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Temporal allocation of metabolic tolerance to transgenic Bt cotton in beet armyworm, *Spodoptera exigua* (Hübner)

GUO JianYing[1*], WU Gang[1,2] & WAN FangHao[1*]

[1]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China;*
[2]*College of Plant Sciences and Technology, Huazhong Agricultural University, Hubei 430070, China*

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Digestive and detoxification enzyme activity and nutrient composition were examined in the body of fourth instar beet armyworms, *Spodoptera exigua* (Hübner), fed on transgenic *Bacillus thuringiensis* (Bt) and non-Bt cotton for different time periods. Nutrient composition and specific enzyme activities differed significantly between the *S. exigua* fed Bt vs. non-Bt cotton. At 1, 6 and 24 h, free fatty acid and glucose levels were significantly lower in *S. exigua* fed on Bt cotton than those fed on non-Bt cotton. *S. exigua* fed on Bt cotton had significantly higher trypsin and total superoxide dismutase (T-SOD) activities and significantly lower lipase, carboxylesterase and acetylcholinesterase activities than non-Bt fed worms for all feeding time periods. Differences were also observed among feeding times within each cotton variety group. Significantly lower free fatty acid and total amino acid were observed in *S. exigua* fed on Bt cotton for 24 h than in those fed for 1 h. Significantly lower activities of lipase and trypsin were detected in *S. exigua* fed on Bt cotton for 24 h than those for 1 and 4 h. However, carboxylesterase and acetylcholinesterase activities in *S. exigua* fed on Bt cotton for 24 h were significantly higher than those for 1, 4 and 6 h. The interaction between cotton variety and feeding time significantly affected the activities of lipase, trypsin, acetylcholinesterase and T-SOD enzymes in *S. exigua*. Measuring the temporal allocation of protection and detoxification enzyme activities in the body of *S. exigua* in response to *B. thuringiensis* can provide a meaningful evaluation on the metabolic tolerance of herbivorous insects under the continuous selection pressure of a toxic protein.

***Bacillus thuringiensis*, *Spodoptera exigua*, detoxification enzyme, carboxylesterase, acetylcholinesterase**

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Crop losses caused by insect pests, estimated at 10%–20% for major crops, are a significant factor limiting food production [1]. Insecticide application against the larval stage of herbivorous insects is the primary control method; however, a high tolerance to most insecticides and associated environmental problems may jeopardize their continued use [2]. Genetically engineered cotton plants, which exhibit enhanced-resistance or tolerance to insect pests, are a major success of transgenic technology. During the past decade, *Bacillus thuringiensis* (Bt) cotton (*Gossypium hirsutum*) plantings in China have expanded quickly and transgenic Bt cotton is currently the main genetically modified (GM) crop commercialized on a large scale in this country [3,4].

Bt plants have great potential in integrated pest management (IPM) programs. They may be used to complement the effects of other biological control agents because of their higher selectivity than most insecticides [4–6]. It has been concluded that the mechanism of action of the Bt *Cry* proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the *Cry* toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion

*Corresponding author (email: guojy@mail.caas.net.cn; wanfh@mail.caas.net.cn)

channels or pores [7]. When ingested by larvae, toxin proteins bind to specific receptors in the midgut region, and toxin binding in susceptible insects disrupts the midgut epithelium, thereby causing overall toxic effects and ultimately resulting in larval death [8]. Transgenic Bt cotton that expresses the Cry1Ac toxin is highly effective against the major lepidopterous cotton insect pests [9]. Multiple field trials have proven that Bt cotton exhibits high insecticidal efficacy against larvae of tobacco budworm, cotton bollworm and other target lepidopterous pests and decreases the population densities of these target pest species in cotton fields [10–12]. However, the evolution of resistance or tolerance to Bt toxins by herbivorous insects is a serious threat to the efficacy of Bt-based insecticides and toxin-producing transgenic plants, which are generally pest specific and environmentally safe [13,14].

