



# Assessing the effects of Cry2Aa protein on *Habrobracon hebetor* (Hymenoptera: Braconidae), a parasitoid of Indian meal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae)

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## ABSTRACT

Transgenic crops express Cry proteins exhibit high resistant to target insect pests. When we evaluate the effects of Cry proteins on the parasitoid of target insect pest via tritrophic experiments (transgenic plant-target insect pest-parasitoid) host quality of parasitoids might decrease because of insecticidal protein ingestion, this would cause host-quality mediated effects and influence the accuracy of biosafety assessment. In the current study, high dose of Cry2Aa protein was injected into the hemolymph of *Plodia interpunctella* by microinjection, and the hemolymph was used as the carrier to deliver Cry protein to *Habrobracon hebetor*, which has been previously reported as an ectoparasitoid of *P. interpunctella* larval, in order to avoid the “host-quality mediated effects”. Results showed that injected Cry2Aa remained at high concentration and bioactive in the hemolymph of *P. interpunctella* parasitized by *H. hebetor*, the hemolymph of *P. interpunctella* could be used as carriers of Cry protein to *H. hebetor*, and high dose of Cry2Aa have no negative impacts on the development time, weight of pupa, sex ratio, adults weight (male and female), adult longevity and fecundity, and the activity of stress-related enzymes of *H. hebetor*. However, the hemolymph of *P. interpunctella* injected into *Galanthus nivalis* agglutinin (the positive control) showed significant negative impact on these parameters measured in the present study of *H. hebetor*. This indicated that Cry2Aa protein had no detrimental effects on the biological parameters of *H. hebetor* measured in the current study. Meanwhile, this study provides a new method for the safety evaluation of the ectoparasitoids of target pest and might be expanded to the other species of ectoparasitoids of target insects of Cry proteins in biosafety risk assessment.

## 1. Introduction

Rice (*Oryza sativa* L.) is a staple food in Asia. During growth season, it is infested by lepidopteran insect pests including stem borers and leafrollers, these pest species account for more than 50% of the annual losses of rice pests and diseases (Zhang, 2007; Schwember, 2008). At present, the control of these pests mainly depends on chemical pesticides. However, wide use of pesticides causes some problems including resistance of pests to insecticide, harmfulness to non-target arthropods and contamination to water and soil (Matteson, 2000; Lou et al., 2013).

Cry protein produced by *Bacillus thuringiensis* (Bt) is encoded by cry gene, has been used as insecticidal protein against lepidopteran insect pests for 50 years (Chen et al., 2005; Raymond et al., 2010; Li et al., 2017). Cry genes are introduced into rice with transgenic technology to develop transgenic rice, which can effectively control lepidopteran pests and reduce the use of chemical pesticides (Huang et al., 2015). Up to date, a series of transgenic rice plants expressing various cry genes (such as cry1Ab, cry1Ac, cry1Ab/1Ac, cry1C, cry2Aa, cry9C, cry1Ac/cry11-like) have been developed in China (Cheng et al., 1998; Xiang et al., 1999; Tu et al., 2000; Ye et al., 2001; Chen et al., 2005, 2008;

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Tang et al., 2006; Yang et al., 2014). However, Cry protein expressed in transgenic rice may be transmitted to non-target arthropods through the food chain. Thus, the biosafety of Cry protein to non-target arthropods has attracted great attentions from public. The Indian meal moth, *Plodia interpunctella* (Hübner) is a worldwide storage pest with wide host range, especially abundant in grain (Vick et al., 1986; Mohandass et al., 2007; Vukajlović et al., 2019). *Habrobracon hebetor* Say (Hymenoptera: Braconidae) is an important ectoparasitoid used as biological agent to control lepidopteran pests including *Sitotroga cerealella* (Oliver), *Ephesia kuehniella* (Zeller), *Corcyra cephalonica* (Staint), *P. interpunctella*, etc. (Akinkulore et al., 2009; Ghimire and Phillips, 2010; Belda and Riudavets, 2013; Ba et al., 2014; Borzoui et al., 2016; Trematerra et al., 2017). Cry protein may be transmitted to the *H. hebetor* through parasitizing the larvae of *P. interpunctella*, which infests rice during storage. Therefore, the biosafety evaluation of transgenic *Bt* rice on *H. hebetor* is worthy of attention.

