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The oral toxicity of the transgenic Bt+CpTI cotton pollen to honeybees (*Apis mellifera*)

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ABSTRACT

Transgenic insect-resistant cotton has been planted in China in a large scale and may have adverse impacts on honeybees. Pollens from the transgenic Cry1Ac+CpTI cotton Zhong-41 and the parental cotton Zhong-23 were collected from the field and their impacts on adult worker bees were assessed. Experimental results showed that Zhong-41 pollen had no acute oral toxic effect on worker bees. No significant differences were observed in the superoxide dismutase activity or in the longevity of worker bees fed with diets containing the two cotton pollens. The main reasons for the outcome may be the low expression level of the transgenic proteins Cry1Ac and CpTI in the pollen of Zhong-41 as well as the substantial equivalence in the amounts of gross protein and soluble saccharides for the two cotton pollens. The implications of these results are discussed and further work to be carried out is put forward.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is a very important crop for China. More than 5 million ha of cotton are planted each year. Over the past two decades, damage caused by the cotton bollworm, *Helicoverpa armigera* (Hübner), had become the most important restricting factor for cotton yield in China. In order to effectively control *H. armigera*, which had become resistant to nearly all chemical insecticides, a series of transgenic cotton varieties expressing toxic proteins encoded by genes from *Bacillus thuringiensis* (Bt) were developed. Transgenic Bt cotton varieties have been proven effective in eliminating outbreaks of some Lepidopteran pests, and have been commercialized in China since 1997 (Xie et al., 1991; Cui and Guo, 2001). At the same time, the possibility of resistance to transgenic Bt cotton in *Helicoverpa armigera* was considered, and transgenic cotton expressing both the Bt protein (Cry1Ac) and the CpTI (cowpea trypsin inhibitor protein, hereafter referred as Bt+CpTI cotton) was developed and commercialized in 2001 (Guo et al., 1999). In recent years, the planted area of transgenic insect-resistant cotton varieties has reached more than 70% of the total cotton planted area in China (Clive, 2007; Stone, 2008). Though it has led to economic benefit, concerns have arisen regarding the potential adverse impacts of transgenic crops on biodiversity and human health (Dale et al., 2002; Doblhoff-Dier and Collins, 2001). In addition, the Yangtze and Yellow River Valleys are the main areas for both the planting of transgenic cotton and the apiculture industry in China. In these areas, the flowering period of cotton is from June to September, and the period when *Apis mellifera*, one of the main pollinators for cotton, collects pollen from April to October. With the commercialization of transgenic cotton on a large scale, pollinating insects such as *A. mellifera* have a greater exposure to pollen from transgenic insect-resistant cotton, lasting about 3 months annually.

There are seven million colonies of honeybees in China and honey production is the highest in the world at about 210 thousand tons per year (Chen, 2001). Furthermore, at least one-third of all crops are pollinated by insects or other animals. Pollinating insects, including bees, have an important role in crop yield, quality and maintenance of biodiversity in nature (Klein et al., 2007). Concerns of a worldwide decline in pollinators over the recent decades have now been acknowledged internationally (Steffan-Dewenter et al., 2005; Stokstad, 2007). Some studies have been conducted to evaluate the impact of transgenic plants on insect pollinators such as *A. mellifera* and bumblebees (*Bombus*

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sp.) (Brodsgaard et al., 2003; Girard et al., 1998; Hanley et al., 2003; Pierre et al., 2003; Huang et al., 2004; Malone et al., 2004, 2005; Dechaume-Moncharmont et al., 2005; Sagili et al., 2005; Tian et al., 2006). These studies, however, only investigated the impact of a single transgenic protein, or transgenic plants expressing single transgene proteins on bees. To our knowledge, no reports have been published on the impact of transgenic plants expressing two or more transgene proteins on *A. mellifera*. Purified Bt toxins and pollen expressing Bt toxins have been proven to have no acute lethal effects on *A. mellifera* (Sims, 1995; Arpaia, 1996; Benedict et al.,1996; Malone et al., 1999, 2001, 2004; Hanley et al., 2003; Babendreier et al., 2005; Ramirez-Romero et al., 2008). However, the CPTI protein may decrease learning performance in *A. mellifera* (Picard-Nizou et al., 1997), and the food consumption or learning processes of the tested honeybee could be affected by Cry1Ab protein (Ramirez-Romero et al., 2008). More importantly, it is unknown if there are synergistic effects of the two toxic proteins on bees. Therefore, it is of economic and ecological significance to study the impacts of Bt+CpTI cotton on *A. mellifera*.