Not all lepidopteran pest species are equally susceptible to *B. thuringiensis*. Some lepidopteran species can survive after feeding on transgenic Bt cotton expressing Cry1Ac toxin [15,16]. Some non-lepidopteran herbivores are also unaffected by the toxin. Beet armyworm, *Spodoptera exigua* (Hübner), is an important pest in numerous crops, including cotton, and causes economic damage in China. However, *S. exigua* is not a target pest of currently commercialized transgenic Bt cotton varieties in China. It was added to the prediction list of outbreaking insect pests in China in 2001 [17]. The failure of chemical measures to control this insect has shifted the emphasis towards other effective strategies (e.g., transgenic crops or natural enemies) in an IPM program. However, the effect of Bt transgenic plants on non-target organisms is becoming increasingly important in agro-ecosystems [5]. There is concern about the widespread development of Bt resistance or tolerance in beet armyworm due to reports of field resistance to Bt toxins in several lepidopteran families [13]. Guo *et al*. [18] reported that significantly lower carboxylesterase (CarE) was observed in the larvae of *Micromelalopha troglodyte* fed on the leaves of transgenic poplars and revealed that these plants disturbed the insect metabolism mainly by restraining the activities of CarE in the midgut of larvae and subsequently caused larval death. Because the control efficacy of Bt cotton depends on the expression of *Cry* genes through synthesis of insecticidal protein [19], the temporal variation of efficacy may lead to insufficient control of herbivorous insects and the evolution of resistance or tolerance [20]. To date, there is limited information regarding the temporal variation of enzymes activities in the body of *S. exigua* in response to *B. thuringiensis* and further evaluation of these factors is necessary.

In this study, nutrient composition (protein, total amino acid, free fatty acid and glucose) and activities of digestive enzymes (i.e., lipase, trypsin and amylase) and protection and detoxification enzymes (i.e., CarE, acetylcholinesterase (AChE) and total superoxide dismutase (T-SOD)) were examined in *S. exigua* fed on transgenic Bt cotton (cv. GK-12) or non-Bt cotton (cv. Simian-3) for different time periods. This study aimed to (i) quantify the temporal allocation of metabolic tolerance in the body of *S. exigua* in response to *B. thuringiensis*; (ii) evaluate the interaction between cotton variety (GK-12 vs. Simian-3) and beet armyworm feeding time (i.e., 1, 4, 6 and 24 h) on digestive and detoxification enzymes and nutrient composition in *S. exigua*.

1 Materials and methods

1.1 Cotton variety and growth conditions

Two cotton cultivars were used in the study, including a transgenic Bt cultivar 'GK-12' and its parental non-transgenic cultivar 'Simian-3'. Both cultivars were planted in plastic pots (15 cm in diameter, 13 cm in height, one plant per pot) in a thermostatic chamber, in which the temperature was maintained at (28±1)°C and relative humidity (RH) was maintained at 70%–80%. Eighty pots for each cotton cultivar were randomly placed in the thermostatic chamber and re-randomized once a week to minimize position effects. No chemical fertilizers or insecticides were used throughout the entire experiment.

1.2 Beet armyworm stocks

Egg masses of *S. exigua* were obtained from the Insect Virology Laboratory, Institute of Zoology, Chinese Academy of Sciences (CAS), and hatched in a growth chamber (PRX-500D-30; Haishu Safe Apparatus, Ningbo, Zhejiang Province, China). The chamber was maintained at 75%±5% RH, (28±0.5)°C, with a photoperiod of 14-h light/10-h dark at 30000 LX of active radiation supplied by 26 W fluorescent lamps (n=39). The newly hatched larvae were fed on artificial diet as described by Li *et al*. [21].

1.3 Beet armyworm feeding treatments

The beet armyworm feeding experiment was carried out in another thermostatic chamber, using the same conditions as mentioned above for the cotton growth.

During the seven-leaf stage (approximately 35–40 d after planting) of cotton plants, the fourth instar *S. exigua* larvae from laboratory cultures were randomly collected from the growth chamber as described above. Larvae were placed on the fourth leaf from the base of the plant, with one larva per pot. Each set was placed in a cubic cage (60 cm for each side) covered with a fine mesh net to prevent the incursion of other insects. Five pots were used with four replications per cotton cultivar for four treatments of *S. exigua* (i.e., feeding time for 1, 4, 6 and 24 h), with a total of 20 insects per feeding time. After *S. exigua* inoculation for 1, 4, 6 and 24 h, beet armyworm larvae were collected to measure di-

gestive and detoxification enzyme activities and nutrient compositions.