Previous reports indicated that the quality of target lepidopteran pests infesting Cry proteins would decrease, which will affect the growth and development of parasitic wasp (Baur and Boethel, 2003; Liu et al., 2005, 2011; Ramirez-Romero et al., 2007), i.e. the “prey-quality mediated effects”. Because of “prey-quality mediated effects”, even for a same Cry protein, the results of the potential effects on the same species of parasitoid from different reports would be controversial (Schuler et al., 2004; Wang et al., 2017). This will influence the accuracy of biosafety assessment of transgenic *Bt* plants on non-target parasitoids. Using *Bt*-resistant lepidopteran target insect strains as hosts to evaluate the potential effects of Cry protein to parasitoids can avoid the “prey-quality mediated effects” (Schuler et al., 2004; Chen et al., 2008; Tian et al., 2014). However, the construction of a resistant strain requires a lot of manpower, material resources and time. Therefore, more convenient and efficient method to overcome “prey-quality mediated effects” needs to be developed.

T2A-1 is a transgenic rice variety expressing Cry2Aa protein driven by maize ubiquitin promoter, and has high resistance to the lepidopteran pests (Chen et al., 2005; Yang et al., 2016). In the current study, in order to eliminate “prey-quality mediated effects”, high dose of Cry2Aa protein was directly injected into the hemolymph of the *P. interpunctella* by microinjection and the hemolymph of *P. interpunctella* was used as the carrier of Cry2Aa protein to *H. hebetor*. The potential effects of high dosage of Cry2Aa protein, which was transmitted via the hemolymph of *P. interpunctella* to *H. hebetor*, on the development, survival, fecundity and the activity of stress-related enzymes of *H. hebetor* were evaluated.

## 2. Materials and methods

### 2.1. Plant materials

Transgenic rice line T2A-1 and its corresponding non-transgenic Minghui 63 were used as rice lines. T2A-1 expressed a synthetic *cry2Aa* gene driven by maize ubiquitin promoter (Chen et al., 2005). Minghui 63 was served as the control. T2A-1 is homozygous and highly resistant to lepidopteran rice pests. Both rice lines were kindly provided by Prof. Lin Yongjun (National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China).

These two rice lines were planted in the rural area of Hanchuan city, Hubei Province, China (30.63°N 113.59°E) without any pesticide application. Rice seeds were harvested and dried by sun for three days (water content of seeds < 8% measured by cereal moisture tester), then sealed in plastic bags and stored in the refrigerator at -20 °C.

### 2.2. Insects

*P. interpunctella* and *H. hebetor* were collected from rice seed warehouse of Huazhong Agricultural University and reared in our laboratory for 30 generations. Twenty couples of *P. interpunctella* adults (< 24 h

after eclosion) were put into an empty glass jar (height 10 cm, diameter 15 cm) to lay eggs for 1 day. Then 50 g of artificial diet (1000 g cracked wheat, 1000 g wheat shorts, 100 g wheat germ, 80 g brewer's yeast, 4 g methyl-p-hydroxybenzoate, 4 g sorbic acid, 240 ml glycerine, 240 ml pure honey and 120 ml water) (Oluwafemi et al., 2007) was added into glass jar to feed the larvae of *P. interpunctella*. The 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella* were used as the hosts for *H. hebetor*. The adults of *H. hebetor* were fed with 10% of honey water.

All insects were cultured and all experiments were conducted in a climatic chamber at 27 ± 1 °C, RH 70 ± 10% and with a 14 L: 10 D photoperiod.

### 2.3. Compounds

Cry2Aa protein was purchased from the Biochemistry Department Laboratory, School of Medicine, Case Western Reserve University, USA. The Cry2Aa protoxin was trypsinized and lyophilized before use. Bovine serum albumin (BSA), which was purchased from Dalian Meilun Biotech Technology Co., Ltd (Dalian, China), used as negative control. *Galanthus nivalis* agglutinin (GNA), which was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), used as positive control. The three proteins were dissolved in PBS (NaCl 136.89 mM; KCl 2.67 mM; Na<sub>2</sub>HPO<sub>4</sub> 8.1 mM; KH<sub>2</sub>PO<sub>4</sub> 1.76 mM). All solutions were prepared at a concentration of 0.5 mg/ml before use and stored at 4 °C.

### 2.4. Determine the realistic dose of Cry2Aa exposure to *H. hebetor*

T2A-1 rice seeds (about 500 g) were ground and mixed with glycerol in a ratio of 9:1 to be rice powder. Two hundred of 3<sup>rd</sup> instar larvae of *P. interpunctella* were starved for 48 h and fed with T2A-1 rice powder. Two days later the living larvae were collected for hemolymph collection. The larvae were washed three times with 1 × Extraction buffer (Provided by QuantiPlate Kit) to remove Cry2Aa contamination on the surface. Then the anal legs were cut with dissecting scissors, and the hemolymph was collected with capillary tube. At the same time, *P. interpunctella* fed T2A-1 rice seeds were parasitized for *H. hebetor* (15 *P. interpunctella* with 5 pair of *H. hebetor*), and Cry2Aa protein content in *H. hebetor* was detected. T2A-1 seeds were ground to detect Cry2Aa protein content. The concentrations of Cry2Aa protein were determined by using EnvironLogix kits (AP005, EnvironLogix, USA). The minimum detection limit of this kit is 0.52 ng/g, and the detection wavelength is 450 nm.

