Analysis of Cry1Ab toxin bioaccumulation in a food chain of Bt rice, an herbivore and a predator

Mao Chen · Gong-yin Ye · Zhi-cheng Liu · Qi Fang · Cui Hu · Yu-fa Peng · Anthony M. Shelton

Abstract Lessons from organophosphorus pesticides, which could be bioaccumulated in non-target organisms at different trophic levels and caused unexpected negative impacts, necessitate a study of the possibility of biotransfer and bioaccumulation of *Bacillus thuringiensis* (Bt) insecticidal toxin(s) expressed in Bt plants. Using ELISA, we evaluated the transfer of Cry1Ab toxin in a food chain of Bt rice (KMD1 and KMD2), the target insect, *Cnaphalocrocis medinalis,* and its predator, *Pirata subpiraticus*. Cry1Ab was detected in *C. medinalis* and *P. subpiraticus*. However, the concentration of Cry1Ab detected from *C. medinalis* and *P. subpiraticus* did not increase as feeding or preying time increased. A binding study of Cry1Ab to the brush border membrane vesicle of *C. medinalis* and *P. subpiraticus* indicated that *P. subpiraticus* does not have binding receptors in its midgut to Cry1Ab, while *C. medinalis* does. Survivorship and fecundity of *P. subpiraticus* preying on Bt rice-fed *C. medinalis* were not significantly different from those preying on non-Bt rice-fed *C. medinalis*. Developmental time of *P. subpiraticus* was significantly longer when it preyed on Bt rice-fed *C. medinalis* than on non-Bt rice-fed prey. However, a 3-year field trial indicated that Bt rice did not significantly affect the density of *P. subpiraticus*.

Keywords Bt rice · Non-target effect · Bioaccumulation · Tritrophic interactions · Ecological risk assessment

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Introduction

Rice (*Oryza sativa* L.) is the most widely consumed cereal grain and was grown on over 152 million ha worldwide in 2004 (FAO 2007). The main lepidopteran pests of rice in China are the striped stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae), yellow stem borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae), pink stem borer, *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae), and the leaffolder, *Cnaphalocrocis medinalis* (Gueneé) (Lepidoptera: Pyralidae). Collectively these insects annually cause a 3–10% yield loss equal to ca. $1 US billion, despite the intense use of insecticides (Sheng et al. 2003). Furthermore, the broad-spectrum insecticides commonly used in rice production have disrupted many biological control agents in the rice ecosystems and reduced their effectiveness for control of the Lepidoptera and other pest herbivores (Matteson 2000).

Because of the prominent pest status of stem borers and leaffolders, the limited source of resistant germplasm, and the success of genetically modified (GM) maize and cotton with *Bacillus thuringiensis* (Bt) insecticidal genes, there have been extensive investments in insect-resistant GM

rice research and development in China (Wang and Johnston 2007). Since 1993, numerous genotypes of GM rice with insecticidal *Bt* gene(s) (referred as Bt rice hereafter) have been developed that confer strong resistance against rice stem borers and leaffolders (reviewed by High et al. 2004; Chen et al. 2006; Wang and Johnston 2007). Bt rice field trials were first conducted in China in 1998 and large field trials of several Bt rice lines have continued in China (Chen et al. 2006; Wang and Johnston 2007). Field trials have confirmed that Bt rice effectively controls stem borers and leaffolders (Chen et al. 2006). In a study in farmers' fields in China, a Bt rice line resulted in yield increases of 6–9%, and 80% reduction in insecticide use, in comparison to conventional varieties (Huang et al. 2005). Because of the effectiveness of Bt rice for pest control and the extensive research on Bt rice in China, it is likely that China will be the first in the world to commercialize Bt rice (Cohen et al. 2008).

A major concern about the deployment of Bt rice is its potential impact on non-target arthropods, especially natural enemies through tritrophic interactions. Obrist et al. (2006) reported that Bt toxin could be transferred to arthropod predators (*Orius* spp., *Chrysoperla* spp., and *Stethorus* sp.) in Bt maize field. Chen et al. (2005) also found that Bt toxin could be taken up by the predator, *Pirata subpriaticus* (Bösenberg et Strand) (Araneae: Lycosidae) when it fed on Bt rice-fed *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae). Thus, it is possible that Bt toxins in Bt rice can appear in natural enemies through tritrophic interactions, although it is not clear what ecological consequences, if any, may arise from this phenomenon. Another question is whether Bt toxins could be bioaccumulated when consumed by natural enemies in a food chain, similar to organophosphorus pesticides being bioaccumulated in invertebrates, fish, and birds at different trophic levels and cause unexpected negative impacts (Serrano et al. 1997a, b).

