Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin

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Here we describe development of transgenic elite rice lines expressing a *Bt* fusion gene derived from *cryIA(b)* and *cryIA(c)* under the control of rice *actinI* promoter. The lines used in the study were indica CMS restorer line of Minghui 63 and its derived hybrid rice Shanyou 63. The level of *Bt* fusion protein CryIA(b)/CryIA(c) detected in Minghui 63 (T51-1) plants was 20 ng/mg soluble protein. The *Bt* Shanyou 63 was field-tested in natural and repeated heavy manual infestation of two lepidopteran insects, leaffolder and yellow stem borer. The transgenic hybrid plants showed high protection against both insect pests without reduced yield.

Keywords: Hybrid rice, fusion *Bt* protein, transgenic indica restorer line, field performance, pest control

Hybrid rice was first cultivated commercially in China in 1976, and now its planting area has expanded to more than 13 million Ha annually. Hybrid rice, with a 20% yield advantage over inbred varieties, has helped China produce 300 million tons more paddy from 1976 to 1994, thereby significantly increasing national food production[1]. Hybrid rice has now become a commercial success in many Asian countries[2], and an estimated 6 million Ha of production area would be required if hybrid rice had not been developed[3].

Hybrid rice not only has a distinct yield advantage over inbred varieties, it is more responsive to fertilizer and more adaptable to different environments than conventional varieties. These attributes, however, are closely associated with vulnerability to disease epidemics and insect outbreaks[4], with the incidence of insect pests, especially stem borers, and diseases more frequent in hybrid rice than in inbred varieties[5]. To date, stem borer–resistant varieties of rice hybrid and inbred varieties have not been released in China and elsewhere. Chemical control of these insect pests is ineffective because stem borer larvae remain for only a short time on the outer surface of the rice plant before they penetrate the stem[6]. Furthermore, chemical sprays also pollute land and water, are toxic to nontarget organisms, accumulate in food chains, and can cause human health problems[7].

Bacillus thuringiensis (*Bt*) is a bacterium used for more than 50 years as a biological insecticide. The insecticidal activity resides in crystalline inclusion bodies that are produced during sporulation of the bacteria and are composed of δ-endotoxin[8]. The mode of action of the δ-endotoxin involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of endotoxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores that lead to disruption of osmotic processes[9]. *Bt* insecticidal activity is highly specific in that the endotoxins are nontoxic to nontarget insects, birds, and mammals[10].

Recent advances in plant biotechnology[11–16] and the reservoir of cloned *cry* genes[17,18] that encode *Bt* δ-endotoxins have made it possible to express this novel insecticidal protein in crop plants[19–22], including rice[23–27]. To date, however, the only commercialized *Bt* crops have been cotton[10], maize[28], and potato[29].

We report here the field performance of the transgenic indica rice CMS restorer line Minghui 63 (T51-1) and its derived hybrid plant expressing a *Bt* fusion protein derived from CryIA(b) and CryIA(c) sequences[30]. The transgenic CMS restorer rice plant and its hybrid exhibited excellent protection against extremely high, repeated infestations of yellow stem borer and natural outbreaks of leaffolder. There have previously been no reported field evaluations of a commercial hybrid *Bt* rice in which the yield was higher than that of non-*Bt* commercial hybrid rice.

Results

Production of a homozygous transgenic line for field evaluation. A construct containing the rice actinI promoter driving expression of the Bt fusion gene, consisting of the 1,344 bp encoding the N terminus of cryIA(b) and 486 bp encoding the C terminus of cryIA(c)[30] (Fig. 1), was inserted into the genome of elite indica restorer line Minghui 63, using biolistic transformation[31]. Fusion of two toxin genes were used as a strategy to improve duration of pest resistance. We obtained several transgenic plants containing a 1.8 kb DNA fragment corresponding to two intact copies of the coding sequence of the hybrid Bt fusion gene and four rearranged copies (data not shown). Homozygous lines from these transgenic Minghui 63 plants were produced in the T_2 generation, as confirmed by molecular analysis and insect bioassay.

The transgenic line showed a 3:1 segregation pattern for the transgene. Ten T_1-derived progenies with 20 plants for each progeny were grown in the IRRI containment greenhouse under 29°C and 85% humidity at daytime and 25°C and 90% humidity at night. Ten samples from each progeny were screened by western blot analysis. The T_2 progeny line showing an identical expected size of 60 kDa *Bt* protein and a truncated polypeptide were primarily identified as homozygous (Fig. 2). Twenty plants of these homozygous lines were then further

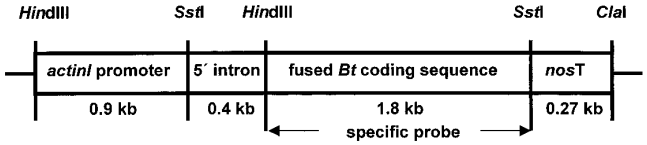

Figure 1. Diagram of plasmid construct pFHBT1, which contains a *Bt* fusion gene consisting of *cryIA(b)* and *cryIA(c)* sequences under the control of the rice *actinI* promoter with its first intron and *nos* terminator.