1.4 Beet armyworm enzyme activity assays

Biochemical assays were conducted to test for significant changes in enzyme activities in *S. exigua* in response to *B. thuringiensis*. Four nutrient composition types (protein, total amino acid, free fatty acid and glucose), three digestive enzymes (lipase, trypsin, amylase) and three protection and detoxification enzymes (CarE, AChE, T-SOD) were used to test for metabolic changes in *S. exigua* larvae fed on transgenic Bt cotton (cv. GK-12) or non-Bt cotton (cv. Simian-3). CarE activity was determined by the method of Van Asperen [22]. Nutrient compositions and other enzymes activities were measured according to the manufacturer's instructions for assay kits (Nanjing Jiancheng Co., Ltd., Nanjing, Jiangsu Province, China). Enzyme activities were presented relative to protein concentration, which was determined using the method of Bradford [23] with bovine serum albumin (Nanjing Jiancheng Co., Ltd., Nanjing, Jiangsu Province, China) as the standard.

1.5 Data analysis

One-way analysis of variance (ANOVA) [24] was used to compare differences in nutrient composition and enzyme activity in *S. exigua* after being fed on transgenic Bt cotton (cv. GK-12) and non-Bt cotton (cv. Simian-3) for different time periods. Two-way ANOVAs were used to analyze the impacts of cotton cultivar and beet armyworm feeding time, along with their interactions, on the above indices. Differences between means were compared using the least significant difference (LSD) test.

2 Results

2.1 Nutrient compositions in the fourth instar *S. exigua* larvae fed on transgenic Bt cotton vs. non-Bt cotton

2.1.1 Protein and total amino acid

Cotton variety significantly affected the protein ($P<0.001$) and total amino acid ($P<0.01$) levels in the body of *S. exigua*. Interactions between cotton variety and *S. exigua* feeding time significantly influenced protein ($P<0.05$) and total amino acid ($P<0.01$) levels (Table 1).

Protein level in *S. exigua* increased significantly after being fed on Bt cotton for 6 ($P<0.01$) and 24 h ($P<0.001$), compared with those fed on non-Bt cotton for the same time periods. Significantly lower protein levels were detected in *S. exigua* fed on Bt cotton for 1 h than those for 4, 6 and 24 h ($F=3.74$, $df=3, 12$, $P=0.0415$) (Figure 1A). Total amino acids in *S. exigua* also decreased significantly after being fed on Bt cotton for 6 ($P<0.01$) and 24 h ($P<0.001$), compared with those fed on non-Bt cotton for the same period. Significantly higher total amino acids were detected in *S. exigua* fed on Bt cotton for 1 and 4 h than those fed for 6 and 24 h ($F=15.84$, $df=3, 12$, $P=0.0002$) (Figure 1B).

Table 1 Effects of cotton variety, beet armyworm feeding time and their interactions on nutrient compositions and the activities of digestive enzymes and detoxification enzymes in fourth instar *Spodoptera exigua* larvae[a)]

Measured indexes	Variety	Time	Variety×time
Protein	0.0001***	0.4961	0.0218*
Total amino acid	0.0072**	0.066	0.0026**
Free fatty acid	0.0001***	0.0344*	0.1657
Glucose	0.0001***	0.9551	0.3166
Lipase	0.0001***	0.9595	0.0019**
Trypsin	0.0001***	0.0001***	0.0001***
Amylase	0.5619	0.9711	0.8659
CarE	0.0001***	0.0036**	0.1061
AChE	0.0001***	0.7183	0.0018**
T-SOD	0.0001***	0.0925	0.0004***

a) Variety, cotton variety, Simian-3 and GK-12; time, beet armyworm feeding time for 1, 4, 6 and 24 h; CarE, carboxylesterase; AChE, acetylcholinesterase; T-SOD, total superoxide dismutase. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

2.1.2 Free fatty acid and glucose

Cotton variety significantly affected free fatty acid ($P<0.001$) and glucose ($P<0.001$) levels in *S. exigua*. Beet armyworm feeding time also significantly influenced the level of free fatty acid ($P<0.05$) (Table 1).

The free fatty acid level in *S. exigua* decreased significantly after being fed on Bt cotton for 6 ($P<0.001$) and 24 h ($P<0.001$), compared with those fed on non-Bt cotton for the same period (Figure 1C). Significantly lower glucose ($P<0.01$) was detected in *S. exigua* fed on Bt cotton for 1, 6 and 24 h, compared with those fed on non-Bt cotton for the same period (Figure 1D).

