



Screen of *Bacillus thuringiensis* toxins for transgenic rice to control *Sesamia inferens* and *Chilo suppressalis*

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ABSTRACT

Transgenic rice to control stem borer damage is under development in China. To assess the potential of *Bacillus thuringiensis* (Bt) transgenes in stem borer control, the toxicity of five Bt protoxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba and Cry1Ca) against two rice stem borers, *Sesamia inferens* (pink stem borer) and *Chilo suppressalis* (striped stem borer), was evaluated in the laboratory by feeding neonate larvae on artificial diets containing Bt protoxins. The results indicated that Cry1Ca exhibited the highest level of toxicity to both stem borers, with an LC₅₀ of 0.24 and 0.30 µg/g for *C. suppressalis* and *S. inferens*, respectively. However, *S. inferens* was 4-fold lower in susceptibility to Cry1Aa, and 6- and 47-fold less susceptible to Cry1Ab and Cry1Ba, respectively, compared to *C. suppressalis*. To evaluate interactions among Bt protoxins in stem borer larvae, toxicity assays were performed with mixtures of Cry1Aa/Cry1Ab, Cry1Aa/Cry1Ca, Cry1Ac/Cry1Ca, Cry1Ac/Cry1Ba, Cry1Ab/Cry1Ac, Cry1Ab/Cry1Ba, and Cry1Ab/Cry1Ca at 1:1 (w/w) ratios. All protoxin mixtures demonstrated significant synergistic toxicity activity against *C. suppressalis*, with values of 1.6- to 11-fold higher toxicity than the theoretical additive effect. Surprisingly, all but one of the Bt protoxin mixtures were antagonistic in toxicity to *S. inferens*. In mortality-time response experiments, *S. inferens* demonstrated increased tolerance to Cry1Ab and Cry1Ac compared to *C. suppressalis* when treated with low or high protoxin concentrations. The data indicate the utility of Cry1Ca protoxin and a Cry1Ac/Cry1Ca mixture to control both stem borer populations.

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1. Introduction

Rice, *Oryza sativa* L., is one of the most important crops worldwide, the primary staple food for nearly 3 billion people. In a rice ecosystem, many pest species can cause economic loss. The four major Lepidoptera pests of rice are the striped stem borer (SSB), *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae); the yellow stem borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae); the pink stem borer (PSB), *Sesamia inferens* Walker (Lepidoptera: Noctuidae), and the rice leaf roller, *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae). In China alone, these pests occurred in 15 million hectares in 2002 (Sheng et al., 2003), about half the acreage of rice planted annually in China, resulting in significant economic loss.

Transgenic *Bacillus thuringiensis* (Bt) rice varieties expressing single Cry toxins have demonstrated excellent control of SSB, yellow stem borer, and rice leaf roller both in laboratory and field trials (Cheng et al., 1998; Shu et al., 2000; Ye et al., 2001; Zhao

et al., 2004; Ho et al., 2006). However, the control of PSB by Bt transgenic rice varieties is the lowest among targeted pests. Several field investigations revealed that some PSB could complete their life cycle on Bt transgenic rice. For example, a field survey was designed to investigate the field performance of two Bt varieties, MSA and MSB, each producing Cry1Ac toxin (Gao et al., 2006). The results indicated that the control of SSB was 99% in both early and late season rice, while the control of PSB varied from 93% in early to 44–64% in late season rice. Of particular concern was the finding that some PSB individuals completed development in the Bt rice field (Gao et al., 2006). This phenomenon has also been reported for other transgenic Bt rice lines (Han et al., 2006, 2009). We speculated that the inefficacy of Bt transgenics expressing Cry1Ac toxin was due to insufficient toxin concentration in transgenic Bt rice and/or insensitivity of PSB to the transgenic toxin. The survival of PSB larvae on transgenic rice suggests that these larvae have genetic resistance to Cry1Ac, and therefore widespread planting of these transgenic rice varieties may result in control failures in the field, and more importantly the rapid selection of Cry1Ac-resistant PSB populations. Thus, it is important to consider how to develop and deploy Bt transgenic rice that would delay the evolution of stem borer resistance before transgenic varieties are

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released for commercial use. In fact, two Bt rice varieties (Huahui1 and Bt Shanyou63), both expressing Cry1Ac/Cry1Ab toxins, received an environmental safety license in Hubei province of China in August 2009 (<http://www.stee.agri.gov.cn/biosafety/spxx>). Therefore, Bt transgenic rice is nearing approval for commercial planting in China, the largest rice-producing nation in the world.

