM containing NAA, regeneration coupled with extensive callus induction was observed whereas on PCM with IAA, callus induction was less and few shoots regenerated directly from cut ends of the hypocotyl segments. More than 90% of the shoots originated from the cut ends of hypocotyl segments within two weeks of incubation. Regeneration of transformants was initiated by *A. tumefaciens* mediated transformation. Putative transformants were selected on medium containing 2 mg/l BAP, 0.2 mg/l NAA and 20 mg/l kanamycin. For further growth and development of putative transformants, 0.2 mg/l BAP, 300 mg/l cefotaxime and 0.8% agar were used. Deletion of kanamycin and AgNO₃ in SGM enabled small green shoots selected on kanamycin medium to grow healthy. Although Phytigel promoted initiation of large number of shoots (20-24 per hypocotyl segments) agar was a better gelling agent for shoot growth and development. Agar as gelling agent did not cause vitrification and shoot-tip necrosis when small green shoots were transferred to SGM medium.

Southern Hybridization

Putative transformants (12) selected on medium containing kanamycin were analyzed for the presence of *cryIAb* gene. PCR analysis carried out with primers specific for *cryIAb* gene revealed that all the putative transformants were positive but not the wild type cabbage plants (Results not shown). Preliminary insect bioassays carried out with the leaf discs

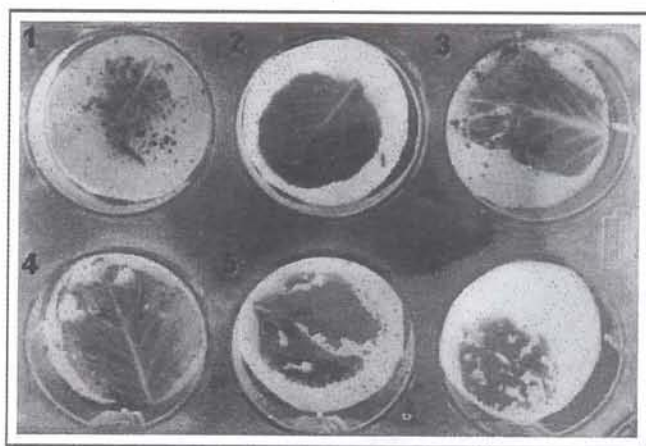


Fig. 2—Insect bioassay of putative transformants using first instar larvae of diamondback moth (DBM). 1- Wild type cabbage plant; 2 - a putative transformant of cabbage showing resistance to infestation by DBM larvae and also showed Southern hybridization signal; 3 to 6 - putative transformants susceptible to infestation by DBM larvae and did not show Southern hybridization signal.

punched from the PCR-positive plants and neonate larvae of DBM revealed that one of the transformants was completely resistant to repeated infestation by DBM larvae (Fig. 2). Genomic DNA isolated from the resistant transgenic plant was digested with *Hind* III. *Hind* III cuts at a single site within T-DNA (Fig. 1), downstream of the Octopine synthase-poly (A) sequence and so provided information regarding the insertion position and number of the transgene copies. Southern analysis with *cryIB-cryIAb* fusion gene probe revealed that the copy number of the fusion gene was three (Fig. 3a). The third copy (4.0 kb) was smaller than the expected molecular weight of T-DNA, pointing out the possibility of truncation of T-DNA. The transgenic plants were selfed and the seeds were collected. Inheritance of the transgene was investigated in the T₁ generation by PCR analysis for the presence of *npt* II, *cryIB* and *cryIAb* fusion gene. Eight transgenic plants of T₁ generation, which gave PCR positive signal for *npt* II, *cryIB* and *cryIAb* genes (results not shown) were chosen for Southern analysis. The analysis confirmed the inheritance of the fusion gene (Fig. 3b). Two of the plants were found to carry single copy of the transgene, which segregated in a Mendelian ratio of 3:1 in T₂ generation (data not shown). Thus, it was possible to obtain plants with single copy transgene from plants carrying multiple transgene copies through segregation.

Quantification of CryIB-CryIAb Fusion Protein by ELISA

Double-antibody sandwich ELISA analysis was performed with leaves taken from T₀ transgenic plant and T₁ population using immunoaffinity-purified polyclonal rabbit antibodies specific for CryIB and CryIAb proteins to detect the levels of fusion protein expression in the transgenic plants. The transgenic plant (T₀) expressed the fusion protein to a level of 0.15% of TSP. Toxin levels as measured by ELISA were converted to microgram toxin per milligram total soluble protein (TSP) (Table 1). Expression of the fusion protein ranged from 0.08 to 0.16% in young leaves (upper whorls), which is about 10 to 20 µg fusion protein per gm leaf fresh weight. The level of expression ranged from 0.04 to 0.13% TSP (2.96 to 10.0 µg per g leaf fresh weight) in mature leaves (lower whorls).