In China, though there are many native honeybee species such as *Apis cerana* F., *Apis mellifera* L. is the dominant species for apiculture. *A. mellifera* is a model organism for the toxicological testing of chemicals in China, the US and most European countries. Here we report on the acute oral and long-term toxicity of Bt+CpTI cotton pollen on *A. mellifera*, the main nutritional contents of the pollen compared to the parent cotton variety, and the expression levels of Cry1Ac and CpTI in Zhong-41 Bt+CpTI cotton pollen. Some issues regarding the evaluation methods of the impact of transgenic plants on honeybees are also addressed in this paper.

2. Materials and methods

2.1. Cotton varieties

Transgenic Bt+CpTI cotton variety Zhong-41 and its parent variety Zhong-23 were developed and provided by the Institute of Cotton Research Institute, Chinese Academy of Agricultural Sciences (CAAS). The transgene proteins of Cry1Ac and CpTI are expressed in all parts of Zhong-41 under the regulation of the CaMV35S promoter (Guo et al., 1999; Kang et al., 2005; Chen and Guo, 2006). Both cotton varieties were planted in April 2006, and the pollens and leaves were collected on July 20, August 10, August 30 and September 20. Fresh pollen collected from the four sampling dates was tested for the presence of Cry1Ac and CpTI using the ELISA method and then mixed for the acute oral toxicity bioassays. No pesticides were applied to either variety throughout the growing season. Fresh pollen was collected from the fields and mixed into sucrose solution (50%, w/v) for feeding the worker bees. Extra pollen was stored at −20 °C for later experimental use.

2.2. Worker bees

Worker bees of *A. mellifera* were obtained from the Jiangning Apiary, Nanjing, China, and colonies were kept at the Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection of China. Newly emerged adult worker bees were collected from the same colony and assigned randomly to groups in wooden cages (10 cm × 8.5 cm × 5.5 cm) covered with mesh on two sides. The bees were held in an incubator at 25 ± 2 °C temperature, 55% r.h., in the dark for 6 h and fed with sucrose solution for 72 h to let them adapt the experimental conditions prior to the formal experiments. No antibiotic was used to treat the tested bees before and during the experiment.

According to the result of our preliminary experiment, one bee in the test stage consumes 3.09 ± 0.28 mg/d Zhong-41 pollen and 3.11 ± 0.33 mg/d Zhong-23 pollen. A glass tube (50 mm long, 10 mm diameter narrowing to ca. 2.5 mm) was fitted for each cage to supply bees with the pollen-sucrose solution. Worker bees were starved for 2 h prior to the initiation of all tests so that all bees were equal with respect to gut content at the start of the tests.

2.3. Detection of Cry1Ac and CpTI proteins in the cotton pollen using the enzyme-linked immunosorbent assay (ELISA)

The Cry1Ac in the leaf and pollen of cotton was quantified as described by Wang et al. (2002), using ELISA polyclonal kits, prepared and provided by Center of Crop Chemical Control, China Agricultural University, Beijing. The quantitative detection limit of Cry1Ac kit was at 10 ng/mL.

The CpTI in the leaf and pollen of cotton was detected and quantified according to the method of Rui et al. (2004), using ELISA polyclonal kits, prepared and provided by Center of Crop Chemical Control, China Agricultural University, Beijing. The quantitative detection limit of the CpTI kit was at 20 ng/mL.