To date, many studies have evaluated the impact of Bt toxins through tritrophic interactions (reviewed by Romeis et al. 2006 and Chen et al. 2006), however bioaccumulation of Bt toxins at different trophic levels has not been investigated. Moreover, the ecological impact of the possible biotransfer and bioaccumulation of Bt toxins expressed in Bt rice through tritrophic interactions has not been explored. In this paper, we evaluated (1) biotransfer and bioaccumulation of Cry1Ab toxin in a food chain comprising two Bt rice lines, the herbivore *C. medinalis* and the predator *P. subpriaticus*, (2) binding activity of Cry1Ab to *P. subpriaticus* brush border membrane vesicles (BBMVs), (3) effect of Cry1Ab on the development and fecundity of the predator *P. subpriaticus* under laboratory conditions, and (4) ecological impact of Cry1Ab on *P. subpriaticus* in a 3-year field study.

Materials and methods

Transgenic rice

Two homogenous transgenic Bt rice lines with a synthetic *cry1Ab* gene, KMD1 and KMD2 (Ye et al. 2001) at the tenth generation after transformation, were used together with their non-Bt isoline *japonica* rice cultivar Xiushui11. The Bt rice contained a synthetic *cry1Ab* gene under the control of the maize *ubiquitin* promoter and linked in tandem with *gus* (encoding the β–glucuronidase), *hpt* (encoding the hygromycin phosphotransferase), and *npt* (encoding the neomycin phosphotransferase) genes (Cheng et al. 1998; Xiang et al. 1999). The Bt rice selected through four generations was homozygous for the transgenes (*cry1Ab*, *gus* and *npt*) (Shu et al. 1998), and could effectively control rice stem borers (Ye et al. 2001) and leaffolders (Ye et al. 2003) under field conditions. The three different rice lines were sown then transplanted into pots (dia. 15 cm) in a greenhouse on Zhejiang University's Huajiachi Campus and kept free from insects until needed. Rice plants were used at 40 ± 2 days after transplant for the following experiments.

Arthropods tested

Eggs and early instars of *C. medinalis* were collected from local farmers' rice fields in Hangzhou (120.2°E, 30.3°N) China and maintained on non-Bt rice plants (Xiushui11) in an insect rearing chamber maintained at 25 ± 1°C, RH 75–80% and a photoperiod 14:10 (L:D) h. Two nylon cages were set up in the chamber to maintain the *C. medinalis* colony for experimental use. Twenty pots of rice plants were moved into a rearing chamber and placed in a plastic tub in a nylon netted cage. The cage was 2.0 m long × 1.0 m wide × 1.8 m high with a zippered opening on one side. Potted rice plants were replaced as needed.

Early instars of the predator, *P. subpriaticus,* were also collected from local farmers' rice fields in Hangzhou and kept in another rearing chamber under the same conditions. Because of their cannibalistic characteristic, *P. subpriaticus* were individually put into glass vials (length 9.5 cm, dia. 2.5 cm) lined with a water-soaked sponge at the bottom and sealed with fine mesh for ventilation. Each *P. subpriaticus* was supplied with two *C. medinalis* larvae daily and water was added to the sponge as necessary.

Biotransfer and bioaccumulation of Cry1Ab from Bt rice to *C. medinalis*

Cnaphalocrocis medinalis third–fourth instars were collected from the insect culture. Each larva, without

starvation treatment, was placed into a Petri dish (dia. 9.5 cm) lined with a filter paper (Whatman, England). One piece of KMD1 rice leaf (cut to 6 cm length) was put into the petri dish with the two cut ends covered with moistened non-absorbent cotton (Ye et al. 2000). A *C. medinalis* larva was kept in the Petri dish for 1, 2, 3, 4 and 5 days, respectively. KMD1 leaves were changed daily. Meanwhile, the feeding areas were recorded with a flexible transparent grid (1 mm^2 grid squares) and the excretions of the *C. medinalis* larvae were collected to indicate how much they fed. Five *C. medinalis* samples (insects and excretions) were collected at each inoculation time interval with each sample being a replicate. After the larvae and excretions were weighed, they were immediately put into different Eppendorf vials (1.5 ml) and stored at −70°C until ELISA (Enzyme-linked Immunosorbent Assay) assays were carried out using Cry1Ab/Cry1Ac QuantiPlate kit (EnviroLogix, Portland, ME) to detect the presence of Cry1Ab in *C. medinalis* larvae and excretions.

Experimental designs for detecting the biotransfer and bioacuumulation of Cry1Ab toxin from KMD2 rice to *C. medinalis* larvae were the same as described above. Five *C. medinalis* fed on non-Bt Xiushui11 rice were used as the controls at each time interval.