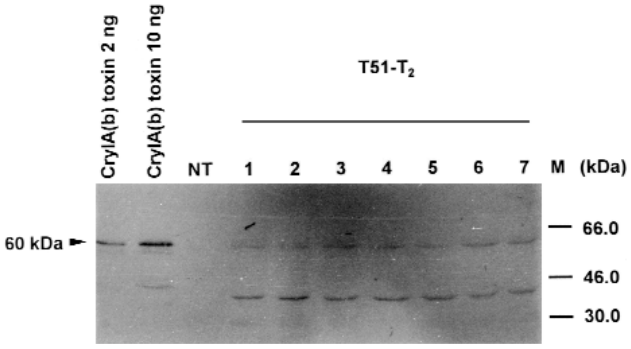

Figure 2. Western blot analysis of stem protein extracts from T_2 progenies homozygous for the transgenes analyzed by SDS–PAGE. Into each lane were loaded 50 μg of total soluble protein. The arrow marks the expected size of the 60 kDa *Bt* fusion protein. M, Molecular weight marker; NT, protein samples extracted from nontransgenic Minghui 63 control plants; T51-1 T_2, protein samples extracted from seven T51-1 T_2 homozygous plants; CryIA(b) toxin, purified *Bt* endotoxin.

confirmed by the petri dish insect bioassay[32] at the maximum tillering stage (data not shown). One homozygous line, designated as transgenic line T51-1 (thereafter referred to as T51-1) and with 100% larval mortality of each plant, was developed and selected at IRRI and the seeds were sent to Wuhan, China, for field evaluation. The transgenic hybrid seeds used in the field trial were subsequently produced by pollination of elite indica CMS line Zhenshan 97A with line T51-1.

Field evaluation of homozygous Bt rice plants. A natural outbreak of the third generation of leaffolder occurred at the testing site in 1999, providing a good opportunity to evaluate transgene efficiency. The parameters used for measuring the severity of damage to the plants by the leaffolder larvae were the average number of damaged leaves per plant, the percentage of damaged tillers per plant, and percentage of affected plants. The results showed that the means of parameters measured at the maximum tillering stage on T51-1 were 0.3 ± 0.9, 0.7 ± 2.6, and 15.6, in contrast to 30.5 ± 10.5, 85.9 ± 11.4, and 100 on the nontransgenic Minghui 63 control plants, respectively (Table 1). These results indicate that the transgenic line T51-1 is highly resistant to the infestation of leaffolder at the natural outbreak level.

After heavy and repeated manual infestation at maximum tillering and booting stages in the field, the T51-1 plants exhibited even better resistance against larvae of yellow stem borer. The mean percentages of deadhearts (caused by infestation during the vegetative stage) per plant and plants with deadheart measured at the booting stage of T51-1 were 0.2 ± 1.1 and 4.3, whereas those on the nontransgenic Minghui 63 control plants were 41.8 ± 13.2 and 100, respectively (Table 2). Moreover, the mean numbers of whiteheads (caused by infestaion during reproductive stage) per plant and of plants with whitehead measured at the grain-filling stage on T51-1

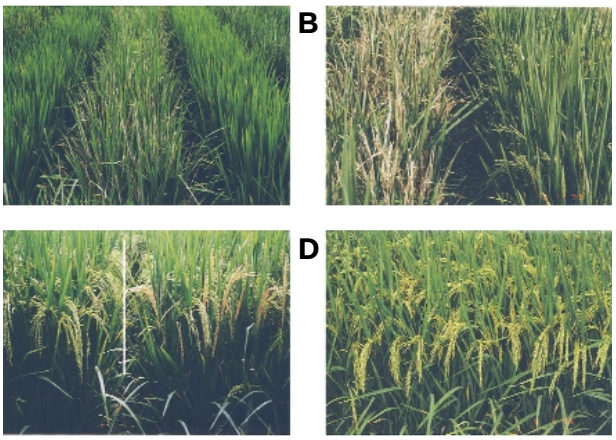

Figure 3. Pest reactions and phenotype of the transgenic indica CMS restorer line T51-1 and its hybrid plants expressing the *Bt* fusion protein derived from CryIA(b) and CryIA(c). (A) Initial stage of Minghui 63 control (middle row) and T51-1 (both flanking rows) plants against natural infestation of leaffolder. (B) Pest reaction of T51-1 (right) and Minghui 63 control (left) plants against heavy manual infestation of yellow stem borer. (C) Pest reaction of *Bt* hybrid (left) and non-*Bt* hybrid Shanyou 63 (right) plants against natural infestation of yellow stem borer. (D) The phenotype of the *Bt* hybrid rice plants at grain-filling stage.

