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Short Communication

Effects of *Bt* transgenic Chinese cabbage pollen expressing *Bacillus thuringiensis* Cry1Ac toxin on the non-target insect *Bombyx mori* (Lepidoptera: Bombyxidae) larvae

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ABSTRACT

We evaluated the effects of the pollens of transgenic Chinese cabbage (*Brassica campestris* subsp. *napus* var. *pekinensis* Makino), expressing *Bacillus thuringiensis* Cry1Ac toxin (*Bt* cabbage), on *Bombyx mori* larvae. Decreased survival rate and body weight of *B. mori* larvae were observed when fed with an artificial diet containing *Bt* cabbage pollens. ELISA test using Cry1Ac-antibody revealed that the Cry toxin was detected not only in the alimentary canal but also in the hemolymph and remaining body parts, suggesting that the ingested Cry1Ac protein is distributed inside the body likely through the damaged midgut epithelial membrane of *B. mori*. Taken together, our results suggested that *Bt* cabbage pollens adversely affect non-target insect *B. mori* larvae when consumed. Considering the low possibility of *Bt* cabbage pollen exposure to *B. mori* in the actual field condition, however, the risk would be minimal.

Introduction

Examination of potential adverse effects on non-target organisms is an essential element in the environmental risk assessment of transgenic crops expressing *Bacillus thuringiensis* Cry toxin (*Bt* crops). Impacts of *Bt* crop pollens, in particular, have been a major concern due to the high potential of pollen dispersion to nearby habitats of non-target insects. A number of studies have been conducted to address the potential effects of *Bt* crop pollens on non-target butterflies and moth, and produced controversial results. For instance, higher mortality and slower growth were observed in monarch butterfly, *Danaus plexippus*, and black swallowtail, *Papilio polyxenes*, when fed with *Bt* corn pollens (Losey et al., 1999; Laura et al., 2000; Hellmich et al., 2001; Zangerl et al., 2001). In other experiment, however, *Bt* corn pollens did not significantly affect the larval mortality of *P. polyxenes* (Wraight et al., 2000). In addition, no significant effects by the pollens of *Bt* corn (Li et al., 2002, 2005), cotton (Li et al., 2002, 2003) and rice (Yao et al., 2006) were observed on the larvae of silkworms, *Bomyx mori*, and *Antheraea pernyi*. In particular, any of the developmental indexes, including larval mortality, cocoon weight, pupal weight, cocoon shell weight, pupation rate, emergence rate and fecundity, was not affected when *B. mori* neonates were fed with pollens of *Bt* cotton and *Bt* corn for 72 h (Li et al., 2002). No significant differences were found in larval mortality and other growth indexes when *A. pernyi* larvae were treated with *Bt* cotton pollen (Li et al., 2003).

A transgenic Chinese cabbage, *Brassica campestris*, strain expressing *B. thuringiensis* Cry1Ac toxin (*Bt* cabbage) has been recently developed in Korea for the control of diamondback moth, *Plutella xylostella*. Since the *Bt* cabbage strain has the cauliflower mosaic virus 35S promoter (Rural Development Administration, 2008), the Cry1Ac toxin is expressed in all plant tissues including pollen. It is essential, therefore, to assess the ecological impacts of *Bt* cabbage pollen on non-target insects prior to eventual commercialization. Although silkworm culture has been diminished recently, *B. mori* is still one of the most important beneficial insect species in Korea (Ministry of Agriculture and Forest, 2007). Considering the possibility of *Bt* cabbage pollen dispersion in cabbage–mulberry mixed cultivation area, resulting in *Bt* cabbage pollen deposition on nearby mulberry leaves, it is imperative to evaluate the potential hazards of *Bt* cabbage pollen to *B. mori* larvae when exposed.

To this end, we have investigated the adverse effects of *Bt* cabbage pollen on *B. mori* larvae by comparing the larval mortality and weight. In addition, we have analyzed the distribution of Cry1Ac toxin inside *B. mori* larval body to investigate the Cry1Ac-mediated toxicity.