Biotransfer and bioaccumulation of Cry1Ab from Bt rice-fed *C. medinalis* to *P. subpiraticus*

Pirata subpiraticus adults, without starvation treatment, were individually put into glass vials. Each *P. subpiraticus* was daily supplied with two *C. medinalis* third–fourth instars that had fed on KMD1 rice plants for 1 day. *C. medinalis* (either dead or live) were taken with forceps when new ones were supplied to *P. subpiraticus* each day. After continuously feeding on KMD1 rice-fed *C. medinalis* larvae for 1, 2, 3, 4 and 5 days, respectively, five *P. subpiraticus* adults (as five replicates) were sampled at each inoculation time interval, immediately weighed and stored at −70°C until ELISA assays were conducted to detect the presence of Cry1Ab. In order to detect whether *P. subpiraticus* adults excrete Cry1Ab toxin, the white excretions of *P. subpiraticus* adults were washed from the glass vial's inner wall with 1 ml Extraction/Dilution Buffer (Supplied in Cry1Ab/Cry1Ac QuantiPlate kit) after *P. subpiraticus* had fed on KMD1 rice plant-fed *C. medinalis* larvae for 5 days. Samples were stored at −70°C before analyzing the presence of Cry1Ab.

Experimental designs were the same as above for detecting the biotransfer and bioaccumulation of Cry1Ab from KMD2 rice-fed *C. medinalis* larvae to *P. subpiraticus*. Five *P. subpiraticus* preying on Xiushui11 rice-fed *C. medinalis* were used as controls at each time interval.

Binding of Cry1Ab to *C. medinalis* and *P. subpiraticus* midgut BBMVs

Preparation of BBMVs

Cnaphalocrocis medinalis and *P. subpiraticus* midguts were prepared as described by Wolfersberger et al. (1987). Their BBMVs were extracted by differential magnesium precipitation in a procedure modified from Wolfersberger et al. (1987). The final vesicles were resuspended in binding buffer (8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 150 mM NaCl, pH 7.4, containing 0.1% bovine serum albumin) to a final protein concentration of 1 mg/ml and stored at −70°C for blotting analysis.

Ligand blotting of binding proteins

A total of 30 µg BBMV proteins of *C. medinalis* and *P. subpiraticus* were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by Bio-Rad Trans Blot apparatus. The membrane was incubated with activated Cry1Ab (1 µg/ml) for 2 h (Liao et al. 2005). The bound Cry1Ab was detected using Cry1Ab monocolonal antibody (1:2000 dilution, v/v) followed by a second antibody (HRP-conjugated goat anti-mouse antibody, Sigma, USA). Color development was induced by incubation with TMB solution (Promega, USA). A negative control of each treatment was processed the same as above except without incubation with Cry1Ab.

Effect of Bt rice on survival, developmental time and fecundity of *P. subpiraticus*

Pirata subpiraticus has eight instars before reaching adulthood under the rearing chamber conditions. *Pirata subpiraticus* fifth instars were selected for survival and development tests, because the earlier instars have high natural mortalities (data not shown). Fifth instars were placed into glass vials individually and divided into three groups (group I, II and III). Each group had 60 *P. subpiraticus* fifth instars. Group I individuals were supplied with *C. medinalis* larvae that had fed on KMD1 rice for 1 day. Group II and III individuals were supplied *C. medinalis* larvae that had fed for 1 day on KMD2 and Xiushui11, respectively. Each *P. subpiraticus* nymph was supplied with two *C. medinalis* larva daily in each group until adult stage. The number of dead *P. subpiraticus* in each group was recorded daily. The total developmental time of each *P. subpiraticus* nymph from fifth instar to adult stage was also recorded. After *P. subpiraticus* reached the adult stage, females and males were paired within each group. After mating, females were put into glass vials individually and supplied with KMD1-fed *C. medinalis* larvae for group I,

KMD2-fed *C. medinalis* larvae for group II, and Xiushui11-fed *C. medinalis* larvae for group III until the first egg bag of each *P. subpiraticus* was laid and hatched. The number of eggs per egg bag and egg hatching rate were recorded.

Effect of the Bt rice on *P. subpiraticus* population in fields

Bt rice, KMD1 and KMD2, were used for field studies at the Experimental Farm of Zhejiang University in Hangzhou City (120.12°E, 30.13°N), China in 2002, 2003 and 2004, together with their non-Bt isoline rice cultivar Xiushui11. Each year, Bt and non-Bt rice were transplanted 1 month after sowing. In 2002 and 2003, rice seeds were sown on May 27, and the seedlings transplanted on June 27 at the Experimental Farm. In 2004, seeds were sown on April 25, and the seedlings were transplanted on May 25. Each year, the field was divided into nine experimental plots in a three (treatments, KMD1, KMD2, and non-Bt) × 3 (replications) completely randomized design. Each experimental plot was 20 m × 25 m. Each plot was bordered on all sides by an unplanted 50 cm-wide earthen walkway. Seedlings were hand transplanted at one seedling per plant or hill spaced 16.5 cm × 16.5 cm apart, and the entire experimental field was surrounded by five border rows of the untransformed control plants. Normal cultural practices for growing rice, such as fertilization and irrigation, were followed during the course of the experiment except that no insecticides were applied after sowing and transplanting.