The fresh leaf pieces or pollen were homogenized with hand model homogenizers in 2 mL extraction buffer to extract the Cry1Ac toxin or CpTI protein. Homogenized samples were washed with 3 mL extraction buffer and then kept in 10 mL glass tubes at 4 °C for 4 h. The glass tubes were centrifuged at 5000 r.p.m. for 5 min. The supernatants were used for the analyses of the Cry1Ac and CpTI using different ELISA polyclonal kits. The optical density (OD) was measured at 450 nm.

2.4. Detection of the contents of gross protein and soluble saccharides in the cotton pollen

The concentration of soluble saccharides in the cotton pollen was measured using the Anthrone method as described by Cai and Yuan (1982). Pollen was dried at 70–80 °C for 10 h and crushed into powder, and aliquots of 0.2 g were added into 10 mL of 80% ethanol and boiled for 30 min. The solution was centrifuged at 1000g for 10 min and the supernatant was collected. Pollen residues were repeatedly extracted twice over 80% ethanol. Supernatants were pooled, and adjusted to 50 mL with 80% ethanol. Suitable volumes of each supernatant were added to 3 mL of Anthrone reagent and incubated at 90 °C for 15 min. The reaction solutions were then cooled and the optical density measured at 620 nm.

The gross protein content in the pollen was measured using the Micro-Kjeldahl method as described by Cai and Yuan (1982). Cotton pollen was dried at 70–80 °C for 10 h, and 0.5 g of pollen was put into a Kjeldahl flask containing 5 g of catalytic agent (a mixture of $CuSO_4$ and K_2SO_4) and 10 mL of sulfuric acid. The flask was heated for about 6 h until the pollen grains and foam were completely dissolved. After the solution in the flask turned blue-green and transparent, the volume was adjusted to 100 mL for gross protein detection.

2.5. Oral acute toxicity

The standard procedure for the evaluation of the acute oral toxicity of chemical pesticides was adapted for the safety tests of some transgene proteins and the transgenic cotton pollen in the present study (Girard et al., 1998; Malone et al., 1999; OECD, 1998). In preliminary tests, the mouth of the glass tube was blocked by the pollen if more than 0.16 g of the pollen was added into 0.4 mL of the sucrose solution. Therefore, the doses of the pollen in the experiment were set to 0.16, 0.08 and 0.04 g into 0.4 mL of sucrose solution, the corresponding pollen doses were 0.4, 0.2 and 0.1 g/mL, respectively. This artificial diet was infused into the glass tube and supplied to 20 bees of each group. To prevent the microbial fermentation, the artificial diet was renewed each day. Control groups received the sucrose solution only. A pesticide of triazophos (Xinnong Chemical Ltd., Zhejiang Province) was diluted in the sucrose solution to 6.67 mg/L, this was the minimal lethal dose of this pesticide for honeybees, and served as the positive control for the acute toxicity assays. After the above-mentioned diets were consumed, sucrose solutions were provided for the bees *ad libitum*. For each group of bees, mortality was recorded at 24, 48 and 72 h after the start of the experiment. All abnormal behavioral effects (buzzing and creeping) observed during the experiment were recorded. All assays were independently replicated four times.

2.6. Long-term toxicity evaluation

It is difficult to collect enough cotton pollen to conduct the long-term toxicity experiment of all the three pollen doses. Therefore, the cotton pollen dose of 0.2 g/mL was selected to test the long-term toxicity of transgenic Bt+CpTI cotton pollen on *A. mellifera*. The feeding solutions were renewed every 1 or 2 d. The number of dead bees per cage was recorded and then removed daily until all bees had died. Survival curves, plotting the mean numbers of surviving individuals against days from the beginning of the experiment, were generated for each cage of worker bees. All assays were independently replicated four times.

