

Bt rice expressing Cry1Ab does not stimulate an outbreak of its non-target herbivore, *Nilaparvata lugens*

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Abstract In this study, the non-target effects of *Bt* rice “KMD2” expressing a Cry1Ab protein on the performance of the brown planthopper (BPH), *Nilaparvata lugens*, over multiple generations were evaluated under laboratory and field conditions. In the laboratory, BPH was reared to observe the impact of the *Bt* rice as compared to its parental non-*Bt* cultivar Xiushui 11, while the population dynamics and oviposition performance of BPH were investigated in the field. The survival of BPH nymphs fed *Bt* and non-*Bt* rice did not differ significantly. The nymph developmental duration of BPH was

significantly delayed by the *Bt* rice by comparison with the non-*Bt* rice for the 1st and 2nd but not the 4th generation. Most importantly, the fecundity of BPH on the *Bt* rice was significantly decreased in every generation when compared with the non-*Bt* rice. In the field investigations, the population density of BPH nymphs was significantly lower in the *Bt* rice field. However, the temporal pattern of population dynamics of BPH adults was similar between the *Bt* and non-*Bt* rice, presumably due to migratory interference of the adults. In the *Bt* rice field, the percentage of tillers with eggs and the number of eggs per tiller were also significantly lower from tillering to mature stage. Additionally, Cry1Ab protein could not be detected in guts from single BPH adults. In general, our results suggest that the *Bt* rice “KMD2” could not stimulate an outbreak of BPH.

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Introduction

One of the risks associated with the growing of insect-resistant genetically modified (IRGM) crops is their potential impacts on non-target organisms. One concern is that pests which are not directly targeted by the introduced trait could gain importance and would require the application of additional control

measures. This could, for example, be the case if the GM plants are more susceptible to these non-target herbivores (Faria et al. 2007), if the biological control of the non-target herbivores is disrupted (Romeis et al. 2008) or when the pests are benefiting from the fact that chemical pest control of the target pest is reduced on the GM crop (Lu et al. 2010). Consequently, with the introduction of a new pest management approach, including a new insecticide or insect-resistant cultivar, it is necessary to determine any impact on other members of the ecosystem so that ecological problems can be avoided. For example, rice planthoppers were only a secondary insect pest complex of rice in China before the 1970s. However, the adoption of hybrid rice cultivars with insect susceptibility and the overuse of broad-spectrum chemical insecticides have resulted in the outbreak of the rice planthopper populations, making them become major pests since 1980s (Sogawa et al. 2003). Whether the introduction of *Bt* rice would lead to a similar problem is a major concern that need to be addressed prior to the release of *Bt* rice to the farmers for commercialization.

To date, numerous transgenic rice lines carrying single genes derived from the soil bacterium *Bacillus thuringiensis* (*Bt*), namely *cry1Aa* (Breitler et al. 2004), *cry1Ab* (Ye et al. 2001), *cry1Ac* (Bashir et al. 2004), *cry1B* (Breitler et al. 2004), *cry1C** (Ye et al. 2009), *cry1Ca1* (Zaidi et al. 2009), *cry2A* (Riaz et al. 2006) and *cry9C* (Chen et al. 2008), have been obtained to develop high resistance to lepidopteran pests of rice. One *Bt* rice cultivar (Huahui 1) and its hybrid line (Shanyou 63) have recently been approved to have a limited commercialization trial in Hubei Province in China for a 5-year period (2009–2014) (MAPRC 2009). Thus, it is important to evaluate the potential effects on non-target herbivore insects, particularly on rice planthoppers which are major pests of rice in China, and to define whether the introduction of Lepidoptera-resistant *Bt* rice could lead to the outbreak of non-target rice planthoppers. A lot of research has been conducted over the past decade to evaluate direct non-target effects on herbivores, predators, parasitoids and decomposers in laboratory and field conditions (Cohen et al. 2008).

There is considerable evidence available today that shows that *Cry1Ab* expressed in GM crop varieties do not cause any effects on species outside the order of Lepidoptera (Naranjo 2009). Consequently, direct

toxic effects of *Cry1Ab* expressed in rice on BPH are highly unlikely. However, other changes on the plant level as a consequence of the transformation process might cause effects. For example, in a recent study we found that compared with the non-transgenic rice variety Xiushui 11, both immature development duration and preoviposition duration of the thrips *Stenchaetothrips biformis* (Bagnall) (Thysanoptera: Thripidae) fed on the transgenic *cry1Ab* rice “KMD2” were longer, eggs laid were fewer and the total number of *S. biformis* collected from the KMD2 field was smaller than that in the non-*Bt* rice parental control (Xiushui 11) field (Akhtar et al. 2010). This study suggests that the *Bt* rice plants and their nearest non-transgenic control differ in their susceptibility to other herbivores and might also benefit rice planthoppers such as the brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae).

BPH is a superior species of rice planthoppers and one of the most notorious insect pests of rice in temperate and tropical Asia (Kanaoka et al. 1996), feeding mainly on the stem and sucking the assimilates from the phloem (Sogawa 1982), thus leading to the leaves drying and the tiller wilting, called “hopperburn” (Bae and Pathak 1970). In India, BPH has become the one of the most important pests since the 1970s, and epidemics caused by BPH have occurred in many countries (Chatterjee 1978). In China, the ratio of the BPH occurrence area to the total paddy plantation area has been about 50% every year since the 1980s (Cheng et al. 2003). Some reports regarding non-target effects of *Bt* rice on insects were related to BPH, specifically concerning about the feeding and oviposition behavior (Chen et al. 2003a, b, 2004) or field population dynamics (Li et al. 2007; Chen et al. 2006, 2007) of BPH between *Bt* rice and their non-*Bt* parental rice, as well as the detection of *Bt* toxin in BPH (Bernal et al. 2002; Chen et al. 2005; Bai et al. 2006). However, long-term effects of *Bt* rice on BPH for multiple generations have not been addressed. Thus, it is worth evaluating whether the *Bt* rice plantation would induce or reduce the emergence of BPH population.

