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Development of insect-resistant transgenic indica rice with a synthetic *cry1C** gene

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Abstract Stemborers and leaffolders are two groups of lepidopteran pests that cause severe damage to rice in many areas of the world. In this study, a *cry1C** gene encoding *Bacillus thuringiensis* (*Bt*) δ-endotoxin was synthesized by codon optimization as the first step towards gene stacking in our resistance management strategy of transgenic rice. *Agrobacterium*-mediated transformation of this gene into Minghui 63 (*Oryza sativa* L.), an elite indica CMS restorer line, produced 120 independently transformed plants, 19 of which had a single-copy transgene. Preliminary screening of T_1 families of these 19 transformants in the field identified five lines showing a high level of resistance to leaffolders (*Cnaphalocrocis medinalis*) and stemborers. Hybrids were produced by crossing these five lines with Zhenshan 97A, the male-sterile line for Shanyou 63, the most widely cultivated hybrid in China. These five lines and their hybrids were highly resistant to yellow stemborer (*Tryporyza incertulas*) as revealed by an insect bioassay. The content of Cry1C* protein varied considerably among the five lines as well as among the corresponding hybrids. T1c-19, a line showing the highest content of Cry1C* protein, and its hybrid were tested in the field for insect resistance and agronomic performance and found to be highly resistant to stemborers and leaffolders throughout the growth period, resulting in a significantly increased grain yield compared with the respective controls. These results indicate that T1c-19 can be used for production of insect-resistant hybrid rice and as a germplasm for gene stacking to produce rice plants with two toxins.

Keywords *cry1C** · Leaffolders · Resistance management · Transgenic field trials · *Oryza sativa* · Stemborers

Abbreviations

Bt	*Bacillus thuringiensis*
CaMV	Cauliflower Mosaic Virus
CMS	Cytoplasm male sterile
ELISA	Enzyme-linked immunosorbent assay

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Introduction

Stemborers and leaffolders are two groups of major pests in rice that cause severe yield loss in most rice-producing countries. For a long time the control of these pests has depended chiefly on the

use of large amounts of poisonous chemical insecticides, mostly as sprays, which cause considerable environmental pollution and represent a health hazard to farmers as well as significantly increasing the costs of rice production. Consequently, alternative means of pest control have been sought.

One alternative to chemical pesticides is *Bacillus thuringiensis* (*Bt*). Its insecticidal properties have long been recognized, and *Bt* has been applied as an insecticide for several decades. By 1995, 182 *Bt* products were registered by the United States Environmental Protection Agency. However, in 1999, *Bt* formulations made up only 2% of the total sales of all insecticides (http://www.epa.gov/pesticides/biopesticides/), mainly due to the toxicity instability of *Bt* insecticides as an insecticidal spray.

During the last two decades, considerable research efforts have been invested to introduce insecticidal crystal protein genes from *Bt* into plants by transgenic approaches. *Bt*-genes were first introduced and expressed in tobacco (Barton et al. 1987; Vaeck et al. 1987) and tomato (Fischhoff et al. 1987), and the transgenic plants exhibited a certain level of insect resistance. Since then, insect-resistant crops harboring *Bt* genes have been developing at very fast pace. By 2004, the global area planted in commercial transgenic crops had reached 81 million ha, of which *Bt* crops accounted for 28%, or 22.5 million ha (James 2005). These crops have benefitted the growers and environment by greatly reducing the use of chemical insecticides (Ferré and Van Rie 2002).

However, there is also an increasing concern that widespread adoption of *Bt* crops may lead to the development of resistance to the insecticidal genes in the pest populations (Tabashnik et al. 1994; Gould 1998; Frutos et al. 1999). Indeed it has already been shown that some insects have developed resistance to *Bt* proteins under laboratory and greenhouse conditions (McGaughey 1985; Hama et al. 1992; Gould et al. 1995; Sayyed et al. 2000; Ferré and Van Rie 2002; Kain et al. 2004). For resistance management, high-dose/refuge and gene stacking have been proposed as two effective strategies to prevent or delay the emergence of pest resistance to *Bt* toxins (Frutos et al. 1999; Ferré and Van Rie 2002; Shelton et al. 2002). The high-dose/refuge strategy requires the dosage of *Bt* toxins to be sufficiently high to kill all or most heterozygotes when the mutation occurs. However, this strategy seems not to be applicable in most rice-producing countries where the majority of the rice growers are small-scale farmers and it is therefore not feasible to designate certain areas of non-transgenic crops as refuge. Alternatively, the gene stacking strategy implies that plants containing two or more dissimilar *Bt* toxins have the potential to delay resistance more effectively than ones having only single toxin, as the insects have to develop simultaneous resistance to two or more *Bt* toxins in order to survive. This latter strategy seems to be more practical for most rice-producing areas. However, one requirement for this strategy to work is that the ''stacked'' toxins have different modes of action. Combinations of toxins that share a common binding site are not likely to be useful for managing resistance.