To develop effective transgenic rice varieties incorporating genes encoding either a single Bt toxin or multiple (stacked) Bt toxins, it is important to evaluate the toxicity of Cry toxins against the target pests. The identification of toxin mixtures that have synergy against the target pest also can help to reduce or delay the development of insect resistance to individual Bt toxins (Zhao et al., 2003).

In this report, we determined the individual toxic effect of five Cry protoxins on SSB and PSB. In addition, we determined the interactions between protoxin pairs in stem borer larvae. These studies were undertaken to identify the most effective single Bt toxin or the most effective toxin combination for development of new Bt rice varieties that can manage the evolution of resistant PSB populations and simultaneously provide effective protection of both SSB and PSB damage.

2. Materials and methods

2.1. Insects

From March–May of 2005, more than 400 late instar larvae and more than 100 pupae of SSB and PSB were collected from the rice fields (30.4°N, 119.5°E) at the China National Rice Research Institute, Zhejiang province, China. The collected larvae were reared on rice planted in 15 cm pots until pupation. Pupae were moved to glass dishes ($d = 10$ cm, $h = 2$ cm) before eclosion and were placed inside an oviposition cage ($1 \times 1 \times 1.5$ m). Moths were provided a cotton swab dipped in 10% honey solution and renewed daily. All cages were placed in a greenhouse under a photoperiod of 14:10 h (L:D) and 75% RH at 27 ± 1 °C. Rice plants loaded with egg masses were replaced by fresh pots after 4 days. Harvested egg masses were kept in a chamber at 27 ± 1 °C until hatching. Newly hatched neonates (<24 h) were used for our tests.

2.2. Preparation of protoxins

Five protoxins, Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba and Cry1Ca, were tested against both SSB and PSB. All protoxins were kindly provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Protoxins were purified and solubilized as previously described (Lee et al., 1992). Solubilized protoxins were dialyzed against 50 mM Na_2CO_3 (pH 10). The concentration of protoxin was estimated by measuring total protein by the Bradford method (Bradford, 1976), with bovine serum (BSA) as the standard, and determining the percent toxin composition after proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All protoxin suspensions were stored at -20 °C before use.

2.3. Bioassays

2.3.1. Artificial diet and exposure of neonates to Bt protoxin

Bioassays involved the exposure of PSB and SSB neonates to a single Bt toxin or to mixtures of two toxins. All toxins were incorporated into a modified artificial diet (0.25 g of sucrose, 1.5 g of bran coat starch, 0.75 g of wheat germ, 0.75 g of casein, 0.50 g of yeast, 0.50 g of rice starch, 0.10 g of Weber's salt mixture, 0.01 g of cholesterol, 0.05 g of sorbic acid, 0.50 g of agar and 0.10 g of ascorbic acid in 27.5 ml distilled water). Each protoxin was diluted

serially in distilled water and blended into the diet thoroughly with a blender to achieve the appropriate concentration. The artificial diet without Bt protoxin was used as control. All bioassays were performed in glass tubes ($d = 10$ cm, $h = 0.8$ cm). Approximately 1.8 g diet was placed into the bottom of each tube, and 10 neonates were placed on the surface of the diet with a fine brush. A wet paper was put on the inner wall of the tube to maintain suitable humidity for stem borers. The top of the tube was blocked by a cotton plug to prevent escapes. All tubes containing diet and neonates were kept in a 27 ± 1 °C chamber and covered with clear cotton cloths to allow the bottom of the tube to be exposed to light.

2.3.2. Single Bt protoxins

We tested 5–7 concentrations of each protoxin to calculate the LC_{50} for each protoxin to SSB and PSB larvae. For each Bt concentration, 40 SSB or PSB neonates, 10 neonates per glass tube, formed one experiment unit. All toxicity trials of a given protoxin were repeated two to four times. All glass tubes containing inoculated neonates were kept at 27 ± 1 °C, a photoperiod of 14:10 h (L:D), and 75% R.H chamber. The artificial diets were renewed after 2 days inoculation. Mortality was recorded after incubation for 4 days. The larvae were recorded as dead when no movement was observed after probing with a fine brush.

