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Transgenic IR72 with Fused *Bt* Gene *cry1Ab/cry1Ac* from *Bacillus thuringiensis* is Resistant Against Four Lepidopteran Species Under Field Conditions

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Abstract

Two transgenic IR72 lines, TT9-3 and TT9-4, carrying a fused *Bt* gene *cry1Ab/cry1Ac* from *Bacillus thuringiensis* Berliner were field tested to evaluate their multiple resistance against four lepidoptera and their agronomic performance in Hangzhou, China, in 1999. The results demonstrated that both transgenic lines were highly resistant against natural infestation and artificial infestation of four lepidopteran species: striped stem borer (*Chilo suppressalis*), pink stem borer (*Sesamia inferens*), leaffolder (*Cnaphalocrocis medinalis*), and green semilooper (*Naranga anescens*). In contrast, the non-transformed IR72 control showed serious damage symptoms of deadhearts, whiteheads, folded leaves or leaf notches. Field performance data showed that *Bt* transgenic lines may provide excellent opportunity for *Bt* rice deployment for commercial scale in Asia.

Key words: Transgenic rice, *Bacillus thuringiensis*, fused *Bt* gene, stem borers, leaffolder, semilooper, field testing, agronomic traits

Abbreviations

Bt: Bacillus thuringiensis; SB: stem borer; PSB: pink stem borer; SSB: striped stem borer; YSB: yellow stem borer; LF: leaffolder; GS: green semilooper; BPH: brown planthopper; WHPH: whitebacked planthopper; IPM: integrated pest management.

Introduction

Larvae of lepidopta are the most destructive insect pests of rice throughout the world (Kan *et al.*, 1991). Annual yield loss of 5 to 10% can result from these larvae feeding at various stages of plant growth (Pathak *et al.*, 1994). Feeding of stem borer (SB) larvae, including yellow stem borer (YSB, *Scirpophaga incertulas*), striped stem borer (SSB, *Chilo suppressalis*), and pink stem borer (PSB, *Sesamia inferens*) results in deadheart if the feeding damage occurs at the tillering stage or whitehead if the feeding damage occurs at the booting stage. Both cases lead to a complete loss of tillers or spikelets. On the other hand, feeding of foliage-attacking larvae, including leaffolder (LF, *Cnaphalocrocis medinalis*) and green semilooper (GS, *Naranga anescens*), results in a severe reduction in photosynthetic area, finally causing a lower plant yield (Pathak *et al.*, 1994). Chemical pesticides are effective against lepidopteran moth and larvae, but their use is limited because of their high cost, persistence in the environment, and increasing rates of application due to decreasing effectiveness (Alam *et al.*, 1998). These concerns may increase further in the future because of a negative public perception of current insecticide use (Spencer, 1989) and the lack of alternative, novel approaches for pest control without damage to the bio-environment.

Lepidopteran larvae are susceptible to various insecticidal crystal proteins, or δ-endotoxins, produced by a number of strains of the spore-forming soil bacterium *Bacillus thuringiensis* (*Bt*) (Frutos *et al.*, 1999; Höfte *et al.*, 1989). The mode of action of the δ-endotoxins involves solubilization of the crystal in the insect midgut, proteolitic processing

of the protoxin by midgut proteases, binding of the endotoxin to midgut receptors, and insertion of the toxin into the apical membrane to create pores. Finally, the insect dies by losing of its cellular osmotic balance in the midgut (Grochulski *et al.*, 1995; Höfte *et al.*, 1989). This insecticidal activity is highly specific, however, in that the endotoxins are nontoxic to non-target insects, birds, and mammals (Beegle and Yamamoto, 1992; Perlak *et al.*, 1990). A variety of genes that encode δ-endotoxins have now been transferred to higher plants including tobacco (Vaeck *et al.*, 1987), tomato (Delannay *et al.*, 1989), cotton (Perlak *et al.*, 1990), canola (Stewart *et al.*, 1996), maize (Koziel *et al.*, 1993), rice (Alam *et al.*, 1999; Cheng *et al.*, 1998; Datta *et al.*, 1998; Fujimoto *et al.*, 1993; Nayak *et al.*, 1997; Tu *et al.*, 1998), and potato (Perlak *et al.*, 1993), resulting in insect-resistant phenotypes. Two commercialized *Bt* crops, cotton and maize, had their protection area increased to 11.8 million hectares in 1999, accounting for one-third of the global area of transgenic crops (James, 1999).

We previously used a fused *Bt* gene truncated from *cry1Ab* and *cry1Ac* under the control of the rice *actin 1* promoter to transform the elite indica rice IR72 via the biolistic method (Christou *et al.*, 1991; Datta *et al.*, 1998; Tu *et al.*, 1998) and an insecticidal effect against YSB was demonstrated under transgenic greenhouse conditions. We report here the results of field trials of two selected transgenic lines, TT9-3 and TT9-4, both of which were derived from the same transformant of IR72. These field trials were conducted in Hangzhou, China, in 1999 and the levels of resistance of two transgenic lines against four lepidopteran species (SSB, PSB, LF, and GS) as well as their yield performance were evaluated. Our transgenic lines exhibited season-long protection of plants against natural and/or artificial infestation of the four lepidopteran species.