### 2.5. Determine the optimal injection volume for micro-injection

To determine the optimal micro-injection volume, 300, 400 and 500 nl PBS was injected into 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella* with Nanoliter 2010 (WPI, USA). Then the injected larvae were putted in a culture dish (9 cm in diameter) and reared in the climatic chamber with artificial diets. Survival rate within 7 days after injection was recorded. Three replicates per treatment, 20 larvae per replicate.

### 2.6. The uniformity of Cry2Aa protein in *P. interpunctella* hemolymph at different time points after injection

We mix the ink and Cry2Aa protein solution (0.5 mg/ml) in a 1:1 volume ratio. Then the mixture was injected into *P. interpunctella* hemolymph to observe the diffusion of Cry2Aa proteins. After injection, the distribution of the mixture in the larvae was quickly observed under the Olympus SZX 16 microscope (Olympus, Japan).

### 2.7. The stability of Cry2Aa protein in the hemolymph of *P. interpunctella* larvae parasitized by *H. hebetor*

Fifteen 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella* were injected with Cry2Aa solution, and placed in a glass tube (diameter 2.5 cm, height

7.5 cm). After that, five pairs of *H. hebetor* adults (12 h after eclosion) were mated for 48 h, and were immediately introduced into the glass tube to parasitize the larvae of *P. interpunctella* for 6 h. Larval hemolymph of *P. interpunctella* was collected at different time points after injection to determine the content of Cry2Aa. Fifteen larvae of *P. interpunctella* per replicate, and three replicates were set up.

## 2.8. Biological activity of Cry2Aa in *P. interpunctella* hemolymph during wasp parasitization

The 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella* were injected with Cry2Aa solution and parasitized by *H. hebetor* as described in 2.7. The hemolymph of *P. interpunctella* larvae was collected three days after injection and parasitization. The hemolymph of non-injected and non-parasitized larvae of *P. interpunctella* was used as control. The artificial diet was mixed with the hemolymph in a ratio of 1 g: 1 ml, the mixture was lyophilized. The *P. interpunctella* neonates (< 24 h) were fed with the mixture. The mortality of *P. interpunctella* larvae was recorded one week later. Forty *P. interpunctella* neonates were used for one replicate, and three replicates for each treatment.

## 2.9. Western blot analysis

5  $\mu$ l hemolymph was diluted with 5  $\mu$ l H<sub>2</sub>O, then mixed with 2  $\times$  SDS buffer. The mixture was heated at 95  $^{\circ}$ C, for 5 min. The proteins in the sample were resolved by 10% SDS-PAGE and electro-transferred (20 V, 4  $^{\circ}$ C overnight) to PVDF membranes. Membrane were blocked at room temperature for 2 h in blocking buffer (PBS plus 3% BSA, 0.1% Tween-20), then washed by washing buffer (PBS plus 0.1% BSA, 0.1% Tween-20) for 3 times, 15 min each time. Then the rabbit polyclonal anti-Cry2Aa sera hybridized with PVDF membrane in blocking buffer (1:5,000, 2 h, 25  $^{\circ}$ C). After washing as described in the previous step, the PVDF membrane were hybridized with polyclonal HRP-conjugated goat anti-rabbit sera (1:5,000, 1 h, 25  $^{\circ}$ C). After washing, filters were developed using the ECL chemiluminescence detection kit (Thermo Fisher Scientific).

## 2.10. The effects of high dosage of Cry2Aa in the hemolymph of *P. interpunctella* larvae on the life-table parameters of *H. hebetor*

The same concentration and volume of BSA, GNA and Cry2Aa solution were injected into the 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella*. The injected larvae were parasitized by *H. hebetor* as described in 2.7. The injected and parasitized *P. interpunctella* larvae (one egg of wasp per *P. interpunctella* larval) were transferred to a new culture dish (diameter 2.5 cm). In order to ensure that the wasp was always exposed to high-dose protein, we changed the injected *P. interpunctella* larvae every three days to feed the wasp larvae. The larval development time, weight of pupa and adult, sex ration of *H. hebetor* were recorded based on daily observation. The newly emerged *H. hebetor* adults were paired and supplied with 10% honey. The survival of adults was observed and recorded daily until all the adults died. The host for *H. hebetor* was renewed every day and the total number of eggs laid by parasitic wasps within six days was recorded. The 4<sup>th</sup> - 5<sup>th</sup> instar larvae injected with BSA were used as negative control, and GNA injection was used as positive control.