Materials and methods

Collection of cabbage pollens

The *Bt* cabbage cultivar genetically engineered to express the Cry1Ac *Bt*-toxin was provided by the National Institute of Agricultural Biotechnology (NIAB), Rural Development Administration (RDA), Korea. Flowers of *Bt* or non-*Bt* cabbage cultivated in the isolated glass house of NIAB were collected, immersed in ample amount of water in a 500-ml round bottle, and gently rotated for 3 h at room

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temperature to separate pollens. The pollen-containing water was filtered through a 604 μm sieve and the filtrate was centrifuged at 13,000 *g* for 2 min. After removing supernatant water, pollen pellets were examined by microscope to confirm whether pure pollens were isolated without contamination with other tissues, and dried for 3 days at 37 °C. Dried pollen pellets were gently ground with a mortar/pestle to separate individual pollens and stored at −20 °C until use.

Effects of Bt cabbage pollens on B. mori larvae

B. mori eggs and artificial diets were obtained from the Applied Sericulture and Apiculture Division, RDA, Korea. All stages of *B. mori* were maintained at 25±3 °C, 60±5% RH and a photoperiod of L:D 16:8 h. Insects were supplied with artificial diets and replaced as needed.

Thirty 2-day-old first instar larvae of *B. mori* were kept individually in plastic containers (3.5-cm diameter, 4-cm high) containing the artificial diets mixed with the pollens of *Bt* or non-*Bt* cabbage (designated *Bt* pollen diet and non-*Bt* pollen diet, respectively, hereafter) at a 1000:1 ratio (w/w). Artificial diets without pollens were also supplied as a control (control diet). Larval survivorship was checked daily for 12 days and larval weight was measured at 9 days post-feeding.

Detection of Cry toxin in the body of B. mori

Forth instar *B. mori* larvae were starved for 24 h to empty gut and inoculated on the *Bt* pollen diet or non-*Bt* pollen diet each mixed at a 20:1 ratio (w/w). Diet-fed *B. mori* larvae were collected at 24 and 72 h post-feeding. The larval body was divided into three parts (hemolymph, alimentary track and remaining organs). Hemolymph was removed first by puncturing the dorsal part of the larval body with a razor blade and gently squeezing the body. Then the larval body was dissected with microscissors in the phosphate-buffered saline (PBS) to remove remaining hemolymph and the alimentary track was pulled out from remaining body parts with a pair of fine forceps. The tissue samples were rinsed with an ample amount of PBS to avoid cross-contamination with Cry toxin. Hemolymph was diluted with a 3-fold volume of PBS–Tween (PBST). The alimentary tracks and remaining body parts were separately homogenized in a 10-fold volume, and the feces, artificial diet containing pollens and *Bt* cabbage pollens in a 20-fold volume of PBST (fresh weight to volume ratio) using a glass–glass tissue grinder (1 ml volume; Radnoti, Monrovia, CA, USA). The homogenates and diluted hemolymph were centrifuged at 13,000 rpm for 1 min and supernatants were collected. Diluted or homogenized samples were quick-frozen with liquid nitrogen and stored at −75 °C until the ELISA test.

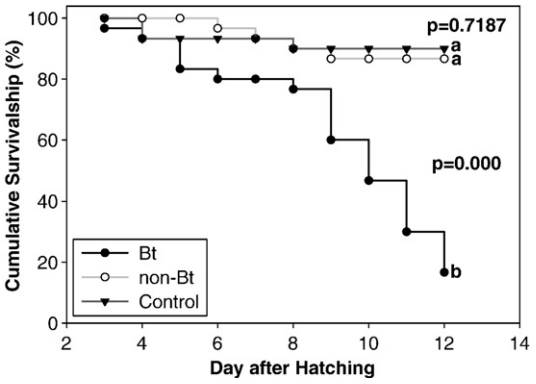

Fig. 1. Survivorship of *B. mori* larvae fed with *Bt* pollen, non-*Bt* pollen or control diets. Survivorship was compared by Kaplan-Meier survival analysis (p=0.000, n=30 in *Bt*, non-*Bt* and control group). Means with different letters are significantly different (p<0.05).

