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# Herbicide and insect resistant Bt cotton pollen assessment finds no detrimental effects on adult honey bees☆

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ABSTRACT

One important concern regarding the use of transgenic cotton expressing insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) is its potential detrimental effect on non-target organisms. The honey bee (*Apis mellifera*) is the most important pollinator species worldwide and it is directly exposed to transgenic crops by the consumption of genetically modified (GM) pollen. However, the potential effects of Bt cotton on *A. mellifera* remain unclear. In the present study, we assessed the effects of two Bt cotton varieties; ZMSJ expressing the Cry1Ac and Cry2Ab insecticidal proteins, and ZMKCKC producing Cry1Ac and EPSPS, on *A. mellifera*. Feeding on pollen from two Bt cotton varieties led to detection of low levels of Cry toxins (<10 ng/g fresh weight) in the midgut of *A. mellifera* adults, yet expression of detoxification genes did not change significantly compared to feeding on non-Bt cotton. Binding assays showed no Cry1Ac or Cry2Ab binding to midgut brush border membrane proteins from *A. mellifera* adults. Taken together, these results support minimal risk for potential negative effects on *A. mellifera* by exposure to Bt cotton.



## 1. Introduction

Genetically modified (GM) crops play an important role in pest control (Lu et al., 2012a). In 2015, China successfully planted ~3.7 million hectares of insect-resistant transgenic cotton plants expressing insecticidal protein genes from the bacterium *Bacillus thuringiensis* (Bt), representing 96% of the cotton acreage (James, 2015). The wide use of transgenic Bt cotton varieties has proven benefits including control of cotton bollworm (Lu et al., 2012a) and reductions in the application of insecticidal sprays (Brookes and Barfoot, 2012). However, evolution of insect resistance threatens the future utility of transgenic Bt cotton expressing a single Bt toxin. Cotton varieties expressing multiple (pyramided) insecticidal genes are expected to delay the onset of resistance (Lu et al., 2012b). Two new Bt cotton varieties producing Cry1Ac and Cry2Ab or Cry1Ac and 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) have been developed and will be commercially available soon in China (Luo et al., 2012; XingHua et al., 2012). The potential unintended effects of these two new Bt cotton varieties need to be tested for ecological risks assessment.

Insect pollination plays an important role in agriculture, improving production in 75% of global crops (Klein et al., 2007). The honey bee *Apis mellifera* is the key pollinator insect around the world, contributing approximately ¥304.22 billion to Chinese agriculture per year (Liu et al., 2011). Pollen provides protein for *A. mellifera* colonies (Decourtye et al., 2010) and both adults and larvae are exposed to pollen from transgenic Bt crops (Hendriksma et al., 2011). This is especially true in the case of Bt cotton, which is planted in mass monocultures in China (Han et al., 2010a). Therefore the honey bee is a key non-target insect for risk assessment evaluation of transgenic Bt cotton on pollinators (Duan et al., 2008; Lima et al., 2011; Malone and Burgess, 2009).

A few studies have evaluated the effects of transgenic Bt cotton on *A. mellifera* (Han et al., 2010a, b; Jiang et al., 2010; Liu et al., 2009; Niu et al., 2013). For example, the survival, learning capacities and the midgut proteolytic enzymes or the development of their hypopharyngeal glands were not affected when fed with Bt cotton pollen (Han et al., 2010a, b, 2012). Transgenic Cry1Ac cotton had no detrimental effects on the microbial diversity in *A. mellifera*

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intestines after short exposures (Jiang et al., 2010). No adverse effects were observed in the superoxide dismutase activity or in the longevity of worker bees after being fed with the Bt cotton pollen (Liu et al., 2009). Hendriksma et al. found that neither different Bt proteins nor EPSPS expressed in Bt corn were harmful to the honey bee larvae (Hendriksma et al., 2012). In addition, our previous results also showed that the transgenic Cry1Ac/EPSPS and Cry1Ac/Cry2Ab cotton hybrids have no detrimental effects on the survival, pollen consumption or total hemocyte count (THC) of *A. mellifera* adults (Niu et al., 2013). However, most of the studies regarding the risk assessment of Bt crops on *A. mellifera* have focused on biology and ecology (Han et al., 2010a, b; Hendriksma et al., 2011) and the molecular response of the honey bee to Bt crops still remains uncharacterized.

Insects have an ability to protect themselves from pathogens, toxic chemicals and secondary plant metabolites by regulating expression of detoxification-related genes (Bao et al., 2012). Three major enzyme families, cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and esterases (ESTs), are involved in the xenobiotic metabolism of insects (Enayati et al., 2005; Feyereisen, 1999; Ramsey et al., 2010). Zhao et al. reported that the expression levels of the detoxification-related genes of non-target *Aphis gossypii* and *Propylea japonica* did not change after treatment with Cry1Ah or Cry2Ab proteins (Zhao et al., 2016). Our previous study showed that transgenic Bt rice producing Cry1Ab/Cry1Ac, Cry2Aa or Cry1Ca had no detrimental effects on detoxification responses of the non-target insect *Nilaparvata lugens* (Mannakkara et al., 2013).