## 2.11. Detection of stress-related enzymes activities in *H. hebetor* larva

The activity of glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) of *H. hebetor* larvae at 4<sup>th</sup> instar was determined using Glutathione Reductases assay kit, Superoxide Dismutase (SOD) assay kit (WST-1 method) and Catalase (CAT) assay kit (Visible light) (Nanjing Jiancheng Bioengineering Institute, China). The enzyme activity was determined according to manufacturer's instructions.

## 2.12. Data analysis

Determination of LD<sub>50</sub> was analyzed using REG procedure after the mortality data were transformed into probit and the concentration data were subjected to logarithmic transformation. The data except those from LD<sub>50</sub> determination test, were tested for its normality and homogeneity of variance by univariate and glm procedure of SAS software. Percentage data were arcsine-square root transformed. All count data were square root ( $x + 1$ ) or log<sub>10</sub> ( $x + 1$ ) transformed before test. Untransformed means are presented in results.

Kruskal-Wallis *H* test and Nemenyi method were used to analyze the statistical significance of larval development time, weight of pupa, weight of female/male, eggs laid per female within six days and adult longevity between different treatments. The data of sex ratio were compared by *Chi*-square test. The mortality for detecting Cry2Aa protein activity were analyzed using Student's *t*-test. Survival response of *H. hebetor* exposed to host hemolymph was analyzed by the Kaplan-Meier procedure. One-way ANOVA and LSD were used to compare survival rates after injection of different volumes of PBS on the seventh day and enzyme activities among treatments.

All statistical analyses were conducted using SAS (version V8 for windows).

## 3. Results

### 3.1. Determining the optimal dose and volume for microinjection

Cry2Aa content in T2A-1 seeds was 12.87  $\pm$  0.23  $\mu$ g/g. Two days after 3rd-instar larvae feeding T2A-1 rice powder, the contents of Cry2Aa in *P. interpunctella* hemolymph was 176.0  $\pm$  9.1 ng/g. Compared to T2A-1 seeds, the Cry2Aa content in the hemolymph of *P. interpunctella* larvae decreased sharply. However, Cry2Aa protein was not detected in *H. hebetor* adult which parasitized *P. interpunctella* larvae feeding T2A-1 seed powder. This indicated that Cry2Aa protein was not transformed to *H. hebetor* via food chain. Therefore, the realistic dose of *H. hebetor* larvae exposed to Cry2Aa protein is 176.0 ng/g, and the dose of Cry2Aa in *P. interpunctella* hemolymph, which was used as Cry protein carrier to *H. hebetor* larvae in Tier-1 test, should be at least 1760 ng/g in the Tier-1 bioassay.

Different volume of PBS had significant effect on the survival of the 4<sup>th</sup> - 5<sup>th</sup> instar larvae of *P. interpunctella* ( $F = 39.00$ ;  $df = 2,6$ ;  $P < 0.01$ ). There was no significant difference in survival rate between 300 nl and 400 nl PBS injection on the seventh day after injection, the larvae injected with 300 or 400 nl PBS survived more than 80% at seventh day after injection. The survival rate of *P. interpunctella* larvae injected with 500 nl PBS was significantly lower than those of 300 and 400 nl PBS injection on the same day after injection (Fig. 1). In order to inject as much Cry2Aa as possible into the *P. interpunctella* hemolymph, we need to choose as great a volume as possible to ensure enough dosage of Cry2Aa protein exposure to *H. hebetor* larvae. Thus, comprehensively considering survival rate and injection volume, we chose 400 nl as the optimal injection volume for the next bioassay.

### 3.2. The uniformity of Cry2Aa protein in *P. interpunctella* hemolymph at different time points after injection

After the injection of the ink-Cry2Aa protein mixture, it could be seen under the microscope that the ink-Cry2Aa protein mixture diffused very rapidly in the hemolymph of *P. interpunctella*. Within 30 s, the ink-Cry2Aa protein mixture diffused to the most part of the larval body. It was clear that the ink-Cry2Aa protein mixture entered into the dorsal blood vessel and diffused with blood circulation (Fig. 2A and B). Two minutes later, the ink-Cry2Aa protein mixture spread uniformly in the hemolymph of *P. interpunctella* larvae (Fig. 2C). Thus, 2 min after injection is long enough to assure that *H. hebetor* larvae could be exposed to the same dosage of Cry2Aa no matter where *H. hebetor* adults