In 2007, we first observed that the symptoms of hopperburn caused by BPH were more serious in the non-*Bt* rice field compared with that in the *Bt* rice field. Subsequently, we investigated the density of BPH on three sampling dates. The preliminary data of

three samplings indicated that the density of BPH in the *Bt* rice field was lower than that in the non-*Bt* rice field (Online Resource 1). Therefore, we evaluated the impact of *Bt* rice “KMD2” on the development and reproduction of BPH for the 1st, 2nd and 4th generations in the laboratory, in light of the fact that the BPH has about 4 generations annually in the rice fields of Zhejiang province, China, commonly with overlapped generations (Cheng et al. 2003). Meanwhile, the effects of *Bt* rice on the population density and egg-laying performance of BPH during the entire rice growth season in the field were investigated. The overall aim of the study was to investigate whether *Bt* rice “KMD2” could stimulate an outbreak of BPH.

Materials and methods

Plant materials and insects

Bt rice line, KMD2 at the 16th generation after transformation, was selected for rearing BPH in the laboratory and field conditions while its non-*Bt* parental *japonica* rice cultivar Xiushui 11 was used as control. The *Bt* rice line 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). The *Bt* rice line effectively controls the target lepidopteran species including rice stem borers (Ye et al. 2001) and leaf-folders (Ye et al. 2003) under field conditions. For the laboratory experiment, both *Bt* and non-*Bt* rice plants were grown under natural lighting and ambient temperature in a screen-house to avoid any interference from other non-target insect species.

A colony of BPH was collected from paddy fields at the Farm of Zhejiang University (120.12°E, 30.13°N), in Hangzhou City, Zhejiang Province, China, in 2008, and was reared on rice seedlings (7–10 days after germination) of the susceptible variety “Taichung Native 1” (TN1) (*Oryza sativa* L.) for 5 generations in plastic containers (30 × 25 cm, 30 cm in height), using a method similar to that described by Wada et al. (2007), until use in the laboratory experiments.

Effects of *Bt* rice on BPH survival

Insecticidal activity of transgenic plants toward BPH was assayed using a feeding chamber as described previously (Heinrichs et al. 1985). One-month-old plants were used for the bioassay. We collected newly hatched nymphs of BPH (within 12 h after hatching) and transferred those (five newly hatched nymphs) to another plastic cup containing freshly potted plants. Feeding continued for 10 days at 28°C under the light and dark regime of 14:10 h. For each KMD2 and Xiushui 11 line, 10 replicates were carried out. After 10 days, the experiment was terminated and the number of surviving insects was recorded.

Effect of *Bt* rice on BPH development and reproduction

Both the *Bt* rice (KMD2) and non-*Bt* control (Xiushui 11) plants at the tillering, heading, and filling stages were used to test the development and reproduction of the 1st, 2nd and 4th generations of *N. lugens*, respectively. For the 1st generation, 10 nymphs within 12 h after hatching were confined on a potted individual rice plant of *Bt* rice and non-*Bt* rice at the tillering stage. The individual plant was covered with a transparent plastic cylindrical cage (diameter 9 cm; 16 cm in height) with a pair of mesh windows (diameter 6 cm). For each tested rice type, there were 8 replications. The plants were changed every week until adult emergence. Then, at least 30 pairs of newly emerged females and males (within 12 h after adult emergence) were transferred to a new potted plant of the same rice type, allowing them to mate and lay eggs. The plants were also covered with a transparent plastic cylindrical cage, and changed every week until the adults were dead. For each rice type (*Bt* and non-*Bt*), the 1st generation eggs on plants were randomly collected, and freshly hatched nymphs (<12 h old) were moved to the heading stage rice plants to study the performance of the 2nd generation. Similarly, nymphs that hatched from the 2nd generation on *Bt* and non-*Bt* rice plants were shifted to their corresponding rice plants at the flowering stage for the 3rd generation test, and nymphs that hatched from the 3rd generation on *Bt* and non-*Bt* rice plants were shifted again to their corresponding rice plants at the filling stage for the 4th generation test. For each generation except the 3rd generation (within

this generation, BPHs were continually reared on Bt and non-Bt rice plants, respectively, but biological parameters of BPHs were not recorded so as to minimize the disturbance and conserve manual labor), both nymph development (from hatching to adult emergence) and adult longevity were recorded. To count the number of eggs laid by per BPH female, each plant were dissected and observed under a dissecting microscope. All experiments were conducted at $27 \pm 1^\circ\text{C}$, 70–80% RH, and under a photoperiod of 14:10 h (light:dark).

Detection of *Bt* insecticidal protein in BPH

BPH newly hatched nymphs continually fed on *Bt* rice plants which were 30 days old after transplantation until eclosion. Then, adults (within 2 h after eclosion) were collected. All samples were transferred into 1.5-ml Eppendorf tubes and immediately frozen at -70°C . To detect the Cry1Ab protein, ELISA was carried out using a QualiPlate kit for Cry1Ab/Cry1Ac (EnviroLogix, Portland, OR, USA). According to the protocol of the kit, samples (30 males and 30 females) were individually homogenized in 0.2 ml phosphatebuffered saline solution in 0.05% Tween-20 and centrifuged for 5 min at 12,000g, and the supernatant was analyzed using the kit. The positive control was added into the plate. The spectrophotometric measurements were taken using a multidetection microplate reader (Bio-Tek, Winooski, VT, USA) reading at 450 nm.