Materials and methods

Genetic materials

Two transgenic IR72 lines, TT9-3 and TT9-4, both in their sixth generation, and the non-transgenic IR72 control were used for the present study. A screenhouse test was conducted in 1998 to evaluate the multiple resistance of these two transgenic lines against artificial infestation of Lepidoptera (data not shown) and the homozygous state of these two lines was also confirmed by Southern blot analysis (**Fig. 1**). Transgenic seeds at homozygous state were transferred to China for experimental field evaluation.

Fusion of Bt protein

The fused *Bt* gene was made from *cry1A*(b) and *cry1A*(c) as reported previously (Tu *et al.*, 1998). The first 448 amino acids of this fused *Bt* gene are identical to the analogous region of the Cry1A(b) protein excepting C5-R6, D304 and D385, instead of P5-N-I-N-E-C-I11, A309 and Y390 respectively. The remaining sequences of amino acids 449-615 are truncated from Cry1A(c) without any change. Before fusion, the codon usage of this fused *Bt* gene was optimized to replace A and T bases at the third position with G or C to meet high G + C content in rice genome. As a result, the overall G + C content in the fused *Bt* gene is 47.8%, while that of the original DNA sequences from the corresponding parts of *cry1A*(b) and *cry1A*(c) genes is 37.2%.

Experimental plot design

A field evaluation of the two transgenic lines against four lepidoptera was done at the Zhejiang University Research Farm, Hangzhou, China, in 1999. Transgenic plants were sowed and transplanted in two batches. The first batch was sown on May 20 and transplanted on June 25, 1999, for evaluation of insect resistance as well as for observation of yield. The second batch was sown on June 5 and transplanted on July 5 for evaluation of insect resistance only. The plot for the insect test on the first batch of materials representing the three test entries was divided into three subplots and each subplot consisted of 5 rows 8.3 m long and 1.0 m wide with 50 plants spaced at 16.5 cm within a row. The experimental layout for the insect test on the second batch of materials contained the same number of subplots and each subplot consisted of 5 rows 6.0 m long and 1.0 m wide with 36 plants spaced at 16.5 cm within a row. Non-transgenic IR72 was

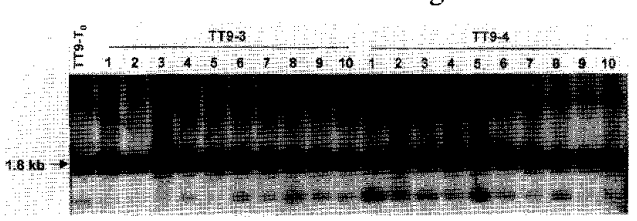

Fig. 1 Southern blot analysis of transgenic T_2 plants. A total of 5 μg of plant genomic DNA was digested with *Hin*dIII and *Sst*I and hybridized with the same enzyme-digested plasmid DNA fragment. The arrow marks the expected size of the hybridizing band (1.8 kb), which appears in the positive transgenic T_0 and T_2 plants. TT9-T_0 : positive transgenic T_0 plants. TT9-3 and TT9-4: two homozygous lines. The roman number 1-10 under TT9-3 and TT9-4 means 10 individual plants from each homozygous line in its T_8 generation.

planted as a susceptible control. Normal cultural practices for growing rice were followed during the course of the experiment except that no chemical treatment was applied after transplanting to allow evaluation of the resistance reaction of the test materials.

The plot for the yield test of the first batch of materials was arranged in a field separated from that for the insect test. The size of each subplot was 2.0 m^2 containing 72 plants. The rest of the designs and the field management were the same as those of the insect test except that chemical sprays were applied twice. The first was on July 28, when a 100 cc dosage of 50% methanidophos emulsifiable mixed with 30 g buprofezin wettable powder was used for control of SB, LF, and planthoppers (PH). The second spraying was on August 15, when 200 cc 25% Sha Chong Suan ($C_5H_{11}NS_4O_6Na_2$) was used. All materials from the yield trials were harvested on October 25 and data were scored.

Insect infestation

The insects used in the field test were SSB, LF, PSB, and GS. SSB and LF are two major insect pests in the local area (latitude 120°20′ East; longitude 30°30′ North) and they cause much more severe yield loss than the other two insect pests.

Artificial infestation of SSB and GS

Moths of SSB and GS were collected at nightfall from a farmer's field and ten pairs of healthy moths per batch were transferred into a nylon mesh oviposition cage (20 x 20 x 35 cm) containing a rice plant, and fed with 10% honey solution. Egg masses from SSB were collected daily from the caged plant and transferred into a test tube (20 x 2.5 cm) containing pieces of moist filter paper and plugged with non-absorbent cotton. The egg masses were incubated at 27 ℃ and 10 newly hatched larvae were transferred into a plastic straw 2 cm in length with both ends plugged with cotton. Each larvae-containing straw was stacked on the middle portion of a rice stem in selected hills and then the cotton plug of the upper end was removed to allow the neonate larvae to move to the plant. For the first batch of materials, infestation was done at both the tillering stage and booting stage but in separate plants, whereas for the second batch of materials the infestation was done only at tillering stage. The damage symptoms on infested plants as well as on the non-infested neighboring plants were scored one month after infestation.

On the other hand, the egg masses of GS were directly hatched and fed on the rice leaves of the caged plants up to their third instar stage. Ten larvae at this instar stage were used to infest each of 30 randomly selected plants. The damage symptoms, including the total number of damaged leaves as well as the number of pupae on sampled plants were scored 2 wk after infestation.