Although a large number of *Bt* toxins are known (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/), only a small fraction of these are currently used in developing transgenic crops to control lepidopteran pests. The most commonly used *Bt* genes in transgenic crops, including rice, are *cry1Ab*, *cry1Ac*, and fusion gene *cry1Ac/cry1Ab* (Fujimoto et al. 1993; Nayak et al. 1996; Wünn et al. 1996; Ghareyazie et al. 1997; Wu et al. 1997; Cheng et al. 1998; Datta et al. 1998; Alam et al. 1999; Tu et al. 2000; Wang et al. 2002). The results of binding tests of midgut brush border membrane vesicles indicated that the *cry1Aa*, *cry1Ab*, and *cry1Ac* toxins share a common binding site (Escriche et al. 1997; Ballester et al. 1999; Karim and Dean 2000), thus a mutant that is able to overcome one of the *cry1A* genes is also likely to be resistant to other *cry1A* genes. Consequently, combinations of *cry1A* genes with other groups of *Bt* genes should be explored as a means of preventing or delaying the emergence of pest resistance.

The Cry1C toxin is effective against a variety of lepidopteran pests, including rice stemborers, and does not share a common binding site with Cry1A toxins (Alcantara et al. 2004). Strizhov et al. (1996) expressed a modified *cry1Ca* gene in

alfalfa and tobacco, and the transgenic plants showed enhanced resistance to Egyptian cotton leafworm (*Spodoptera littoralis*) and the beet armyworm. Cao et al. (1999) transformed broccoli with a modified *cry1C* gene, and the transgenic plants showed high resistance to diamond back moth (*Plutella xylostella*). Therefore, it would appear that the Cry1C toxin can be a potential alternative to Cry1A toxins and that it can also be combined with other *cry1A* genes to develop two-toxin *Bt* crops.

We report here the introduction of a codon-optimized *cry1C* gene, referred to as *cry1C**, into elite indica rice line Minghui 63 via *Agrobacterium*-mediated transformation. The transgenic plants were examined for both insect resistance and agronomic traits under field conditions, with the goal of obtaining transgenic lines that may be useful for developing insect-resistant hybrids.

Materials and methods

Synthetic *cry1C** and transformation construct (pC-1C*)

The *cry1C** gene was synthesized on the basis of a wild-type *cry1Ca5* gene of *B. thuringiensis*. The original sequences that may affect gene expression in plant cells, such as AT-rich sequences (ATTTA and AATGAA) and inverted repeats, were eliminated, and the codons were optimized based on preferred codons in plants. The synthetic *cry1C** gene, 2.3 kb in length, consists of three main parts: the 5′-end leader sequence, the coding sequence, and the 3′-end tailing signal and terminating sequences. The modified sequence has an overall GC content of 44.65% and shares 84% nucleotide sequence homology with the original *cry1Ca5* gene.

The transformation construct (pC-1C*) was made using the backbone of the vector plasmid pCAMBIA1300 (kindly provided by the Center of the Application of Molecular Biology to International Agriculture, Australia). The *cry1C** gene driven by a maize ubiquitin promoter was cloned into the polylinker of the plasmid pCAMBIA1300 (Fig. 1). The hygromycin phosphotransferase gene (*Hph*) of the T-DNA region of the plasmid was replaced by the phosphinothricin acetyltransferase gene (*Bar*) under the control of the CaMV 35S promoter that was used as the selection marker for transformation (Block et al. 1987; Thompson et al. 1987).

Generation of transgenic plants

An indica cultivar Minghui 63, the restorer line for a number of widely grown hybrids – including the most widely cultivated hybrid in China Shanyou 63 (a cross between Zhenshan 97A and Minghui 63) – was used for transformation. Callus culture and transformation essentially followed the methods described by Lin and Zhang (2005), except that the selection media contained 25 mg/l of glufosinate ammonium (Basta) for subculture and 20 mg/l of Basta for differentiation in place of the antibiotic hygromycin.

DNA assay of the transgenic plants

Total cellular DNA of the plants was isolated from fresh leaf tissue and digested with *Dra*I, blotted onto a nylon membrane, and hybridized with a probe prepared from a fragment of the *cry1C** gene. DNA isolation, electrophoresis, blotting, and hybridization essentially followed procedures described elsewhere (Liu et al. 1997).