2.7. Measurement of superoxide dismutases (SODs) activity (U/mg protein) in worker bees

Assays of SOD activity was performed according to Zou et al., (1986). After 48 h from the onset of the acute oral toxicity experiments, living individuals were chosen at random for SOD activity measurement. Eight bees were selected from

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each treatment and their legs and wings were removed. Two bees were dissected and homogenized together at 4 °C in physiological saline (0.7% NaCl). The total biomass of the worker bees was 0.1 g/0.9 mL of physiological saline. The homogenized solution was centrifuged at 1000*g* for 12 min at 2 °C. The Superoxide Dismutases kit and Coomassie Brilliant Blue kit, developed by the Jiancheng Bio-engineering Institute of Nanjing, Jiangsu, were used to measure the SOD activities in the supernatants.

2.8. Data analysis

The cumulative survival data of worker bees at each sampling time were compared using a *t*-test in PROC GLM for acute oral and long-term toxicity bioassays between Bt+CpTI and control cotton pollen ~~(SAS, 1998)~~. Shapiro–Wilk tests were performed to check the cumulative survival data for normal distribution before tested in the GLM. Similarly, the toxic protein contents from the ELISA tests, the SOD activity measurements from worker bees, and the gross protein and soluble saccharide contents in pollen were analyzed using a simple ANOVA and a *t*-test. The significant levels were set to $P<0.05$.

3. Results

3.1. The contents of Cry1Ac and CpTI proteins in the fresh leaf and pollen of cotton

The Cry1Ac and CpTI proteins were found in the fresh leaves and pollen of Zhong-41 during the flowering period from July 20 to September 20 in 2006, while no Cry1Ac and CpTI proteins were detected in the non-transgenic cotton variety Zhong-23 (Table 1). For the transgenic Zhong-41 cotton, the Cry1Ac protein expression in fresh leaf was significantly higher than that in fresh pollen during the four flowering times. The CpTI protein in Zhong-41 cotton pollen was detectable at only low concentrations and varied a lot between samples, which indicated that the content of the CpTI protein in Zhong-41 cotton pollen was below the quantitative threshold of the kit.

Pollen collected from the four sampling dates used for detection of the transgene proteins was mixed and pooled for the acute oral toxicity bioassays. For the mixed pollen from Zhong-41, the contents of Cry1Ac and CpTI proteins were 67.1±12.43 ng/g and below 10 ng/g. No Cry1Ac and CpTI proteins were detected in the mixed Zhong-41 cotton pollen.

3.2. Content of gross protein and soluble saccharides in cotton pollen

The average content of gross protein in transgenic cotton pollen was found to be consistently higher than that of pollen from the control cotton in all four sampling stages, especially in samples collected on August 30 and September 20 (Table 2). For the soluble saccharides, the average contents were variable, with one sample (July 20) being much higher than the other three samples. Except in July 20, the contents of soluble saccharides were much higher in pollen from transgenic cotton than the control cotton in the other three times. Since no pesticides were applied throughout the growing season, there was less cotton bollworm damage in Zhong-41 than in Zhong-23, and Zhong-41 cotton grew better than Zhong-23 in the field. This might account for the higher contents of gross protein and soluble saccharides in pollen of Zhong-41 than Zhong-23.

For the mixed pollen from Zhong-41, the contents of gross protein and soluble saccharides were 27.15±1.32 and 565±12.3 mg/g. For Zhong-23, the pollen contained 25.17±1.45 mg/g gross protein and 557±13.2 mg/g of soluble saccharides.

3.3. Acute oral toxicity to worker bees

According to the OECD guidelines for acute oral toxicity testing of chemicals, the acute oral toxicity testing of honeybee lasts typically 24–72 h (OECD, 1998). All individuals in the group treated with triazophos died within 24 h, while after 48 h none had died in the control group fed only the sucrose solution, proving that this testing system was stable and usable as it could detect the acute lethal effect of a substance on honeybees, and sustain the normal growth of the tested organisms. Under the experimental conditions, there were no significant differences in mortalities of *A. mellifera* among groups with or without transgenic cotton pollen or in control pollen within 24, 48 or 72 h ($P>0.05$) (Table 3). The results showed that there was no acute oral toxicity of transgenic Bt+CpTI cotton pollen in the doses tested. No abnormal behaviors were observed in *A. mellifera* treated with Zhong-41 cotton pollen, Zhong-23 cotton pollen or from the control group.

