NOTES

Can other host species of cotton bollworm be non-Bt refuges to prolong the effectiveness of Bt-cotton?

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Abstract **The potential ecological risks of *Bacillus thurigiensis* (Bt) insecticides and Bt-crops have caused increasing concern since their commercial release in the field, among which pests' resistance to Bt-crops is the major ecological risk. Refuge tactic, which can produce sensitive populations, has proved to be a key and sound resistance management strategy in USA and Australia; however, no tactics have been performed in China where Bt-cotton is mostly planted with other host crops of cotton bollworm. Genetic variation and gene flow among different host populations of the cotton bollworm *Helicoverpa armigera* were analyzed using PCR fingerprinting method. The results show that maize and castor-oil plant, as well as cotton can take effect as refuges to prevent resistance of cotton bollworm to Bt-cotton, while peanut and sesame are not as suitable for planting with Bt-cotton as refuges in the field as low gene flow was detected among populations on peanut, sesame and Bt cotton.**

Keywords: transgenic Bt-cotton, *Helicoverpa armigera*, host crops, refuge, gene flow, resistance management.

The cotton bollworm *Helicoverpa armigera* (Lepitoptera: Noctuinae) is one of the most serious insect pests on cotton. It has adapted to many different host crops in Asia, Europe, Africa and Australia, and caused huge damage to cotton production in recent years. In the last decade, cotton bollworm broke out frequently and had evolved resistance to many chemical pesticides. The damage caused by this pest has become a key limiting factor in the sustained production of cotton. At the same time the environment has suffered from the pollution with chemical insecticides. With rapid development in genetic engineering, Bt-insecticide and transgenic Bt-crops bring about a new era of pests management because they are especially resistant to Lepidoptera pests as well as environmentally friendly[1,2]. In China, transgenic Bt-cotton has been planted on a large scale in several provinces since 1997.

Despite their potential benefits on the pest management and sustained agricultural production, there are also concerns with these transgenic crops about their possible impacts on the environment. Evolution of resistance by pests is the most serious threat to the continued efficacy of Bt toxins and Bt-crops[1]. With millions of hectares of Bt toxin-producing transgenic cotton grown yearly, pests are likely to evolve resistance quickly under such constant selection pressure unless effective countermeasures are designed and implemented soon[1,2]. Resistance to Bt toxin has already been reported in some insect species in both the field environment and under laboratory selection[3,4]. Several resistance management strategies for transgenic plants had already been developed before their commercial release[4—8]. Refuge tactic, which is considered as an effective resistance management strategy, has been adopted in Australia and America[5,6, 8,9]. Because effective refuge can maintain enough susceptible individuals in the local population of *Helicoverpa,* and random mating between these survivors from Bt-crops and susceptible individuals generated from refuge will ensure that all progeny of any resistant survivors will be heterozygotes. In addition less than 5% of the progeny survive on the plants as Bt resistance are inherited as a recessive or partially recessive trait[3,5,6]. The transgenic cotton has been planted in China on a large scale, no tactics has been performed to prevent cotton bollworm's resistance. It is important therefore to develop effective strategies to manage the resistance. Once the cotton bollworm develops resistance to Bt-cotton in the field it will have catastrophic effects on Bt-insecticides and other Bt-crops, such as Bt-maize, Bt-corn, Bt-potato, Bt-tomato, etc.[5].

In China Bt-cotton is inter-planted with many other host species of *H. armigera*, and the migration ability of this insect specie is strong. It was not thought necessary to construct a refuge tactic in the fields of China[2]. However, can other host species of *H armigera* really take effect as refuges to prevent cotton bollworm's resistance to Bt-cotton? Can individuals from those crops randomly mate with survivors from Bt-cotton? Based on the published data, the Environmental Protection Agency (EPA) of USA suggested that additional research efforts are needed to address the use of alternate cultivated or wild hosts as refuges[5]. This information is valuable in designing an effective refuge strategy that maximizes the probability that susceptible individuals arising from a structured refuge will find and mate with the resistant individuals that survive exposure to the delta endotoxin produced in the Bt-plant. Research data regarding these efforts must be submitted to the registration of a new Bt-crop[5]. Thus it is very important then to investigate the population dynamics of cotton bollworm between different host species. As direct field study on the migration of insects, such as re-captured method, is often difficult and very costly, the analysis of the population genetic structure using molecular genetic markers becomes an important information source for evaluating the spatial dynam-

ics of population. As two are closely related, information about some ecological processes can be inferred from the variation of population's genetic structure, for instance, information about the reproduction pattern of populations from the relationship among individuals. Additionally, levels of gene flow between populations can be inferred from F-statistics (Fst) and Number of Effective Migration (Nem) analysis, which can give information about the population dynamics, speciation, ecological introgression and origins of some species[10,11]. In recent years, molecular genetic marker technology has been progressing rapidly, and has become an efficient tool in the field of ecology and can strengthen the investigation of population genetic divergence, gene flow and migration patterns, and productive strategies[10].