Natural infestation of SSB and PSB

For natural infestation of plant materials in the first batch, duplicate surveys of 30 randomly sampled plants were done weekly from August 10 until October 5. Up to fifty tillers with deadhearts or whiteheads from each batch of 30 sampled plants were likewise randomly selected and dissected to classify two species of larva, SSB or PSB, and the data obtained were then converted into percentage of damage caused by each specie. After that, the examined tillers containing the larvae were replaced in the same plot. For the second batch of materials, however, the percentage of deadhearts caused by each specie was not surveyed since the natural infestation during this time was not high.

Natural infestation of LF and GS

The damage symptoms caused by natural infestation of LF and GS were measured on both batches of plant materials. The parameters used for measuring the severity of damage to the plants caused by LF were the percentage of plants with folded leaves and the percentage of folded leaves out of the total number of sampled leaves. The severity of damage to the leaves scraped by LF was scored with Heinrich's scale (1985). The parameters used to measure the severity of damage caused by GS were the same as those in artificial infestation.

Southern blot analysis

Genomic DNA was isolated from rice plants and processed for Southern blot analysis using the standard procedure described earlier (Tu *et al.*, 2000). The coding sequence of the fused *Bt* gene *cry1Ab/cry1Ac* was used to generate a random-primed ^{32}P probe.

Quantification of the fused Cry1Ab/Cry1Ac protein

Quantification of the fused Cry1Ab/Cry1Ac protein produced in transgenic rice plants was performed using a double-sandwich ELISA technique described before (Datta *et al.*, 1998). A Cry1Ab/Cry1Ac plate kit (ENVIROLOGIX INC., Portland, Maine 04103 USA) was used to determine nanogram quantities of the fused Cry1Ab/Cry1Ac per mg fresh leaf tissue according to the manufacturer's instructions.

Results

Resistance of transgenic lines against natural infestation of SB

Two transgenic lines TT9-3 and TT9-4, confirmed to be homozygous by the Southern analysis (**Fig. 1**), were highly resistant against natural infestation of SSB and PSB. At the tillering stage, the percentage of plants with deadheart ranged from 0.0 to 13.3 and the percentage of deadheart from 0.0 to 0.9, in contrast to those of the nontransgenic IR72 control, which were from 10.0 to 90.0 and 1.1 to 17.5, respectively (**Table 1**). A clearer difference between the transgenic and control blocks at the heading stage was observed, however, for the percentage of plants with whitehead and the percentage of whitehead in the total number of sampled panicles. For instance, from 60 randomly sampled non-transgenic plants with 978 panicles in the control block, these two parameters reached 25% and 3.8%, respectively, whereas from the same number of sampled transgenic plants even with 81 more panicles in each transgenic block such symptoms of whiteheads were not found. Further analysis of the percentage of deadhearts caused by each SB species revealed that the limited damages to the transgenic lines were all caused by PSB and occurred only at the tillering stage. Damages to the control plants, however, were caused by both SB species with the predominance of SSB and occurred at both the tillering and heading stages (**Table 1**). These results implied that PSB was more tolerant of the *Bt* protein that was expressed in the transgenic plants than SSB.

Resistance of the transgenic lines against artificial infestation of SSB

The field evaluation of transgenic lines against artificial infestation of SSB was done on both

Table 1. Resistance reaction of two transgenic lines with the fused *cry1A/cry1Ac* and nontransgenic IR72 control against natural infestation of pink stem borer (PSB) and striped stem borer (SSB) in the field at Hangzhou, China (sown on May 20 and transplanted on June 25, 1999).

Sampled date	Tested line	Sampled tillers[1]	% hills with deadhearts[2] ± S.E.	% deadhearts[3] ± S.E.	% PSB-deadhearts vs. % SSB-deadhearts[4]
Aug. 10	TT9-3	703	0.0 ± 0.0	0.0 ± 0.0	0.0: 0.0
	TT9-4	874	3.3 ± 0.0	0.3 ± 0.0	100 : 0.0
	IR72	1182	69.7 ± 3.5	11.0 ± 0.3	13.0:87.0
Aug. 17	TT9-3	996	0.0 ± 0.0	0.0 ± 0.0	0.0: 0.0
	TT9-4	826	3.3 ± 3.3	0.2 ± 0.2	100 : 0.0
	IR72	1146	80.0 ± 0.0	15.0 ± 3.2	13.8:86.2
Aug. 24	TT9-3	922	13.3 ± 6.7	0.9 ± 0.5	100 : 0.0
	TT9-4	840	6.7 ± 6.7	0.5 ± 0.5	100 : 0.0
	IR72	1124	90.0 ± 3.3	17.5 ± 3.1	10.0:90.0
Aug. 31	TT9-3	822	0.0 ± 0.0	0.0 ± 0.0	0.0: 0.0
	TT9-4	870	3.3 ± 3.3	0.7 ± 0.7	100 : 0.0
	IR72	1000	63.3 ± 3.3	8.4 ± 0.4	7.4: 92.6
Sept. 7	TT9-3	936	13.3 ± 6.7	0.9 ± 0.4	100 : 0.0
	TT9-4	794	3.3 ± 3.3	0.3 ± 0.3	100 : 0.0
	IR72	958	83.3 ± 10.0	16.7 ± 3.8	8.3:92.7
Sept. 14	TT9-3	1005	0.0 ± 0.0	0.0 ± 0.0	0.0: 0.0
	TT9-4	816	0.0 ± 0.0	0.0 ± 0.0	0.0: 0.0
	IR72	855	10.0 ± 0.0	1.1 ± 0.4	20.2:79.8