were 229.8-fold and 11.6-fold less than those on the nontransgenic Minghui 63 control plants (Table 2). Clear differences between the transgenic line T51-1 and nontransgenic Minghui 63 control plants in damage to leaves and panicles caused by both leaffolder and yellow stem borer are shown in Figure 3A and B, respectively.

Field evaluation of Bt hybrid plants. The field evaluation of Bt hybrid plants under natural infestation of both leaffolder and yellow stem borer was done separately from that of the transgenic line T51-1. The results revealed that neither leaffolder-damaged leaves nor deadhearts due to yellow stem borer damage were observed on Bt hybrid plants at the maximum tillering stage, in contrast to non-Bt Shanyou 63 hybrid plants, which showed considerable leaf damage and deadheart as a result of attack by both pests (Table 3).

Furthermore, the percentages of whiteheads per plant and of plants with whitehead caused by yellow stem borer at the grain-filling stage were also significantly lower on the *Bt* hybrid plants than on the non-*Bt* hybrids (Table 3). These results consistently confirm that the *Bt* hybrid plants heterozygous for the transgenes are likewise highly resistant to the larvae of both leaffolder and yellow stem borer. Figure 3C shows the marked difference in the severity of typical whitehead damage by the third generation of yellow stem borer between *Bt* and non-*Bt* Shanyou 63 plants at the grain-filling stage.

Table 1. Resistance reaction of the transgenic indica CMS restorer line T51-1 homozygous for *Bt* transgenes and control against natural outbreaks of leaffolder under field conditions (Wuhan, China, 1999)

Line	Replication	Number of plants examined	Plants affected (%)	No. of tillers per plant	Damaged tillers per plant (%)	No. of damaged leaves per plant
Transgenic T51-1	I	62	17.7	21.4	0.5 ± 3.9	0.5 ± 1.3
	II	62	12.9	25.1	0.6 ± 1.8	0.2 ± 0.5
	III	62	16.1	21.4	1.0 ± 2.2	0.3 ± 0.7
	Mean	62	15.6[a]	22.6	0.7 ± 2.6[a]	0.3 ± 0.9[a]
Control Minghui 63	I	62	100.0	21.7	87.0 ± 9.2	34.3 ± 12.7
	II	62	100.0	21.4	85.0 ± 13.7	30.8 ± 10.3
	III	62	100.0	19.4	83.7 ±.11.4	26.4 ± 8.6
	Mean	62	100.0	20.8	85.9 ±.11.4	30.5 ± 10.5

[a]Significantly different from control at $P < 0.01$.

Table 2. Resistance reaction of the transgenic indica restorer line T51-1 homozygous for *Bt* transgenes and control against heavy manual infestation of yellow stem borer under field conditions (Wuhan, China, 1999)

Line	Replication	No. of plants examined	No. of tillers per plant	Booting stage: Plants with deadheart	Booting stage: Deadheart per plant (%)	Grain-filling stage: Plants with whitehead	Grain-filling stage: Whitehead per plant (%)
Transgenic T51-1	I	62	21.4	4.8	0.3 ± 1.3	9.7	0.4 ± 1.6
	II	62	25.1	6.5	0.2 ± 0.9	12.9	0.7 ± 2.1
	III	62	21.4	1.6	0.1 ± 1.1	3.2	0.1 ± 0.7
	Mean	62	22.6	4.3[a]	0.2 ± 1.1[a]	8.6[a]	0.4 ± 1.5[a]
Control Minghui 63	I	62	21.7	100.0	35.7 ± 11.2	100.0	97.0 ± 5.7
	II	62	21.4	100.0	45.1 ± 13.5	100.0	90.5 ± 11.6
	III	62	19.4	100.0	44.6 ± 14.9	100.0	95.2 ± 9.3
	Mean	62	20.8	100.0	41.8 ± 13.2	100.0	94.2 ± 8.9

[a]Significantly different from control at $P < 0.01$.