2.2 Activities of the digestive enzymes in fourth instar *S. exigua* larvae fed on transgenic Bt cotton vs. non-Bt cotton

2.2.1 Lipase

Cotton variety significantly affected *S. exigua* lipase activity ($P<0.001$). Interactions between cotton variety and feeding time significantly influenced the lipase activity in *S. exigua* ($P<0.01$) (Table 1).

Lipase activity in *S. exigua* decreased significantly after being fed on Bt cotton for 24 h ($F=6.13$, $df=3, 12$, $P=0.009$) than those for 1, 4 and 6 h. Significantly lower lipase activity was detected in *S. exigua* fed on Bt cotton for 1 ($P<0.001$), 4 ($P<0.01$), 6 ($P<0.001$) and 24 h ($P<0.001$), compared with those on non-Bt cotton for the same periods (Figure 2A).

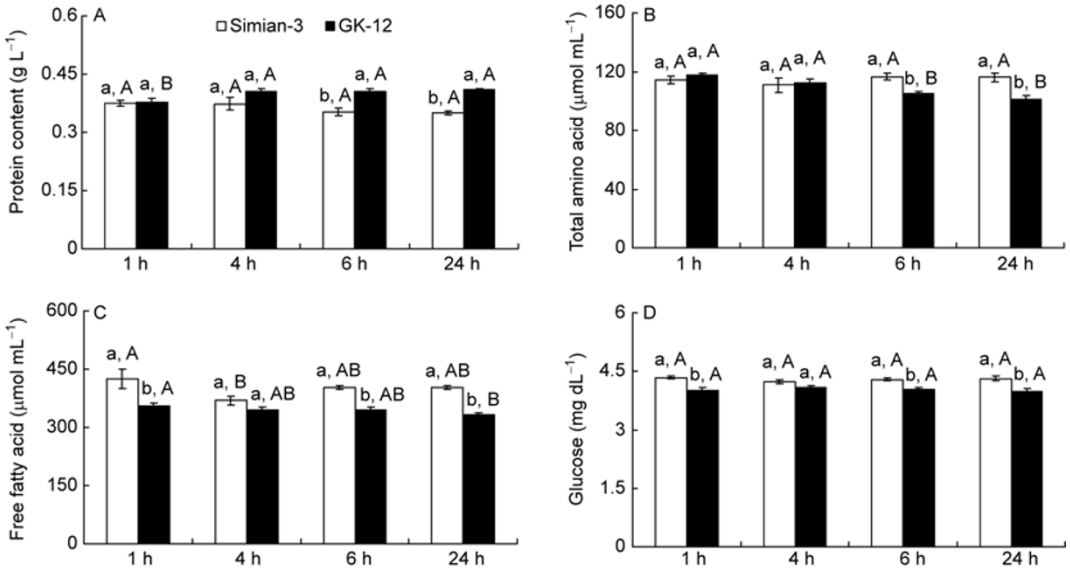

Figure 1 Mean±SE nutrient compositions in the body of fourth instar *Spodoptera exigua* larvae fed on transgenic Bt cotton (cv. GK-12) or non-Bt cotton (cv. Simian-3) for different times (1, 4, 6 and 24 h). Data within the same group that are indicated with different lowercase letters are significantly different (P<0.05) between different cotton varieties; data within the same group that are indicated with different uppercase letters are significantly different (P<0.05) among different feeding times. A, protein; B, total amino acid; C, free fatty acid; D, glucose.