Insect Bioassay

DBM is highly susceptible to Bt toxins CryIAb (LC₅₀: 1.45 µg/cm²) and CryIB (LC₅₀: 0.26 µg/cm²)¹⁵. In combination, the two toxins exhibit LC₅₀ value of

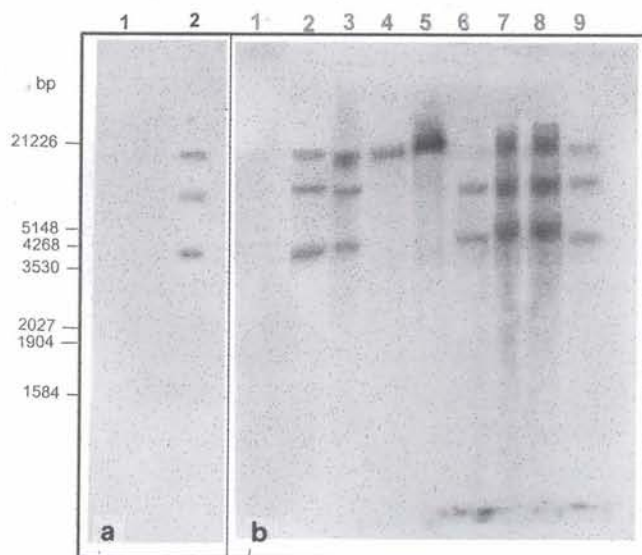


Fig. 3—Southern blot analysis of T₀ and T₁ Bt transgenic cabbage plants. Total genomic DNA was isolated from the transgenic plants and wild type cabbage plants and complete digested with *Hind* III. Blots were hybridized to the entire *cryIB-cryIAb* fusion gene (3.795 kb) that was radiolabelled with [α^{32} P]; a. Southern blot of T₀ Bt transgenic cabbage plant showing hybridization signal at three loci; b. Southern blot of T₁ Bt transgenic cabbage plants. Two plants were showing hybridization signal at single locus (lane 4 and 5).

0.09 µg/cm². To ascertain the effect of CryIAb-CryIB fusion protein *in planta*, insect bioassays were carried out with leaf discs, detached leaves and whole-plants expressing the fusion gene. All four stages of DBM larvae were utilized in insect bioassays. Toxicity of the Bt fusion protein, CryIB-CryIAb to DBM was evident from all the insect bioassays (Table 2; Fig. 4). Even Bt-transgenic plants expressing low levels of CryIB-CryIAb fusion protein (0.04% TSP) in mature leaves caused 100% mortality to all the four larval stages of DBM. Complete mortality of neonate larvae was observed within 24 hrs and within 48 hrs in the case of other three stages of larvae. These studies clearly demonstrated high lethality of CryIB-CryIAb fusion protein to DBM larvae. These results also support our previous observations made from experiments with CryIAb-CryIB fusion protein expressed in *E. coli* and tested against *P. xylostella*¹⁵. The fusion protein exhibited enhanced insect mortality when compared to that of CryIAb or CryIB alone. Similar observations were made by Honee *et al*¹⁶ by developing CryIAb-CryIC fusion protein to broaden insecticidal spectrum.

Table 1—Quantification of Cry1B-Cry1Ab fusion protein expressed in Bt transgenic cabbage plants (T₁ population)

Transgenic Lines	Fusion gene insertion sites	Young leaves		Mature leaves		Fusion protein $\mu\text{g/g}$ leaf fresh weight	
		TSP (mg)	Fusion protein content (%)	TSP (mg)	Fusion protein content (%)	Young leaves	Mature leaves
BtC-1	2	12.5	0.08	7.3	0.04	10.00	2.92
BtC-2	3	11.5	0.09	7.1	0.06	10.35	4.26
BtC-3	1	12.8	0.16	7.8	0.13	20.48	10.14
BtC-4	1	12.5	0.12	6.9	0.08	15.00	5.52
BtC-5	2	11.9	0.1	7.1	0.07	11.90	4.97
BtC-6	3	11.8	0.15	8.0	0.09	17.70	7.20
BtC-7	3	12.1	0.15	7.2	0.11	18.15	7.92
BtC-8	3	12.5	0.11	7.6	0.08	13.75	6.08
Homozygous	1	12.2	0.12	7.9	0.09	19.85	9.10
Wild type	-	12.0	-	7.0	-	-	-

Note: Leaf segments were collected from 50 d old T₁ transgenic plants for ELISA analysis

Table 2—Toxicity effect of Cry1B-Cry1Ab fusion protein expressed in Bt transgenic cabbage plants on DBM larvae

Type	Stages of larvae released	No. of larvae released	Leaf damage	Mortality (%)	Time (hrs) taken to kill larvae
Leaf disc bioassay	Neonate	10	Nil	100	24
	Second instar	10	Nil	100	48
Detached leaf bioassay	Second instar	15	Nil	100	48
	Third instar	15	Nil	100	48
	Fourth instar	15	Nil	100	48
Whole plant bioassay	Neonate	20	Nil	100	48
	Second instar	40	Nil	100	48
	Third instar	40	Nil	100	48

Note: Data were collected 5 d after releasing DBM larvae on Bt-transgenic cabbage and wild type plants. Both young and mature leaves of T₀ and T₁ Bt-transgenic cabbage plants (50 d old) used in insect bioassay exhibited similar toxicity effect on all stages of larvae, invariably to difference in fusion protein levels in leaf tissues of various transgenic plants. In wild type plants, there was severe infestation by DBM and no mortality was observed.