Table 3. Resistance reaction of the *Bt* hybrid Shanyou 63 against natural infestation of both leaf-folder and yellow stem borer under field conditions[a] (Wuhan, China, 1999)

Hybrid	Replication	Plants affected by leaffolder (%)	Yellow stem borer[b]: Deadheart per plant (%)	Yellow stem borer[b]: Whitehead per plant (%)	Yellow stem borer[b]: Plants with whitehead (%)
Bt Shanyou 63	I	0.0	0.0	0.0	0.0
	II	0.0	0.0	0.0	0.0
	III	0.0	0.0	0.1 ± 0.0	3.3
	Mean	0.0[c]	0.0[d]	0.0 ± 0.0[d]	1.1[c]
Shanyou 63	I	57.4 ± 18.98	0.9 ± 0.4	4.9 ± 1.1	25.0
	II	60.3 ± 22.91	0.5 ± 0.0	6.2 ± 1.3	40.0
	III	56.0 ± 15.90	2.0 ± 0.9	19.8 ± 2.5	66.7
	Mean	57.9 ± 19.26	1.1 ± 0.4	10.3 ± 1.6	43.9

[a]Measured from 30 randomly sampled plants per test material per replication.
[b]The data for yellow stem borer damage were converted to square root before applying the *t*-test.
[c]Significantly different from control at $P < 0.01$.
[d]Significantly different from control at $P < 0.05$.

Agronomic traits and yield performance of Bt hybrids in the field. The agronomic traits and yield performance of the Bt hybrid Shanyou 63 were observed in the same plots that were used for the insect test. The non-Bt hybrid Shanyou 63 was set as a control for phenotypic comparison. The data showed that the Bt Shanyou 63 was 2.0 cm taller but had the same number of days to flower as the non-Bt control (Table 4). It grew 0.38 more panicles per plant but developed panicles with 3.7 fewer filled grains. Its seed-setting rate was 4.3% higher, but the weight of 1,000 of its filled grains was 0.76 g lower. These observations imply that the introduced transgenes have some effects on the yield components of their recipient plants. The yield of the Bt hybrids based on these yield components, however, was exactly the same as that of the non-Bt hybrid that was sampled from healthy nonaffected plants. The high yield potential of Bt hybrid plants at a later stage is shown in Figure 3.

Finally, the yield of the *Bt* hybrid was 28.9% more than that of the non-*Bt* hybrid (Table 4). Considering that the field trial was conducted without the use of chemicals after transplanting, these results demonstrate that expression of the *Bt* fusion protein in the genome of the transgenic restorer line and its hybrid plants provided season-long protection for these plants from the natural outbreak or heavy manual infestation of the two lepidopteran insects.

Discussion

Our results also demonstrate that plant protection by random insertion of the hybrid *Bt* gene could be achieved without reducing the restorer line's combining ability and its hybrid's yield potential. In contrast, previous studies of transgenic Taipei 309 with *nptII*[33] and IR72 and Koshihikari with *bar*[34] indicated poor field performance, which limited their potential commercial value.

The typical level of the fusion CryIA(b) and CryIA(c) detected in the T51-1 plants was about 20 ng/mg soluble protein. A half dose of this endotoxin expressed in the T51-1-derived hybrid rice plants was much less than the levels of CryIA(b) expressed in transgenic hybrid maize plants[35], but showed comparable plant protection against the insect attack. It is also reported that the LD_{50} for yellow stem borer neonate is 7.58 μg/ml diet, whereas that for striped stem borer is 7.41 μg/ml diet[36]. Therefore, the expression level of the *Bt* fusion gene in the genome of both Minghui 63 and its hybrid is sufficient to control the lepidopteran insects.

The use of rice heterosis is now becoming an important technology applied by many tropical Asian countries such as India, Vietnam, Indonesia, Malaysia, the Philippines, and Thailand[2], where lepidopteran insects occur more frequently and cause more serious yield losses to rice than in China. The successful expression of the *Bt* fusion gene in the genome of an elite indica CMS restorer line and its hybrid provides a good resource for management of rice pests in China and tropical Asian countries.

Experimental protocol

Genetic materials and transformation. The genetic materials used in this field trial were the transgenic line of Minghui 63 (T51-1), nontransgenic Minghui

Table 4. Agronomic traits of the *Bt* and non-*Bt* hybrids under field conditions (Wuhan, China, 1999)

Hybrid	Days to flower	Plant height (cm)	Panicles per plant	Filled grains	Nonfilled grains per panicle	Total grains per panicle	Seed-setting per panicle	1000 yield grain rate (%)	Expected yield weight (g)	Observed yield (t/Ha)	[a](t/Ha) ±(%)
Bt Shanyou 63	95	108.6	9.98	140.0	21.9	161.7	87.6	28.00	7.44	8.69	+28.9[b]
Shanyou 63	95	106.4	9.60	143.7	28.8	172.5	83.3	28.76	7.44	6.74	–

[a]The observed yield was measured based on the average production per unit of four subplots after harvesting and then converted into tons per hectare.
[b]Significantly different from control at $P < 0.01$.