3.4. Impacts of the pollen of the transgenic cotton on sod activities in worker bees

After the tests of acute oral toxicity, individuals were chosen randomly from the groups tested with pollen doses of 0.4 and 0.2 g/mL for the test of SOD activities (Table 4).

Superoxide dismutase acts as a scavenger that removes superoxide radicals. It was reported that levels of this enzyme were related to animal resistance to stresses such as pollution and cold (Abele-Oeschger, 1996). The results in Table 4 show that the SOD activity of *A. mellifera* in the control group (without pollen) were significantly higher than those in groups with pollen of transgenic or non-transgenic cotton varieties (df = 6, $F=4.398$, $P=0.0105$). Pollen is an essential food source for honeybees for protein acquisition, and honeybees without pollen may have

Table 1
Cry1Ac and CpTI protein content in cotton leaf and pollen from the transgenic Bt+CpTI variety Zhong-41 and the non-transgenic variety Zhong-23 as assayed by ELISA method in 2006.

Transgene proteins	Cotton tissue	Cotton variety	Contents of transgene proteins (ng/g fresh tissue) (±SD)			
			Jul. 20	Aug. 10	Aug. 30	Sep. 20
Cry1Ac	Leaf	Zhong-41	685.4±81.5	2475.3±331.9	1735.7±143.1	753.7±63.6
		Zhong-23	0	0	0	0
	Pollen	Zhong-41	75.2±31.2	65.9±24.5	59.7±15.3	79.3±40.6
		Zhong-23	0	0	0	0
CpTI	Leaf	Zhong-41	258.6±65.1	885.7±136.7	586.2±57.8	345.8±91.5
		Zhong-23	0	0	0	0
	Pollen	Zhong-41	+[a]	+	+	+
		Zhong-23	0	0	0	0

[a] Detectable qualitatively but very low and variable.

Table 2
Contents of gross protein and soluble saccharides in fresh pollen from the transgenic Bt+CpTI variety Zhong-41 and the non-transgenic variety Zhong-23 at different growth stages in 2006.

Items	Cotton variety	Contents (mg/g fresh pollen) (±SD)			
		Jul. 20	Aug. 10	Aug. 30	Sep. 20
Gross protein	Zhong-41	25. 71±0.43	29.44±0.82	27.93±0.68	26. 84±0.22
	Zhong-23	25. 27±0.84	28.19±0.93	25. 98±0.92	24.71±0.35
Soluble saccharides	Zhong-41	781.18±26.54	629.01±17.67	427.0±22.18	575.46±14.15
	Zhong-23	861.57±35.83	578.25±12.01	376.4±18.33	540.9±11.03

Table 3
Acute oral toxicity of pollen from the transgenic Bt+CpTI variety Zhong-41 and the non-transgenic variety Zhong-23 on adult worker bees of *A. mellifera*.

Treatments	Test doses (g/mL)	Cumulative mortality (±SD)					
		24 h		48 h		72 h	
		#	%	#	%	#	%
CK (sucrose only)	0	0	0	0.25±0.5	1.25±2.5	1.25±0.5	5.0±4.1
Pollen of Zhong-23	0.1	0	0	0	0	0.5±0.6	2.5±2.8
	0.2	0	0	0.5±0.6	2.5±2.8	0.25±0.5	1.25±2.5
	0.4	0	0	0	0	0.5±0.6	2.5±2.8
Pollen of Zhong-41	0.1	0	0	0.25±0.5	1.25±2.5	0.5±0.6	2.5±2.8
	0.2	0	0	0	0	0.75±0.5	3.75±2.5
	0.4	0	0	0	0	0.25±0.5	1.25±2.5
Triazophos	6.67 mg/L	20	100	20	100	20	100

# indicates the actual average number of dead individuals out of one group of 20 bees; % = percent mortality.

Table 4
The effect of pollen from the transgenic Bt+CpTI variety Zhong-41 and the non-transgenic variety Zhong-23 on the SOD activities in adult worker bees *A. mellifera*.