Population density of *P. subpiraticus*, a predominant predator, in different rice plots was evaluated using a vacuum-suction machine. The machine was based on a description by Carino et al. (1979), supplemented with a square sampling frame (50 cm × 50 cm × 90 cm high with a planar area of 2,500 cm^2) made of Mylar sheets to enclose nine rice hills. Each year, samples were taken in all plots on a 15 ± 2 days schedule starting ca. 1.5 month after transplanting. On each sampling date, a square sampling frame was placed at random along a diagonal line of each tested plot, with five samples per plot. Arthropods inside the frame enclosure were removed using a vacuum-suction machine, and then transferred into a coded glass vial containing 75% ethanol. All samples were returned to the laboratory for counting the number of *P. subpiraticus*.

There were six sampling dates (from August to October) in 2002 and five sampling dates (from July to September) in 2003 and 2004.

Statistical analysis

Data on the accumulative feeding areas and excretions of *C. medinalis* larvae on different rice plants and concentration of Cry1Ab in *C. medinalis* and *P. subpiraticus* were analyzed using GLM PROC in SPSS and Fisher's protected LSD means separation test. Developmental time, the number of the eggs, egg hatching rate of *P. subpiraticus* supplied with Bt rice-fed *C. medinalis* larvae were also analyzed using GLM PROC. Survival analysis of *P. subpiraticus* on Bt rice-fed *C. medinalis* larvae was conducted using the Kaplan–Meier procedure and Logrank Test (Norusis 2005). Survival data were recorded until all *P. subpiraticus* nymphs either died or reached adulthood stages. All live *P. subpiraticus* adults were recorded as "surviving until the last recorded day".

Population density of *P. subpiraticus* (means by sampling date) in different rice fields within each year was analyzed using GLM PROC. The rice type (Bt treatments) and sampling date variables were entered as fixed factors. Since Bt treatment may have a possible chronic effect on *P. subpiraticus* population, rice type × sampling date interaction was considered for population dynamics of *P. subpiraticus*, and was analyzed using two-way ANOVA (rice type verses sampling date). All count data were square root $(X + 1)$ or log 10 $(X + 1)$ transformed, as necessary, before univariate analysis, but untransformed means are presented. All statistical analyses were conducted using SPSS for Windows version 11.5 (SPSS Inc., Chicago, USA).

Results

Biotransfer and bioaccumulation of Cry1Ab from Bt rice to *C. medinalis*

Bt rice lines KMD1 and KMD2 significantly reduced *C. medinalis* larval food consumption, compared to non-Bt rice Xiushui 11 ($F = 321.46$, $df = 2,135$; $P < 0.001$). (Fig. 1). At day one, feeding areas of *C. medinalis* larvae on Xiushui11 rice plants were 276 mm^2, and 11.38 and 7.24 mm^2 on KMD1 and KMD2 rice plants, respectively (Fig. 1). The feeding areas of *C. medinalis* larvae on each type of rice did not significantly vary as the feeding times increased from 1 to 5 days ($F = 1.706$, $df = 4,135$; $P = 0.152$). Similarly, average excretion weight of *C. medinalis* larvae after feeding on different rice plants was significantly affected by rice type ($F = 28.135$, $df = 4,135$; $P < 0.001$) and feeding time ($F = 10.022$, $df = 2,135$; $P < 0.001$) (Fig. 2).