Using PCR fingerprinting method, the present note reports data on the genetic variation and gene flow among different local host crops of cotton bollworm in China. And the possibilities of other host species of cotton bollworm to take effect as refuges to prevent or delay resistance to Bt-cotton were analyzed as well.

1 Materials and methods

(ⅰ) Sampling of the cotton bollworm. Larvae of cotton bollworm in the fourth to sixth instars were collected from their different host crops in the suburb of Cheng'an County, Hebei Province, China (table 1). The larvae were brought to laboratory and fed with leaves of host crops or stored in ethanol (95%). 15—30 individuals of each population were collected from the field and 15 of each population were used in the following analysis.

(ⅱ) Extraction of genomic DNA. Genomic DNA of individual for PCR amplification was extracted according to the method of Chen[12].

Table 1 Cotton bollworms for this study

Population	Host species	Number	Collection time
C	transgenic Bt-cotton	30	August 1999
M	normal cotton	30	August 1999
Y	maize	30	August 1999
H	peanut	30	August 1999
Z	sesame	15	August 1999
B	castor-oil plant	15	August 1999

(ⅲ) PCR reaction. PCR fingerprinting, also called amplified polymorphism with repeated sequence primers, was developed from the combination of PCR and DNA fingerprinting methods. It has been applied successfully to amplifying hypervariable repetitive DNA sequence in a wide range[13]. The results of this technique are more reproducible than RAPD-PCR method, for these primers are specific to microsatellite or minisatellite DNA sequence, longer than random primers used in RAPD-PCR, and they need higher annealing temperature in PCR reaction. Furthermore this technique can indicate more genetic information than DNA fingerprinting and it avoids the complicated selection of probes. In our study three single primers specific to microsatellite DNA sequence, and a 16-bp long core sequence of 33.15 microsatellite DNA (synthesized by Cyber Syn Co., table 2), were used as single primers in PCR amplification.

Table 2 Primers used for PCR reaction

Primer	Sequence (5′-3′)	Annealing temperature/℃
$(CAC)_5$	CACCACCACCACCAC	57
$(GATA)_4$	GATAGATAGATAGATA	40
$(GT)_8$	GTGTGTGTGTGTGTGT	57
33.15 core sequence	AGAGGTGGGCAGGTG	57

Polymerase chain reaction (PCR) amplification was carried out in a Perkin-Elmer 480 Cetus thermal cycler in 25 μL of reaction mixture including about 20—40 ng of template DNA, 0.2 mmol/L dNTPs, 0.5 μmol/L of each primer, ddH_2O, 1 × PCR buffer (50 mmol/L KCl, 2 mmol/L MgCl, 10 mmol/L Tris-HCl), and 1 unit of Taq DNA Polymerase (Promega Co.). After initially denaturation at 94℃ for 5 min, the reaction underwent 35 cycles with 94℃ for 1 min, specific annealing temperature (listed in table 2) for 30 s, 72℃ for 40 s and a final extension at 72℃ for 5 min. The product of PCR was run on 1.2% agarose gel and stained with ethidium bromide, then visualized and photographed under UV light.

(ⅳ) Data analysis. According to their molecular weight, all bands of each individual were transformed to 1/0 data. Based on these data genetic distance[14], F-statistic index (Fst)[15] and number of effective migration (Nem)[14] were calculated. UPGMA method was used to perform clustering analysis of populations based on genetic variation indexes[14].

2 Results

Using single repetitive DNA primers, clear and stable amplification results were revealed (shown in fig. 1). And polymorphism among individuals can be indicated from the results of PCR amplification. The genetic dis-

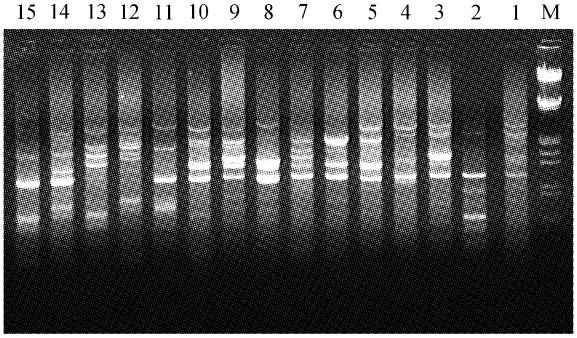

Fig. 1. Result of PCR with primer $(CAC)_5$. M, Standard molecular weight marker λ DNA/*Hin*d Ⅲ+ *Eco*R Ⅰ; 1—5, individuals from non-transgenic cotton population; 6—10, individuals from maize population; 11—15, individuals from castor-oil plant population.

tance between different populations is shown in table 3, with the average genetic distance 0.114. And the Fst and Nem indexes are shown in table 4, with the average 0.155 and 1.36 respectively.

