Theor Appl Genet (2005) 111: 1330–1337
DOI 10.1007/s00122-005-0062-8

ORIGINAL PAPER

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Transgenic indica rice plants harboring a synthetic *cry2A** gene of *Bacillus thuringiensis* exhibit enhanced resistance against lepidopteran rice pests

Received: 15 March 2005 / Accepted: 13 July 2005 / Published online: 27 September 2005

Abstract A novel synthetic *cry2A** gene was introduced into the elite indica rice restorer line Minghui 63 by *Agrobacterium*-mediated transformation. A total of 102 independent transformants were obtained. Among them, 71 transformants were positive *cry2A** plants according to PCR analysis. Four highly insect-resistant lines with single-copy insertion (designated as 2A-1, 2A-2, 2A-3, and 2A-4) were selected based on field assessment and Southern blot analysis in the T_1 generation. All four transgenic lines showed Mendelian segregation by seed germination on 1/2 MS medium containing Basta. Homozygous transgenic plants were selected according to germination ratio (100%) in the T_2 generation. Cry2A* protein concentrations were determined in homozygous transgenic lines, their derived hybrids, and their backcross offspring. The Cry2A* protein concentrations of four homozygous transgenic lines ranged from 9.65 to 12.11 μg/g of leaf fresh weight. There was little variation in the hybrids and backcross offspring. Insect bioassays were conducted in both the laboratory and field. All four transgenic lines were significantly resistant to lepidopteran rice pests. These *cry2A** transgenic lines can be used to produce insect-resistant hybrids and serve as a resistant source for the development of two-toxin Bt rice.

Communicated by D. J. Mackill

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Introduction

Formulations of *Bacillus thuringiensis* (Bt) have been used as a biological insecticide in agricultural production for more than 50 years. As a good alternative to synthetic insecticides, these formulations are safe to the user and environment. Due to limited field stability, inability to reach cryptic insects, and narrow spectrum of activity, however, Bt products represent only a small portion of the insecticide market (Ferre and Van Rie 2002). With the development of plant genetic engineering, genes expressing Bt insecticidal crystal protein can be introduced into plants for insect control. Transgenic plants containing Bt genes control pests more effectively than Bt formulations. So far, three species of Bt crops (cotton, maize, and potato) have become commercially available worldwide. In 2004, the estimated global area of Bt crops reached 22.4 million hectares (James 2004). Commercialization of Bt crops has significantly reduced the use of synthetic insecticides (Ferre and Van Rie 2002).

Although no insect species resistant to Bt crops have been reported under natural conditions, the potential of insects to evolve resistance against Bt toxins is a serious threat to this technology. In fact, a number of resistant insect strains have been selected under laboratory, greenhouse, and/or field conditions (Frutos et al. 1999; Ferre and Van Rie 2002; Shelton et al. 2002; Tabashnik et al. 2003), indicating the potential for evolution of resistance in most pests. To meet this challenge, several strategies have been proposed to manage insect-resistance, such as the high-dose/refuge strategy, gene-stacking, and temporal- or spatial-specific expression of the toxin (Roush 1998; Frutos et al. 1999; Ferre and Van Rie 2002; Shelton et al. 2002; Bates et al. 2005). Among these strategies, only the high-dose/refuge strategy has been used in developed countries such as the United States and Australia. It appears, however, that this strategy is not applicable to Bt rice crops in Asia, for two main reasons. First, the isolated and small scale of rice