[1] Sampled tillers are the sum of total tillers of all sampled hills in two sub-blocks of each tested line;

[2] % hills with deadhearts = total number of sampled hills encountered with one or more deadhearts divided by total number of sampled hills × 100;

[3] % deadhearts = total number of deadhearts sampled divided by total number of tillers sampled × 100;

[4] % PSB- or SSB- deadhearts = total number of deadhearts damaged by PSB or SSB divided by total number of observed deadhearts. S.E. = standard error; Similarly hereinafter in Tables 2 and 3.

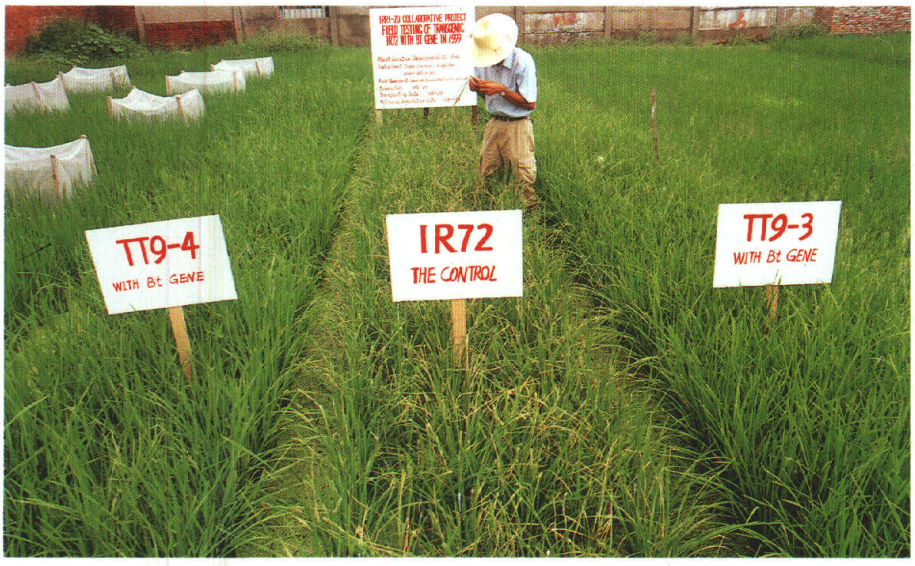

Fig. 2 Control IR72 in the middle row showing leaffolder (LF) damage, whereas TT9-4 (left) and TT9-3 (right) show LF resistance in the field.