63, the nontransgenic hybrid produced from the hybridization of Zhenshan 97A × Minghui 63 (Shanyou 63), and the transgenic hybrid from the cross Zhenshan 97A × T51-1 (Bt Shanyou 63). Minghui 63 is an elite indica CMS restorer line developed in China in the early 1980s, and Zhenshan 97A is an elite indica CMS line. Shanyou 63 has been the most widely used hybrid in rice production in China for the past 15 years. Currently, Minghui 63 is still considered the best restorer and Shanyou 63 the best hybrid for yield potential. Immature embryos from Minghui 63 grown at IRRI greenhouse were used for transformation by biolistic method. Bt fusion gene cryIA(b)/cryIA(c)[30] driven by actin1 promoter[37] shown in Figure 1 was used for rice transformation. Further development, molecular characterization, and selection of homozygous lines were carried out at IRRI as described [26,30-32], and the seeds of selected transgenic lines were sent to China for field evaluation.

Plot design. Field evaluation of the genetic materials was performed at the experimental farm at Huazhong Agricultural University, Wuhan, China (latitude 30°34' North; longitude 114°17' East), where three rice-cropping seasons are practiced annually. The early-season rice starts in early April and the late-season rice ends in the middle of November, with the mid-season rice in between. For rice leaffolder, the peak damage time corresponds to its third generation in this area, which usually occurs in late July to early August, when most of the mid-season rice is at maximum tillering to booting stages. On the other hand, the peak damage time of yellow stem borer, corresponding to its third generation, is in early to mid-August, at which time the mid-season rice is at the heading stage. The transgenic restorer line, its hybrid plants, and the nontransgenic controls (Minghui 63 and Shanyou 63) were planted in accordance with the normal mid-season rice growing time, but with a delay of 10 days to ensure that the maximum tillering and booting stages coincided with the peak damage times of these two lepidopteran insects.

The seeds of the transgenic plants were sown in a seedling bed for four weeks and transplanted in a well-isolated paddy field on 10 June 1999. The plots for insect testing consisted of two rows 6.9 m long and 0.8 m wide with 31 plants (a single plant per hill) spaced at 19.8 cm within a row. The space distance between plots was 52.8 cm. Nontransgenic Minghui 63 was planted as a susceptible control, and three replications were arranged for each test material. 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 for an objective evaluation of the resistance reaction of the test materials.

For the hybrid's yield evaluation, the plots were arranged in a field separate from that for insect testing and contained four replications. The *Bt* and non-*Bt* hybrid plants in each replication were randomized subplots. Each subplot consisted of eight rows 6.33 m long and 2.11 m wide with 32 plants (a single plant per hill) spaced at 19.8 cm within a row. The space distance between subplots was 52.8 cm. The size of the subplot was 13.3 m^2. The non-*Bt* hybrid Shanyou 63 was planted as a control. The rest of the design and field management were the same as those of insect testing.

Insect infestation. Leaffolder. Natural infestation was used. Plant reaction to the natural infestation of leaffolder was scored five to seven days after its peak damage appeared. Leaves with visible scrapes and tillers with one of these visible scraped leaves were scored as damaged leaves and tillers, respectively.

Yellow stem borer. Both natural and manual infestation were used. For manual infestation, eggs of yellow stem borer were collected from a local rice field one to three days before field infestation, and one egg mass was put into each finger-shaped testing tube (9 cm in length) containing 1 ml distilled water, then covered with black cloth. The neonate larvae were hatched after incubation at 28°C in the dark overnight to 24 h. Within 6 h of hatching, neonate larvae from one egg mass were placed at the base of each test plant at maximum tillering for deadheart symptoms and booting stage for whitehead symptoms. The average number of the neonate larvae hatched from one egg mass was about 80. Damage symptoms were checked 7 to 10 days after infestation.

Protein extraction and western blot analysis. The Bt toxin extraction and western blot analysis for identifying T_2 progenies homozygous for the transgenes were done following the modified procedures described earlier[30].

Statistical analysis. Data on leaf, tiller, or plant damage, deadheart and whitehead from subplots were analyzed by a paired comparison Student's *t*-test, whereas the data for final yield of *Bt* and non-*Bt* hybrid plants were analyzed by a F-test. Statistically significant results at $P < 0.01$ or $P < 0.05$ are indicated in the tables.

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