2.3.3. Bt protoxin mixtures

To evaluate interaction among Bt protoxins in this experiment, we tested seven mixtures with two Bt protoxins mixed at 1:1 (weight/weight) ratio. Mixtures were Cry1Aa/Cry1Ab, Cry1Aa/Cry1Ca, Cry1Ac/Cry1Ca, Cry1Ac/Cry1Ba, Cry1Ab/Cry1Ac, Cry1Ab/Cry1Ba and Cry1Ab/Cry1Ca. According to a study conducted by Xue et al. (2005), a 1:1 ratio mixture of Cry1C/Cry1Aa was most effective against the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and the cotton bollworm, *Helicoverpa armigera*. Bioassays were conducted as for single protoxins.

2.3.4. Time-mortality response

We also tested the relationship of time and Bt protoxins (Cry1Ab and Cry1Ac) against both SSB and PSB. Currently, most of the developed Bt rice varieties in China contain either Cry1Ab or Cry1Ac toxin. In this experiment, Bt protoxin concentrations were the data against SSB only (LC_{50} (0.66 $\mu\text{g/g}$) and LC_{90} (5.60 $\mu\text{g/g}$) of Cry1Ab, and LC_{50} (4.37 $\mu\text{g/g}$) and LC_{90} (21.2 $\mu\text{g/g}$) of Cry1Ac). We did not use LC_{50} and LC_{90} values for PSB because these concentrations were toxic to SSB and would result in rapid mortality. Neonates of SSB and PSB were exposed to Bt diet continuously as previously described, and dead neonates were removed and recorded every 48 h for 8 days (LC_{50}) and 6 days (LC_{90}). Three replications of each concentration were tested.

2.4. Data analysis

The corrected mortality value for each bioassay was calculated by dividing the percentage of mortality on the Bt-treated diet by that of the control. The mean lethal concentration (LC_{50}), 95% fiducial limit (FL_{95}), and slopes were estimated by probit analysis (Finny, 1971) using POLO-PC software (LeOra Software, Berkeley, CA). LC_{50} values were considered to be significantly different if their FL_{95} did not overlap.

The expected median lethal concentration ($\text{LC}_{50(m)}$) of protoxin mixtures was calculated by the equation (Eq. (1)) described by Tabashnik (1992):

$$\text{LC}_{50(m)} = \left[\frac{r_a}{\text{LC}_{50(a)}} + \frac{r_b}{\text{LC}_{50(b)}} \right]^{-1} \quad (1)$$

In this equation, $LC_{50(m)}$ is the expected LC_{50} of the mixture, which is the harmonic mean of the LC_{50} observed for protoxins a and b acting separately, and r_a and r_b are the relative proportions of protoxin a and protoxin b in the mixture, respectively.

The synergism factor (SF) of protoxin mixtures was calculated by dividing the $LC_{50(m)}$ by the observed LC_{50} values, based on Tabashnik (1992). The value of SF greater than 1 indicates a positive synergistic effect (synergism), while less than 1 indicates a negative synergistic effect (antagonism). SF equal to 1 indicates an additive effect.

The data for the time-mortality response test were assessed by using analysis of variance (ANOVA), and means were separated using the protected least significant difference (LSD) test (SAS Institute, 1998).

3. Results

3.1. Toxicity of Bt protoxins

The toxicity of five different Cry protoxins was evaluated against neonate larvae of SSB and PSB (Table 1). Cry1Ca ($LC_{50} = 0.24 \mu\text{g/g}$) had twofold higher toxicity than Cry1Ab protoxin ($LC_{50} = 0.66 \mu\text{g/g}$) in SSB larvae (Table 1). Cry1Ba, Cry1Ac, and Cry1Aa protoxins had lower toxicity against SSB (LC_{50} values ranged from 3.02 to 9.08 $\mu\text{g/g}$). In PSB larvae, Cry1Ca was the most toxic protoxin ($LC_{50} = 0.30 \mu\text{g/g}$), followed by Cry1Ab and Cry1Ac with somewhat lower toxicity. However, Cry1Aa and Cry1Ba were only marginally toxic to PSB, with LC_{50} values of 32.9 and 154 $\mu\text{g/g}$, respectively. The results indicate that Cry1Ca exhibited the highest level of toxicity to both SSB and PSB, indicating that Cry1Ca protoxin may be a good candidate for transgenic rice to provide effective protection from both SSB and PSB damage.