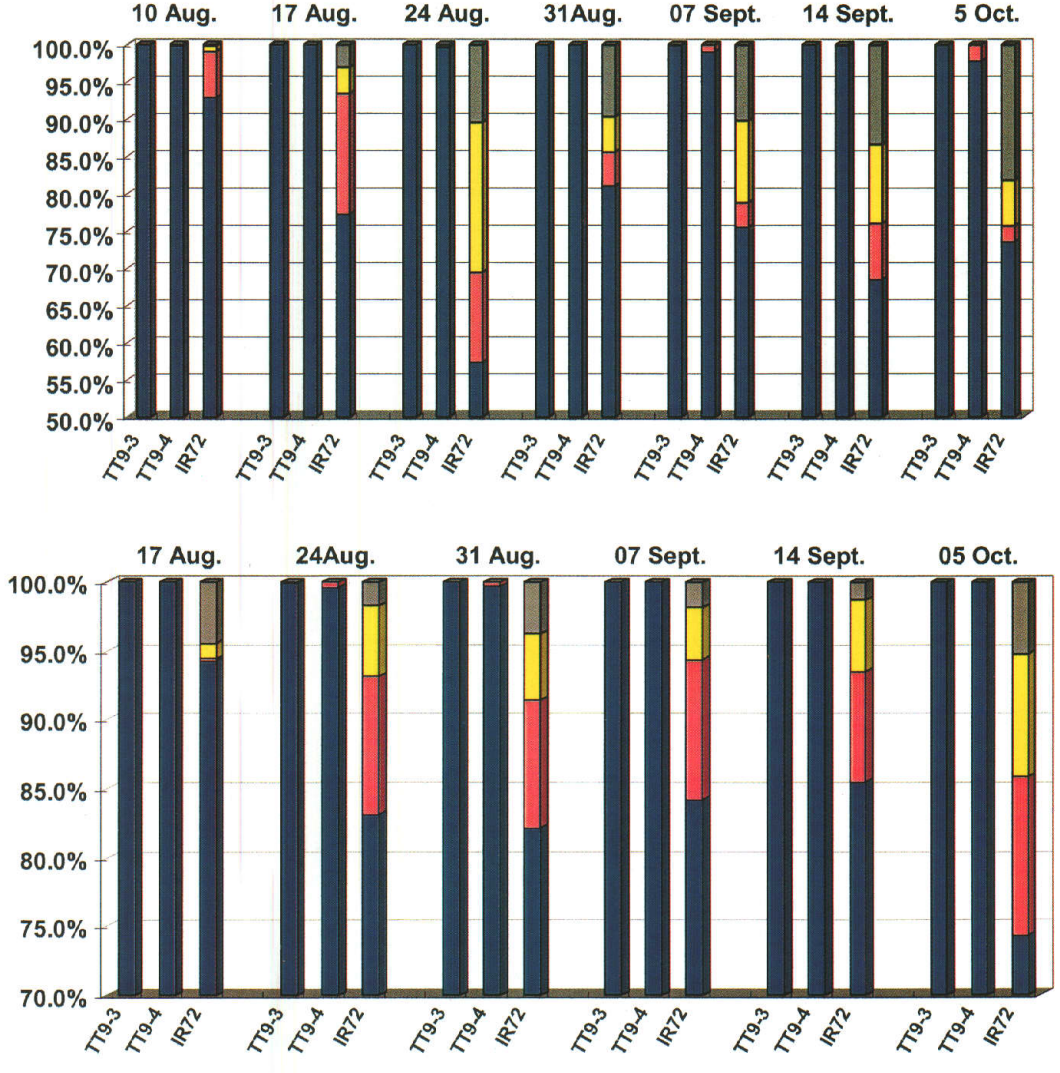

Fig. 3 Heinrich's score of leaf damage of transgenic lines and control plants caused by leaffolder (LF). A. Data from the first batch of materials. B. Data from the second batch of materials. Y axis indicates the percentage of LF-scraped leaves in each damage grade. X axis indicates the varieties (bottom line) and checking date (top line). : grade 0, leaf with no scrape. : grade II, leaf with scraped area >0 to 1/3. : grade III, leaf with scraped area >1/3 to 1/2. : grade IV, leaf with scraped area >1/2.

Table 2. Resistance reaction of two transgenic lines with the fused *cry1Ab/cry1Ac* and nontransgenic IR72 control against artificial infestation of striped stem borer (SSB) at tillering stage in the field at Hangzhou, China

Test no.[1]	Tested line	Artificially infested hills			Neighboring hills		
		Total tillers	% hills with deadhearts ± S.E.	% deadhearts ± S.E.	Total tillers	% hills with deadhearts ± S.E.	% deadhearts ± S.E.
I	TT9-3	948	3.3 ± 3.3	0.2 ± 0.2	980	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	952	10.0 ± 10.0	0.8 ± 0.5	962	3.3 ± 3.3	0.2 ± 0.2
	IR72	1086	96.7 ± 3.3	14.0 ± 0.2	976	50.0 ± 10.0	8.6 ± 1.4
II	TT9-3	1167	0.0 ± 0.0	0.0 ± 0.0	1022	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	904	0.0 ± 0.0	0.0 ± 0.0	926	0.0 ± 0.0	0.0 ± 0.0
	IR72	1113	77.5 ± 8.2	10.4 ± 0.9	988	52.5 ± 16.8	6.6 ± 1.9

[1] Test I: seeds were sown on May 20 and seedlings were transplanted on June 25; Test II: seeds were sown on June 5 and seedlings were transplanted on July 5. S.E. = standard error.

Table 3. Resisitance reaction of two transgenic lines with the fused *cry1Ab/cry1Ac* and nontransgenic IR72 control against artificial infestation of striped stem borer (SSB) at booting stage in the field at Hangzhou, China.

Tested line	Artificially infested hills			Neighboring hills		
	Total panicles	% hills with whiteheads ± S.E.	% whiteheads ± S.E.*	Total panicles	% hills with whiteheads ± S.E.	% whiteheads $\bar{\chi}$ ± S.E.
TT9-3	903	5.0 ± 5.0	0.4 ± 0.4	1176	5.0 ± 5.0	0.3 ± 0.3
TT9-4	1233	5.0 ± 5.0	0.2 ± 0.2	1242	0.0 ± 0.0	0.0 ± 0.0
IR72	969	85.0 ± 15.0	10.9 ± 4.9	924	55.0 ± 5.0	4.2 ± 0.2

*S.E. = standard error

batches of plant materials. The results showed that the percentage of hills with deadheart and the percentage of deadheart measured at the tillering stage on two transgenic lines ranged from 0.0 to 10.0 and 0.0 to 0.8 in contrast to 77.5 to 96.7 and 10.4 to 14.0, respectively, of the control materials (**Table 2**). Similarly, these indices of whitehead measured at the heading stage on the two transgenic lines artificially were infested 17-fold and 35-fold less than those of the nontransgenic IR72 materials on average (**Table 3**). For neighboring plants surrounding infestation sites, a sharp difference in the severity of plant damage was also observed between the transgenic and nontransgenic samples (**Tables 2 & 3**). These results further demonstrated that the two transgenic lines were highly resistant against SSB.

Resistance of transgenic lines against LF

The field evaluation of transgenic plants against LF was performed under natural infestation from August 10 to October 5 (**Table 4**). For the first batch of materials, the results on August 17 showed that 100% of the plants in the control plot had their foliage attacked by leaffolder larvae. Two weeks after that, the folded leaves per plant reached a peak of 34.0% (**Table 4**). The plants in the transgenic plots had their foliage attacked by leaffolder larvae at a maximum of 15% and the folded leaves per plant were less than 1% (**Fig. 2 & Table 4**). Comparing the two transgenic lines, the percentage of TT9-3 plants with folded leaves ranged from 0.0 % to 3.3%, whereas those of TT9-4 ranged from 0.0% to 15%. Similar results were observed with the second batch of materials (**Table 4**). These results indicate that the two transgenic lines were highly resistant against LF and that line TT9-3 had better resistance than TT9-4.