farming in Asia causes difficulties in establishing and enforcing a mandatory policy of planting refuges. Second, with a few exceptions, the yellow stem borer (*Tryporyza incertulas*) and the striped stem borer (*Chilo suppressalis*) only feed on rice, and hence there is no natural refuge provided by alternative host plants (High et al. 2004). A plan has been developed to produce novel Bt cotton that expresses two different Bt toxins (Cry1Ac + Cry2A) to manage insect-resistance (Tabashnik et al. 2002). Gene stacking is also a feasible strategy for Bt rice development. This strategy is based on the assumption that a single mutation in a pest is unlikely to confer simultaneous resistance to two different Bt toxins, and thus two-toxin rice cultivars would require smaller refuges and have the potential to delay the development of resistance more effectively than single-toxin cultivars (Gould 2003; Zhao et al. 2003). The *Bt* genes usually used in rice are *cry1Ab*, *cry1Ac*, and fused *cry1Ac*/*cry1Ab* (Nayak et al. 1996; Cheng et al. 1998; Tu et al. 2000; Ye et al. 2001; Ramesh et al. 2004). However, producing two-toxin rice by *cry1Ab* and *cry1Ac* does not appear promising due to high-protein homology (>90%) and the probability of cross-resistance to *cry1Ab* and *cry1Ac*. The Bt gene *cry2A* has low homology with *cry1A* (<45%). It also produces a δ-endotoxin crystal protein that is active against both lepidopteran and dipteran insects. In addition, researchers have confirmed that Cry2A is toxic to several of the main lepidopteran pests such as yellow stem borer, striped stem borer, and two species of rice leaf folder (*Marasmiapatnalis* and *Cnaphalocrocis medinalis*) when the insects are fed the toxin in an artificial diet (Karim and Dean 2000; Alcantara et al. 2004). Furthermore, biochemical studies showed that Cry2A did not share binding sites with Cry1A in brush border membrane vesicles (BBMV) from rice leaf folder, striped stem borer or yellow stem borer, indicating that it would be effective to combine Cry2A with Cry1Ab or Cry1Ac (Karim and Dean 2000; Alcantara et al. 2004).

There are few reports about *cry2A* rice (Maqbool et al. 1998, 2001; Bashir et al. 2004). In this study, a modified novel *cry2A** gene designed on the basis of rice preference codons was introduced into the elite indica rice restorer line Minghui 63. Several insect-resistant plant lines were obtained. The objectives of this study were: (1) to confirm that the novel synthetic *cry2A** can be effectively expressed in transgenic rice; (2) to examine the field performance of *cry2A** plant lines and evaluate their toxicity to the main lepidopteran rice pests; and (3) to produce breeding sources for two-toxin (Cry1Ac plus Cry2A, or Cry1C plus Cry2A) Bt rice.

Materials and methods

The foreign gene and the recipient variety

The *cry2A** gene was synthesized based on the amino acid sequence of the corresponding wild-type *cry2Aa* genes of *B. thuringiensis*. It possessed an overall GC content of 59.04% and shared 69.45% nucleotide sequence homology with the original *cry2Aa* gene. The elite indica rice CMS restorer line Minghui 63 was used as the recipient of the *cry2A** gene.

Construction of expression vector and transformation

The expression vector used in this study is displayed in Fig. 1. Plasmid pCAMBIA1300 (provided by the Center for the Application of Molecular Biology in International Agriculture, Australia) was used to construct the vector. The *cry2A** gene driven by the maize ubiquitin promoter was cloned into the polylinker of the plasmid and the hygromycin phosphotransferase (*hph*) gene was replaced by the phosphinotricin acetyltransferase (*bar*) gene.

A strain of *Agrobacterium tumefaciens*, *EHA105*, harboring a disarmed plasmid pTiBO542 was used for the transformation experiment.

The callus culture and transformation procedures were as described by Lin and Zhang (2005). Scutellum-derived embryonic calli were cocultured for 3 days with the *Agrobacterium* strain *EHA105* that carried the expression vector and then transferred to the selection medium containing 25 mg/l Basta. After 8–9 weeks, resistant calli produced on the selection medium were transferred to the pre-regeneration medium containing 20 mg/l Basta. After 7 days, the resistant calli were transferred to the regeneration medium without herbicide to regenerate transformed plantlets.