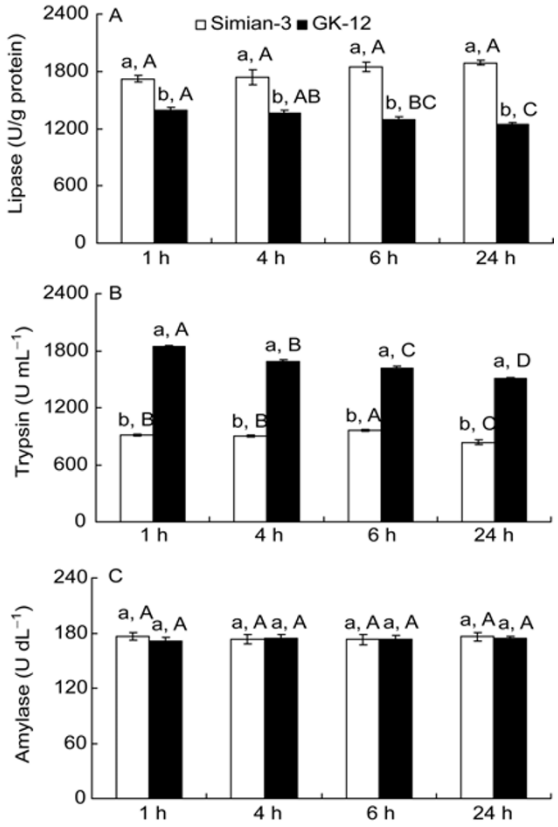

Figure 2 Mean±SE digestive enzyme activities in the body of fourth instar *Spodoptera exigua* larvae fed on Bt cotton (cv. GK-12) or non-Bt cotton (cv. Simian-3) for different times (1, 4, 6 and 24 h). Data within the same group that are indicated with different lowercase letters are significantly different (P<0.05) between different cotton varieties; data within the same group that are indicated with different uppercase letters are significantly different (P<0.05) among different feeding times. A, lipase; B, trypsin; C, amylase.

2.2.2 *Trypsin*

Cotton variety and feeding time significantly affected the trypsin activity in *S. exigua* (P<0.001). Interactions between cotton variety and feeding time also significantly affected *S. exigua* trypsin activity (P<0.001) (Table 1).

Trypsin activity in *S. exigua* decreased significantly after being fed on Bt cotton (F=85.52, df=3, 12, P=0.0001) and non-Bt cotton (F=12.19, df=3, 12, P=0.0006) for 24 h, compared with those fed on the same cotton variety for 1, 4 and 6 h. Significantly higher trypsin activity (P<0.001) was detected in *S. exigua* fed on Bt cotton for 1, 4, 6 and 24 h, compared with those on non-Bt cotton for the same periods (Figure 2B).

2.2.3 *Amylase*

Cotton variety, feeding time and their interactions had no effect on the *S. exigua* amylase activity (P>0.05) (Table 1).

Amylase activity in *S. exigua* did not differ significantly (P>0.05) after being fed on Bt cotton for different periods. This was also the case for those fed on non-Bt cotton for different time periods. Amylase activities in *S. exigua* also did not differ significantly (P>0.05) between those fed on Bt cotton or non-Bt cotton for the same periods (Figure 2C).

2.3 Activities of protection and detoxification enzymes in fourth instar *S. exigua* larvae fed on transgenic Bt cotton vs. non-Bt cotton

2.3.1 *AChE*

Cotton variety significantly affected the *S. exigua* AChE activity (P<0.001). Interactions between cotton variety and feeding time significantly influenced *S. exigua* AChE activ-

ity (P<0.01) (Table 1).

AChE activity in *S. exigua* increased significantly after being fed on Bt cotton (F=7.58, df=3, 12, P=0.0042) for 24 h, compared with those fed for 1, 4 and 6 h. Significantly lower AChE activities (P<0.001) were detected in *S. exigua* fed on Bt cotton for 1, 4, 6 and 24 h, compared with those fed on non-Bt cotton for the same periods (Figure 3A).

2.3.2 CarE

Cotton variety significantly affected *S. exigua* CarE activity (P<0.001). Feeding time also significantly influenced *S. exigua* CarE activity (P<0.01) (Table 1).

CarE activity in *S. exigua* increased significantly after being fed on Bt cotton (F=6.14, df=3, 12, P=0.009) or non-Bt cotton (F=3.74, df=3, 12, P=0.0416) for 24 h, compared with those fed on the same cotton variety for 1, 4 and 6 h. Significantly lower CarE activities (P<0.001) were detected in *S. exigua* fed on Bt cotton for 1, 4, 6 and 24 h, compared with those fed on non-Bt cotton for the same periods (Figure 3B).