Treatments	Test dose (g/mL)	SOD activities (U/mg protein) (±SD)
CK (sucrose only)	0	175.28±16.83
Pollen of Zhong-23	0.1	134.66±11.37
	0.2	137. 52±14.39
	0.4	128.15±18.05
Pollen of Zhong-41	0.1	128.66±9.07
	0.2	127. 95±15.32
	0.4	129.53±11.47

responded to the poor nutrition through elevated SOD activities, while those fed pollen had lower SOD levels. SOD activities were not statistically different for *A. mellifera* in the groups with pollen from transgenic or non-transgenic cotton, even though the SOD activity in bees fed with a dose of 0.2 g/mL non-transgenic pollen was higher than that from *A. mellifera* fed with the same dose of transgenic cotton pollen. Similarly, no significant differences were found in the SOD activities between *A. mellifera* fed with pollen doses of 0.4 g/mL for the two cotton lines (df = 5, $F = 0.2637$, $P = 0.924$). Therefore, pollen from the non-transgenic cotton variety Zhong-23 and pollen from the transgenic cotton variety Zhong-41 had no significant adverse effects or differences on the SOD activity in *A. mellifera*.

The gross protein and soluble saccharides in the mixed pollen were similar in the two cotton varieties. Moreover, the contents of the Cry1Ac or CpTI toxic proteins in Zhong-41 cotton pollen were very low, and the two toxic proteins have been proven to have no acute effects on the activity of honeybees (Sims et al., 1995; Picard-Nizou et al., 1997). These factors might account for the results observed in the SOD activity testing.

3.5. Impact of the pollen of transgenic cotton on the longevity of worker bees

Pollen dose of 0.2 g/mL was used for the longevity tests on *A. mellifera*. The results indicated that *A. mellifera* in the control group without pollen died gradually within 38 d, while bees in the groups with transgenic and non-transgenic pollen died within 49 and 46 d, respectively (Fig. 1). The survival time of work bees in each Shapiro–Wilk test is a normal distribution (The Shapiro–Wilk statistic df significances are Ck = 0.188; Zhong-23 = 0.534; zhong-41 = 0.074). The mortality curve in Fig. 1 shows that the death rate in the control group was much higher from 14 to 38 d than in groups fed pollen. The survival curve in both groups fed pollen indicated that there was no adverse effect of transgenic cotton pollen on the longevity of bees. Pollen is the main source of protein for adult bees, and the absence of protein significantly reduces the longevity of worker bees (Chen, 2001). The importance of pollen for *A. mellifera* may explain the significantly shorter life span and higher SOD activity of bees fed without cotton pollen.

4. Discussion

Honeybees (*A. mellifera* L.) are the most important pollinators of many agricultural crops worldwide. The concentration of toxic proteins expressed in pollen from transgenic cotton is the important factor in the assessment of its non-target effect on pollinators (Liu and Xu, 2003). The expression levels of transgenic proteins in the pollen of transgenic Bt+CpTI cotton have not been

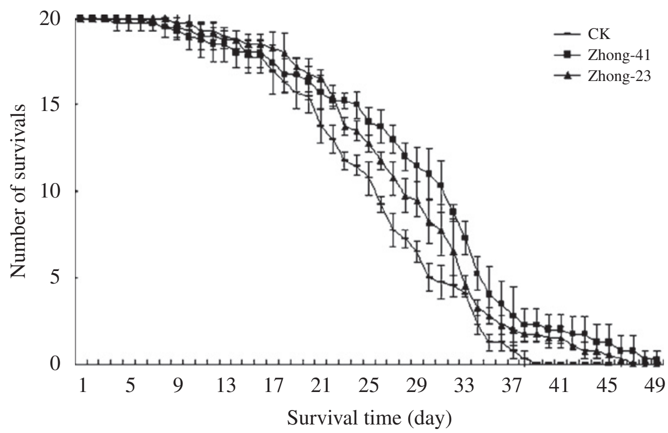

Fig. 1. Survival curves for caged *Apis mellifera* worker bees fed with pollen (at dose of 0.2 g/mL sucrose solution) of non transgenic cotton variety Zhong-23, transgenic Bt+CpTI cotton variety Zhong-41 and without pollen (±SD).