Cry1Ab was detected using ELISA in both KMD1 rice-fed and KMD2 rice-fed *C. medinalis* larvae (Fig. 3a). However, no Cry1Ab was detected from Xiushui11 rice-fed *C. medinalis* larvae. Concentrations of Cry1Ab in different Bt rice-fed *C. medinalis* larvae were not significantly affected by Bt rice type (KMD1 and KMD2) ($F = 1.188$, $df = 1,20$; $P = 0.289$), feeding time ($F = 0.769$, $df = 4,20$;
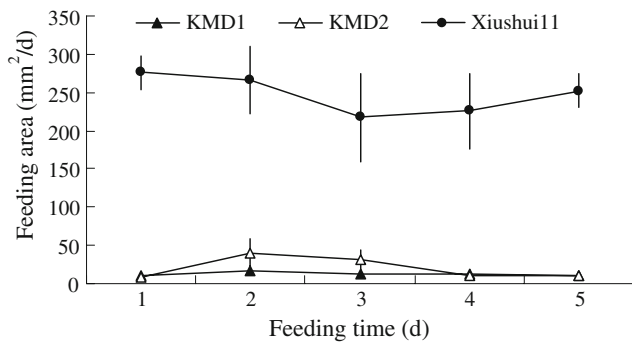
Fig. 1 Feeding area of *C. medinalis* on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice plants for different time periods
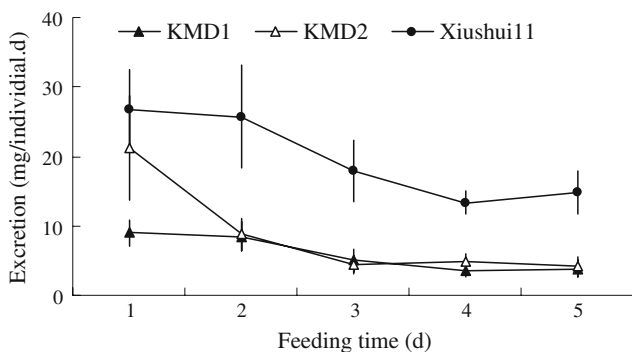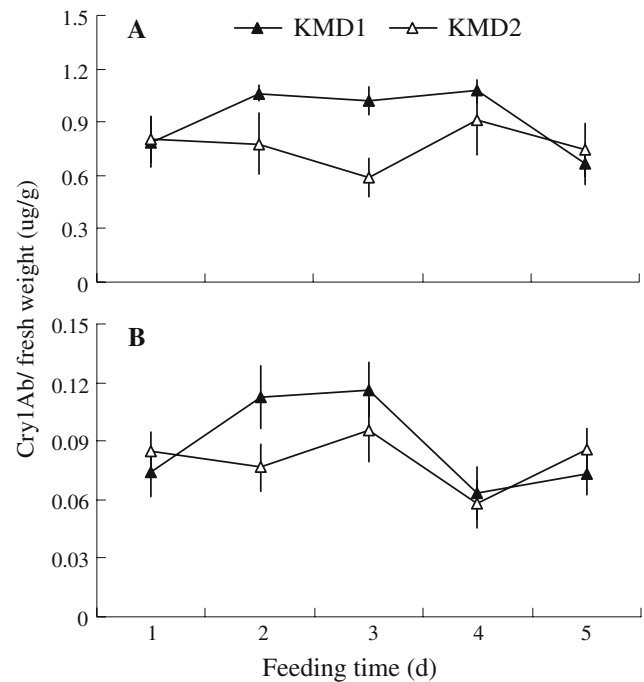

Fig. 2 Excretions of *C. medinalis* after feeding on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice plants for different time periods
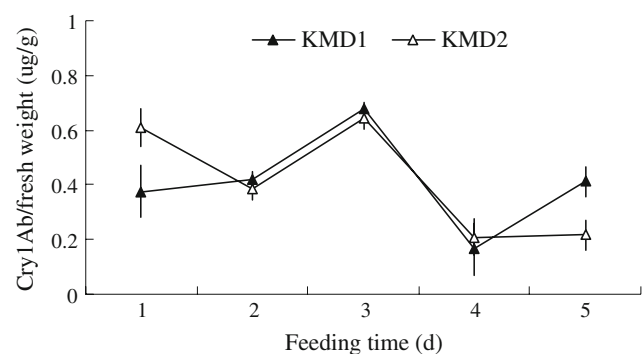

Fig. 3 Presence of Cry1Ab toxin in *C. medinalis* (**a**) after feeding on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice and *P. subpriaticus* (**b**) preying on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice-fed *C. medinalis* for different time periods. No Cry1Ab was detected in Xiushui11-fed *C. medinalis* and *P. subpriaticus* preying on Xiushui11-fed *C. medinalis*


Fig. 4 Presence of Cry1Ab toxin in excretions of *C. medinalis* after feeding on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice for different time periods. No Cry1Ab was detected in the excretion of Xiushui11-fed *C. medinalis*

$P = 0.558$) and the interactions of Bt rice and feeding time ($F = 0.680$, $df = 4,20$; $P = 0.614$). No Cry1Ab accumulation was found in *C. medinalis* as feeding time increased from 1 to 5 days. Cry1Ab was also detected from the excretions of KMD1 rice-fed and KMD2 rice-fed *C. medinalis* larvae. Rice type ($F = 0.136$, $df = 1,20$; $P = 0.716$), feeding times ($F = 0.819$, $df = 4,20$; $P = 0.528$) and interactions of rice type and feeding time ($F = 0.167$, $df = 4,20$; $P = 0.953$) did not significantly affect the presence of Cry1Ab in excretions (Fig. 4).