PCR analysis and Southern blot analysis

PCR and Southern blot analyses were used to determine the presence of the *cry2A** gene and the copy number of the transgene. PCR analyses were performed using the primers *cry2A**-F (5′-cgtgtcaatgctgacctgat-3′) and *cry2A**-R (5′-gatgccggacaggatgtagt-3′). A 20 μl mixture of 30 ng of template DNA, 2.0 μl of 10× buffer, 1.0 μl of 2 mM dNTP, 1.5 μl of 25 mM $MgCl_2$, 0.4 μl each of 10 μM primers, and 1 U of *Taq* DNA polymerase was prepared for the PCR assay. The PCR reaction was

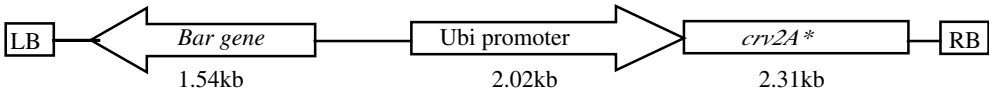

Fig. 1 T-DNA region of the expression vector for transformation. The *cry2A** gene was controlled by a maize ubiquitin promoter and nos terminator. The *bar* gene was used as a selective gene under the control of CaMV 35S promoter and CaMV 35S polyA tail

performed at 94°C for 5 min; then for 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; followed by 72°C for 5 min.

Plant genomic DNA was extracted by the CTAB method (Murray and Thompson 1980). For Southern blot analysis, 4 μg of genomic DNA per sample were digested with *Hind*III and separated on a 0.8% agarose gel, and then transferred to a nylon membrane. The probe was prepared from a PCR-amplified fragment of *cry2A**. The procedures for hybridization were as described in Liu et al. (1997).

Selection of homozygous *cry2A** plants with single copy insertion

In 2003, T_1 transgenic plant families derived from fertile T_0 transgenic plants were grown (50 plants per family) on the experimental farm of Huazhong Agricultural University in Wuhan, China. No insecticide was sprayed during the entire rice growth period. Those highly insect-resistant families without significant phenotypic changes were selected based on visual observation. The resistant families were subjected to Southern blot analysis to determine copy number of the transgene. Mature seeds were harvested from individual plants from those families with high resistance and single-copy insertion. A seed germination assay was carried out to select the plants homozygous for the *cry2A** gene. The procedure of the seed germination assay was as follows: the husked mature seeds were sterilized in 0.15% $HgCl_2$ for 15 min and rinsed twice with sterile distilled water. Then the sterilized seeds were placed on fresh 1/2 MS medium (Murashige and Skoog 1962) supplemented with 10 mg/l Basta for germination. The germination percentage was recorded 7 days later.

Detection of Cry2A* protein expressed in *cry2A** plants

Approximately 20 mg samples of fresh leaves from homozygous transgenic plants, their hybrids Shanyou 63 (F_1) crossed with Zhenshan 97A (a sterile line), and backcross offspring crossed with the original Minghui 63 (BC_1F_1) were collected separately in the field. Cry2A* protein content in transgenic plants was determined by ENVIRONLOGIX kits APP005 (ENVIRONLOGIX, USA). The protein assay procedures exactly followed the protocol provided by the manufacturer.

Bioassay of *cry2A** plants in the laboratory

Egg masses of yellow stem borer were collected from the rice field and allowed to hatch in the laboratory. Fresh rice stems were harvested at the later tillering stage and cut into 5–6 cm pieces. Five pieces of freshly cut stems with 12 first-instar larvae of yellow stem borer were placed into a sealed glass bottle and incubated in the controlled environmental chamber (28 ± 1°C, 80% relative humidity, and darkness) for 5 days. The stem cuttings were then dissected and insect mortality was recorded. Five replicates were performed for each treatment.

Evaluation of insect-resistance in the field

Evaluation of the insect-resistance of transgenic plants in the field was conducted by artificial infestation combined with natural infestation of yellow stem borer at the experimental farm of Huazhong Agricultural University in Wuhan, China. The materials tested included four homozygous *cry2A** transgenic lines (T_4 generation), the source cultivar Minghui 63 as a susceptible control, and a homozygous *cry1Ac* line developed previously in our laboratory for comparison. The seeds of these lines were sown in a seedling bed in early June 2004 and transplanted to the paddy field in early July. The layout of the plots in the field followed a completely randomized block design with three replications. Each plot consisted of 100 plants grown in ten rows with 20 cm within rows and 28 cm between rows. No insecticide for lepidopteran pests was applied during the entire growing season. Each rice plant was infested with five first-instar larvae of yellow stem borer at the tillering stage. The number of leaves damaged by leaf folders and the number of deadhearts and whiteheads damaged by stem borers were counted in the field.

