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RESEARCH

Influences of Cry1Ac Broccoli on Larval Survival and Oviposition of Diamondback Moth

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ABSTRACT. Larval survival and oviposition behavior of three genotypes of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), (homozygous Cry1Ac-susceptibile, Cry1Ac-resistant, and their F_1 hybrids), on transgenic *Bacillus thuringiensis* (Bt) broccoli expressing different levels of Cry1Ac protein were evaluated in laboratory. These Bt broccoli lines were designated as relative low, medium, and high, respectively, according to the Cry1Ac content. Untransformed brocccoli plants were used as control. Larval survival of diamondback moth on non-Bt leaves was not significantly different among the three genotypes. The Cry1Ac-resistant larvae could survive on the low level of Bt broccoli plants, while Cry1Ac-susceptible and F_1 larvae could not survive on them. The three genotypes of *P. xylostella* larvae could not survive on medium and high levels of Bt broccoli. In oviposition choice tests, there was no significant difference in the number of eggs laid by the three *P. xylostella* genotypes among different Bt broccoli plants. The development of Cry1Ac-susceptible and Cry1Ac-resistant *P. xylostella* on intact Bt plants was also tested in greenhouse. All susceptible *P. xylostella* larvae died on all Bt plants, while resistant larvae could survive on broccoli, which expresses low Cry1Ac protein under greenhouse conditions. The results of the greenhouse trials were similar to that of laboratory tests. This study indicated that high dose of Bt toxins in broccoli cultivars or germplasm lines is required for effective resistance management.

Key Words: *Bacillus thuringiensis*, *Plutella xylostella*, larval survival, oviposition behavior, resistance management

Genetically modified plants expressing *Bacillus thuringiensis* (Bt) insecticidal proteins have been developed in many crops. Some transgenic Bt varieties have been deployed successfully on a commercial scale for pest control (James 2012). These Bt crops afford enormous economic and environmental benefits (Shelton et al. 2002, Bates et al. 2005). However, widespread cultivation of Bt crops exerts strong selection pressure on pest populations. It would result in evolution of resistance to Bt plants and control failure (Alstad and Andow 1995, Gould 1998, Tabashnik et al. 2008). To keep the long-term sustainability and durability of Bt crops, it is necessary to manage the resistance of insects targeted by the technology.

Several cases of field resistance in target insect species to Bt crops have been documented including resistance of *Spodoptera frugiperda* to Cry1F corn in Puerto Rico (Storer et al. 2010), resistance of *Pectinophora gossypiella* to Cry1Ac cotton in western India (Dhurua and Gujar 2011), and resistance of *Diabrotica virgifera* to Cry3Bb1 corn in Iowa (Gassmann et al. 2011). The reasons for control failure are generally believed to be caused by inadequate refuge size and non–high-dose Bt plants (Tabashnik et al. 2009, Huang et al. 2011).

Currently, refuge strategy is the main method to counter the evolution of resistance in insect populations to Bt crops. Simulation models show that three factors contribute in delaying resistance evolution. These include large areas of refuge, low initial frequency of resistance alleles, and recessive inheritance of resistance (Roush et al. 1998, Carrière et al. 2012). Results from many models and small-scale experiments suggest that refuges could delay resistance substantially (Gould 1998, Shelton et al. 2000, Carrière et al. 2004). To implement refuge strategy, a high expression level of Bt toxin is necessary, which would reduce the fitness of heterozygote, providing that the inheritance of resistance is functionally recessive (Pereira et al. 2008).

Nevertheless, oviposition preference behavior of insects was not considered in these models (Jongsma et al. 2010). If moths were able to avoid laying on Bt crops, then evolution of physiological resistance should be slow, as a smaller fraction of the population will be subject to selection (Zalucki et al. 2012). The oviposition behavior of some lepidopteran pests to Bt and non-Bt plants has been compared in some studies. Both resistant and susceptible diamondback moth adults could not discriminate Bt and non-Bt brassica crops (Ramachandran et al. 1998, Tang et al. 1999, Kumar 2004). In contrast to these results, *Helicoverpa armigera* adults preferred to lay eggs on non-Bt cotton rather than on Bt cotton (Men et al. 2005). However, none of these studies incorporate heterozygous genotype whose behavior plays an important role in the evolution of insect resistance (Roush et al. 1994).

Diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), is a destructive pest in Brassica crops throughout the world. A recent study (Furlong et al. 2013) estimated that annual *P. xylostella* control alone in Brassica crops costs US$1.4 billion worldwide, rising to US$2.7 billion if yield losses are included. The use of insect-resistant cultivars is the most effective and economical method to control the pest. Despite extensive screening, high levels of natural resistance to *P. xylostella* have not been found in Brassica germplasms. An attractive alternative for protection against *P. xylostella* is the production of insecticidal toxins within the Brassica plant itself through genetic engineering.

The primary objective of this study was to determine the oviposition preference of different *P. xylostella* strains to transgenic broccoli with different Bt toxin levels and non-Bt control. This was achieved by comparing the eggs laid on Bt and non-Bt plants from Cry1Ac-resistant, Cry1Ac-susceptible, and F_1 progeny from crossing of resistant with susceptible adults. The larval survival of different genotypes of *P. xylostella* on Bt broccoli was used to assess the inheritance of resistance. The development of the Cry1Ac-resistant and Cry1Ac-susceptible *P. xylostella* on different Bt broccoli lines was studied to compare their adaptability to Bt broccoli under greenhouse condition. These results could enhance the general understanding of Bt resistance and provide useful information for insect resistance management strategy using Bt crops.

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Materials and Methods

Plant and Insect Materials

Three Cry1Ac broccoli plants derived from different T_0 transformants were kindly provided by Prof. Hanxia Li from Huazhong Agricultural University and the plants were already homozygous for the transgene. Vegetative propagation was conducted to enlarge population of each homozygous plant in the laboratory.

Cry1Ac-susceptible (Cry1Ac-SS) and Cry1Ac-resistant (Cry1Ac-RR) strains of *P. xylostella* were provided by the Laboratory of Entomology, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. The Cry1Ac-resistant strain showed 1,000-fold higher resistance than the Cry1Ac-susceptible strain (Yang et al. 2012). F_1 hybrids (Cry1Ac-RS) were developed by mass reciprocal crossing between Cry1Ac-SS and Cry1Ac-RR individuals.

Protein Assays

Seedlings from vegetative propagation were transplanted in the greenhouse under normal operating conditions at 25°C and 85% RH. Detection and quantitative determination of the amount of Cry1Ac protein produced in different Bt broccoli lines at six-leaf stage were measured by enzyme-linked immunosorbant assays (ELISA) kit (kit lot 280101) purchased from Envirologix company (Maine, USA). Three plants of each line were randomly chosen to measure the amount of Cry1Ac toxin, and ELISA was performed according to the manufacturer' instruction with Cry1Ac kit. Untransformed broccoli plants were used as control. The optical density (OD) values of sample were measured by a microplate reader with wave length set at 450 nm. The amount of Cry1Ac was calculated from standard curves obtained with Cry1Ac protein.

Insect Bioassays

Isolated leaves from three Bt broccoli lines were tested for the lethality to different genotypes of *P. xylostella* larvae. Bt broccoli leaves were cut into leaf disks (6 cm in diameter) and placed individually in Petri dishes lined with moistened filter paper. Ten second-instar Cry1Ac-resistant, Cry1Ac-susceptible, or F1 larvae were inoculated on each disk, respectively. The Petri dishes were sealed with parafilms. The number of larvae survived on each disk was recorded on the third day. The damage grade of leaves was assessed following the grading criteria described by Liang et al. (2003): grade 0: no apparent damage; grade 1: <25% leaf area eaten; grade 3: 25–50% leaf area eaten; grade 5: 50–75% leaf area eaten; and grade 7: >75% leaf area eaten. All insect bioassays were conducted with three replications at 26 ± 1°C and a photoperiod of 16:8 (L:D) h.

Oviposition Choice Tests in Laboratory

One undamaged plant from each broccoli line was randomly arranged in a mesh cage (0.7-m long, 0.65-m wide, and 0.65-m high) in laboratory. There was no physical contact among the plants. Five pairs of *P. xylostella* adults (5 females and 5 males) were released into each cage. The adults were allowed to mate in the cage and oviposit on plants. The eggs laid on each plant were counted after 2 days. Oviposition choice tests for Cry1Ac-SS, Cry1Ac-RR, and Cry1Ac-RS strains were carried out respectively with five replications at 26 ± 1°C and a photoperiod of 16:8 (L:D) h.