Biotransfer and bioaccumulation of Cry1Ab from Bt rice-fed *C. medinalis* to *P. subpiraticus*

Cry1Ab was detected from *P. subpiraticus* after preying on Bt rice (KMD1 and KMD2)-fed *C. medinalis* for 1 day (Fig. 3b). No Cry1Ab was detected from *P. subpiraticus* that fed on Xiushui11 rice-fed *C. medinalis*. Cry1Ab did not accumulate in *P. subpiraticus* as feeding time increased from 1 to 5 days. Rice type ($F = 0.1.134$, $df = 1,20$; $P = 0.283$), preying time ($F = 0.768$, $df = 4,20$; $P = 0.579$) and interactions of rice type and preying time ($F = 0.432$, $df = 4,20$; $P = 0.712$) did not significantly affect the presence of Cry1Ab in *P. subpiraticus*. A trace amount of Cry1Ab was also detected using ELISA from the excretions of

P. subpiraticus that had fed on KMD1 and KMD2 rice-fed *C. medinalis* larvae (data not shown).

Binding of Cry1Ab to *C. medinalis* and *P. subpiraticus* midgut BBMVs

BBMV proteins (30 μg) of *C. medinalis* and *P. subpiraticus* were prepared and separated by 10% SDS–PAGE (Fig. 5a), then transferred to a polyvinylidene difluoride
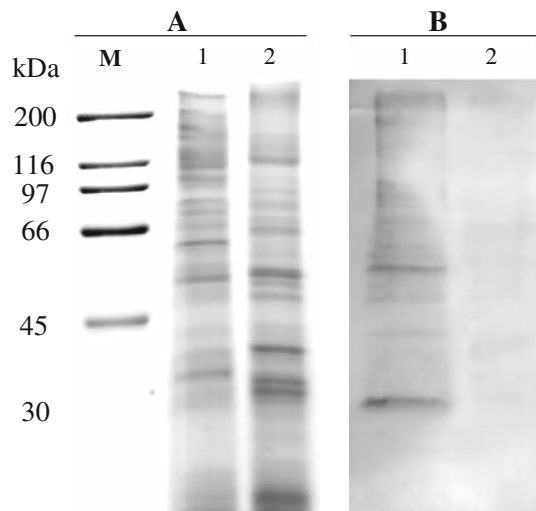
Fig. 5 Binding of Cry1Ab toxin to BBMV proteins of *C. medinalis* and *P. subpriaticus*. **a** BBMV proteins SDS-PAGE analysis; **b** Ligand blotting of Cry1Ab binding to BBMV proteins; *Lane 1*: *C. medinalis* BBMV proteins; *Lane 2*: *P. subpriaticus* BBMV proteins; *M*: molecular weight marker

membrane. In vitro binding of activated Cry1Ab (1 μg/ml) to *C. medinalis* and *P. subpiraticus* BBMV proteins is shown in Fig. 5. The results indicate that Cry1Ab binds to several BBMV proteins in the midgut of *C. medinalis*; however, Cry1Ab does not have binding receptors in BBMVs of *P. subpiraticus* (Fig. 5b).

Survival, developmental time and fecundity of *P. subpiraticus*

Survival probability of *P. subpiraticus* from the fifth instar to adult stage was not significantly affected by rice type ($\chi^2 = 2.240$, $df = 2$, $P = 0.326$) (Table 1). Total developmental time of *P. subpiraticus* preying on KMD1-fed and KMD2-fed *C. medinalis* larvae was significantly longer than that on Xiushui11 rice-fed *C. medinalis* larvae ($F = 69.588$, $df = 2,82$; $P < 0.001$) (Table 1). In total 12, 14 and 16 pairs of *P. subpiraticus* adults were collected

from group I (KMD1), II (KMD2) and III (Xiushui11), respectively. After *P. subpiraticus* adults were mated within each group, females were reared until eggs were laid. The number of eggs per egg bag ($F = 0.133$, $df = 2,40$; $P = 0.876$) and egg hatching rate ($F = 2.244$, $df = 2,40$; $P = 0.112$) of *P. subpiraticus* between Bt rice treatments and the control were not statistically different (Table 1).