Results

Transformation and PCR assay of T_0 transgenic plants

Since the bar gene has been approved for use in commercialized herbicide-tolerant crops, it was used as a selectable marker gene in this study. A total of 102 independent transgenic plants were obtained at a transformation frequency of 5%. Putative T_0 transgenic plants were detected by PCR analysis. The expected band was amplified in 71 transformed plants, which indicated that about 70% of the transformants, were positive.

Selection of insect-resistant homozygous transgenic plant lines

Thirty-six T_1 transgenic plant families derived from fertile T_0 transgenic plants were grown in the field. No synthetic insecticide was used during the entire rice growth period. The transgenic families with significantly higher resistance to rice pests were selected. Theoretically, more than 75% of the plants in any T_1 transgenic family should display insect-resistance. However, abnormal segregations (1:1 or less than 1:1) in T_1 transgenic progeny were found in this study, and similar

results were also observed in previous studies (Wang et al. 2001; Husnain et al. 2002). Based on the following selection criteria—(1) high insect-resistance, (2) no significant phenotypic changes, (3) single-copy insertion, and (4) Mendelian segregation, 4 out of 36 T_1 transgenic families were chosen and designated 2A-1, 2A-2, 2A-3, and 2A-4. The insect-resistant inheritance of all four selected transgenic families met the Mendelian model in the T_1 generation in the field (Table 1). Furthermore, Southern blot analysis confirmed that these resistant lines had a single copy of the transgene inserted (Fig. 2).

To obtain homozygous transgenic plants, the mature seeds from selected T_1 transgenic plants were collected separately and germinated on fresh 1/2 MS medium containing 10 mg/l Basta. Homozygous transgenic plants, heterozygous plants and negative plants were differentiated by germination ratio (100%, 75% or 0%). Statistical analysis also confirmed that the four selected families represented single site of insertion (Table 1).

Cry2A* protein quantification in *cry2A** plants

Cry2A* protein content of fresh leaves at the tillering stage from homozygous T_3 transgenic lines was measured. The Cry2A* protein concentrations of homozygous transgenic lines ranged from 9.65 to 12.11 μg/g leaf fresh weight, indicating that Cry2A* was expressed in transgenic rice lines at a similar level. Cry2A* protein was also detected in hybrid (F_1) and backcross offspring (BC_1F_1) derived from their transgenic parents and the concentrations varied little from their transgenic parents (Fig. 3). These results indicate that the insect-resistant Minghui 63 containing Cry2A* could be used for rice hybrid production.

Bioassay of *cry2A** plants in the laboratory

The feeding assay of *cry2A** plants showed that all the yellow stem borer larvae in transgenic stem cuttings were killed within 5 days after infestation (Fig. 4), whereas the larvae infesting the stem cuttings of Minghui 63 grew normally and developed into second-instar larvae (Fig. 5). Thus, the transgenic lines were highly toxic to the first-instar larvae of the yellow stem borer.

Insect-resistance of *cry2A** plants in the field

To assess the insect-resistance of the *cry2A** transgenic plants in the field, two independent field investigations were performed after artificial infestation. The insect-resistance performance of the four *cry2A** transgenic lines was very similar and were all significantly superior to that of the original Minghui 63 recipient (Table 2). During the entire growing period, Minghui 63 was damaged seriously by the rice leaf folder, while the transgenic cry2A* lines did not show any symptoms

Table 1 Segregation of resistance in four transgenic lines in the T_1 generation and matured T_1 seeds (Wuhan, China, 2003)