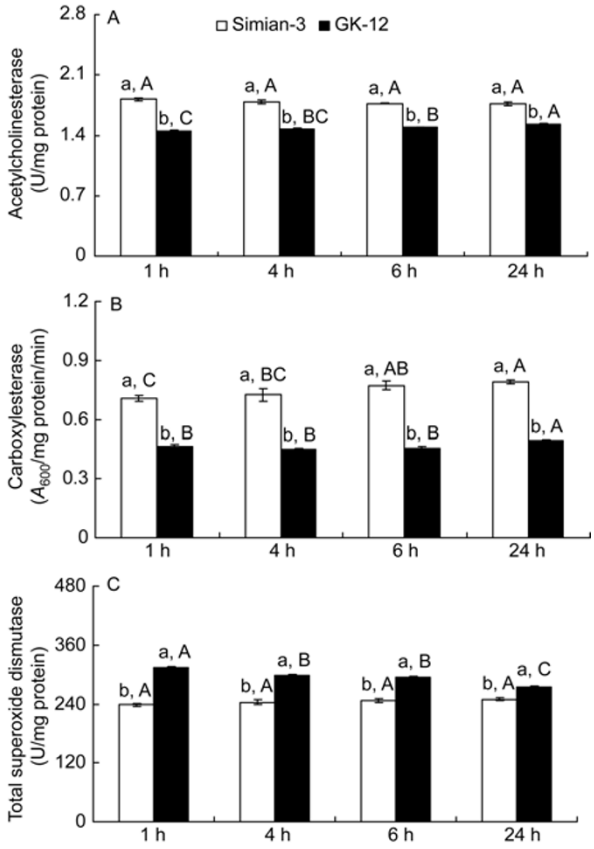

Figure 3 Mean±SE activities of protection and detoxification enzymes in fourth instar *Spodoptera exigua* larvae fed on Bt cotton (cv. GK-12) or non-Bt cotton (cv. Simian-3) for different times (1, 4, 6 and 24 h). Data within the same group indicated with different lowercase letters are significantly different (P<0.05) between different cotton varieties; data within the same group indicated with different uppercase letters are significantly different (P<0.05) among different feeding times. A, acetylcholinesterase; B, carboxylesterase; C, total superoxide dismutase.

2.3.3 T-SOD

Cotton variety significantly affected the activities of T-SOD in *S. exigua* (P<0.001). Interactions between cotton variety and feeding time also significantly influenced *S. exigua* T-SOD activity (P<0.001) (Table 1).

T-SOD activity in *S. exigua* decreased significantly after being fed on Bt cotton (F=33.29, df=3, 12, P=0.0001) for 24 h, compared with those fed for 1, 4 and 6 h. Significantly higher T-SOD activities were detected in *S. exigua* fed on Bt cotton for 1 (P<0.001), 4 (P<0.001), 6 (P<0.001) and 24 h (P<0.001), compared with those fed on non-Bt cotton for the same periods (Figure 3C).

3 Discussion

Annual production of GM crops exceeded 1×10^8 hm^2 in 2006. This included Bt cotton expressing insecticidal proteins that occupied 1.34×10^7 hm^2, or 13% of the total [3]. In China, planting of Bt cotton has grown from 6.3×10^4 hm^2 in 1998 to 3.5×10^6 hm^2 in 2006, which is equivalent to approximately 60% of the total cotton area in this country [3,25]. Transgenic Bt cotton is considerably effective in controlling lepidopteran pests, and is highly beneficial to growers and the environment by reducing chemical insecticide sprays and preserving populations of beneficial arthropods [26,27]. Currently, there are many publications that have focused on the development and reproduction of herbivorous insects in response to *B. thuringiensis*. For example, Dutton *et al*. [28] reported that larval mortality and development of the Egyptian armyworm, *Spodoptera littoralis* (Boisduval), were negatively affected by Bt maize. Liu *et al*. [29] reported that larval growth of the cotton bollworm, *Helicoverpa armigera* (Hübner), was prolonged and its pupation rate and pupal weight decreased when fed on a diet containing Cry1Ac toxin. Liu *et al*. [30] reported that aphids fed on transgenic Bt+CpTI cotton had a shorter reproduction period, longer lifespan, lower survival rate, and earlier occurrence of peak daily mortality in the first or second generation when compared with those fed on non-transgenic cotton.

Insect resistance or tolerance problems is another issue that should be viewed as a part of integrated management strategies including IPM and integrated resistance management (IRM) programs [27]. Since the first commercial release of GM crops expressing Bt genes, there have been environmental impact concerns. In particular, the continuous expression of the insecticidal protein in most tissues of the plant throughout the growing season has raised concerns regarding the development of resistance or tolerance in the target pest, and the possible impacts of this new pest control technology on various groups of non-target organisms of ecological and economic values [20,31,32]. Most herbivores are affected by the physiological and nutritional states of their host plants [33], such as cotton plants expressing

Cry1Ac toxin. Some pest species have also developed resistance or tolerance to Bt toxin under the continuous selection pressure of the toxic protein [2,34].

