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Comparison of susceptibility of *Chilo suppressalis* and *Bombyx mori* to five *Bacillus thuringiensis* proteins

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ABSTRACT

Transformation of rice with genes encoding insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) should confer high resistance to target lepidopteran pests, such as *Chilo suppressalis*, and low toxicity to non-target organisms, such as silkworm *Bombyx mori*. Five purified Cry proteins that have been used for plant transformation were tested using dietary exposure assays. The susceptibility of *C. suppressalis* larvae to the five insecticidal proteins in the decreasing order was: Cry1Ca > Cry1Ab > Cry1Ac > Cry2Aa > Cry1Fa. However, the toxicities of the Cry proteins to *B. mori* were in the order: Cry1Fa > Cry1Ca > Cry2Aa > Cry1Ab > Cry1Ac. The Cry1Ca, Cry1Ab and Cry1Ac proteins exhibited relatively high toxicity to *C. suppressalis* larvae, with EC_{50} values of 16.4, 45.8 and 89.6 ng/g, respectively. The toxicities of the three Cry proteins to *B. mori* larvae were 8, 14, and 22 times lower, with EC_{50} values of 138.3, 628.4 and 1939.2 ng/g, respectively. The Cry1Fa and Cry2Aa proteins showed high toxicity to *B. mori* larvae, with EC_{50} values of 135.7 and 373.9 ng/g, respectively, but low toxicity to *C. suppressalis* larvae, with EC_{50} values of 6092.1 and 1208.5 ng/g, respectively. We thus conclude that Cry1Ab, Cry1Ac and Cry1Ca are appropriate for transforming rice to control lepidopteran rice pests. In contrast, Cry1Fa and Cry2Aa are not appropriate due to their high toxicity to silkworm larvae and low activity against the target pest.

1. Introduction

Rice, *Oryza sativa* L., is one of the most important food crops worldwide, and China is the largest rice producer and consumer in the world (Li et al., 2016). It was estimated that approximately 20% of arable Chinese land is planted with rice (Chen et al., 2011). In rice production, insect pests are a serious constraint upon yield, especially lepidopteran pests, such as the rice stem borers *Chilo suppressalis* (Family Crambidae), *Scirpophaga incertulas* (Pyralidae), and *Sesamia inferens* (Noctuidae), and the rice leaf roller *Cnaphalocrocis medinalis* (Pyralidae) (Chen et al., 2011). Damage results in a direct loss of $US 1.9 billion each year in China (Sheng et al., 2003a,b).

The development of insect-resistant rice varieties is a promising strategy for controlling insect pests. In the recent years, genetic engineering technology has provided a new tool for developing insect-resistant crops. To efficiently control rice pests and to reduce the need for conventional broad-spectrum insecticides, China has devoted great effort to develop insect-resistant genetically engineered (IRGE) rice lines. Multiple lines have been developed that produce Cry proteins derived from the bacterium *Bacillus thuringiensis* (*Bt*) (Li et al., 2016). Most of these *Bt* rice lines, such as KMD (expressing the *cry1Ab* gene), Huahui 1 and *Bt* Shanyou 63 (both expressing a *cry1Ab/1Ac* fusion gene), TIC-19b (expressing a *cry1C* gene) and T2A-1 (expressing a *cry2A* gene) have proven to be effective against lepidopteran pests (Tu et al., 2000; Wang et al., 2016; Ye et al., 2001). In addition to the *Bt* genes currently used, other *Bt* genes, such as *cry1Fa*, have the potential to be applied in future IRGE rice lines.

Despite great benefits, the risks associated with the growing of GE plants have to be carefully addressed prior to the commercialization of any novel GE plant. The potential effect of IRGE plants on non-target organisms is an important concern that is addressed in environmental risk assessment (Li et al., 2016; Li et al., 2014b). Discussions of the impacts on non-target organisms have been focused primarily on terrestrial organisms such as natural enemies (Li et al., 2014a, 2013; Li et al., 2015; Wang et al., 2012), economically important insects (Wang et al., 2015; Yang et al., 2014), and soil microorganisms (Yang et al., 2015; Yuan et al., 2011, 2013). The silk worm *Bombyx mori* (Lepidoptera: Bombycidae) is an economically and culturally important insect in China, which is a

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world center of silk production (Liu et al., 2010). The larvae feed exclusively on mulberry (*Morus atropurpurea*) leaves. In Southeast China, mulberry trees are typically planted near or around rice fields in a planting system that is referred to as mulberry-mixed cropping (Fan et al., 2003). Thus, once *Bt* rice is commercially grown in China, mulberry leaves may be covered with *Bt* rice pollen. Consequently, *B. mori* larvae could be exposed to Cry proteins if Cry proteins are expressed in rice pollen and if the larvae consume mulberry leaves covered with *Bt* rice pollen (Wang et al., 2001, 2002; Yang et al., 2014; Yao et al., 2008, 2006; Yuan et al., 2006). Because *B. mori* belongs to the same order, Lepidoptera, as the target pests, it is sensitive to the lepidopteran-active Cry proteins that have been used or may be used for *Bt* rice development. Therefore, the safety of *Bt* rice lines to *B. mori* should be confirmed before they are commercially planted.

In the current study, we aimed to identify *Bt* proteins that exhibit high toxicity to the target lepidopteran pest *C. suppressalis*, but relatively low toxicity to the non-target silkworm *B. mori*. The results from the current study could be useful for decision-making regarding the commercialization of the currently developed *Bt* rice lines. In addition, the information might be valuable for *Bt* rice breeders for the selection of appropriate genes for future rice transformation.