Transgenic Lines	No. of resistant plants	No. of susceptible plants	χ^2 value for 3:1	No. of heterozygous plants	No. of homozygous resistant plants	χ^2 value for 2:1 of resistant plants	No. of Basta positive seeds[a]	No. of Basta negative seeds[a]	χ^2 value for 3:1
2A-1	38	12	0.000	26	12	0.003	939	277	3.080
2A-2	33	16	1.150	25	8	0.852	920	303	0.022
2A-3	33	15	0.694	22	11	0.034	621	240	3.794
2A-4	23	9	0.043	14	9	0.135	426	118	3.002

[a]No. of Basta-positive seeds or no. of Basta-negative seeds is the sum of Basta-positive seeds or Basta-negative seeds from all heterozygous plants of the transgenic line

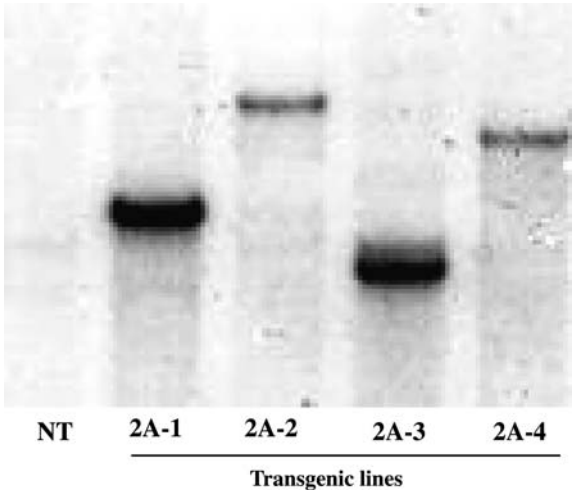

Fig. 2 Southern blot analysis of four *cry2A** transgenic lines in the T_1generation. NT: Minghui 63 control. The genomic DNA was extracted from the mixed leaves of six positive plants in each T_1 families. Four microgram of genomic DNA per sample were digested with *Hind*III and separated on a 0.8% agarose gel for Southern blot analysis. There was unique site of *Hind*III between the bar gene and the ubiquitin promoter in the T-DNA. The probe was a PCR-amplified fragment of *cry2A**. The bands contain the entire *cry2A** gene and the right border of the T-DNA

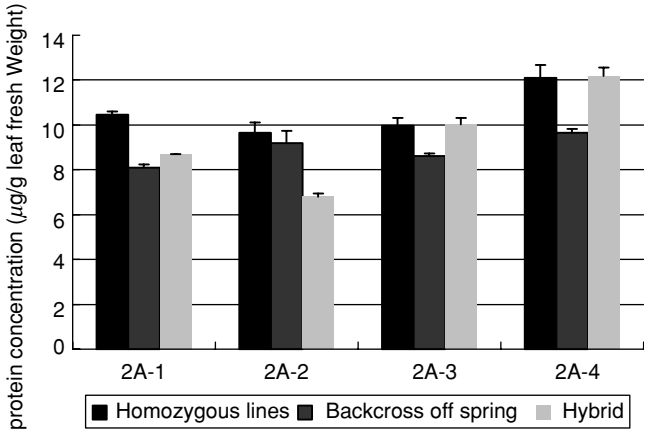

Fig. 3 Cry2A* protein assay of fresh leaves from transgenic rice plants by ELISA. The Cry2A* protein concentration was slightly different in homozygous plants, its backcross offspring and hybrids

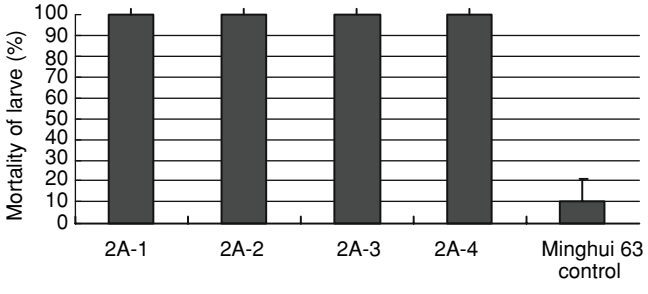

Fig. 4 Laboratory assay using artificial infestation of first-instar larvae of yellow stem borer. Mortality of the larvae in transgenic stem cuttings was 100%. The larvae in stem cuttings of Minghui 63 control exhibited low natural mortality (10.1 ± 10.9%)