Field experiments

Field experiments were conducted at the Experimental Farm of Zhejiang University, in Hangzhou, China, in 2008 and 2009. The field was divided into 6 experimental plots in a 2 (treatments, *Bt* rice KMD2 vs. non-*Bt* rice Xiushui 11) \times 3 (replications) completely randomized design (Chen et al. 2007). Each experimental plot was 20 by 35 m. Each plot was bordered on all sides by a 50-cm-wide unplanted walkway. Seedlings were hand transplanted at one seedling per hill spaced 16.5 by 16.5 cm apart, and the entire experimental field was surrounded by five border rows of non-*Bt* (Xiushui 11) plants. The planting in 2008 was sown on 2 July, and transplanted on 2 August, and in 2009 sown on 18 June and transplanted on 19 July. Normal cultural

practices, such as fertilization and irrigation, for growing rice were followed during the course of the experiment except that no insecticide was applied after sowing and transplanting.

Field investigation

Every 7 days from 2 September to 28 October 2008 and from 19 August to 14 October 2009, rice plants were individually sampled with a beat white porcelain plate (36 cm \times 46 cm \times 3.5 cm) made of metal, of which the surface is painted with white paint, modified from Joost and Riley (2004) to monitor the density of BPH. On each sampling date, 30 plants were sampled at random along a diagonal line of each tested plot. When sampling, the plate was held at a 45° angle to the ground and a single plant was carefully grasped at the lower stem and then quickly bent into the plate. The sampled plant was beaten vigorously against the side of the plate for 4- to 5-s periods (about 13–15 beats). Subsequently, the dislodged BPH nymphs and adults were counted.

In 2009, to determine the egg-laying performance and oviposition site of BPH in the field, 10 plants of the *Bt* and non-*Bt* rice type were cut from the base at random along a diagonal line of each tested plot at the tillering, jointing, heading, filling and mature stage of rice, respectively. Then, the plants were individually dissected under a dissecting microscope with the aid of a dissecting needle to count the number of eggs laid by BPH. For each sample, total number of tillers per plant, the number of tillers with eggs per plant, the number of eggs laid per tiller, and the position of eggs laid on the tillers (including the lower, middle and upper part of tillers) were recorded. In addition, the height of tillers and the height of oviposition site for each egg mass on the tillers were measured. The percentage of tillers with eggs and the relative height of oviposition (%) were calculated as the following: % tillers with eggs = the number of tillers with eggs per plant/total number of tillers per plant \times 100, the relative height of oviposition (%) = the height of oviposition site on the tillers/the height of tillers \times 100.

Statistical analysis

The data on the nymph developmental duration, adult longevity and fecundity of BPH of the 1st, 2nd and 4th generations were analyzed using two-way

analysis of variance (ANOVA) followed by Tukey's multiple-range test. The mortality and differences in the data for each biological parameter observed between the *Bt* and non-*Bt* rice plants within the same generation were compared using the *t* test. The densities (season means) of BPH were also analyzed using two-way ANOVA and Tukey's multiple-range test. The number of nymphs, and adults sampled from the field in 2008 and 2009, the number of tillers with eggs to total tillers, the number of egg masses per tiller, the percentage of tillers with eggs and the relative height of oviposition (%) were analyzed using repeated measures ANOVA. All percentage data were transformed to arcsine-square root, but untransformed means are presented. 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 calculations were performed using the SAS v.9.1 package (SAS institute 2001). For all tests, α was set at 0.05.

Results

Effects of *Bt* rice on BPH survival

The average mortality of BPH nymphs fed on *Bt* rice "KMD2" and non-*Bt* rice (Xiushui 11) for 10 d was 6 and 4%, respectively, and did not differ significantly ($t = 0.4392$, $df = 18$, $P = 0.629$).

Effects of *Bt* rice on BPH development and reproduction

The performance of BPH on the *Bt* and non-*Bt* rice plants was compared for the 1st, 2nd and 4th generation. The nymphal development duration was significantly affected by rice type and rice type*generation interactions (except female duration) were found to be significant. The factor, generation had no effect on BPH performance. The nymphal development duration on the *Bt* rice plants was significantly prolonged in the 1st and 2nd generations when compared with that on the non-*Bt* rice plants while such an effect was not significant in the 4th generation (Table 1) (1st generation: $t_{\text{male}} = 5.695$, $df = 61$, $P < 0.001$; $t_{\text{female}} = 4.067$, $df = 63$, $P < 0.001$; $t_{\text{total}} = 5.997$, $df = 126$, $P < 0.001$; 2nd

generation: $t_{\text{male}} = 3.937$, $df = 68$, $P < 0.001$; $t_{\text{female}} = 2.621$, $df = 60$, $P = 0.011$; $t_{\text{total}} = 2.010$, $df = 130$, $P = 0.047$; 4th generation: $t_{\text{male}} = 0.791$, $df = 68$, $P = 0.432$; $t_{\text{female}} = 0.545$, $df = 81$, $P = 0.587$; $t_{\text{total}} = 1.939$, $df = 132.8$, $P = 0.055$).

The adult longevity for males and females and total adult longevity were not affected by rice type, generation or rice type \times generation interaction, and did not differ between the *Bt* and non-*Bt* rice plants for each generation tested (Table 1). For example, the adult longevity for males and females and total adult longevity (except the 2nd generation) on the *Bt* rice plants for each tested generation were not significantly different from those on the non-*Bt* rice plants in the *t* test (1st generation: $t_{\text{male}} = 1.373$, $df = 53.6$, $P = 0.176$; $t_{\text{female}} = 0.377$, $df = 63$, $P = 0.708$; $t_{\text{total}} = 0.6629$, $df = 120.4$, $P = 0.509$; 2nd generation: $t_{\text{male}} = 1.194$, $df = 64$, $P = 0.237$; $t_{\text{female}} = 1.696$, $df = 55.2$, $P = 0.096$; $t_{\text{total}} = 1.989$, $df = 122.3$, $P = 0.049$; 4th generation: $t_{\text{male}} = 0.123$, $df = 66$, $P = 0.903$; $t_{\text{female}} = 0.209$, $df = 67$, $P = 0.835$; $t_{\text{total}} = 0.233$, $df = 135$, $P = 0.817$). The fecundity was significantly affected by rice type and generation, but not by rice type \times generation interaction (Table 1). By comparison with the non-*Bt* rice, the fecundity on the *Bt* rice was decreased by 25.6, 21.5 and 29.6% for the 1st, 2nd and 4th generation, respectively. Similarly, within the same generation, the fecundity on the *Bt* rice plants was significantly lower by comparison with the non-*Bt* rice plants according to the *t* test analysis (Table 1) (1st generation: $t = 2.613$, $df = 47.3$, $P = 0.012$; 2nd generation: $t = 2.254$, $df = 58$, $P = 0.028$; 4th generation: $t = 3.172$, $df = 66$, $P = 0.002$).