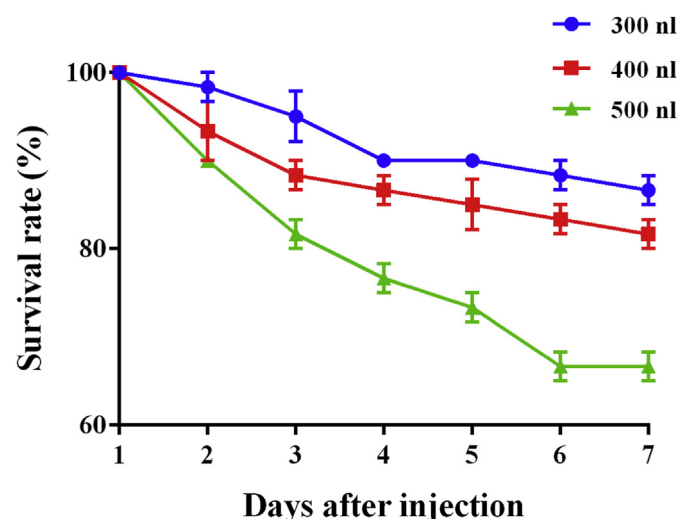


Fig. 1. The effect of injection volume of PBS on the survival rate of *Plodia interpunctella* larvae. Three replicates were done for each volume, 20 larvae for each replicate. Error bars indicate SE of the three replicates. The difference in survival rate between treatments on seventh day was determined using one-way ANOVA and LSD test.

parasitized.

### 3.3. The contents of Cry2Aa protein in the hemolymph of *P. interpunctella* larvae parasitized by *H. hebetor*

After Cry2Aa was injected into *P. interpunctella* larvae for 2 min, *P. interpunctella* larvae were parasitized with parasitic wasps. Then we examined the content of Cry2Aa in the hemolymph of *P. interpunctella* larvae at 1, 2, 3, and 4 d after injection. It was found that Cry2Aa gradually degraded in the hemolymph (Fig. 3). On the third day, the Cry2Aa content in the hemolymph was  $2028 \pm 175$  ng/g. From the fourth day, the Cry2Aa content in the hemolymph ( $1434 \pm 145$  ng/g) was lower than 1760 ng/g, which was the lowest dose the Tier-1 test required. Therefore, in order to ensure that the wasp was always exposed to high-dose protein, we renewed the injected *P. interpunctella* larvae every three days to feed the wasp larvae.

### 3.4. The bioactivity of Cry2Aa purchased from company and in the hemolymph of *P. interpunctella* larvae

The LD<sub>50</sub> of *P. interpunctella* neonates for this batch of Cry2Aa was  $13.17 \mu\text{g/g}$  ( $\chi^2 = 2.98$ ,  $df = 3$ ). The hemolymph of *P. interpunctella* larvae injected with Cry2Aa and parasitized by *H. hebetor* for three days caused  $39.17 \pm 3.63\%$  of mortality, whereas, the control group caused  $16.67 \pm 1.67\%$  of mortality (Table 1). Moreover, the Western blot showed that the injected protein was as same as those in the hemolymph of larva fed with the transgenic *cry2Aa* seed (Fig. S1). These

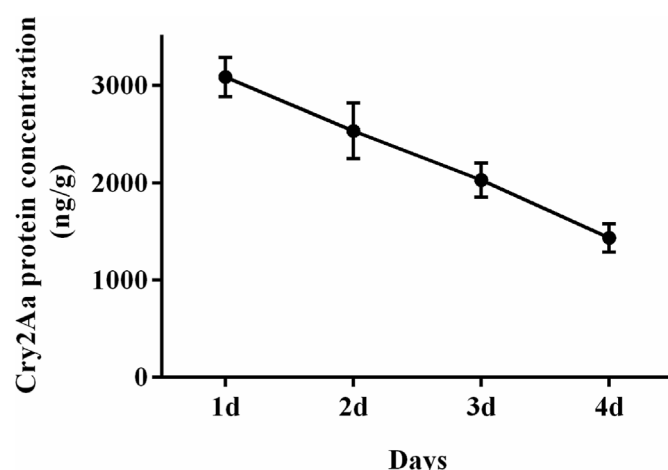


Fig. 3. Content of Cry2Aa protein in the hemolymph of *Plodia interpunctella* larvae, which were injected Cry2Aa and parasitized by *Habrobracon hebetor*. Within 3 days, the content of Cry2Aa in the hemolymph of *P. interpunctella* larvae was 10 times higher than the realistic exposure dosage to *H. hebetor*. Error bars indicate statistical differences between three replications (Mean  $\pm$  SE).

results indicated that Cry2Aa protein could maintain intact and insecticidal activity during the parasitization of *H. hebetor* larvae.