Development of Susceptible and Resistant diamondback moth in greenhouse

In the greenhouse, three different levels of Bt broccoli line and the control line, nine plants in each line, were transplanted into a cage in a randomized complete block design. The cage was made of nylon netting and it was 4.5-m long by 1.2-m wide by 0.7-m high. At the beginning of the experiment, 20 pairs of *P. xylostella* adults (20 females and 20 males) were released into each cage. Eggs on each plant line were counted after 3 days. The number of larvae (all stage) on each line was recorded when the eggs had developed into larvae. Finally, plants were assessed for damage on a 0–7 grade scale: grade 0: no visible damage on leaves; grade 1: <5% of the leaves with damage; grade 2: 6–10% of the leaves with damage; grade 3: 11–20% of the leaves with damage; grade 4: 21–30% of the leaves with damage; grade 5: 31–60% of the leaves with damage; grade 6: 61–80% of the leaves with damage; and grade 7: >80% of the leaves with damage. Separate tests were conducted for susceptible and resistant *P. xylostella* and replicated three times.

Data Analysis

Analysis of variance (ANOVA) was performed to determine treatment (plant line) effects for bioassays and oviposition tests. The least significant difference (LSD) method was used for comparing means. All analyses were carried out with SAS 8.01 (SAS Institute 2000). The percentage of mortality data was transformed with an arsine square-root transformation before analysis.

Results

Quantification of Cry1Ac Protein in Transgenic Broccoli

Three Bt lines expressing different levels of Bt protein were selected for the experiment. They were designated as T_1, T_2, and T_3, which expressed 167 ± 1.83, 224 ± 1.63, and 246 ± 7.76 ng/g fresh weight, respectively. The amount of Cry1Ac varied greatly in different Bt lines ($P < 0.05$).

Insect Bioassays and Assessment of Resistance Inheritance

Larval mortality of *P. xylostella* on non-Bt leaf tissue was not significantly different among the three insect genotypes ($F = 0.52$, df = 3, and $P = 0.68$). The mortality on T_1 broccoli leaves was 100% for Cry1Ac-susceptible *P. xylostella*, $^{b}F_1$ (SS♂×RR♀), and $^{c}F_1$ (SS♀×RR♂). The average mortality of resistant *P. xylostella* on T_1 and control did not differ ($F = 0.44$, df = 1, $P = 0.54$). None of the larvae from different genotypes could survive on T_2 and T_3 (Table 1). The non-Bt leaves suffered serious damage from all the three genotypes of *P. xylostella* (Fig. 1). The T_1 leaves could suppress feeding damage from susceptible and F_1 *P. xylostella* larvae but suffered serious damage from resistant *P. xylostella* larvae (Fig. 2). These results preliminarily suggested that the inheritance of resistance to the low level of Bt broccoli in the Cry1Ac-resistant strain was recessive.

Oviposition Choice Tests in the Laboratory

The number of eggs on different Bt broccoli plants and non-Bt plants showed no significant difference among Cry1Ac-SS ($F = 0.31$, df = 3, and $P = 0.82$), Cry1Ac-RR ($F = 0.41$, df = 3, and $P = 0.74$), $^{b}F_1$ (SS♂×RR♀) ($F = 0.2$, df = 3, and $P = 0.89$), and $^{c}F_1$ (SS♀×RR♂) ($F = 0.41$, df = 3, and $P = 0.75$). The concentration of Cry1Ac had no effect on the oviposition behavior of *P. xylostella* adults (Table 2).

Development of Susceptible and Resistant P. xylostella in Greenhouse

The average number of eggs laid by susceptible *P. xylostella* on T_1, T_2, T_3, and control was 6.2, 6.9, 7.5, and 7.3, respectively. The average number of eggs laid by resistant *P. xylostella* was 5.7, 4.7, 5.8, and 5.7, respectively. There was no significant difference in the number of eggs laid on different Bt plants for resistant ($F = 3.71$, df = 3, and $P = 0.07$) and susceptible *P. xylostella* ($F = 0.55$, df = 3, and $P = 0.59$) (Table 3).