Table 3 Genetic distance between different populations[a)]

	C	M	Y	B	H	Z
C						
M	0.1412					
Y	0.1190	0.0908				
B	0.1539	0.0882	0.0407			
H	0.1613	0.1115	0.0868	0.1130		
Z	0.1738	0.1075	0.0967	0.1339	0.0939	

a) C, Population from Bt-cotton; M, from non-Bt-cotton; Y, from maize; B, from castor-oil plant; H, from peanut; Z, from sesame.

Table 4 Fst and Nem indexes between different populations

	C	M	Y	B	H	Z
C						
M	0.1593					
	1.3196					
Y	0.1507	0.1509				
	1.4086	1.4062				
B	0.1509	0.1017	0.0734			
	1.4062	2.2063	3.1555			
H	0.1921*	0.1561	0.1390	0.1638		
	1.0513	1.3510	1.5476	1.2761		
Z	0.2204*	0.1500	0.1636	0.1528	0.2026*	
	0.8840	1.4157	1.2773	1.3854	0.9837	

* $P \leqslant 0.05$.

3 Discussion

It is widely believed that effective resistance management tactic should be performed to delay the evolution of pests' resistance to Bt-cotton in the field[5,6, 8,9]. However, no strategy has been performed to manage the resistance in China although the Bt-cotton was commercially released on a large scale, which has caused much concern[16]. There was no data showing that cotton bollworm had evolved resistance to Bt-cotton, but 5 populations of *H. armigera* from Yanggu (Shandong Province), Handan (Hebei Province), Xinxiang (Henan Province), Huixian (Anhui Province) and Fengxian (Jiangsu Province) had shown early resistance to Bt toxin[17]. Field investigation indicated that the third and fourth generations of cotton bollworm can survive from the Bt-cotton, and chemical insecticides are needed to reduce the damage[18]. In the Chinese Huanghe Cotton Belt, cotton is mostly inter-planted with other host species of cotton bollworm. Our results show that the genetic distance (table 3) among all populations from different host species is low, with an average of 0.114. But there were differences between some populations, the genetic distance between Bt-cotton with normal cotton and maize populations is lower than those between Bt-cotton and peanut, sesame and castor-oil plant populations. As shown in fig. 2, the populations collected on sesame and peanut were separated from those on Bt-cotton, with normal cotton, maize and castor-oil plant.

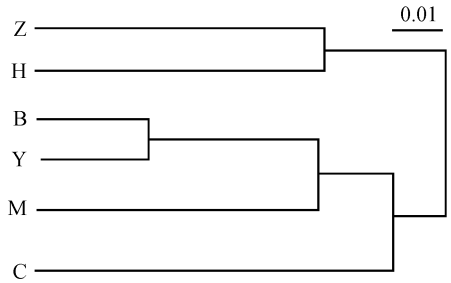

Fig. 2. Cluster diagram of populations from different host species by UPGMA based on genetic distance data. B, Population from castor-oil plant; C, from Bt-cotton; H, from peanut; M, from normal cotton; Y, from maize; Z, from sesame.

The cluster diagram also shows that the population on Bt-cotton differentiated genetically from those on the other host species. Differentiations between populations from Bt-cotton and sesame, Bt-cotton and peanut are larger than those between Bt-cotton and normal cotton, maize, castor-oil plant populations. Gene flow analysis by Fst and Nem indexes[14,15,19] shows that populations from Bt-cotton have frequent gene flow among other populations except those from peanut and sesame, while no significant gene flow was found between populations from Bt-cotton and peanut, Bt-cotton and sesame, sesame and peanut. This suggests that populations from Bt-cotton, normal cotton, maize and castor-oil plant can mate randomly, but moths from Bt-cotton, peanut and sesame may not.