When Cry proteins are not affected by the detoxification responses, they must bind to specific receptors on the brush border membrane of midgut cells to form pores conducive to insect death (Jurat-Fuentes and Crickmore, 2016). However, while binding is required, it is not sufficient to cause toxicity (de Maagd et al., 2001; Wolfersberger, 1990). Despite its importance for toxicity, some studies have reported Bt binding proteins in non-target insects (Ferry et al., 2007; Rodrigo-Simón et al., 2006).

In the present study, we investigated the potential effects of pollen from two Bt cotton hybrids on the honey bee *A. mellifera* adults. We focused our study on three aspects: (1) presence of Cry proteins in midgut of *A. mellifera* after being fed Bt cotton pollen; (2) activation of the *A. mellifera* detoxification response; and (3) the potential existence of binding proteins for Cry1Ac or Cry2Ab in *A. mellifera* midgut brush border membrane vesicles (BBMVs).

## 2. Materials and methods

### 2.1. Insects

Newly emerged adult worker bees (0 d) of *A. mellifera* were obtained from a colony during summer from Huazhong Agricultural University. Worker bees were reared in cages (15 × 10 × 20 cm) (Pain, 1966), and kept in an incubator (Wuhan Ruihua Instrument and Equipment Co. LTD, China) (33 ± 1 °C, 60± 10% relative humidity, darkness). After 1-day of adaptation, they were used for the experiments.

### 2.2. Pollen

The two Bt cotton varieties ZMSJ (producing Cry1Ac/Cry2Ab) and ZMKCKC (producing Cry1Ac/EPSPS), and the near-isogenic cultivar Emian-24 (non-GM cotton) were obtained from the Institute of Cotton Research, the Chinese Academy of Agricultural Sciences (Anyang, China). All cultivars were cultivated at Huazhong Agricultural University on April 30, 2014.

Twenty grams pollen samples from each type of cotton varieties (n = 40 plants) were collected using the multi-point field sampling method described before (Chen et al., 2011; Han et al., 2010a) on July 20th when these two Bt cotton hybrids had pollen with the highest expression levels of Cry1Ac and Cry2Ab proteins (Niu et al., 2013). After being sieved (830 mm mesh size), the fresh collected pollen samples were stored at - 80 °C.

### 2.3. Detection of Bt toxin in A. mellifera guts after feeding on pollen

Worker *A. mellifera* were exposed to non-Bt and Bt cotton pollen. Three different diets (two kinds of Bt cotton pollen and one non-Bt cotton pollen) were prepared (water: honey: pollen = 1:2:7 by weight) without any sugar. On average, a worker consumes about 7–10 mg of cotton pollen per day (Han et al., 2010a). Three replicates (cage) were undertaken for each treatment, with 40 bees per replicate. After being treated for 7 days, the surviving bees (there was no difference of survival rate among different treatments, results see (Niu et al., 2013) were prepared for Cry protein detection.

The concentrations of Cry1Ac and Cry2Ab in *A. mellifera* guts were estimated by ELISA method using the Envirologix Qualiplate Kits (EnviroLogix Quantiplate Kit, Portland, ME, USA) (Han et al., 2010a, 2014). The quantitative detection limits of the Cry1Ac and Cry2Ab kit were 0.1 ng/g and 0.52 ng/g. Three samples (ten dissected guts per sample per cage) were prepared for each treatment. Each sample was homogenized in 0.5 ml of extraction EnviroLogix buffer, and centrifuged at 12,000 × *g* for 10 min. After that, the supernatant was transferred to an ELISA plate for the analyses, following manufacturer's instructions. A xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA) was used to detect the ODs of the sample.

### 2.4. Expression of detoxification response genes

Worker *A. mellifera* bees were reared on three different diets (two kinds of Bt cotton pollen and one conventional cotton pollen) as described above for 7 days. After frozen in liquid nitrogen, the adult bees were stored at - 80 °C for further use. Ten honey bee whole bodies from each treatment were used to extract total RNA, and 1 μg total RNA was used to synthesize cDNA.

qRT-PCR experiments were performed with SYBR PremixExTaq (TaKaRa Biotechnology) in a 20 μl reaction volume. The reactions were performed as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Specific primers (Supplementary Table 1) used for qRT-PCR experiments were provided by Sangon Biotech (Shanghai) Co. Ltd. The honey bee actin gene (Accession number: GB17681) was used as internal reference (Lourenço et al., 2008). Standard curves were prepared using a five-fold dilution series to determine the primer efficiencies (Supplementary Table 2). Each reaction was performed in triplicate (technical) with three biological replicates.

### 2.5. BBMV preparation

Thirty *A. mellifera* midguts were isolated from 7-day old adults fed with non-Bt cotton pollen to prepare BBMV using differential precipitation (Wolfersberger et al., 1987). As positive control we used BBMV prepared from fourth instar *Spodoptera exigua* larvae, as an insect susceptible to Bt cotton producing Cry1Ac and Cry2Ab (Adamczyk et al., 2008). Five milliliters of MET buffer A (0.3 M Mannitol, 5 mM EGTA, 17 mM Tris-HCl pH 7.5) was added to the pooled dissected midguts, and after being homogenized, the homogenate was centrifuged for 5 min at 500 × *g* at 4 °C. Then an equal volume of 24 mM $MgCl_2$ was added to the supernatant, and the mixture was centrifuged for 10 min at 3000 × *g* at 4 °C. After that, the supernatant was transferred to a clean tube and