### 3.5. The effects of high dosage of Cry2Aa in the hemolymph of *P. interpunctella* larvae on the life-table parameters of *H. hebetor*

Survival analyses did not detect significant differences in survival response of *H. hebetor* from egg to adult between Cry2Aa and BSA treatments, while GNA treatment had significantly negative effect on the survival response of *H. hebetor* as compared to BSA treatment ( $P < 0.01$ ) (Fig. 4). As compared to BSA treatment, high dose exposure to Cry2Aa protein had no adverse effect on the life-table parameters of *H. hebetor* including larval development time, weight of pupa, sex ratio, weight of adults, eggs laid per female within six days and adult longevity. However, GNA treatment had obvious detrimental effects on the life-table parameters of *H. hebetor*, as compared to BSA treatment ( $P < 0.05$ ) (Table 2). These results indicated that this testing system could detect insecticidal activity to *H. hebetor* of the proteins injected into the hemolymph of *P. interpunctella* larvae. Via this testing system, it could be concluded that Cry2Aa had no negative effects on the life-table parameters of *H. hebetor* in the present study.

### 3.6. Stress-related enzyme activity in *H. hebetor* larvae exposed to cry proteins

As shown in Fig. 5, the activities of SOD, CAT and GR in the 5th-instar larvae of *H. hebetor* exposed to GNA were significantly higher than those of BSA and Cry2Aa treatment (in the above order,  $df = 2,6$ ;

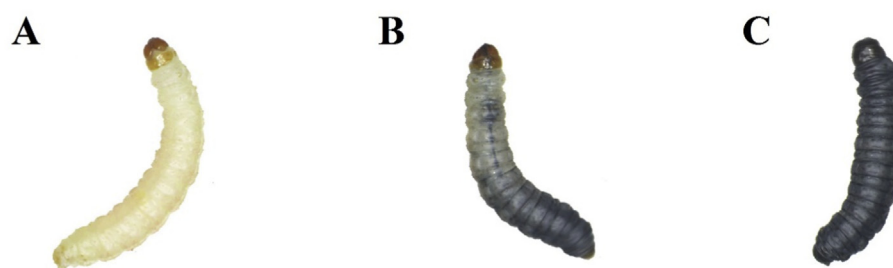


Fig. 2. Diffusion of ink-Cry2Aa protein mixture in the hemolymph of *P. interpunctella* larvae at different time points after injection. A: Before injection; B: 30 s after injection; C: 2 min after injection.

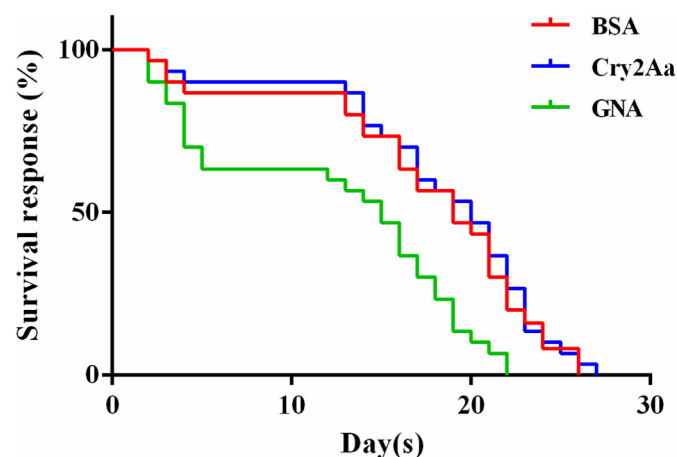


**Table 1**

The mortality of *Plodia interpunctella* neonates fed by artificial diets containing hemolymph of *P. interpunctella* injected with Cry2Aa and parasitized by *Habrobracon hebetor*.

Treatment	Mortality of <i>P. interpunctella</i> neonates (%)
Hemolymph of the non-injected and non-parasitized larvae of <i>P. interpunctella</i>	16.67 ± 1.67
Hemolymph of <i>P. interpunctella</i> larvae injected with Cry2Aa and parasitized by <i>H. hebetor</i>	39.17 ± 3.63 *

Mortality was represented as Mean ± SE. Three replicates were set up for each treatment, and 40 insects per replicate. Significant differences between treatments are indicated with asterisks.



**Fig. 4.** Survival response of *Habrobracon hebetor* exposed to the hemolymph of *Plodia interpunctella* larvae injected into 200 ng of Cry2Aa protein or BSA (control) or GNA (positive control).

$F = 9.08$ ;  $P = 0.02$ ,  $df = 2,6$ ;  $F = 5.90$ ;  $P = 0.04$ ,  $df = 2,6$ ;  $F = 10.84$ ;  $P = 0.01$ , respectively). There were no significant differences in these three enzymes activity between the BSA and Cry2Aa treatment.