2. Materials and methods

2.1. Insects

Neonates of *C. suppressalis* were obtained from a laboratory colony maintained in a climate-controlled chamber at 27 ± 1 °C, 75 ± 5% RH, and 15:9 h L:D photoperiod. The colony has been maintained on an artificial diet for over 60 generations in the laboratory without contact to *Bt* protein (Han et al., 2012).

A hybrid of *B. mori*, qiufeng × baiyu, was used in this study. Eggs of *B. mori* were purchased from Minghe Biotechnology Co., Ltd. (Huzhou City, Zhejiang, China) and were kept in a climate-controlled chamber at 27 ± 1 °C, 75 ± 5% RH, and 12:12 h L:D photoperiod. Newly hatched larvae (<12 h after hatching) were used for all experiments.

2.2. Insecticidal compounds

The five *Bt* proteins Cry1Ab, Cry1Ac, Cry1Ca, Cry1Fa, and Cry2Aa were purchased from Envirotest-China (an agent for EnviroLogix, Inc., Portland, Maine, USA; www.envirotest-china.com). The protoxins from *B. thuringiensis* had been expressed as single-gene products in *Escherichia coli* at Case Western Reserve University (Cleveland, Ohio, USA). The *E. coli*-protoxin inclusion bodies then were dissolved and trypsinized, and isolated and purified by ion exchange HPLC; the pure fractions were desalted and lyophilized. The purity of the purchased proteins was about 94–96% (Marianne Pusztai-Carey, Department of Biochemistry, Case Western Reserve University, personal communication). The respective Cry proteins were loaded into 8% SDS-PAGE gels to check their purity and molecular weight (Fig. 1). The molecular weights of the Cry proteins were between 55 and 72 kDa.

2.3. Bioassays with C. suppressalis larvae

Stock solutions of the five Cry proteins were diluted with distilled water and incorporated into an artificial diet as described in Han et al. (2012) for *C. suppressalis* to obtain the following concentrations: 0, 25, 50, 75, 100, 150 and 200 ng/g fresh weight (FW) of diet for Cry1Ab; 0, 50, 100, 200, 400 and 800 ng/g FW of diet for Cry1Ac; 0, 6.25, 12.5, 25, 50 and 100 ng/g for Cry1Ca; 0, 2000, 4000, 8000, 12,000 and 16,000 ng/g for Cry1Fa; and 0, 500, 1250, 2500, 5000, 6000, 12,000, 24,000 ng/g for Cry2Aa. These concentrations were selected based on the results of preliminary experiments. Since the *C. suppressalis* diet must be heated during preparation, the Cry protein solutions were mixed into the diet when the diet temperature had decreased to <60 °C to avoid degradation. Once the diet was solid, it was cut into slices (5 × 4 × 0.2–0.3 cm) and individually placed in Petri dishes (90 mm diameter, 15 mm height). Fifteen neonates of *C. suppressalis* were transferred into each Petri dish, which was subsequently sealed with Parafilm. The Petri dishes were covered with a black cloth and put in a climate-controlled chamber at 27 ± 1 °C, 75 ± 5% RH, and 15:9 h L:D photoperiod. Four replicates (Petri dishes) were set up for each concentration × protein combination, resulting in a total of sixty neonates tested for each concentration and protein. The diet was not replaced during the bioassay, and after 7 days, the mortality and weight of *C. suppressalis* larvae were recorded. The larvae were judged as dead when no response was observed after touching with a fine brush. Larvae were weighed using an electronic balance (CPA224S, Sartorius, Germany). The EC_{50} (toxin concentration resulting in 50% weight reduction compared to the control) and LC_{50} (toxin concentration resulting in 50% mortality compared to the control) to *C. suppressalis* were estimated for each protein.

2.4. Bioassays with B. mori larvae

An artificial diet for *B. mori* was purchased from Minghe Biotechnology Co., Ltd. (Huzhou City, Zhejiang, China). Using a method similar to that described above, five Cry proteins were uniformly mixed into the artificial diet to obtain the final concentrations: 0, 100, 200, 400, 800 and 1600 ng/g fresh weight (FW) of diet for Cry1Ab; 0, 50, 100, 200, 400 and 800 ng/g FW of diet for Cry1Ac, Cry1Ca, Cry1Fa and Cry2Aa. The prepared diets were placed on the bottom of plastic boxes (17 × 11 × 4 cm) with small holes in the lids. Twenty *B. mori* neonates were introduced into each plastic box, yielding three boxes (replicates) with a total of 60 insects tested for each concentration and protein. The plastic boxes were placed in a climate-controlled chamber at 27 ± 1 °C, 75 ± 5% RH, and 12:12 h L:D photoperiod. The diet was replaced every day. After 7 days, the mortality and weight of *B. mori* larvae in each treatment were recorded. The EC_{50} and LC_{50} for *B. mori* were estimated for each protein.

2.5. Statistical analyses

The EC_{50} and LC_{50}, were estimated with their 95% confidence intervals (CI95) by probit analysis using the software package SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL) (Finney, 1971). EC_{50} and LC_{50} values were considered to be significantly different when their CI95 did not overlap. A relative ratio was calculated according to the following formulas:

$$\text{Relative ratio for lethal effect} = \frac{LC_{50} \text{ of } B.\ mori}{LC_{50} \text{ of } C.\ suppressalis}$$

$$\text{Relative ratio for sublethal effect} = \frac{EC_{50} \text{ of } B.\ mori}{EC_{50} \text{ of } C.\ suppressalis}$$

3. Results

3.1. Lethal effects of Cry proteins to C. suppressalis and B. mori

The lowest toxicity to *C. suppressalis* was observed for Cry1Fa with an LC_{50} value of 16.61 μg/g (Table 1). In contrast, Cry1Ca had the highest toxicity, with an LC_{50} value of 0.10 μg/g. The lethal