Effect of the Bt rice on *P. subpiraticus* population in fields

Results of the 3-year field study indicated that KMD1 and KMD2 did no significantly affect *P. subpiraticus* populations in 2002 ($F = 1.950$, $df = 2,72$; $P = 0.15$), 2003 ($F = 0.098$, $df = 2,60$; $P = 0.907$), and 2004 ($F = 0.246$, $df = 2,60$; $P = 0.783$), compared to the non-Bt control Xiushui11 rice (Table 2). However, *P. subpiraticus* populations in different rice fields were significantly impacted by sampling date in each tested year, i.e., 2002 ($F = 23.329$, $df = 5,72$; $P < 0.001$), 2003 ($F = 7.344$, $df = 4,60$; $P < 0.001$), and 2004 ($F = 3.301$, $df = 4,60$; $P = 0.016$). In order to investigate whether Bt treatment had a possible chronic effect on *P. subpiraticus* population, rice type × sampling date interactions were analyzed. No significant impact was found for rice type × sampling date interaction on *P. subpiraticus* populations in 2002 ($F = 1.360$, $df = 10,72$; $P = 0.216$), 2003 ($F = 1.403$, $df = 8,60$; $P = 0.214$), and 2004 ($F = 0.641$, $df = 8,60$; $P = 0.740$).

Discussion

Development and commercialization of Bt plants have revolutionized insect pest management (Shelton et al. 2002). Since commercialization in 1996, the rate of adoption has been unprecedented in agriculture and in 2007 Bt crops were grown on 42.1 million ha worldwide (James 2007). However, their potential impact on nontarget organisms, especially natural enemies, continues to be the

Table 1 Survival, developmental time and fecundity of *P. subpriaticus* preying on Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice-fed *C. medinalis*

Parameters tested	KMD1-fed	KMD2-fed	Xiushui11-fed
Survival probability* (%)	93.33a	90.12a	96.67a
Developmental time* (days)	72.85 ± 1.18a	74.33 ± 1.12a	58.06 ± 0.55b
No. of eggs/egg bag	51.25 ± 0.71a	50.20 ± 2.60a	51.94 ± 2.93a
Egg hatching (%)	90.08 ± 1.0a	86.13 ± 2.78a	91.44 ± 1.11a

* Survival probability and developmental time were evaluated from 5th instar to adult stage

Means (±SE) followed by same lower-case letters within a row are not significantly different (Survival probability was analyzed using the Kaplan–Meier procedure and Logrank Test; other parameters were analyzed using one-way ANOVA and Fisher's protected LSD means separation test, $P < 0.05$)

Table 2 Population density (mean ± SE) of *P. subpriaticus* collected by vacuum-suction in Bt (KMD1 and KMD2) and non-Bt (Xiushui11) rice fields in China in 2002, 2003, and 2004

Year	Sampling date	No. of *P. subpiraticus*/0.25 m²		
		KMD1	KMD2	Xiushui 11
2002	12-Aug	0.6 ± 0.4Ac	1.4 ± 1.2Ac	0.4 ± 0.2Ac
	27-Aug	0.4 ± 0.2Ac	0.4 ± 0.4Ac	2.2 ± 1.6Abc
	22-Sep	0.4 ± 0.4Ac	0.8 ± 0.5Ac	0.2 ± 0.2Ac
	24-Sep	3.8 ± 1.0Aab	5.4 ± 1.0Aab	8.2 ± 2.3Aa
	9-Oct	4.2 ± 0.9Aab	1.2 ± 0.9Ac	4.2 ± 0.7Aab
	26-Oct	5.8 ± 1.0Aa	6.2 ± 1.0Aa	7.6 ± 2.5Aab
2003	28-Jul	0.8 ± 0.8Ab	0.2 ± 0.2Ab	0.2 ± 0.2Ab
	12-Aug	0.2 ± 0.2Ab	0.6 ± 0.6Aab	0.2 ± 0.2Ab
	27-Aug	0.6 ± 0.4Ab	0.8 ± 0.5Aab	0.8 ± 0.4Ab
	9-Sep	1.2 ± 0.6Aab	2.2 ± 0.6Aa	3.6 ± 0.7Aa
	24-Sep	2.6 ± 0.9Aa	1.4 ± 0.9Aab	1.2 ± 0.7Ab
2004	8-Jul	0.8 ± 0.2Aa	1 ± 0.3Aa	1 ± 0.3Aa
	23-Jul	1 ± 0.3Aa	1.2 ± 0.2Aa	1.2 ± 0.2Aa
	7-Aug	1.6 ± 0.5Aa	1 ± 0.3Aa	1.2 ± 0.5Aa
	22-Aug	2 ± 0.8Aa	2.2 ± 0.5Aa	2.2 ± 0.6Aa
	6-Sep	2 ± 0.4Aa	1 ± 0.3Aa	1 ± 0.3Aa

Means (±S.E.) followed by different lower-case letters within a column for each year or different capital letters within a row are significantly different (Fisher's protected LSD means separation test, $P < 0.05$)

focus of considerable debate (Romeis et al. 2006; Marvier et al. 2007). Although many field studies to date have shown negligible impact on non-target organisms (Romeis et al. 2006; Marvier et al. 2007), some laboratory studies have shown negative effects (Ferry et al. 2003). So far, there are no clear universal guidelines for assessing the effects of Bt plants on selected non-target arthropods. A tiered system that has been adapted from the ecotoxicological discipline of plant protection products is currently suggested for such evaluation, which includes a first tier "worst-case" study under laboratory conditions, with additional studies if needed (Romeis et al. 2008).

