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

Biao Liu [a,*], Chang Shu [b], Kun Xue [c], Kexin Zhou [a], Xiaogang Li [a,d], Doudou Liu [a,d], Yangping Zheng [a], Chongren Xu [b]

[a] State Key Biosafety Laboratory, Nanjing Institute of Environmental Sciences (NIES), Ministry of Environmental Protection of China, Nanjing, Jiangsu 210042, China
[b] College of Life Sciences, Peking University, Beijing 100871, China
[c] College of Life and Environmental Science, the Central University for Nationalities, Beijing 100081, China
[d] College of Forest Resources and Environment, Nanjing Forestry University, Nanjing 210037, China

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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* 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,

* Corresponding author. Fax: +86 25 85287064.
E-mail address: 85287064@163.com (B. Liu).

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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 1000*g* 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 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.