In the experiment, Heinrich's scoring scale (1985) was used to further judge the severity of leaves scraped by LF. The results showed that the scrape symptoms on the leaves of damaged transgenic plants were limited and that all were within the level of grade I, whereas those on the leaves of the control plants were much more severe and mostly up to grade III (**Fig. 3**).

Table 4. Resistance reaction of two transgenic lines with the fused *cry1Ab/cry1Ac* and nontransgenic IR72 control against natural infestation of leaffolder (LF) in the field at Hangzhou, China

Sampled date	Tested line	Sown on May 20 and transplanted on June 25			Sown on June 5 and transpanted on July 5		
		Total leaves[1]	% hills with folded leaf[2] $\bar{\chi} \pm$ S.E.	% folded leaves[3] $\bar{\chi} \pm$ S.E.	Total leaves[1]	% hills with folded leaves[2] $\bar{\chi} \pm$ S.E.	% folded leaves[3] $\bar{\chi} \pm$ S.E.
Aug. 10	TT9-3	3218	0.0 ± 0.0	0.0 ± 0.0	ns	ns	ns
	TT9-4	2814	3.3 ± 3.3	0.0 ± 0.0	ns	ns	ns
	IR72	3519	95.0 ± 1.0	7.0 ± 1.1	ns	ns	ns
Aug. 17	TT9-3	4476	0.0 ± 0.0	0.0 ± 0.0	4148	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	3940	0.0 ± 0.0	0.0 ± 0.0	3588	0.0 ± 0.0	0.0 ± 0.0
	IR72	3996	100.0 ± 0.0	21.6 ± 2.6	4132	96.7 ± 3.3	5.2 ± 0.6
Aug. 24	TT9-3	2580	0.0 ± 0.0	0.0 ± 0.0	3076	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	2744	10.0 ± 3.3	0.4 ± 0.3	3208	6.7 ± 6.7	0.3 ± 0.3
	IR72	3563	100.0 ± 0.0	34.0 ± 1.0	4280	100.0 ± 0.0	15.4 ± 0.9
Tug. 31	TT9-3	3410	0.0 ± 0.0	0.0 ± 0.0	4366	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	3456	0.0 ± 0.0	0.0 ± 0.0	3202	3.3 ± 3.3	0.3 ± 0.3
	IR72	4388	100.0 ± 0.0	16.2 ± 0.7	3606	100.0 ± 0.0	17.1 ± 4.5
Sept. 7	TT9-3	2570	3.3 ± 3.3	0.1 ± 0.1	4652	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	2702	10.0 ± 0.0	0.4 ± 0.0	4311	0.0 ± 0.0	0.0 ± 0.0
	IR72	4008	100.0 ± 0.0	15.2 ± 3.0	3490	100.0 ± 0.0	15.8 ± 1.3
Sept. 14	TT9-3	3177	0.0 ± 0.0	0.0 ± 0.0	3414	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	2805	5.0 ± 5.0	0.1 ± 0.1	3648	0.0 ± 0.0	0.0 ± 0.0
	IR72	2892	100.0 ± 0.0	30.2 ± 1.0	3480	100.0 ± 0.0	13.4 ± 0.2
Oct. 5	TT9-3	2507	0.0 ± 0.0	0.0 ± 0.0	3471	0.0 ± 0.0	0.0 ± 0.0
	TT9-4	2718	15.0 ± 5.0	1.0 ± 0.4	3372	0.0 ± 0.0	0.0 ± 0.0
	IR72	2358	100.0 ± 0.0	24.1 ± 4.1	3460	100.0 ± 0.0	26.0 ± 3.7

[1] Total leaves are the sum of total leaves of all sampled hills in two sub-blocks of each tested line; 2 % hills with folded leaf = total number of sampled hills encountered with one or more folded leaves divided by total number of sampled hills × 100; 3 % folded leaves = total number of folded leaves sampled divided by total number of leaves sampled × 100; ns = not scored.

Resistance of transgenic lines against GS

The field evaluations of both batches of plant materials against GS were conducted under both natural and artificial infestation from August 10 to October 5. The results from natural infestation showed that no damage was observed in all examined plants of the two transgenic lines at each examined date except September 17. The percentage of plants with notched leaves and percentage of notched leaves per plant in the control plot, however, was as high as 10.0 to 30.0 and 3.3 to 15.0, respectively. Moreover, the results from artificial infestation were similar to those from natural infestation. The percentage of leaves damaged by larvae of GS on TT9-3 and TT9-4 was 6.0 and 4.2, respectively, in contrast to 23.5 on the control (**Table 5**). The leaf notches that appeared on transgenic plants were fewer and smaller than those on the control plants (**Table 5**). The number of pupae collected from the transgenic plants examined two weeks after artificial infestation was also much less than that from the control plants (**Table 5**). These results indicate that the two transgenic lines were highly resistant against GS under both artificial and natural infestation.