#### 4. Discussion

In the current study, we successfully constructed a Tier-1 assay for evaluating the potential effects of Cry2Aa on development and fecundity of *H. hebetor*, which is the parasitoid of target arthropod of Cry2Aa. Firstly, the Cry2Aa protein content in the hemolymph of *P. interpunctella* larvae fed the transgenic rice T2A-1 seeds was  $176.0 \pm 9.1$  ng/g, which is the realistic exposure dose of *H. hebetor* parasitizing the *P. interpunctella* larvae. By microinjection method, the exposure dosage of Cry2Aa protein to *H. hebetor* was maintained more than ten times of the actual exposure of parasitic wasps and maintained good insecticidal activity (Table 1). Therefore, the hemolymph of *P. interpunctella* can be used as a Cry2Aa protein carrier to deliver a high dose of Cry2Aa protein to the larvae of the *H. hebetor*. Secondly, GNA

injected into the hemolymph of *P. interpunctella* could prolong the developmental duration of *H. hebetor*, decreased the survival, the weight and fecundity of *H. hebetor*. This indicates that GNA might be used as a positive insecticidal control, and the experimental system can detect the negative impact of insecticidal compounds on the tested organisms. Thirdly, we could use ELISA to monitor the concentration, stability and biological activity of Cry2Aa proteins.

When evaluating the safety of transgenic *Bt* crops to parasitic wasps or predators of target pests, the safety evaluation results of the same Cry protein for the same parasitic wasp or predator are often contradictory. For example, when *Macrocentrus cingulum* parasitizes Cry1Ac-susceptible strain of *Ostrinia furnacalis*, which fed diet containing low concentrations (0.1 µg/g) of Cry1Ac protein, the survival rate, parasitism rate and egg production of *M. cingulum* decreased significantly. However, life-table parameters of *M. cingulum* were not adversely affected when *M. cingulum* parasitizes Cry1Ac-resistant strain of *O. furnacalis*, which fed on diets containing high dose (100 µg/g) of CryAc protein (Wang et al., 2017). When *Chrysoperla carnea* preyed on *Spodoptera littoralis* fed with transgenic maize expressing the *cry1Ab* gene, the mortality rate of *C. carnea* is significantly increased and the developmental duration is significantly prolonged, as compared to the control (Dutton et al., 2002). However, high dose of the Cry1Ab protein was added in the sugar solution to feed *C. carnea*, it was found that there was no significant difference in the growth and development of *C. carnea* as compared to the control (Romeis et al., 2004). These contradictory results are caused by host/prey-mediated quality effects. It is essential to avoid host/prey-mediated quality effects when assessing the safety of transgenic *Bt* rice to parasitic natural enemies or predators of target pests (Romeis et al., 2011). In the current study, high-dose Cry2Aa protein was directly injected into the hemolymph of the *P. interpunctella* by microinjection. *H. hebetor* adults were immediately introduced to parasitize the larvae of *P. interpunctella*. Those larvae were paralyzed and stopped growing quickly, the adverse effects of Cry2Aa protein on the growth and development of *P. interpunctella* were avoided. Therefore, the effect of host quality was limited by this method.

There have been many reports on the effects of Cry2Aa protein on non-target Arthropoda. Han et al. (2015) mixed high-concentration

**Table 2**

Life table parameters of *Habrobracon hebetor* fed by *Plodia interpunctella* larva injected with 200 ng of Cry2Aa protein.

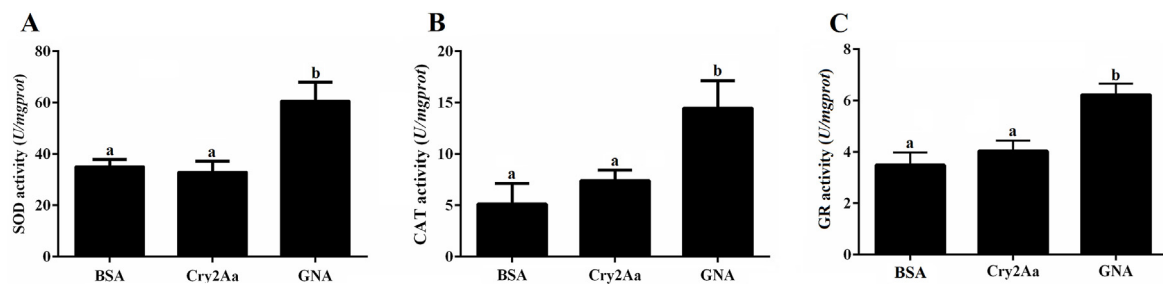
Parameters	Treatments		
	Cry2Aa	BSA	GNA
Larval development time (days) <sup>a</sup>	11.24 ± 0.05 (104)a	11.22 ± 0.05 (100)a	11.74 ± 0.10 (38)b
Weight of pupa (mg) <sup>a</sup>	1.36 ± 0.05 (30)a	1.38 ± 0.06 (30)a	1.19 ± 0.04 (30)b
Sex ratio (female/male) <sup>b</sup>	1.60 (104)a	1.70 (100)a	1.71 (38)a
Weight of female (mg) <sup>a</sup>	0.81 ± 0.02 (30)a	0.84 ± 0.02 (30)a	0.61 ± 0.02 (30)b
Weight of male (mg) <sup>a</sup>	1.17 ± 0.04 (30)a	1.15 ± 0.03 (30) <sup>a</sup>	0.82 ± 0.03 (30)b
Eggs laid per female within 6 days <sup>a</sup>	58.73 ± 1.82 (30)a	56.17 ± 1.69 (30)a	32.10 ± 1.29 (30)b
Adult longevity (days) <sup>a</sup>	13.10 ± 0.58 (104)a	13.03 ± 0.57 (100)a	9.97 ± 0.66 (38)b