Results above indicate that both maize and castor-oil plant can be refuges as normal cotton to produce susceptible individuals and to prolong the effectiveness of Bt-cotton. The four species crops can be inter-planted in the field. However, the inter-planting of maize and Bt-cotton may cause negative effect on the release of Bt-maize in the future. As Bt-maize expresses the same Bt-toxin of Bt-cotton does, the inter-planting of the two species may access the evolution of cotton bollworm's resistance to Bt toxin[2,5,9]. Once the cotton bollworm evolves resistance to Bt-cotton, the Bt-maize may no longer be resistant to cotton bollworm. Thus we strongly suggest that Bt-cotton should be separately, at least 0.5 miles away, planted with maize, peanut and sesame, and a refuge containing normal cotton is needed for the resistance management and the sustainable use of Bt-cotton. According to the refuge strategy, there are two options to prevent the resistance to Bt-cotton[5]. (ⅰ) In-field refuges of at least 10% non-Bt cotton refuge should be implemented. In-field refuges should be planted entirely within the fields as blocks to provide Bt-susceptible moths. Cotton field can be treated with any non-Bt insecticide or other control measures, as long as the entire field is treated in the same manner. (ⅱ) An external refuge of at least 30% non-Bt cotton should be implemented. The

placement of the structured refuge should be planted within 0.5 miles of the farthest Bt-cotton in a field to provide Bt-susceptible moths. The external refuges of non-Bt cotton can be treated with any other registered non-Bt insecticides or other insect control measures.

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References

1. Paoletti, M. G., Pimentel, D., Genetic engineering in agriculture and the environment, assessing risks and benefits, Bioscience, 1996, 46: 665.
2. Zhang, T., Tang, C., Commercial production of transgenic Bt insect-resistant cotton varieties and the resistance management for bollworm (*Helicoverpa armigera* Hubner), Chinese Science Bulletin, 2000, 45(14): 1249.
3. Tang, Z., Zhou, X., Zhang, G., δ—Endotoxin of *Bacillus thuringiensis* and pest-insect resistance, in Molecular Toxicology of Insecticides and Insect Resistance (eds. Leng, X., Tang, Z., Wang, Y.) (in Chinese), Beijing: Chinese Agriculture Publication, 104.
4. Tabashnik, B. E., Evolution of resistance to *Bacillus thuringiensis*, Annual Review of Entomology, 1994, 39: 47.
5. Environmental Protection Agency, The environmental protection agency's white paper on Bt plant-pesticide resistance management, Washington: EPA Publication, 1998, 739-S-98-001.
6. Gould, F., Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology, Annual Review of Entomology, 1998, 43: 701.
7. McGaughey, W. H., Whalon, M. E., Managing insect resistance to *Bacillus thuringiensis* toxins, Science, 1992, 258: 1451.
8. McGaughey, W. H., Gould, F., Gelernter, W., Bt resistance management, a plan for reconciling the needs of the many stakeholders in Bt-based products, Nature Biotechnology, 1998, 16: 144.
9. Fitt, G. P., Risks, development and integration of insect resistant crops expressing genes from *Bacillus thuringiensis*, in Commercialization of Transgenic Crops: Risk, Benefit And Trade Considerations (eds. McLean, G. D., Waterhouse, P. M., Evans, G. et al.), Bureau of Resource Science, Australia, 1997, 273.
10. Avise, J. C., Molecular Marker, Natural History and Evolution, New York: Chapaman & Hall, 1994.
11. Roderick, G. K., Geographic structure of insect populations: gene flow, phylogeography, and their uses, Annual Review of Entomology, 1996, 41: 325.
12. Chen, X. F., Tan, S. J., Liu, R. Y. et al., Study on the genetic variation of the cotton bollworm (*Helicoverpa armigera*) populations in China, Entomologia Sinica, 2000, 7: 243.
13. Meyer, W., Mitchell, T. G., Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: strain variation in *Cryptococcus neoformants*, Electrophoresis, 1995, 16: 1648.
14. Weir, B. S., Methods for Discrete Population Genetic Data, Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers, 1990.
15. Rousset, F., Genetic differentiation and estimation of gene flow from F-statistic under isolation by distance, Genetics, 1997, 145: 1219.
16. Freudling, C., Chinese Bt cotton needs resistance management, Biotechnology and Development Monitor, 1999, 38: 22.
17. Shen, J., Zhou, W., Wu, Y. et al., Early resistance of *Helicoverpa armigera* to *Bacillus thutingiensis* and its relation to the effect of transgenic cotton lines expressing Bt toxin on the insect, Acta Entomologia Sinica, 1998, 41(1): 8.
18. Zhang, H., Wang, M., Dai, H. et al., The fluctuation and damage character of *Helicoverpa armigera* in the field of transgenic Bt cotton, Chinese Entomology Knowledge, 2000, 37(3): 146.
19. Roderick, G. K., Geographic structure of insect populations: gene flow, phylogeography, and their uses, Annual Review of Entomology, 1996, 41: 325.

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