(Fig. 6). In the first investigation, the number of damaged leaves per tiller of transgenic plants ranged from 0.01 to 0.02, whereas that of Minghui 63 was as high as 1.17. The deadheart rates of the transgenic lines ranged from 5.36 to 7.48%, approximately one-third that of Minghui 63 (17.24%). The second investigation showed similar results. The damaged leaves per tiller of transgenic plants ranged from 0.01 to 0.08, while that of the control was 1.13. The deadheart rates of transgenic lines decreased to 0.98–4.18%, while the Minghui 63 control showed no change. This result indicated that the artificially infested yellow stem borer was controlled well in the *cry2A** rice. At the grain-filling stage, the whitehead rate of Minghui 63 was 5.57%, whereas whiteheads (0.00–0.50%) were rarely observed in transgenic lines. The resistant control (*cry1Ac* line) showed complete resistance to rice stem borer and rice leaf folder. However, there were no transgenic *cry2A** lines that were 100% immune to lepidopteran pests (Table 2).

Discussion

Since insects may evolve resistance against Bt toxins, developing a compatible management strategy for the use of Bt rice is necessary. Considering the mode of rice production in China, gene stacking is probably more compatible for Bt rice than the high-dose/refuge strategy. Although field evaluations of several Bt transgenic lines have been conducted in China, all Bt transgenic lines contain a single-toxin (Cry1Ab/Cry1Ac fusion protein or Cry1Ab) (Tu et al. 2000; Ye et al. 2001). There is a lack of resistant rice sources containing other kinds of Bt toxins, such as Cry1C or Cry2A, for development of two-toxin transgenic lines. In this study, a novel *cry2A** gene adapted to express well in rice was synthesized artificially, and four insect-resistant transgenic lines were produced by *Agrobacterium*-mediated transformation. The field trial indicated that the transgenic *cry2A** rice plants are highly resistant to the main lepidopteran rice pests. These *cry2A** transgenic lines can be used for the production of insect-resistant hybrids and can serve as a resistant source for the development of two-toxin Bt rice.

In this study, we used the following selection criteria to select promising transgenic lines: (1) high insect-resistance, (2) no significant phenotypic changes, (3) single-copy insertion, and (4) Mendelian segregation. Only a small portion of transgenic lines (4 out of 36) met the selection criteria. Transgenic silencing and low-level expression of foreign genes are common obstacles in identifying transgenic plants with the expected performance. Undesirable agronomic traits including dwarfism, sterility, and decrease of yield are also often observed in transgenic plants. In addition, singe-copy insertion in transgenic plants is extremely important for transgenic breeding, because multiple gene copies can lead to instability of expression and