Detection of Cry1Ab protein in BPH

The Cry1Ab insecticidal protein cannot be detected in single male or female BPH (for the Envirologix Kit we used in the experiments, the detection limit of the assay was 0.5 ng/g).

Population density of BPH in the *Bt* and non-*Bt* rice fields

In 2008, data from the beat plate samplings showed that the population density of BPH nymphs was significantly affected by rice type ($F = 93.18$, $df = 1, 4$; $P < 0.001$), sampling date ($F = 29.96$,

Table 1 Biological parameters of *Nilaparvta lugens* fed on *Bt* and non-*Bt* rice plants for the 1st, 2nd and 4th generations under laboratory conditions

Generation	Genotypes	Nymphal development duration (days) ^a			Adult longevity (days) ^b			No. of eggs laid by per female ^c
		Male	Female	Total	Male	Female	Total	
1st	<i>Bt</i>	14.0 ± 0.15 a (*) (n = 32)	14.7 ± 0.11 a (*) (n = 34)	14.4 ± 0.10 a (*) (n = 66)	18.8 ± 1.41 a (n = 32)	16.3 ± 1.39 a (n = 34)	17.5 ± 0.99 a (n = 66)	245.7 ± 17.21 c (*) (n = 30)
	Non- <i>Bt</i>	12.9 ± 0.13 c (n = 31)	14.0 ± 0.13 cd (n = 31)	13.5 ± 0.12 cd (n = 62)	16.7 ± 0.85 a (n = 31)	17.0 ± 1.80 a (n = 31)	16.2 ± 1.04 a (n = 62)	330.4 ± 27.47 bc (n = 29)
2nd	<i>Bt</i>	13.8 ± 0.14 a (*) (n = 38)	14.5 ± 0.17 abcd (*) (n = 33)	14.1 ± 0.12 ab (*) (n = 70)	16.8 ± 1.50 a (n = 36)	16.1 ± 1.24 a (n = 32)	16.5 ± 0.98 a (*) (n = 68)	277.3 ± 22.61 bc (*) (n = 30)
	Non- <i>Bt</i>	13.4 ± 0.17 bc (n = 32)	14.0 ± 0.13 d (n = 33)	13.8 ± 0.12 bcd (n = 66)	19.2 ± 1.21 a (n = 30)	18.7 ± 0.86 a (n = 32)	18.9 ± 0.75 a (n = 62)	353.3 ± 25.02 ab (n = 30)
4th	<i>Bt</i>	13.8 ± 0.16 a (n = 37)	14.2 ± 0.15 abcd (n = 41)	13.9 ± 0.10 ab (n = 79)	19.4 ± 1.21 a (n = 38)	18.1 ± 1.06 a (n = 39)	18.8 ± 0.80 a (n = 77)	336.7 ± 26.12 bc (*) (n = 38)
	Non- <i>Bt</i>	13.6 ± 0.2 ab (n = 33)	14.0 ± 0.17 bcd (n = 42)	13.6 ± 0.14 d (n = 74)	19.7 ± 1.74 a (n = 30)	18.4 ± 1.17 a (n = 30)	19.1 ± 1.04 a (n = 60)	478.0 ± 37.66 a (n = 30)

Data are represented as means ± SEM. Values followed by different letters within the same column differ significantly according to two-factor ANOVA and Tukey's multiple-range test. Within the same column, asterisk indicated the significant difference ($P < 0.05$) in the same parameters in the same generation between *Bt* and non-*Bt* rice according to *t* test

^a Two-factor ANOVA: for the males, $F_{\text{rice type}} = 30.52, df = 1, 202, P < 0.001; F_{\text{generation}} = 2.17, df = 2, 202, P = 0.117; F_{\text{rice type} \times \text{generation}} = 4.31, df = 2, 202, P = 0.015$; for the females, $F_{\text{rice type}} = 13.70, df = 1, 209, P < 0.001; F_{\text{generation}} = 1.63, df = 2, 209, P = 0.198; F_{\text{rice type} \times \text{generation}} = 2.07, df = 2, 209, P = 0.129$; for the total, $F_{\text{rice type}} = 33.79, df = 1, 412, P < 0.001; F_{\text{generation}} = 1.17, df = 2, 412, P = 0.311; F_{\text{rice type} \times \text{generation}} = 3.22, df = 2, 412, P = 0.041$

^b Two-factor ANOVA: for the males, $F_{\text{rice type}} = 0.03, df = 1, 196, P = 0.870; F_{\text{generation}} = 1.13, df = 2, 196, P = 0.325; F_{\text{rice type} \times \text{generation}} = 1.38, df = 2, 196, P = 0.253$; for the females, $F_{\text{rice type}} = 1.64, df = 1, 197, P = 0.2022; F_{\text{generation}} = 0.90, df = 2, 197, P = 0.409; F_{\text{rice type} \times \text{generation}} = 0.58, df = 2, 197, P = 0.562$; for the total, $F_{\text{rice type}} = 0.92, df = 1, 394, P = 0.338; F_{\text{generation}} = 2.02, df = 2, 394, P = 0.134; F_{\text{rice type} \times \text{generation}} = 1.69, df = 2, 394, P = 0.186$