The average number of susceptible larvae survived on T_1, T_2, T_3, and control was 1.4, 0.2, 0, and 9.3, respectively ($F = 6.53$, df = 3, and $P = 0.03$). The number of susceptible larvae survived on Bt broccoli was less than that on non-Bt control. Few susceptible larvae could survive on all kinds of Bt plants. The average number of resistant larvae survived on T_1, T_2, T_3, and control was 5.7, 0.4, 0, and 8.6, respectively

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Table 1. Larval mortality of *Plutella xylostella* larvae and damage levels of each broccoli line assayed with detached leaves

Plant type	Cry1Ac-SS		Cry1Ac-RR		[b]F_1 (Cry1Ac-RS)		[c]F_1 (Cry1Ac-RS)	
	Mortality (%)	Damage level	Mortality (%)	Damage level	Mortality (%)	Damage level	Mortality (%)	Damage level
Control	6.7 ± 1.3a	5.5 ± 1a	10 ± 0a	5 ± 0a	6.7 ± 5.7a	5 ± 0a	3.3 ± 5.7a	4.3 ± 1.6a
T_1	100 ± 0b	0 ± 0b	16.7 ± 1.3a	4.3 ± 1.1a	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b
T_2	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b
T_3	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b	100 ± 0b	0 ± 0b
F	160.02	72.25	105.70	66.0	160.02	42.25	186.32	42.25
P	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Means within column followed by same letters are not significantly different at $P > 0.05$ (LSD).
[b]F_1: progeny from crossing between CryAc-RR female and Cry1Ac-SS male; [c]F_1: progeny from crossing between Cry1Ac-SS female and CryAc-RR male.

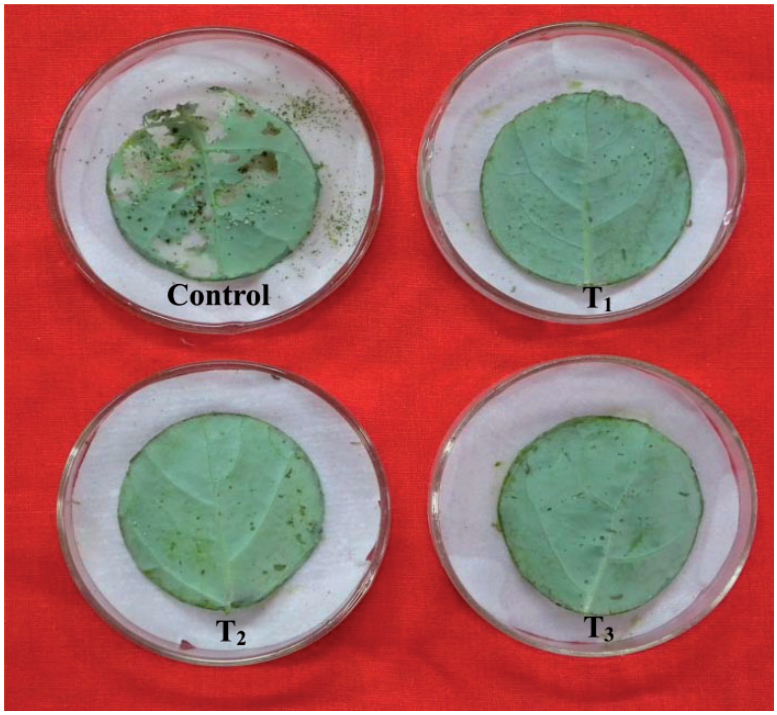

Fig. 1. In vitro bioassay of the transgenic and nontransgenic broccoli plants with susceptible *Plutella xylostella*. Each leaf disk was exposed to 10 susceptible *P. xylostella* larvae. Mortality and damage levels were assessed after 3 days. T_1, T_2, and T_3 were three Bt lines, which expressed 167 ± 1.83, 224 ± 1.63, and 246 ± 7.76 ng/g fresh weight Bt toxin, respectively.

($F = 26.3$, df = 3, and $P < 0.05$). The number of resistant larvae survived on T_1 was lower than that survived on control ($F = 10.21$, df = 1, and $P < 0.05$). The average number of resistant larvae survived on T_2 and T_3 was 0.4 and 0, respectively. Few resistant larvae were found on T_2 and T_3 plants (Table 3).