reported yet. In this study, the Cry1Ac protein in transgenic Bt+CpTI cotton pollen ranged from 59.7 to 79.3 ng/g fresh pollen as tested by ELISA, while the content of the CpTI protein was too low to be quantitatively detected. The levels of the two transgene proteins expressed in the pollen were extremely low in comparison to that in the leaf of transgenic Bt+CpTI cotton Zhong-41. The cotton bollworm (*H. armigera*) and the silkworm (*Bombyx mori* L.) are both the target pests of the Cry1Ac and CpTI proteins. Pollen from transgenic Bt+CpTI cotton Zhong-41 showed neither acute lethal effects nor adverse impacts on the growth and development of the *B. mori* (Li et al., 2002), which also demonstrated that the expression levels of the Cry1Ac and CpTI proteins in Zhong-41 cotton pollen were below the efficacy level to the target pests. Expression levels of toxic proteins in transgenic plants are controlled by promoters. The promoter used in Zhong-41, CaMV 35S, is a constitutive promoter with high activity in leaves, but with little or no activity in the pollen (Wilkinson et al., 1997), which might account for the low transgene protein levels in the pollen of Zhong-41 cotton. However, the fact that both the Cry1Ac and CpTI proteins had been detected demonstrated that the CaMV 35S promoter was active in the Zhong-41 pollen.

During genetic manipulations, the practice of cell and tissue culturing (especially when antibiotics are used as the selection agent) can provoke unintended alterations in plant characteristics. The position effect from the sites of the inserted gene(s) and the expression of the inserted genes disturbs the normal expression of genes in the parental plants (Filipecki and Malepszy, 2006). This can cause unexpected changes in physiological and biochemical characteristics, which may have indirect impacts on non-target organisms such as honeybees (Stelly et al., 1989; Zhang et al., 2001). Some unintended alterations in physiological, biochemical or morphological properties have been observed in transgenic Bt cotton. This includes lower condensed tannin and phenols, higher levels of gossypols, new volatile chemicals and leaf trichome variations (Wu et al., 2000; Shen and Rui 2004; Yan et al., 2004; Lu et al., 2005; Xue et al., 2008). No significant changes in soluble proteins and sugars, however, were found in the pollen of transgenic Bt+ CpTI cotton compared to its isogenic line in the present research. Therefore, the main nutrients in Bt+CpTI cotton pollen and the parental non-transgenic cotton were found to be substantially equivalent, which means that transgenic cotton pollen provides similar nutrients for honeybees and other non-target organisms as the parental non-transgenic cotton.

Direct and indirect impacts of transgenic plants on honeybees and other pollinators have been reported (Malone and Pham-Delègue, 2001; Liu and Xu, 2003). The direct impacts of transgenic plants on bees come from the transgene proteins expressed in the tissues (e.g., pollen) or excretions (e.g., nectar). In this case, the level of the impact on non-target pollinators depends largely on the biological traits and the expression amounts of the transgene proteins in the pollen. Experiments have been conducted on the impact of CrylAc and CpTI on bees, but no reports have been found on transgenic crops simultaneously expressing both proteins on bees. This is the first report regarding the effects of transgenic crops expressing the two toxic proteins on honeybees. The results showed that Bt+CpTI cotton pollen had neither acute toxic effects nor adverse influences on SOD enzymes and the longevity of adult honeybees. The reasons for these results might include the following: (1) There is no acute toxicity of the Bt protein and the CpTI protein expressed in the pollen of transgenic cotton on honeybees (Sims, 1995; Arpaia, 1996; Benedict et al., 1996; Picard-Nizou et al., 1997; Hanley et al., 2003; Malone et al., 2004; Babendreier et al., 2005; Ramirez-Romero et al., 2008). (2) The Cry1Ab protein in concentration of 5000 ng/mL did not cause lethal effects on honeybees (Ramirez-Romero et al., 2008). In the present oral toxicity experiments, three pollen doses (0.4, 0.2 and 0.1 g/mL) were set, the corresponding Cry1Ac doses were calculated to be 26.84±4.97, 13.42±2.48, 6.71±1.24 ng/mL, which were all far below the concentration of 5000 ng/mL. Therefore, the second reason might be that the expression levels of the two toxic proteins in the pollen of Zhong-41 cotton are too low to cause an effect or a combined toxicity. (3) The main nutrition (e.g., soluble proteins and sugar) in transgenic cotton pollen is similar or equivalent to non-transgenic cotton pollen.