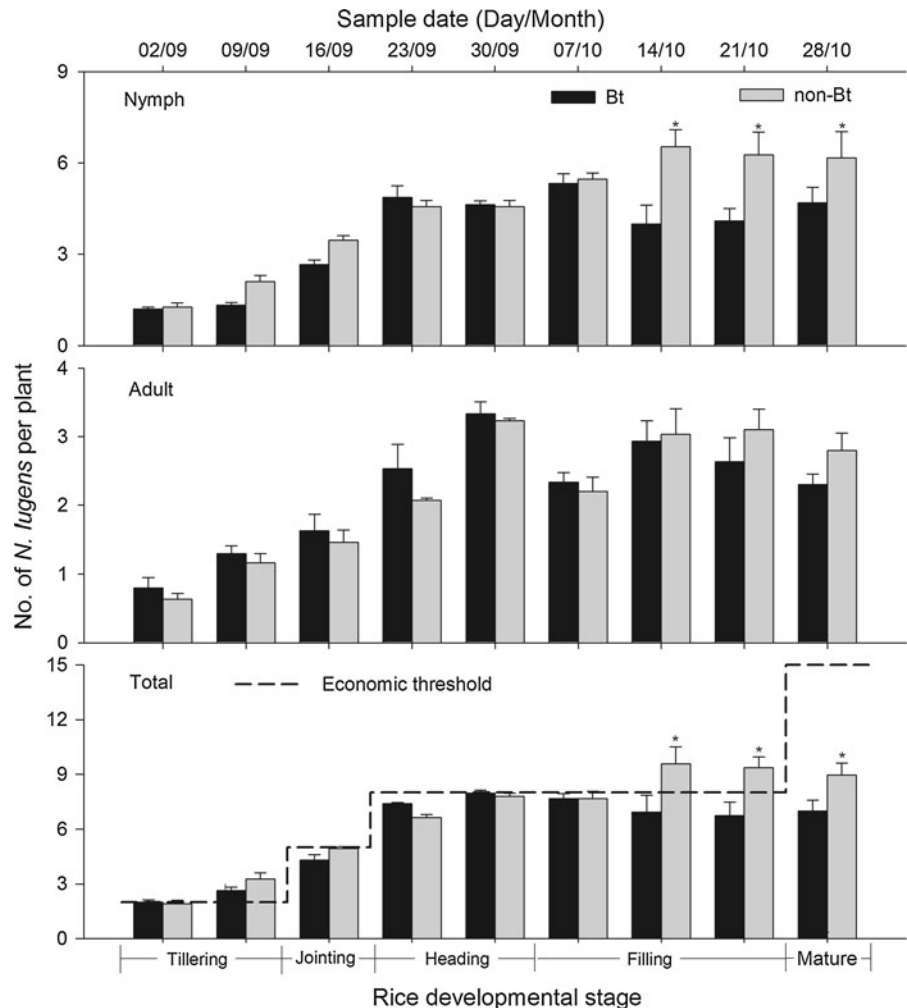
^c Two-factor ANOVA: $F_{\text{rice type}} = 20.97, df = 1, 186, P < 0.001; F_{\text{generation}} = 9.55, df = 2, 186, P < 0.001; F_{\text{rice type} \times \text{generation}} = 0.90, df = 2, 186, P = 0.408$

$df = 8, 32; P < 0.001$) and rice type \times sampling date interaction ($F = 2.86, df = 8, 32; P = 0.016$) (Fig. 1). In contrast, the population density of BPH adults was not affected by the rice type ($F = 0.37, df = 8, 32; P = 0.574$) or rice type \times sampling date interaction ($F = 0.96, df = 1, 4; P = 0.487$), although it was significantly affected by sampling date ($F = 22.59, df = 8, 32; P < 0.001$). The population density of nymphs and adults was significantly affected by rice type ($F = 171.84, df = 1, 4; P < 0.001$), sampling date ($F = 45.80, df = 8, 32; P < 0.001$) and rice type \times sampling date ($F = 3.26, df = 8, 32; P = 0.008$). The density of BPH nymphs in the *Bt* rice field showed a significant decrease during the period from 14 to 28 October when compared with that in the non-*Bt* rice field (Fig. 1), where 63.33, 52.85 and 31.21% more nymphs were

recorded, respectively. Similarly, in the *Bt* rice field, the seasonal average density of the nymphs and total of nymphs and adults were significantly lower and the seasonal average density of adults was not different when compared with the non-*Bt* rice (Online Resource 2). In this year, the occurrence of BPH in the *Bt* was always under the economic control threshold during the entire rice stage, while was markedly over on 14 and 21 October in non-*Bt* rice field according to the criteria of Cheng et al. (2003): 2 BPHs per plant at the tillering stage, 5 BPHs per plant at the jointing stage, 8 BPHs per plant at the heading and filling stages, 15 BPHs per plant at the mature stage (Fig. 1).