Contrasting effects of Bt toxin on herbivorous insects, such as pink bollworm and tobacco budworm, have been reported [35,36]. Chen *et al*. [37] reported that the population-trend index of cotton bollworm decreased significantly when fed on Bt cotton, compared with the non-Bt cotton fed control. *Plutella xylostella* fed on a diet containing Bt laid fewer eggs with lower viability, had reduced larval survival and adult eclosion rates, and ultimately reduced fecundity [38,39]. Carriere *et al*. [40] reported that resistance to Cry1Ac in *Pectinophora gossypiella* was associated with over-wintering fitness costs. Other pest species have also been reported to develop Bt resistance under the continuous selection pressure of the Bt toxin [2,34]. However, not all lepidopteran pest species are equally susceptible to Bt. Some experiments emphasized on the direct negative effects of Bt toxins on non-target herbivores [15,16,41].

Beet armyworm, a non-target herbivorous insect of Bt cotton, is an important pest in numerous crops and causes economic damage in China. Failure to chemically control this species has shifted the emphasis towards the use of other effective strategies (e.g., transgenic crops) as part of an IPM program. However, widespread concerns about the development of Bt resistance in beet armyworm exist due to previous field resistance to Bt toxins reported in several lepidopteran families [13]. Most research was conducted using Bt toxins against *S. exigua* [42–44], including the highly effective Cry1C toxin [45,46]. A high level of Cry1C resistance (>500-fold) was found to develop in *S. exigua* [45].

Transgenic Bt cotton plants are expected to affect certain enzyme activities in lepidopteran herbivores. These include AChE, T-SOD and CarE, which are important protection and detoxification enzymes in herbivorous insect bodies. T-SOD plays an important role in balancing the herbivorous insects' oxidation and anti-oxidation effects, while also protecting the insects' cell from damage caused by harmful environmental factors. AChE directly participates in the functional adjustment of an insect's nervous system. Reduced AChE activity occurs when the insect is exposed to unfavorable environmental stimuli. In our study, the AChE activities in *S. exigua* decreased significantly by 25.3%, 21.4%, 18.6% and 15.3% when fed on transgenic Bt cotton for 1, 4, 6 and 24 h, respectively, compared with those fed on non-Bt cotton for the same periods. However, the activities of T-SOD increased significantly by 31.5%, 22.3%, 18.6% and 10.2% after 1, 4, 6 and 24 h, respectively. These results indicate that *S. exigua* can develop significant resistance or tolerance to Bt toxin under continuous selection pressure. We can further speculate that Bt cotton induces changes in the detoxification enzymes of *S. exigua* under the continuous selection pressure of Bt toxin protein. Such enzymatic changes may promote *S. exigua* survival on Bt cotton and induce the development of larval resistance or tolerance to Bt toxins [6].

Our results indicated that cotton variety significantly affected the activities of the tested digestive enzymes (i.e., lipase and trypsin) except for amylase and the protection and detoxification enzymes (i.e., AChE, CarE and T-SOD) in *S. exigua*. This indicated that Bt cotton expressing Cry1Ac toxin acted effectively against the tested *S. exigua* enzymes in a similar way to other target pests. Beet armyworm feeding time only significantly affected the activities of trypsin and CarE enzymes in *S. exigua*. This indicated that the difference between Bt and non-Bt cotton varieties compared with the beet armyworm feeding time had an overwhelming effect on *S. exigua* enzymatic activity. The interaction between cotton variety and beet armyworm feeding time significantly affected the AChE and T-SOD enzyme activities in *S. exigua*. However, this interaction had no effect on the CarE activity. These results indicated that the activities of different protection and detoxification enzymes in *S. exigua* were not equally susceptive to Bt cotton after different feeding times.

The development and implementation of effective IRM programs is critical to ensure the long-term durability of Bt plants [47]. Our study successfully provides a profile to exemplify the direct effects of Bt cotton on nutrient composition and enzyme activities in *S. exigua* and the responses of this pest species to Bt after different feeding times. Measuring the temporal allocation of protection and detoxification enzyme activities in the body of *S. exigua* in response to Bt can provide a meaningful evaluation of metabolic resistance or tolerance of herbivorous insects under the continuous selection pressure of a toxic protein.

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