The primary purpose of the development and application of transgenic crops is to replace chemical insecticides to control pests. The chemical insecticide used in our study had an acute lethal effect on *A. mellifera*, but transgenic cotton pollen had neither lethal effects nor significant adverse influences on the growth and development of *A. mellifera*. Therefore, in comparison to chemical insecticides, the adverse impact of Bt+CpTI cotton on honeybees is minor and can be neglected, which is consistent with the results of other studies (Bailey et al., 2005; Liu et al., 2005; Ramirez-Romero et al., 2005; Ludy and Lang 2006). Other reports also indicated that the application of transgenic crops expressing insecticidal proteins can not only decrease the use of chemical insecticides but also protect biological control components, and is thus more compatible with the development of integrated pest management (IPM) against pests than chemical pesticides (Reed et al., 2001; Musser and Shelton, 2003; Cattaneo et al., 2006; Ferry et al., 2006; Mulligan et al., 2006; Romeis et al., 2006; Wossink and Denaux, 2006). However, chemical insecticides still were used in transgenic insect-resistant cotton (Wu et al., 2008), which means that honeybees will be influenced jointly by chemical insecticides and the transgene proteins such as Bt toxins and CpTI in the field. Therefore, the results of our study and other reports cannot guarantee the safety of the transgenic insect-resistant cotton to honeybees. With the commercialization of more and more transgenic insect-resistant crops in China (Stone, 2008), it is important to assess the joint effects on pollinating bees of chemical insecticides and the transgene proteins.

Pollination and honey production by bees is ecologically and economically beneficial. Social insects like bees search and locate host plants by plant volatiles. Any changes in plant volatiles and other characteristics can greatly change the pollination behaviors of bees. As such, alteration of plant characters by the insertion of transgenes into plants needs to be studied very carefully in the assessment of the ecological consequences of transgenic crops. Although similar volatile profiles were found from transgenic corn and its parental line, the amount of volatiles released from transgenic corn were greatly higher than those from the non-transgenic line (Turlings et al., 2005). Also, there were some

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differences in the amounts and constitutions of volatiles between transgenic Bt cotton and non-transgenic control cotton (Yan et al., 2004). There might be some differences in the physiological or biochemical characters in transgenic Bt+CpTI cotton associated with the pollination behaviors of bees, such as the amount of nectar secreted, nutritional changes in nectar, the amount of pollen produced, flower color and flowering periods. In addition, some adverse impacts of CpTI on olfactory learning behaviors had been observed (Picard-Nizou et al., 1997), and the behaviors of food consumption or learning processes for honeybees were impacted adversely by Cry1Ab protein at 5000 ppb (Ramirez-Romero et al., 2008), thus nectar collection and pollination behaviors may be influenced by Bt+CpTI cotton.

The results in the present study indicate only that there are no significant direct adverse impacts of transgenic Bt+CpTI cotton pollen on the survival and growth of adult *A. mellifera*. Future research should be conducted on the joint impacts on honeybees (especially bees in the larval stages) of transgenic insect-resistant cotton and chemical pesticides. Moreover, more studies should be conducted to determine the ecological and behavioral impact of transgenic Bt+CpTI and other transgenic crops on bees.

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