3.2. Synergism of Bt protoxin mixtures

The toxicity of Bt protoxin mixtures also was tested. All two-protoxin mixtures demonstrated a synergistic effect against SSB larvae (Table 2). In these tests, all LC_{50} values of Bt protoxin mixtures were greater than expected values, ranging from 0.08 $\mu\text{g/g}$ for Cry1Ab/Cry1Ca to 0.84 $\mu\text{g/g}$ for Cry1Ac/Cry1Ba, an 11-fold difference. In contrast to the synergy of Bt protoxins with SSB, all but one of the Bt protoxin mixtures demonstrated an antagonistic effect against PSB (Table 2). The exception was the combination of Cry1Aa/Cry1Ab with a SF value of only 1.6. The LC_{50} values of protoxin mixtures against PSB ranged from 0.97 $\mu\text{g/g}$ for Cry1Ac/Cry1Ca to 63.91 $\mu\text{g/g}$ for Cry1Ac/Cry1Ba, a 66-fold difference.

3.3. Time-mortality response

We tested the time-mortality response of SSB and PSB Cry1Ab and Cry1Ac using the LC_{50} and LC_{90} for SSB. Although mortalities of both SSB and PSB larvae increased over time, there were significant differences in the mortality response to Cry1Ab and Cry1Ac ($P < 0.05$, Fig. 1).

When exposed to the LC_{50} of Cry1Ab (Fig. 1, A1), the corrected mortality of SSB larvae increased as exposure time increased. At this dose, almost all treated SSB larvae were dead when exposed to Cry1Ab protoxin for 6 days. As for PSB larvae, the corrected mortalities increased at a relatively slower rate as the exposure time increased. The corrected mortalities varied between 20% and 30% after exposure to Bt protoxins after 8 days. When exposed to the LC_{50} of Cry1Ac (Fig. 1, A2), the corrected mortality of SSB and PSB larvae also increased with exposure time. At this dose, almost all treated SSB larvae were dead when exposed to Cry1Ac toxin for 8 days. However, the corrected mortality of PSB was again lower than that of SSB larvae. At 8 days of Cry1Ac exposure, the mortality of PSB larvae was only 60%. These results indicated that PSB larvae are more tolerant to Cry1Ab and Cry1Ac.

In contrast to exposure to Cry1Ab protoxin LC_{50} values, when SSB and PSB larvae were exposed to the LC_{90} of Cry1Ab, the corrected mortality of both pests increased rapidly (Fig. 1, B1). Similar to the previous experiment, most SSB larvae were dead after 4 days of Cry1Ab exposure, but mortalities of PSB larvae were only 75% at this dose. However, after a 6 days exposure, almost all PSB larvae were dead. The results of SSB and PSB larvae exposed to the LC_{90} of Cry1Ac (Fig. 1, B2) were similar to the outcomes from the Cry1Ab LC_{90} test. These results also indicated that PSB larvae are more tolerant to Cry1Ab and Cry1Ac than SSB when exposed to the LC_{90} dose of both Bt toxins.

4. Discussion

It is known that insects have varying susceptibility to different Cry protoxins (Liao et al., 2002; Avilla et al., 2005; Sauka et al., 2007; Zhang et al., 2007). In this study we tested five different Cry protoxins against neonate larvae of SSB and PSB, and we found that Cry1Ca was the most active Bt protoxin against both stem borers. In contrast, Cry1Ba, Cry1Aa, and Cry1Ac had relatively low or marginal toxicity against both stem borers.

In the present study, variations also were observed in the response of SSB and PSB to a median and high dose of Bt protoxins over time. In our mortality-time response experiment, a direct comparison was made to the susceptibility of Cry1Ab and Cry1Ac between the two rice stem borers. The survival rate of SSB was greatly reduced when treated with either a median or high dose concentration of Cry1Ac or Cry1Ab compared to that of PSB, demonstrating that PSB larvae were more tolerant to Cry1Ac and Cry1Ab. Concomitantly, the efficacy of transgenic Bt rice expressing Cry1Ac or Cry1Ab was much lower in PSB than SSB in a field plot experiment, and a relatively higher number of PSB larvae survived on Bt rice during the growing season (Gao et al., 2006).