For the PCR analysis, a pair of primers was designed according to the *cry1C** gene sequence:

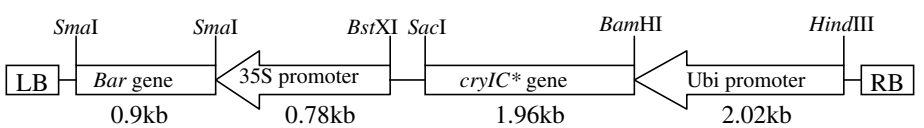

Fig. 1 T-DNA region of transformation construct pC-1C*. The *cry1C** gene was driven by a maize ubiquitin promoter and terminated by the nopaline synthase (nos) terminator. The *Bar* gene as a selectable marker was under the control of CaMV 35S promoter and tailed by the CaMV 35S polyA. *LB* Left border of T-DNA region, *RB* right border of T-DNA region

*cry1C**-F (5′-ttctactggggaggacatcg-3′); *cry1C**-R (5′-cggtatctttgggtgattgg-5′). A 20- μl mixture containing 30 ng of template DNA, 2.0 μl of 10× buffer, 1.0 μl of 2 m*M* dNTP, 1.5 μl of 25 m*M* $MgCl_2$, 0.4 μl of each of the 10 μ*M* primers, 1.5 U *Taq* DNA polymerase was prepared for PCR assay. The PCR reaction was carried out as follows: one cycle at 94°C for 3 min; one cycle at 57°C for 1 min, 72°C for 1.5 min, and 94°C for 1 min for 30 cycles, followed by extension at 72°C for 10 min. The products were then checked by electrophoresis.

Selection of insect-resistant transgenic plants and the production of *cry1C**-containing hybrids

Bagged seeds of transgene-positive T_0 plants were planted among T_1 families in the field, approximately 90 plants per family, with the original Minghui 63 planted as a control. Target transgenic plant families in the T_1 generation were selected based on three criteria: (1) high insect resistance, (2) no obvious phenotypic changes, and (3) a single copy of the transgene.

Seeds of each plant of the selected T_1 families were harvested individually. For determining the genotype of the selected T_1 plants, we first sterilized 50 seeds from each plant and then placed them on germination media (which is the same as the rooting medium except that it contained 10 mg/l of Basta) for 7 days to induce germination . A plant was considered to be homozygous for the transgene if all (or nearly all) the seeds germinated, negative if none of the seeds germinated, and heterozygous (or hemizygous) if a large proportion (around 70%) of the seeds germinated.

Insect bioassay

Insect bioassays were performed at the booting stage of the plants. Egg masses of yellow stemborer collected from paddy rice fields were hatched in an incubator. Five cuttings (roughly 5 cm in length) of the culms were placed into an insect-raising bottle, and 20 first-instar larvae were swept into the bottle with a soft hairy brush. The bottle was tightly sealed with one layer of cotton cloth and a piece of black plastic membrane to prevent the insects from escaping. A feeding experiment consisted of three bottles for each genotype. After 5 days of incubation at 28°C at 85% relative humidity, the culms were dissected for investigating growth status and survival of the insects.

Quantification of the Cry1C* protein

The amount of Cry1C* protein was measured at the heading stage using the enzyme-linked immunosorbent assay (ELISA) kit from Enviro-Logix (Portland, Me.). For the protein preparation, approximately 20 mg of fresh leaves from the plant was homogenized by grinding in 500 μl of extraction/dilution buffer. After 30 min at room temperature, a 20-μl aliquot of the supernatant was transferred to a tube to which 480 μl of the buffer was added. The enzyme-linking reaction was conducted following the manufacturer's instructions. The optical density values of the diluted samples were measured using a microplate reader (Multiskan MK3, Labsystem, P.R. China) at 450-nm wavelength, and the Cry1C* content was calculated based on the reading.

Field experiments

For evaluating the insect resistance and agronomic performance, we planted the selected transgenic line T1c-19, its hybrid with Zhensha 97, Hy-19-Zh, the corresponding negative segregant, and its hybrid Hy-0-Zh in 2004 fields of the experimental farm of Huazhong Agricultural University at Wuhan, China in the 2004 rice growing season. The original Minghui 63 and Shanyou 63 lines were also planted as additional controls. The seeds of the plant materials to be tested were sown in a seeding bed on June 3, and the seedlings were transplanted to the paddy field on July 1. The field layout followed a randomized complete block design with three replications. Each plot consisted of 121 plants in 11 rows, with distances of 20.0 cm between plants within a row and 26.67 cm between rows. The experimental plots were bordered by four rows of non-transgenic japonica rice plants.