In 2009, the results also indicated that the population density of BPH nymphs was significantly affected by rice type ($F = 69.52, df = 1, 4;$

Fig. 1 The population dynamics of *Nilaparvata lugens* in *Bt* and non-*Bt* rice fields in 2008. The data in all samples are expressed as mean \pm SE ($n = 3$). Values followed by an asterisk represent significant differences ($P < 0.05$; repeated measures analysis of variance)



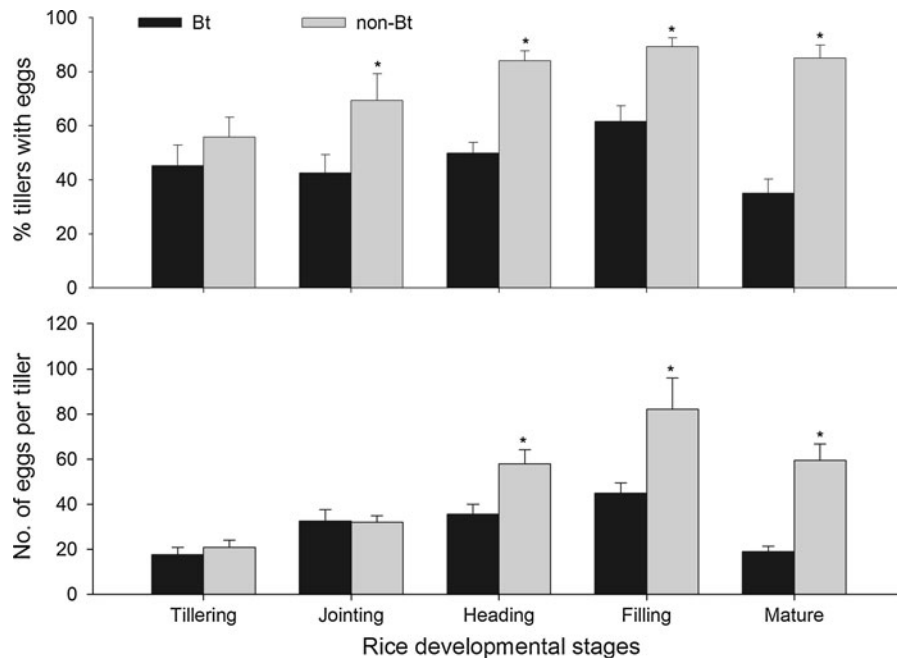


Fig. 2 Oviposition performance of *Nilaparvata lugens* on *Bt* and non-*Bt* rice plants. The data in all samples are expressed as mean \pm SE ($n = 3$). Values followed by an asterisk represent

significant differences ($P < 0.05$; repeated measures analysis of variance)

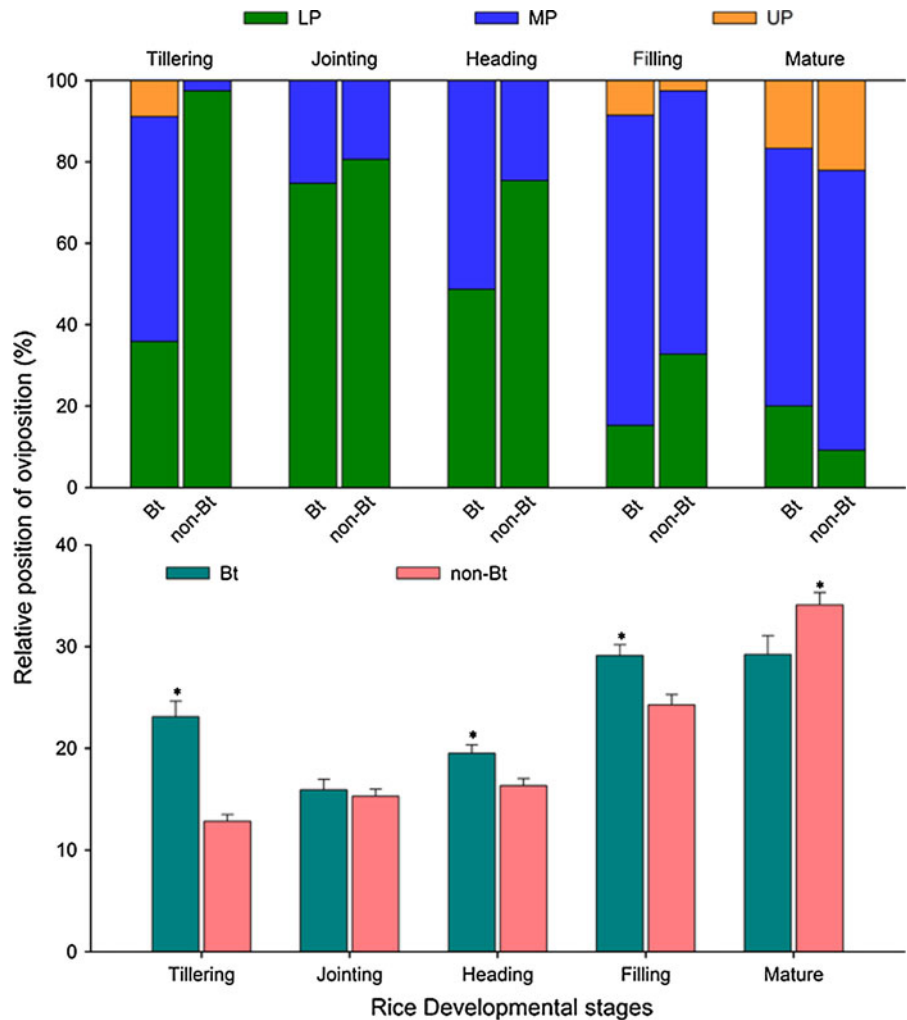
$P = 0.001$), sampling date ($F = 138.32$, $df = 8, 32$; $P < 0.001$) and rice type \times sampling date interaction ($F = 52.72$, $df = 8, 32$; $P < 0.001$). However, the population density of BPH adults was not affected by rice type ($F = 0.27$, $df = 1, 4$; $P = 0.634$) and rice type \times sampling date interaction ($F = 0.29$, $df = 1, 4$; $P = 0.964$), although it was significantly affected by sampling date ($F = 11.06$, $df = 8, 32$; $P < 0.001$). The population density of nymphs and adults was significantly affected by rice type ($F = 50.71$, $df = 1, 4$; $P = 0.002$), sampling date ($F = 92.20$, $df = 8, 32$; $P < 0.001$) and rice type \times sampling date ($F = 33.74$, $df = 8, 32$; $P = 0.008$). The density of BPH nymphs in the *Bt* rice field was lower than that of the non-*Bt* rice in most of the time periods examined, and was significantly lower than that of the non-*Bt* rice from 23 September to 14 October (Online Resource 3), where 296.02, 240.00, 236.5 and 308.78% more nymphs were recorded, respectively. The density of BPH adults showed no significant difference between the *Bt* and non-*Bt* rice (Online Resource 3). Similarly, the seasonal average densities of nymphs in the *Bt* rice field showed to be significantly lower while the adult density remained similar, when compared with the non-*Bt* rice (Online

Resource 2). The occurrences of BPH in both the *Bt* and non-*Bt* rice fields were under the economic control threshold except that the density of BPH in the non-*Bt* rice field was clearly over the economic control threshold at the mature stage according to the criteria of Cheng et al. (2003) (Online Resource 3).