Plant injury was highly correlated with the larval survivorship. The damage grade of T_1, T_2, T_3, and control caused by susceptible *P. xylostella* was 0.5, 0.1, 0, and 2.2, respectively. The damage grade of Bt broccoli was less than that of non-Bt broccoli ($F = 7.58$, df = 3, and $P = 0.014$). Three Bt broccoli lines suffered little damage from susceptible *P. xylostella*. The damage grade of T_1, T_2, T_3, and control caused by resistant *P. xylostella* was 1.7, 0.7, 0.1, and 2.6, respectively ($F = 20.68$, df = 3, and $P = 0.0007$). The damage extent of non-Bt plants caused by resistant and susceptible *P. xylostella* did not differ significantly ($F = 2.06$, df = 1, and $P > 0.05$). T_1 suffered serious damage, while T_2 and T_3 suffered little damage from resistant *P. xylostella* (Table 3).

Discussion

With the widespread cultivation of transgenic crops, several cases of resistance to Bt crops have been documented (van Rensburg 2007, Storer et al. 2010, Dhurua and Gujar 2011). Concerns have been expressed that field resistance to transgenic Bt plants could develop rapidly and reduce the sustainability of Bt technology (Shelton et al. 2000, Tabashnik et al. 2005). To counter the challenge of insect resistance, it is important to understand the mechanism of resistance alleles in insects to Bt crops because the refuge strategy was based on the basis of recessive inheritance. The mode of resistance mechanism in *P. xylostella* is complicated. It has been assumed that resistance to Bt toxins is inherited mainly as a recessive trait in *P. xylostella* (Tang et al. 1997; Zhao et al. 2000, 2003). Incomplete dominant inheritance to Cry1C and Cry1Ac toxins have been reported in *P. xylostella*, and the dominance of resistance was associated with Bt concentration (Liu and Tabashnik 1997, Sayyed et al. 2000). In the bioassays, the Cry1Ac-resistant larvae could survive on the low level of Bt broccoli and caused severe damage. The Cry1Ac-susceptible and F_1 larvae could not survive on the three Bt broccoli lines. The inheritance of resistance to Bt broccoli in the Cry1Ac-resistant strain was assessed as recessive. No maternal effects were associated with the resistance. However, the three genotypes of *P. xylostella* larvae could not survive on relative medium and high levels of Bt broccoli.

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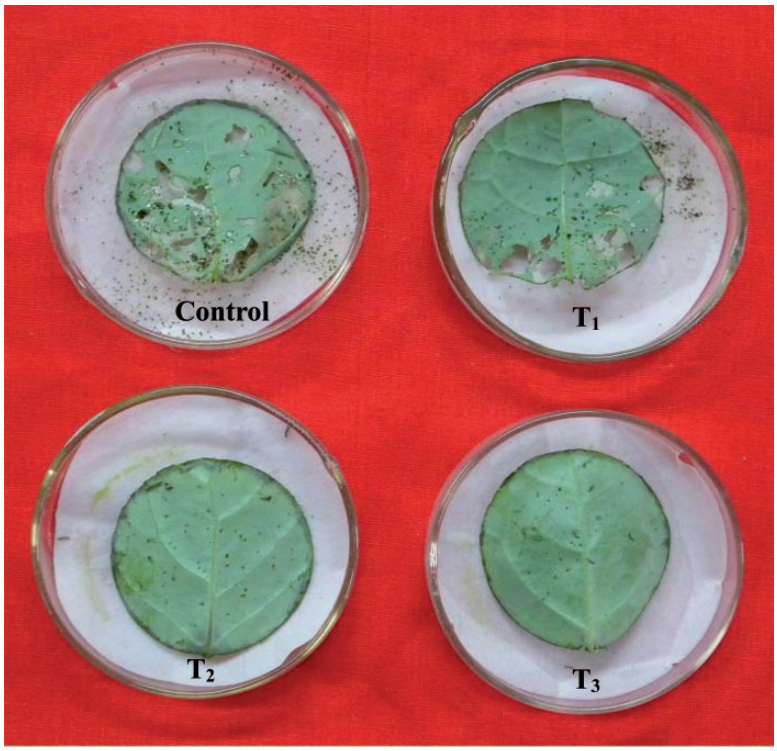

Fig. 2. In vitro bioassay of the transgenic and nontransgenic broccoli plants with Cry1Ac-resistant *P. xylostella*. Each leaf disc was exposed to 10 resistant *P. xylostella* larvae. Mortality and damage levels were assessed after 3 days. T_1, T_2, and T_3 were three Bt lines which expressed 167 ± 1.83, 224 ± 1.63, and 246 ± 7.76 ng/g fresh weight Bt toxin, respectively.