Fig. 4—Whole plant bioassay. Bt transgenic plants expressing fusion protein were highly resistant to all stages of DBM larvae. Wild type cabbage was severely infested by DBM larvae. *Left* Wild type cabbage; *Right* Bt transgenic cabbage.

Since Cry1B and Cry1Ab proteins are highly toxic and bind to different receptors in the midgut epithelial membranes of *P. xylostella*¹⁷, both could cause mortality. According to a theoretical model, it has been proposed that pyramiding two dissimilar toxin genes in the same plant has the potential to delay the onset of resistance much more effectively than single toxins expressed spatially or temporally¹⁸. In a recent study, Zhao *et al*¹⁹ have observed that pyramiding of *cry1Ac* and *cry1C* genes in transgenic Broccoli has resulted in significant delay in resistance development in *P. xylostella* populations resistant to either Cry1Ac or Cry1C toxins.

The Bt-transgenic cabbage expressing Cry1B-Cry1Ab, as translational fusion protein shall serve as an excellent transgenic genotype to study the resistant management strategies designed to curtail the evolution of resistance in *P. xylostella*.

References

- 1 CIE, *Distribution maps of pests - Map No. 32 (Revised)*, (Commonwealth Institute of Entomology, London) 1967
- 2 Talekar N S & Shelton A M, Biology, ecology and management of the diamondback moth, *Annu Rev Entomol*, 38 (1993) 275-301.
- 3 Chelliah S, & Srinivasan K, Biology and management of diamondback moth in India, in. *Proc First Int Workshop Diamondback moth management*, edited by N S Talekar & T D Griggs (Asian Vegetable Research and Development Center, Shanhua, Taiwan) 1985, 63-76.
- 4 Tabashnik B E, Cushing N L, Finson N, & Johnson M W, Field development of resistance to *Bacillus thuringiensis* in diamond back moth (Lepidoptera:Plutellidae), *J Econ Entomol*, 83 (1990) 1671-1676.
- 5 Zhao J Z, Collins H L, Tang J D, Cao J, Earle E D *et al*, Development and characterization of diamondback resistance to transgenic broccoli expressing high levels of Cry1C, *Appl Environ Microbiol*, 66 (2000) 3784-3789.
- 6 Sardana R, Dukiandjiev S, Giband M, Cheng X, Cowan K *et al*, Construction and rapid testing of synthetic and modified toxin gene sequences *cryIA (b & c)* by expression in maize endosperm culture. *Plant Cell Rep*, 15 (1996) 677-681.
- 7 Hofgen R & Willmitzer L, Biochemical and genetic analysis of different putative isoforms expressed in various organs of potato (*Solanum tuberosum*), *Plant Sci* 66 (1990) 221-230.
- 8 Hood E E, Gelvin S B, Melchers L S & Hoekema A, New *Agrobacterium* helper plasmids for gene transfer to plants, *Transgenic Res*, 2 (1993) 208-218.
- 9 Hofgen R & Willmitzer L, Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res*, 16 (1988) 9877.
- 10 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473-497.
- 11 Doyle J J & Doyle J I, Isolation of plant DNA from fresh tissue, *Focus* 12 (1990) 13-15.
- 12 Sambrook J & Russell D W, *Molecular cloning: A laboratory manual*, 3rd ed (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) 2000.
- 13 Bradford M M, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding, *Anal Biochem*, 72 (1976) 248-254.
- 14 Kumar P A, Mandaokar A, Sreenivasu K, Chakraborti S K, Bisaria S *et al*, Insect-resistant transgenic brinjal plants, *Mol Breed*, 4 (1998) 33-37.
- 15 Mandaokar A, Chakraborti S K, Rao N G V, Kumar P A & Sharma R P, A fusion gene coding for two different δ -endotoxins of *Bacillus thuringiensis* toxic to *Plutella xylostella* and useful for resistance management, *World J Microbiol Biotechnol*, 14 (1998) 599-601.
- 16 Honee G, Vriezen W & Visser B, A translational fusion product of two different insecticidal crystal protein genes of *Bacillus thuringiensis* exhibit an enlarged insecticidal spectrum, *Appl Environ Microbiol*, 56 (1990) 823-825.
- 17 Ferre J, Real M D, Van Rie J, Jansens S, & Peferoen M, Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor, *Proc Natl Acad Sci USA*, 88 (1991) 5119-5123.
- 18 Gould F, Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology, *Annu Rev Entomol*, 43 (1998) 701-726.
- 19 Zhao J Z, Cao J, Li Y, Collins H L, Roush R T *et al*, Transgenic plants expressing two to *Bacillus thuringiensis* toxins delay insect resistance evolution, *Nature Biotechnol*, 21 (2003) 1493-1497.