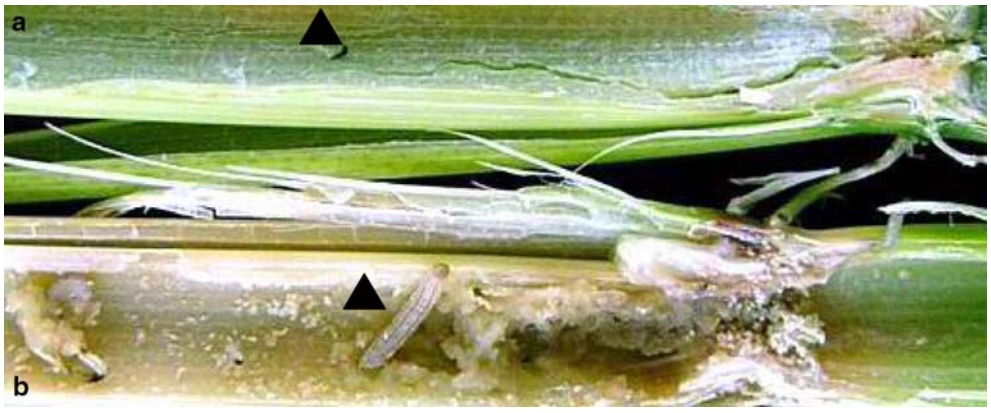

Fig. 5 Laboratory assay using artificial infestation of first-instar larvae of yellow stem larvae. The stem cuttings were dissected and insect mortality was recorded 5 days after artificial infestation. **a** Dissected stem cuttings of transgenic plants. **b** Dissected stem cuttings of Minghui 63 control plants. All the larvae in transgenic stem cuttings were killed, whereas the larvae infesting the stem cuttings of Minghui 63 control grow normally and developed into second-instar larvae

inheritance of the transgene (Stam et al. 1997) or even gene silencing. Thus, to select promising lines, it is crucial to produce an adequate number of independent transgenic plants.

The expression level of a wild-type Bt gene in transgenic plants is usually very low. Therefore, in this study, the principal objective of gene modification was to obtain a novel *cry2A** gene that could be expressed effectively in transgenic rice. The average protein concentration of four *cry2A** transgenic lines reached approximately 10 μg/g leaf fresh weight. Bt protein level expressed in commercial Bt transgenic cultivars (cotton, maize, and potato) is generally 1–11 μg/g leaf fresh weight, which is adequate to meet the requirement of the "high-dose/refuge" strategy (Cohen et al. 2000). Bashir et al. (2004) analyzed the Cry2A protein content of a highly insect-resistant *cry2A* transgenic rice line; the protein concentration of the transgenic line was less than one-tenth of our *cry2A** transgenic lines (< 1 μg/g leaf fresh weight). Based on these data, the *cry2A** gene in the four rice transgenic lines produced in this study is expressed at a very high level (Fig. 3), and the development of the synthetic *cry2A** gene was successful.

Concentrations of foreign protein usually show great differences among different independent transgenic plants with the same Bt construct (Mabqool et al. 1998, 2001; Breitler et al. 2000; Husnain et al. 2002; Ramesh et al. 2004). However, in this study, the four selected transgenic lines contained very similar and high Cry2A* protein concentrations. It appears that the Cry2A* concentration in the transgenic lines reached a level high enough to ensure effective insect-resistance in the field. In Bt plants,

Table 2 Damage symptoms as a measure for field resistance in four *cry2A** transgenic lines (artificial infestation at tillering stage plus natural infestation in Wuhan, China, 2004)

Plant lines	Tillers/ plant	Folded leaves/ plant	Folded leaves/ tiller	Deadhearts/ plant	Rate of deadheart (%)	Whiteheads/ plant	Rate of whitehead (%)
MH63[a]	18.1	20.80	1.17	3.00	17.24		
1Ac[a]	15.9	0.00**	0.00**	0.00**	0.00**		
2A-1[a]	19.3	0.37**	0.02**	1.13**	5.61**		
2A-2[a]	18.4	0.43**	0.02**	1.27**	7.48**		
2A-3[a]	20.1	0.43**	0.02**	1.00**	5.36**		
2A-4[a]	19.5	0.23**	0.01**	1.10**	5.52**		
$LSD_{0.05}$		1.00	0.14	0.46	1.70		
$LSD_{0.01}$		1.42	0.20	0.66	2.41		
MH63[b]	16.8	15.7	1.13	2.34	17.37	0.71	5.57
1Ac[b]	17.9	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
2A-1[b]	18.2	1.49**	0.08**	0.62**	3.48**	0.11**	0.50**
2A-2[b]	16.6	0.16**	0.01**	0.16**	0.98**	0.04**	0.21**
2A-3[b]	19.2	0.55**	0.03**	0.74**	4.18**	0.00**	0.00**
2A-4[b]	16.5	0.14**	0.01**	0.25**	1.65**	0.02**	0.10**
$LSD_{0.05}$		1.74	0.19	0.58	4.21	0.17	0.91
$LSD_{0.01}$		2.48	0.26	0.82	5.99	0.24	1.30