Expression of the fused Cry1Ab/Cry1Ac in the transgenic lines

To determine expression levels of the fused Cry1Ab/Cry1Ac in the two transgenic lines TT9-3 and TT9-4, an ELISA analysis was performed from the plants grown in the containment greenhouse at IRRI. The data showed that the two transgenic lines produced levels of the fused Cry1Ab/Cry1Ac up to 0.01% of total soluble protein. Field evaluation was also conducted in 2000

Table 5. Resistance reaction of two transgenic lines with the fused *cry1Ab/cry1Ac* and nontransgenic IR72 control against artificial infestation of green semilooper (GS) at tillering stage in the field at Hangzhou, China.

Tested line	Sampled leaves	% leaves damaged[1] $\bar{\chi} \pm$ S.E.	Pupae/hill[1] $\bar{\chi} \pm$ S.E.
TT9-3	2778	6.0 ± 2.5 bB	0.1 ± 0.0 bB
TT9-4	2579	4.2 ± 0.5 bB	0.1 ± 0.1 bB
IR72	2386	23.5 ± 2.2 aA	5.5 ± 2.3 aA

[1] In the same column, means were compared by using the least significant difference (LSD) test, and means followed by the common small or capital letter are not significantly different from each other.

and confirmed the data obtained in 1999 (data not shown). A preliminary data on agronomic traits and yield performance of two *Bt* lines were evaluated. More extensive work has been planned for the future experiment to evaluate materials for comprehensive agronomic evaluation with and without treatment of chemicals. The present study showed that transgenic *Bt* lines have greater advantage over the control non *Bt* rice in terms of plant protection (**Tables 1-5**).

Discussion

The present results demonstrate that the two transgenic lines, TT9-3 and TT9-4, were highly resistant against natural and/or artificial infestation of four lepidoptera under field conditions, whereas the nontransgenic IR72 control plant was not. This high resistance was probably due to efficient expression of the fused Cry1Ab/Cry1Ac in the recipient genome.

The use of *Bt* rice to protect plants from lepidopteran attack can be one of the effective components of an integrated pest management (IPM) program which requires the use of refuges (Reissig *et al.*, 1986; Roush *et al.*, 1994). To achieve durable and effective pest control, however, the compatibility of insect-resistant *Bt* rice with other IPM components should be addressed. Especially, the ecological impact of the use of *Bt* rice on parasites and predators of target and non-target insects such as PH and leafhoppers (LH) in the rice ecosystem can now be evaluated (Ye *et al.*, 1998).

Bt proteins are non-toxic to insect predators (Perlak *et al.*, 1990; Pilcher *et al.*, 1997). Therefore, the use of *Bt* rice may result in an enhanced natural control of non-target pests such as PH and LH by increasing the population of their natural enemies because of minimized chemical control. In these experiments, we noticed that the populations of brown planthopper (BPH, *Nilaparvata lugens*) and whitebacked planthopper (WBPH, *Sogatella furcifera*) in the test plots of the two transgenic lines never increased up to the control threshold for application of chemical control. In contrast, an increase in the PH population was observed in an adjacent insecticide-treated normal field and this caused severe damage to the rice plants. These observations showed that the use of *Bt* rice instead of insecticides benefits not only control of *Bt*-target insects but also natural control of some non-*Bt*-target insects.

For yield, however, we noticed a difference in resistance levels and yield performance between the two transgenic lines. TT9-3 exhibited slightly better resistance against the four lepidopteran species in most cases and produced far higher grain yield than TT9-4, although they were derived from the same transformant. Further molecular analysis revealed that some rearranged copies in addition to the expected copy was maintained in progeny of line TT9-4 but they were absent in T_6 progeny of line TT9-3 perhaps by recombination (T_2 generation's data were shown in **Fig. 1**; T_6 generation's data were not shown). The correlation between the phenotypic performance and the maintaining of those rearranged copies may suggest that the randomly inserted copy of the transgene had some effects on the yield performance of the recipient plant. This is why reduction of some particular rearranged copies of the transgene in some progeny plants could result in increased grain production of TT9-3 line. Similar effects of the inserted transgene interfering in the native genes in the recipient genome and negatively affecting their phenotypic performance were often observed (Lynch *et al.*, 1996; Oard *et al.*, 1996). Currently, transgenic rice, IR72 with *Xa21* and hybrid *Bt* rice "Shan You 63" with *Bt* gene showed excellent field performance in China (Tu *et al.*, 2000 a&b). Thus, a cultivar and progeny selection of transgenic plants in a given environment is needed to utilize the potential of improved genetically modified *Bt* rice lines. The present results indicate that *Bt* rice will have a great potential in plant protection against insect pests in Asia.

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References

Alam, M. F., Datta, K., Abrigo, E., Vasquez, A., Senadhira, D., Datta, S. K., 1998. Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiensis cryIA(b)* gene with enhanced resistance to yellow stem borer. Plant Science, **135**: 25-30.

Alam, M. F., Datta, K., Abrigo, N., Oliva, N., Tu, J., Virmani, S. S, Datta, S. K., 1999. Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice. Plant Cell Rep., **18**: 672-575.

Beegle, C. C., Yamamoto, T., 1992. History of *Bacillus thuringiensis* Berliner research and development. Can. Entomol., **124**: 587-616.

Cheng, X. Y., Sardana, R., Kaplan, H., Altosaar, I., 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA, **95**: 2767-2772.

Christou, P., Ford, T. F., Kofron, M., 1991. Production of transgenic rice (*Oryza sativa*) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology, **9**: 957-962.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M. F., Oliva, N., Abrigo, E., Khush, G. S., Datta, S. K., 1998. Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. Theor. Appl. Genet., **97**: 20-30.