Data in the table were Mean ± SE.

Number of replicates is given in parentheses per treatment.

<sup>a</sup> Statistical significance based on Nemenyi test.

<sup>b</sup> Chi square test. Different letters above bars indicate significant differences among groups.



**Fig. 5.** Enzyme activities of SOD (A), CAT (B) and GR (C) in *Habrobracon hebetor* larvae after exposure to BSA (blank control) or Cry2Aa or GNA (positive control). Values are the mean  $\pm$  SE from three replicates. Different letters above bars indicate significant differences among groups (one-way ANOVA and LSD test;  $P < 0.05$ ).

Cry2Aa protein into honey water to evaluate the safety of Cry2Aa protein against adults of *Anagrus nilaparvatae*. The results showed that Cry2Aa protein did not adversely affect the life table parameters of *A. nilaparvatae*. Similarly, Cry2A protein does not have any adverse effects on *Apis mellifera* (Wang et al., 2015). In the current study, we evaluated the effect of Cry2Aa protein on the life table parameters and stress-related enzymes activity of the larvae of *H. hebetor*. Compared with the control, the larval development time, weight of pupa, sex ratio, weight of female and male, fecundity and adult longevity of the larvae of *H. hebetor* ingested high doses of Cry2Aa protein, larval were not significantly affected. The stress-related enzymes activities of SOD, CAT, and GR in the mature larvae did not change significantly. However, when GNA (positive control) was ingested into the host hemolymph, the life-table parameters of *H. hebetor* were negatively affected, and stress-related enzymes of *H. hebetor* was significantly increased. In summary, high dose of Cry2Aa protein had no adverse effects on *H. hebetor*.

According to the previous reports, after the Cry proteins are ingested into midgut of target insect, protoxins are dissolved and processed by proteinase, then the active toxic fragments (toxins) are released. These activated toxins bind with gut Cry-binding proteins and insert into midgut brush border membrane, this binding results in pore-formation and cell lysis by producing an osmotic shock (Pardo-López et al., 2006; Bravo et al., 2011; Adang et al., 2014). In the current study, the Cry2Aa protein used for injection and ingestion bioassay was trypsinized and activated. The active Cry2Aa toxin was ingested by *P. interpunctella* larvae, and the midgut brush border membrane cells of *P. interpunctella* was killed by Cry2Aa, and pores were formed on the midgut membrane. Therefore, we speculated that the follow-up Cry2Aa proteins ingested into the midgut could diffuse into the hemolymph of *P. interpunctella* freely and intactly. The results of Western blots analysis of Cry2Aa proteins accumulated by ingestion and injection into the hemolymph of *P. interpunctella* larvae verified our speculation. This provide strong evidence that the direct injection method for the Cry2Aa protein is valid as the carrier to deliver Cry protein to the *H. hebetor*. On the other hand, we could infer that there are no Cry2Aa-binding proteins in the midgut of *H. hebetor*, the ingested Cry2Aa protein in midgut was digested as other proteins from food and could not accumulate, thus, Cry2Aa had no detrimental effects on the development and fecundity of *H. hebetor*. This hypothesis needs to be further verified.

## 5. Conclusion

By microinjecting Cry protein into the hemolymph of host, we established a new method to avoid host-mediated quality effects in bio-safety evaluation of Cry protein on ectoparasitoids. The results indicated that Cry2Aa protein has no negative effect on *H. hebetor*. At the same time, our experiment provides new ideas for the biosafety evaluation of Cry protein on ectoparasitoid of target pests.

## CRediT authorship contribution statement

**Zhengjie Wang:** Writing - original draft. **Wanlun Cai:** Formal analysis. **Wenjun Wang:** Methodology. **Jing Zhao:** Methodology. **Yifeng Li:** Resources. **Yulan Zou:** Validation. **Karam Khamis Elgizawy:** Validation. **Hongxia Hua:** Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoenv.2020.110380>.

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