Table 2. Number of eggs laid by different genotypes of *Plutella xylostella* adults in laboratory

Plant type	Mean number of eggs (±SEM)			
	Cry1Ac-SS	Cry1Ac-RR	[b]F_1 (Cry1Ac-RS)	[c]F_1 (Cry1Ac-RS)
Control	68.4 ± 10.8a	55.4 ± 10.1a	59.2 ± 9.1a	62.2 ± 13.6a
T_1	62 ± 12.8a	60.6 ± 14.8a	58.8 ± 12.1a	57.8 ± 10.2a
T_2	66 ± 6.9a	54.8 ± 11.2a	55.2 ± 10.4a	63.8 ± 10.9a
T_3	64 ± 12.4a	62.2 ± 15a	59.2 ± 6.1a	64.4 ± 5.5a
F	0.31	0.41	0.20	0.41
P	0.82	0.74	0.89	0.75

Means within column followed by same letters are not significantly different at $P > 0.05$ (LSD).

[b]F_1: progeny from crossing between CryAc-RR female and Cry1Ac-SS male; [c]F_1: progeny from crossing between Cry1Ac-SS female and CryAc-RR male.

Table 3. Oviposition and survival of susceptible and resistant *Plutella xylostella* in greenhouse tests

Plant type	Number of eggs (±SEM)		Number of larvae (±SEM)		Plant injury (±SEM)	
	Cry1Ac-SS	Cry1Ac-RR	Cry1Ac-SS	Cry1Ac-RR	Cry1Ac-SS	Cry1Ac-RR
Control	7.3 ± 1.2a	5.7 ± 0.4a	9.3 ± 1.1a	8.6 ± 1.2a	2.2 ± 0.7a	2.6 ± 0.4a
T_1	6.2 ± 0.5a	5.7 ± 1.0a	1.4 ± 0.3b	5.7 ± 1.0b	0.5 ± 0.1b	1.7 ± 0.2a
T_2	6.9 ± 0.8a	4.7 ± 1.0a	0.2 ± 0.3c	0.4 ± 0.5c	0.1 ± 0.1c	0.7 ± 0.1b
T_3	7.5 ± 0.8a	5.8 ± 0.2a	0 ± 0c	0 ± 0d	0 ± 0c	0.1 ± 0.1c
F	3.71	0.55	6.53	26.3	7.58	20.68
P	0.07	0.59	0.03	0.0003	0.014	0.0007

Means within column followed by same letters are not significantly different at $P > 0.05$ (LSD).

The results of the greenhouse trial were similar to that of laboratory bioassays. The number of Cry1Ac-resistant and Cry1Ac-susceptible *P. xylostella* that survived on untransformed broccoli was not significantly different. It indicated that the adaptability of resistant *P. xylostella* to normal broccoli did not reduce. Cry1Ac-resistant *P. xylostella* could complete development on low level of Bt broccoli and caused significant plant injury. But the number of the resistant larvae survived on low level of Bt broccoli was fewer than that survived on control. Few live resistant larvae or pupae were found on broccoli with high levels of Bt toxin. It suggested that this strain was not able to complete its larval development on high levels of Bt broccoli.

Oviposition behavior of *P. xylostella* is affected by a lot of factors, including host plant species, chemical cues, and ecological conditions (Thompson and Pellmyr 1991, Henniges-Janssen et al. 2011, Liu et al. 2012). The effects of Bt plants on oviposition preference of Cry1Ac-susceptible, Cry1Ac-resistant, and F_1 adults were assessed in this study. At the beginning of cultivation of Bt crops, the frequency of resistant alleles is low in insect populations and resistant alleles of the population will be most prevalent in hybrid genotype of insects (Roush et al. 1997). Therefore, the behavior of heterozygous pests may have enormous effects on the evolution of resistance to Bt crops. For example, it would result in nonrandom oviposition and accelerating resistance evolution if heterozygous females preferred to oviposit on Bt plants. Fortunately, heterozygous *P. xylostella* showed no preference between Bt and non-Bt broccoli in oviposition. The data seem reasonable to assume that eggs laid by different *P. xylostella* strains are distributed randomly between transgenic and nontransgenic crops.

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