In this study, we also analyzed the nature of the interaction of selected Bt protoxins to SSB and PSB. Interaction between toxins in the target insect may result in an increase or decrease in the degree of pest control, and both synergistic and antagonistic interaction of Bt toxins have been observed in lepidopterans (Lee et al., 1996; Sayyed et al., 2001; Liao et al., 2002; Ibagutxi et al., 2008). Based on our study, all Bt protoxin mixtures demonstrated

Table 1
Susceptibility of *Sesamia inferens* and *Chilo suppressalis* larvae to Bt protoxins.

Protoxin	<i>Chilo suppressalis</i> (SSB)			<i>Sesamia inferens</i> (PSB)		
	<i>n</i>	LC_{50} (95%FL)	Slope (\pm SE)	<i>n</i>	LC_{50} (95%FL)	Slope (\pm SE)
Cry1Aa	700	9.08 (7.60–10.7)	1.76 (\pm 0.13)	720	32.9 (28.3–37.6)	2.31 (\pm 0.18)
Cry1Ab	900	0.66 (0.54–0.79)	1.39 (\pm 0.08)	720	3.73 (3.12–4.36)	1.78 (\pm 0.13)
Cry1Ac	960	4.36 (3.77–5.04)	1.87 (\pm 0.12)	840	5.09 (4.23–6.04)	1.49 (\pm 0.11)
Cry1Ba	550	3.29 (2.87–3.70)	3.28 (\pm 0.28)	720	154 (81.6–253)	1.79 (\pm 0.13)
Cry1Ca	480	0.24 (0.19–0.30)	1.80 (\pm 0.16)	720	0.30 (0.23–0.38)	1.33 (\pm 0.10)

Table 2
Synergism of Bt protoxin mixtures against *Chilo suppressalis* and *Sesamia inferens*.

Protoxin mixtures	<i>Chilo suppressalis</i> (SSB)					<i>Sesamia inferens</i> (PSB)				
	Expected LC ₅₀	n	LC ₅₀ (95%FL) ^b	Slope (±SE)	SF ^a	Expected LC ₅₀	n	LC ₅₀ (95%FL) ^b	Slope (±SE)	SF ^a
Cry1Aa/Cry1Ab	1.23	560	0.25 (0.19–0.33)	1.41 (0.13)	4.92	6.70	840	4.18 (3.62–4.86)	2.02 (0.15)	1.60
Cry1Aa/Cry1Ca	0.47	550	0.29 (0.17–0.42)	1.27 (0.12)	1.60	0.59	660	4.44 (3.80–5.07)	2.34 (0.18)	0.10
Cry1Ab/Cry1Ac	1.15	400	0.45 (0.32–0.59)	1.47 (0.15)	2.56	4.31	660	6.08 (5.19–6.99)	2.15 (0.16)	0.70
Cry1Ab/Cry1Ba	1.10	550	0.10 (0.07–0.13)	1.39 (0.12)	11.0	7.29	480	22.8 (18.4–27.2)	1.29 (0.19)	0.30
Cry1Ab/Cry1Ca	0.35	500	0.08 (0.05–0.12)	1.16 (0.11)	4.40	0.56	660	3.56 (3.12–4.03)	2.53 (0.19)	0.20
Cry1Ac/Cry1Ca	0.46	480	0.15 (0.12–0.17)	2.50 (0.23)	3.10	0.57	480	0.97 (0.83–1.12)	2.83 (0.37)	0.60
Cry1Ac/Cry1Ba	3.75	720	0.84 (0.66–1.06)	1.34 (0.10)	4.46	9.90	480	63.9 (35.2–98.8)	2.06 (0.17)	0.20

^a Synergistic factor (SF) equals the theoretical LC₅₀ divided by the observed LC₅₀.

^b 95% Confidence limits are given.