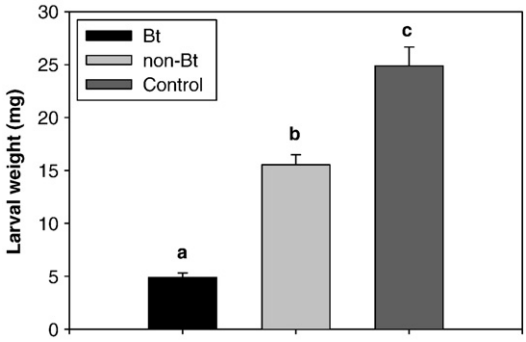

Fig. 2. Larval weight of *B. mori* fed with *Bt* pollen, non-*Bt* pollen or control diets. Larval weight was compared by one-way ANOVA with LSD as multiple caparison test (p=0.000, F=31.479, df=2; n=9 in *Bt*, 26 in non-*Bt*, 27 in control). Data points are shown in mean±SE. Means with different letters are significantly different (p<0.05).

Concentrations of Cry1Ac in collected samples were determined using a PathoScreen Kit for *Bt*-Cry1Ab/1Ac protein according to the manufacturer's protocol (Agdia Inc., Elkhart, IN, USA). Optical density was measured at 650 nm with a microplate reader (Emax Precision Microplate Reader; Molecular Devices, Sunnyvale, CA, USA). Purified Cry1Ac protein solution (31.3 ng/ml) was serially diluted 2-fold with PBST to 0.03 ng/ml concentration to generate a standard curve. All experiments were conducted with three replicates.

Statistical analysis

SPSS for Window version 12.0 K (SPSS Inc., Chicago, IL, USA) was used for data analysis. Cumulative survivorship of *B. mori* was compared by Kaplan–Meier survival analysis. One-way ANOVA with LSD as multiple comparison test was used to compare the larval weight. All average values were described in mean±standard error.

Results

Effects of Bt cabbage pollens on B. mori

Larval survivorships were 16.7%, 86.7% and 90.0% in the *B. mori* larvae fed with the *Bt* pollen, non-*Bt* pollen and control diets, respectively, at 12 days post-feeding (Fig. 1). Survivorship of *B. mori* larvae reared on *Bt* pollen diet was significantly lower compared to that on non-*Bt* pollen or control diets (Kaplan–Meier survival analysis, p=0.000, n=30). However, no significant differences were observed between non-*Bt* and control diets (Kaplan–Meier survival analysis, p=0.7187, n=30).

Larval weight measured at 9 days post-feeding with *Bt* pollen diet was significantly lower than with non-*Bt* or control diet (one-way ANOVA with LSD as multiple comparison test; p=0.000; F=31.48; df=2) (Fig. 2). Larval weight of *B. mori* fed with non-*Bt* pollen diet was also statistically lower than that with control diet (p=0.000; F=31.48; df=2) (Fig. 2).

Detection of Cry toxin in the bodies of B. mori

The average Cry1Ac toxin concentration in *Bt* cabbage pollens was 53.7±3.9 ng/g. Cry1Ac was detected not only in the alimentary canal, but also in the hemolymph and remaining body parts of *B. mori* larvae fed with *Bt* pollen diet (Fig. 3). Mean concentrations of Cry1Ac toxin in the *B. mori* larvae collected at 24 h post-feeding were 4.03 ng/g in alimentary canal, 1.17 ng/g in remaining body parts, 1.74 ng/g in hemolymph and 11.8 ng/g in feces. Cry1Ac concentrations were reduced to less than 1 ng/g in all tissues when examined at 72 h post-feeding. Mean concentration of Cry1Ac in feces was 2.29 ng/g at 72 h post-feeding. Average concentrations of Cry1Ac detected in the *Bt*