Oviposition performance of BPH in the *Bt* and non-*Bt* rice fields

We first compared the percentage of tillers with eggs and the number of eggs per tiller of BPH between *Bt* and non-*Bt* rice. The percentage of tillers with eggs was significantly affected by rice type ($F = 14.08$, $df = 1, 4$; $P = 0.020$) with more eggs found on non-*Bt* rice plants, sampling date ($F = 154.15$, $df = 4, 16$; $P < 0.001$) and rice type \times sampling date interaction ($F = 106.43$, $df = 4, 16$; $P < 0.001$) (Fig. 2). Similar results were found for the number of eggs per tiller (rice type: $F = 84.12$, $df = 1, 4$; $P < 0.001$; sampling date: $F = 34.06$, $df = 4, 16$; $P < 0.0001$; rice type \times sampling date interaction: $F = 13.61$, $df = 4, 16$; $P < 0.001$). Similarly, the seasonal average for the percentage of tillers with eggs and the number of eggs per tiller in the *Bt* rice

Fig. 3 Percentage of oviposition site on rice tillers and relative height of oviposition by *Nilaparvata lugens* on *Bt* and non-*Bt* rice plants. *LP* Lower part of tiller (relative height = 0–20.00%), *MP* middle part of tiller (relative height = 20.01–20.00%), *UP* upper part of tiller (relative height ≥ 40.01%). The data in all samples are expressed as mean ± SE ($n = 3$). Values followed by an asterisk represent significant differences ($P < 0.05$; repeated measures analysis of variance)



field was significantly lower when compared with the non-*Bt* rice although there was no significant difference in the seasonal average for the number of egg masses per tiller between the *Bt* and non-*Bt* rice (Online Resource 2).

We next compared the oviposition sites on rice tillers and the relative height of oviposition of BPH between the *Bt* and non-*Bt* rice. By comparison with the non-*Bt* rice, BPH on the *Bt* rice preferred to lay eggs into the higher part of rice tillers during the entire rice growing season except the mature stage (Fig. 3). The relative height of oviposition by BPH was significantly affected by rice type ($F = 8.70$; $df = 1, 4$; $P = 0.042$), sampling date ($F = 41.60$, $df = 4, 16$; $P < 0.001$) and rice type × sampling interaction date ($F = 6.93$, $df = 4, 16$; $P = 0.002$). The relative height of oviposition on the *Bt* rice was

significantly higher than that on the non-*Bt* rice at the tillering, heading and filling stage of rice, while was significantly lower than that on the non-*Bt* rice at the mature stage of rice (Fig. 3). Similarly, the seasonal average for the relative height of oviposition on the *Bt* rice was significantly higher than that on the non-*Bt* rice (Online Resource 2).

Discussion

Laboratory studies

Our results indicated that the average mortality of BPH showed no significant difference between those fed on the *Bt* and non-*Bt* rice plants. When the data on the nymph developmental duration, adult

longevity and fecundity of 1st, 2nd and 4th generations were analyzed using two-way ANOVA, the rice type had a significant effect on the fecundity, but not on the nymph developmental duration and adult longevity of BPH. The difference in the fecundity between the *Bt* and non-*Bt* rice increased much more from the 1st to 4th generation. In contrast, the difference in the nymph developmental duration of BPH between the *Bt* and non-*Bt* rice became negligible from the 1st to 4th generation although the difference was significant at the 1st and 2nd generations according to the *t* test. Thus, it is implied that BPH itself has the ability to adapt *Bt* rice plants for development but not for fecundity. The present results were similar to our previous laboratory results that the BPH female adults did not prefer to lay eggs on *Bt japonica* rice lines with a *cry1Ab* gene (KMD1 and KMD2) or on *Bt indica* rice lines with a fused gene of *cry1Ab/cry1Ac* (TT9-3 and TT9-4), and the number of eggs laid on them within 48 h was greatly decreased by 74.9, 89.2, 82.1 and 53.6%, respectively, as compared with their respective non-*Bt* controls, namely, Xiushui 11 and IR72 (Chen et al. 2003b, 2004). However, these results were not found in our another previous laboratory results that there were no significant differences in the number of eggs laid by BPH within 48 h between *Bt* rice lines (B1 and B6, being derived from KMD1 through conventional hybrid cross with an *indica* cultivar Jiazao 935; MSA with a fused gene of *cry1Ab/CpTI*) and their non-*Bt* controls, Jiazao 935 and Minghui86 (Chen et al. 2004). Similarly, our present results were also different from most previous reports about non-target effects of *Bt* rice on rice-sucking herbivores under laboratory conditions. In one case, no marked effects on non-target sucking insects were detected. For instance, no marked differences in any of reproduction parameters were found for BPH or the white-backed planthopper, *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae) reared on *Bt* rice (MSA and MSB with a fused gene of *cry1Ab/CpTI*) and non-*Bt* rice (Fu et al. 2003). Such was the case for BPH on five *Bt* rice lines with a gene *cry1Ab* or *cry1Ab/cry1Ac*, being controlled by different promoters (Bernal et al. 2002), and the case for *S. furcifera* on *Bt* rice B1 and B6 (Tan et al. 2006). In another case, the positive effects on sucking insects were found as reported by Zhou et al. (2005) that the fecundity of the green leafhopper,

Nephotettix cincticeps (Uhler) (Hemiptera: Cicadellidae), was considerably increased by 4.61 and 2.25 times on *Bt* rice KMD1 and KMD2, respectively, by comparison with non-*Bt* rice Xiushui 11.