Delannay, X., LaVallee, B. J., Proksch, R. K., Fuchs, R. L., Sims, S. R., Greenplate, J. T., Marrone, P. G., Dodson, R. B., Augustine, J. J., Layton, J. G., Fischhof, D. A., 1989. Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. Bio/Technology, **7**: 1265-1269.

Frutos, R., Rang, C., Royer, M., 1999. Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit. Rev. Biotechnol., **19**: 227-276.

Fujimoto, H., Itoh, K., Yamamoto, M., Kyozuka, J., Shimamoto, K., 1993. Insect-resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Bio/Technology, **11**: 1151-1155.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R., Cygler, M., 1995. *Bacillus thuringiensis* Cry1A(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol., **254**: 447-464.

Heinrich, E. A., Medrano, F. G., Rapusas, H. R., 1985. Genetic evaluation for insect resistance in rice, pp. 267-284, International Rice Research Institute, Los Ba_os, Laguna, Philippines.

Höfte, H., Whiteley, H. R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev., June: 242-255.

James, C., 1999. Global status of commercialized transgenic crops: 1999. ISAAA Briefs No. 12 : Preview (ISAAA: Ithaca, NY).

Khan, Z. R., Litsinger, J. A., Barrion, A. T., Villanueva, F. F. D., Fernandez, N. J., Taylo, L. D., 1991. World bibliography of rice stem borers 1794-1990. International Rice Research Institute, Los Baños, Laguna, Philippines.

Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M., Evola, S., 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology, **11**: 194-200.

Lynch, P. T., Jones, J., Blackhall, N. W., Davey, M. R., Power, J. B., Cocking, E. C., Nelson, M. R., Bigelow, D. M., Orum, T. V., Orth, C. E., Schuh, W., 1996. The phenotypic characterization of R_2 generation transgenic rice plants under field and glasshouse conditions. Euphytica, **85**: 395-401.

Nayak, P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrishnan, N. A., Ghosh, M., Sen, S. K., 1997. Transgenic elite indica plants expressing *cryIAc* δ-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc. Natl. Acad. Sci. USA, **94**: 2111-2116.

Oard, J. H., Linscombe, S. D., Braverman, M. P., Jodari, F., Blouin, D. C., Leech, M., Kholi, A., Vain, P., Cooley, J. C., Christou, P., 1996. Development, field evaluation, & agronomic performance of transgenic herbicide resistant rice. Mol. Breed., **2**: 359-368.

Pathak, M. D., Khan, Z. R., 1994. Insect Pests of Rice. International Rice Research Institute, Los Baños, Laguna, Philippines.

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., Fischhoff, D. A., 1990. Insect-resistance cotton plants. Bio/Technology, **8**: 939-943.

Perlak, F. J., Stone, T. B., Muskopt, Y. M., Petersen, L. J., Parker, S. A., Wyman, J., Love, S., Reed, G., Biever, D., Fischhott, D. A., 1993. Genetically improved potatoes, protection from damage by Colorado potato beetle. Plant Mol. Biol., **22**: 313-321.

Pilcher, C. D., Obrycki, J. J., Rice, M. E., Lewis, L. C., 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environ. Entomol., **26**: 446-454.

Reissig, W. H., Heinrichs, E. A., Litsinger, J. A., Moody, K., Fiedler, L., Mew, T. W., Barrion, A. T., 1986. Illustrated Guide to Integrated Pest Management in Rice in Tropical Asia. International Rice Research Institute, Los Baños. Philippines.

Roush, R. T., 1994. Managing pests and their resistance to *Bacillus thuringiensis*: Can transgenic crops be better than sprays? Biocontrol Science and Technology, **4**: 501 - 516.

Spencer, W., 1989. The threat is real. Cotton Grower, 25: 22.

Stewart, C. N. Jr., Adang, M. J., All, J. N., Raymer, P. L., Ramachandran, S., Parrott, W. A., 1996. Insect control and dosage effect in transgenic canola containing a synthetic *Bacillus thuringiensis cry1Ac* gene. Plant Physiol., **112**: 115 - 120.

Tu, J., Datta, K., Alam, M. F., Fan, Y., Khush, G. S., Datta, S. K., 1998. Expression and function of a hybrid *Bt* toxin gene in transgenic rice conferring resistance to insect pests. Plant Biotechnol., **15(4)**: 195 - 203.

Tu, J., Datta, K., Zhang, Q., Datta, S. K., 2000a. Field performance of transgenic indica rice (*Oryza sativa* L.), IR72, with *Xa21*. Theor. Appl. Genet., **101**: 15 - 20.

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., Khush, G. S., Datta, S. K., 2000b. Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ - endotoxin. Nature Biotechnology, **18**: 1101 - 1104.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., Beuckeleer, M. D., Dean, C., Zabeau, M., Montagu, M. V., Leemans, J., 1987. Transgenic plants protected from insect attack. Nature, **327**: 239 - 247.

Ye, G. Y., Hu, C., Shu, Q. Y., 1998. The development of transgenic rice resistant to insect pests and its sustainable use. In: Chen JA, Zhu WJ (eds.), Research and Development of Agriculture in the 21th Century. pp. 406 - 414, Environment Science Publisher of China, Beijing, China.