[a]The results of first field investigation at the later tillering stage (August 2004)
[b]The results of second field investigation at the grain-filling stage (October 2004)
**Significant difference from Minghui 63 ($P < 0.01$)

Fig. 6 Field performance of transgenic *cry2A** lines. **a** Transgenic lines, **b** Minghui 63 control. Plants of Minghui 63 control were damaged seriously by the rice leaf folder, but transgenic lines were highly resistant

the protein concentration is directly related to the level of insect-resistance. We also found that the amount of the foreign protein in hybrids and backcross offspring varied little compared to their homozygous parental transgenic lines (Fig. 3), indicating that heterozygous transgenic plants will have a resistance similar to their homozygous parents. This result differs from previous reports. For example, in the study by Duan et al. (1996), the homozygous transgenic plants produced approximately twice the amount of foreign protein compared to heterozygous transgenic plants, and Wang et al. (2001) found that the Bt protein content of hybrids was sometimes higher than their transgenic parental plants.

In all four *cry2A** transgenic lines, all the first-instar larvae of yellow stem borers were killed within 5 days (Fig. 4). There was almost no damage to laboratory-tested stem cuttings in the *cry2A** transgenic lines (Fig. 5). In the field, however, some *cry2A** transgenic plants showed symptoms after artificial infestation with first-instar yellow stem borers (Table 2). Thus, a laboratory feeding-bioassay cannot accurately represent the situation in the field. The first-instar larvae of yellow stem borers hatched and grown under laboratory conditions were probably weaker than those grown under natural conditions, and more prone to be killed.

The results of our field tests showed that the mean rate of whiteheads of non-Bt Minghui 63 without artificial infestation was 3.82% when no insecticide was used on the fields. The stems of those plants with whiteheads were dissected to identify the species and number of stem borers. There were 14 yellow stem borers, 45 pink stem borers (*Sesamia inferens*), and 68 striped stem borers per 100 damaged plants (ratio = 1:3.2:4.9) under conditions of natural infestation (unpublished data). In 2004, the striped stem borer was the major borer in the Wuhan area. Several years ago, however, the major borer in the region was the yellow stem borer. Thus, it will be important to evaluate the resistance of *cry2A** rice to striped stem borer and pink stem borer as well.

When fed to rice pests in artificial diets, the toxicity of Cry2A* was lower than Cry1Ac (Karim and Dean 2000; Alcantara et al. 2004). In this study, we evaluated the toxicity of these two kinds of Bt rice plants under field conditions. The result showed that the *cry1Ac* transgenic line was completely resistant against lepidopteran pests. No *cry2A** transgenic lines were 100% immune to rice stem borers, although four transgenic lines were highly resistant against first-instar larvae of yellow stem borer in a laboratory bioassay. The Cry1Ac protein concentration of the *cry1Ac* line was assayed using ENVIRONLOGIX kits APP003 under the same condition as the *cry2A** lines; and the protein concentration was 16.28 μg/g leaf fresh weigh, close to that of the four *cry2A** transgenic lines. This finding indicates that resistance of *cry1Ac* rice is indeed superior to *cry2A** rice, at least against rice stem borers, although *cry2A** rice appears to be very effective against rice leaf folder (Table 2). Thus, it is better for the *cry2A** gene to use a strong promoter such as maize ubiquitin promoter to obtain transgenic plants expressing the cry2A* at a high dose. However, over-expression of Cry2A protein might negatively influence growth and development of transgenic rice plants (Maqbool et al. 1998). Thus, it is

important to improve the toxicity of the Cry2A protein. Currently, we are trying to improve the toxicity of the Cry2A protein through modifying *cry2A** gene using DNA shuffling technology, and we hope to obtain a toxicity-enhanced *cry2A** gene without alteration of its recognition mechanism.

Acknowledgements This research was funded by the National Program on Research and Development of Transgenic Plants and the National Natural Science Foundation of China. The authors thank Dr. Sheng Chen in Huazhong Agricultural University (China) and Dr. Yanping Liu at Oregon State University (USA) for many helpful suggestions for revising the manuscript.

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