We cannot detect the Cry1Ab protein in the male and female individuals, although a previous study found that the Cry1Ab protein can be detected in 5 BPHs (Chen et al. 2005). Our recent study showed that transgenic *cry1Ab* or *cry1Ab/cry1Ac* rice resulted in the longer immature duration of *S. bifurmis*, which is not related to the *Bt* insecticidal protein (Akhtar et al. 2010). Similarly, a previous study showed that the BPH will be exposed to *Bt* insecticidal protein from rice lines transformed with some *Bt* gene constructs, but that this exposure might not affect BPH (Bernal et al. 2002). In addition, activities of GUS and NpTII were also not detected in either males or females of BPHs at their individual levels after feeding on our *Bt* rice (Online Resource 4). Thus, it is clear that the changes of biological parameters of BPH fed on KMD2 are not directly caused by Cry1Ab protein, GUS or NpTII.

Field experiment

The current 2-year field studies also demonstrated that our *Bt* rice line “KMD2” had a beneficial effect in suppressing the population increase of BPH because the population density of BPH nymphs was significantly reduced in the *Bt* rice field as compared with the non-*Bt* rice control. As regards the population density of BPH adults, it was not significantly affected by rice type (*Bt* vs. non-*Bt*). This may result from the dispersal of adults between the *Bt* and non-*Bt* rice fields because the size of our trial field and the gap between the *Bt* and non-*Bt* rice fields were not large enough to minimize the influence of adult dispersal on the population density of adults. Whether the population density of BPH adults, like its nymphs, would be significantly reduced needs to be proved after the commercial planting of our *Bt* rice line “KMD2” on a large scale. Our current field results differed distinctly from some previous reports. For example, our previous multiple year–site studies indicated that no significant difference was found in the population density of WBPH in the field between *Bt* rice lines (such as KMD1, KMD2, TT9-3 and TT9-4) and their non-*Bt* correspondent controls (Chen et al. 2006, 2007). Similarly, various *Bt* rice lines with a fused gene of *cry1Ab/CpTI* (MSA,

MSB, MSA4) and their derived hybrid rice lines did not markedly act to alternate the population densities of BPH, *S. furcifera* and *N. cincticeps* as well as rice gall midge, *Orseolia oryzae* (wood-mason) (Diptera: Cecidomyiidae) as compared to their non-*Bt* control MH86 (Fu et al. 2003; Liu et al. 2007). Conversely, another two cases have been reported in which the population density of *N. cincticeps* on *Bt* rice KMD2 was significantly higher than that on non-*Bt* control Xiushui 11 (Zhou et al. 2005), and the population density of BPH in the plots of *Bt* hybrid rice, Shanyou 63 with a fused gene of *cry1Ab/cry1Ac* was significantly higher than in the non-*Bt* control (Wang et al. 2010). Additionally, to our knowledge, our *Bt* rice line “KMD2” is the first one capable of suppressing the fecundity and population increase of its non-target sucking herbivore, namely BPH, under both the laboratory and field conditions. Such a finding has not been reported in *Bt* cotton and *Bt* maize, which showed almost negligible or adverse effects on their non-target sucking insect species such as aphids and thrips (Faria et al. 2007; Lawo and Romeis 2008; Eizaguirre et al. 2006). To summarize our present study and other previous studies about the effects of *Bt* crops on their non-target sucking herbivores, it can be ascertained that the performances of non-target sucking herbivores on *Bt* crops were various depending on the type of *Bt* crops, their lines tested and insect species evaluated, and must be assessed case-by-case, as conducted so far, taking into account the existing experience and knowledge.

Insect oviposition performance is central to investigations of insect population dynamics and life-history evolution (Minkenberg et al. 1992). In our present study, the egg-laying sites and the capacity for egg-laying of BPH were measured. By comparison with non-*Bt* rice, the egg-laying of BPH on the *Bt* rice under field conditions was significantly decreased, and BPH on the *Bt* rice distinctly prefer to lay eggs on the higher part of plants at each tested rice stage except the mature stage, at which the BPH on the non-*Bt* rice prefer to lay eggs on the leaf midribs rather than the leaf sheath. We think that the alternations in the fecundity and egg-laying sites were caused by the differences in chemical constituents that rendered the rice plants better defended and/or less nutritious for BPH females because the Cry1Ab protein is not directly toxic to BPH. The changes in the composition and content of nutrients

and secondary chemical compounds, associated with the oviposition performance of BPH, likely result from unintended effects caused by the process of transgene insertion. Similarly, several studies have shown unintended compositional changes of nutrients or anti-nutrients in transgenic rice as compared with non-transgenic rice (Ye et al. 2000; Jiao et al. 2010; Keymanesh et al. 2009).

Conclusions

To ensure that *Bt* crops play the roles better when incorporated into integrated pest management (IPM), the effects of *Bt* crops on non-target herbivore species, such as planthoppers on rice, need to be addressed. Based on our present study and other previous reports as described above, it is clear that the effects of *Bt* rice on BPH significantly varied among different *Bt* rice lines. It is suggested that the effects of IRGM crops either on their target pests or on non-target herbivores, which are important or sporadic pests, need to be evaluated among different lines, such as developing novel IRGM lines. Moreover, the lines, like our *Bt* rice line “KMD2”, which are desirable to be screened as the candidates for release to farmers must not only show high and stable resistance to the target pests, but also significantly act to inhibit the population increase of the non-target pests under their economic control threshold, so as to reduce the use of chemical insecticides for controlling the non-target pests.

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