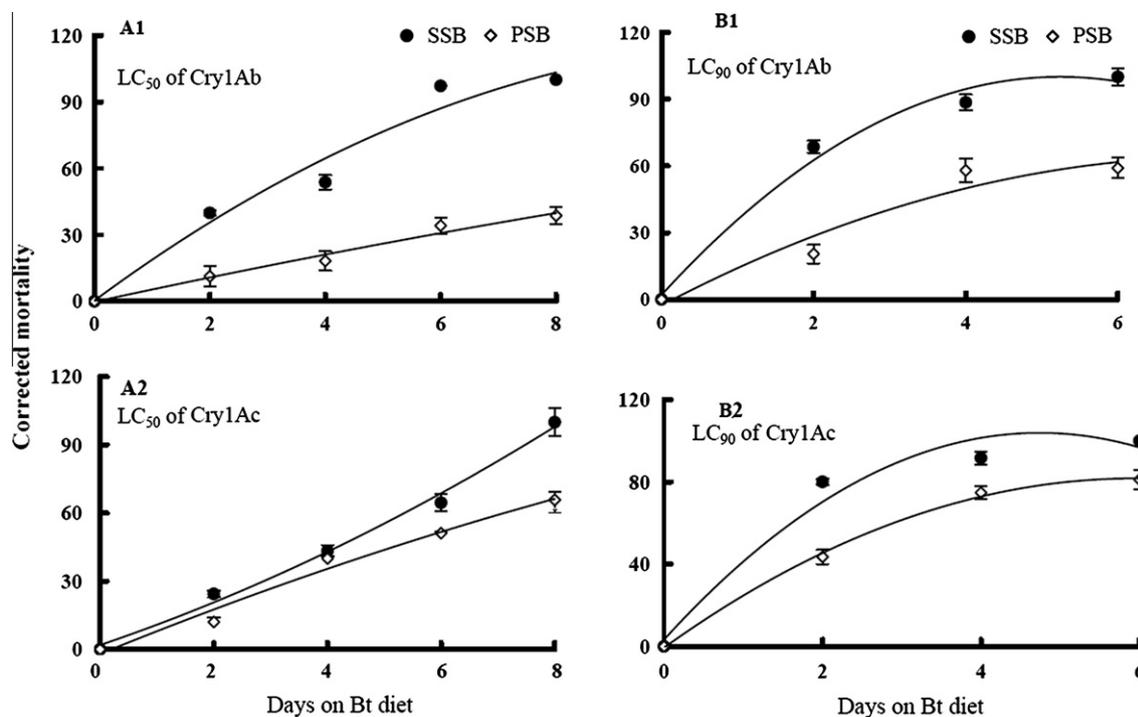


Fig. 1. Relation of the time-mortality response of SSB and PSB neonates fed the LC₅₀ dose (0.66 µg/g for Cry1Ab and 4.37 µg/g for Cry1Ac, A1 and A2) and LC₉₀ dose (5.6 µg/g for Cry1Ab and 21.2 µg/g for Cry1Ac, B1 and B2) for SSB.

a synergistic effect against SSB. However, only the combination of Cry1Aa/Cry1Ab had a synergistic effect against PSB, and the other Bt protoxin mixtures demonstrated an antagonistic effect. In the case of SSB, all protoxin mixtures (Cry1Aa/Cry1Ab, Cry1Aa/Cry1Ca, Cry1Ac/Cry1Ca, Cry1Ac/Cry1Ba, Cry1Ac/Cry1Ca, Cry1Ac/Cry1Ba, Cry1Ab/Cry1Ac, Cry1Ab/Cry1Ba and Cry1Ab/Cry1Ca) are suitable for use in transgenic rice for their high toxicity and synergism against SSB. However, previous studies have found some toxin mixtures may increase the probability of pest cross-resistance and result in failure of transgenic crops. For example, Cry1Aa/Cry1Ac, Cry1Ac/Cry1Ba, and Cry1Ab/Cry1Ac would not be good choices for controlling rice stem borers because they compete for the same binding site, demonstrated in previous studies (Lee et al., 1997; Fiuza et al., 1996; Alcántara et al., 2004). In the case of PSB, although the mixture of Cry1Ac/Cry1Ca demonstrated antagonism against PSB, we propose that this mixture may still be a good candidate to pyramid in Bt transgenic rice crops, because the combination has the highest toxicity to PSB larvae. Lee et al. (1997) also proposed that Cry1C be combined with Cry1A to control rice stem borer due to the lack of cross-resistance.

According to our results, we propose to use Cry1Ca toxin for optimal control of both SSB and PSB pests in transgenic rice. To

prevent the potential development of insect resistance, the mixture of Cry1Ac/Cry1Ca is a good candidate in terms of efficacy against both SSB and PSB. However, our bioassays were with protoxin forms of the Cry proteins, and bioassays with activated Cry toxins are needed to verify these effects. In addition, bioassays of Cry1Ac/Cry1Ca against rice leaf roller and yellow stem borer are needed to verify the potential benefit of this pyramided toxin strategy to control rice field pests.

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