Our "worst-case" study indicated that Cry1Ab toxin expressed in KMD1 and KMD2 rice plants could be transferred from Bt rice to the herbivore, *C. medinalis*, and then to the predator, *P. subpriaticus* (Figs. 3, 4). However, as the feeding time increased from 1 to 5 days, the presence of Cry1Ab did not show a tendency to bioaccumulate in different consumers (primary and secondary) belonging to the food chain comprising Bt rice, *C. medinalis*, and *P. subpriaticus*. In a biomass pyramid, it is possible that any toxin/pollutant in producers (plants) would move into primary consumers (herbivores) by feeding, then into secondary consumers (predators) by preying; thus bioaccumulation and biomagnification would take place (Serrano et al. 1997a, b). However, our results indicate that, instead of being bioaccumulated in the predator, the concentration of Cry1Ab in *P. subpriaticus* was ca. 10-fold lower than that in *C. medinalis*. First this might be due, in part, to the exudation of Cry1Ab by *C. medinalis* and *P. subpriaticus* (Fig. 3). Similarly, Bernal et al. (2002) found Cry1Ab in the honeydew of *N. lugens* after it fed on

Bt rice. These results may suggest that water-soluble toxins (pollutants) usually cannot be bioaccumulated and biomagnified in a food chain because they can dissolve in the bodily fluids of the consumers and are excreted (McShaffrey 1995). Secondly, effective degradation of Cry1Ab toxin by crude protease extracts of *P. subpriaticus* midgut considerably reduced the amount of Cry1Ab in *P. subpriaticus* (Chen et al. 2005). In one previous study, D'Adamo et al. (1997) demonstrated that the differences of the pollutant bioaccumulation in the food chain comprising *Dunaliella tertiolecta* (microalga), *Mytilus galloprovincialis* (mussel) and *Dicentrarchus labrax* (fish) were caused by the efficient detoxification enzymatic system located in the liver of the fish. Similarly, Forcada et al. (1999) found that midgut enzymes of a *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) strain resistant to Bt were able to process and degrade the Bt toxin in vitro, and to minimize the amount of the toxin present in the midgut lumen. Those results are in agreement with our findings that *P. subpriaticus* has an enzyme system in its midgut to prevent itself from being harmed by a Bt toxin.

Movement patterns and impacts on non-target organisms of Bt toxins through tritrophic interactions have been reported in some previous studies (reviewed by Romeis et al. 2006 and Chen et al. 2006). However, the ecological impact of Bt toxin movement has not always been clearly evaluated. Our laboratory feeding experiments demonstrated that Bt rice did not cause higher mortality in *P. subpriaticus* compared to non-Bt rice (Table 1). However, developmental time of *P. subpriaticus* preying on Bt rice-fed *C. medinalis* was significantly longer in comparison with non-Bt rice-fed *C. medinalis*. Since no binding

receptors of Cry1Ab were found in the BBMVs of *P. subpriaticus* midgut (Fig. 5), it may be inferred that longer developmental time of *P. subpriaticus* was due to an indirect effect (i.e., host quality) instead of direct toxicity of Cry1Ab. It is unclear whether a slower developmental time could cause a chronic effect on *P. subpriaticus* population in fields. Our 3-year field trial indicates that *P. subpriaticus* population density was very similar between Bt and non-Bt rice fields. In addition, the interaction of rice type and sampling date did not significantly affect the overall *P. subpriaticus* population (Table 2).

Progress on the research and development of GM rice, especially Bt rice, in China has been rapid in recent years. More than 100 GM rice varieties have been in field testing in China by 2005 (Wang and Johnston 2007). However, to date, none of them has been approved for commercialization not only because of ecological risk concerns from domestic and international bodies, but because of political, economic, and trade concerns. From a standpoint of scientifically sound ecological risk assessments (ERA), the data we presented here used a tier-based method to rigorously evaluate the potential impact of Bt rice on an important non-target predator and did not find a negative direct effect. As the first study to investigate the possibility of bioaccumulation of Cry toxin used in Bt rice in a food chain and its ecological impact in the fields, our results present valuable information that can be used by regulatory agencies to develop scientifically based ERA policies and have broad implications for ecological safety of Bt plants.

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