No chemical insecticides against lepidopteran pests were applied throughout the growth period.

Severity of damage caused by natural infestation of leaffolders was scored within 7 days after its peak damage appeared. Leaves with visible scrapes or folds were considered to be damaged. Dead hearts caused by stemborers were counted at the late maximum tillering stage, and white heads were counted at the flowering stage.

At maturity, plant height was measured in the field, and 20 plants were randomly selected from each plot and harvested for investigating panicle length, number of panicles per plant, number of grains per panicle, 1000-grain weight, and yield per plant.

Results

Transgenic plants

Transformation of 5420 calli resulted in a total of 126 plantlets, of which 120 grew to maturity. PCR analysis identified that 91 of the 120 plants were transgene-positive. Southern blotting analysis showed that the copy numbers of the transgene in the transgenic plants varied from one to six (Fig. 2). Of the 91 transgenic plants, 19 contained a single copy, 46 plants had two to four copies, and 26 plants had five or more copies of the transgene.

T_1 families of all 19 single-copy transgenic plants were planted in the field for preliminary screening. The five families (nos. 3, 7, 11, 17, and 19) showing a high level of resistance were selected for further analyses.

Insect bioassay

Transgene-homozygous lines from the above five families were isolated, and each was crossed to Zhenshan 97A to produce the hybrids. All five lines and their hybrids were tested by bioassay together with the original Minghui 63 and Shanyou 63 lines as controls. The results of the insect bioassay showed that the larvae had developed into the second instar stage in the controls, with large amounts of insect excretions (Fig. 3a). In contrast, neither insect larvae nor feces were observed in the culm cuttings of the transgenic lines and their hybrids in all three replicates (Fig. 3b), with the exception of a few pinhead-sized holes that could be observed in the pith of the culm cuttings; these were presumably created by insects trying to penetrate into the plant. These results confirmed that the transgenic lines and their hybrids were highly resistant to yellow stemborer.

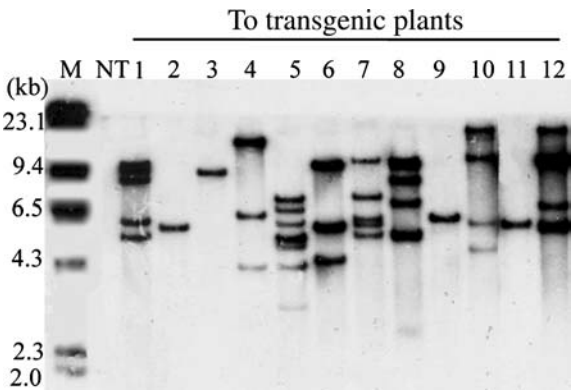

Fig. 2 Southern-blot analysis of total genomic DNA of T_0 transgenic plants. The DNA samples were digested with *Dra*I (no cutting site within T-DNA region) and hybridized with the prepared radioactive probe. The probe size was 2.3 kb and contained the whole encoding region of the *cry1C** gene and nos terminator sequence. *M* DNA marker, *NT* Minghui 63, *lanes 1–12* transgenic plants

Protein assay

At the heading stage, all of the above-mentioned five lines and their hybrids had detectable Cry1C* protein as measured by ELISA. However, these protein contents varied considerably from one line to another (Fig. 4). T1c-19 and its hybrid had 1.38 and 1.32 μg per gram fresh leaf at the heading stage, the highest Cry1C* protein contents among the five lines and the corresponding hybrids. It is also clear from Fig. 4 that Cry1C* protein contents did not seem to be dependent on the dosage of the gene; the hybrids produced approximately the same amounts of the Cry1C* protein as the restorer lines.

Field resistance of T1c-19 and its hybrid to lepidopteran insects

Yellow stemborer, striped stemborer, and leaffolders are the major lepidopteran pests of rice in most rice-producing areas. Yellow stemborer and

Fig. 3 The resistance of T1c-19 against leaffolders and stemborers under laboratory and field conditions. **A**, **B** Insect bioassays with the homozygous transgenic T1c-19 and the control were conducted in the insect-rearing room. **a** Original Minghui 63 (control): extensive damage was visible. Many feces were left on the surface of the inner tissue, and the larvae were much larger than those originally present at the start of the bioassay. **B** T1c-19: cutting stems were not damaged by yellow stemborer. No visible damage or living larvae was found on the inner surface of the cutting stems. **C** The field resistance of the T1c-19 against leaffolders at the tillering stage. *Left* Control: original Minghui 63 was heavily damaged by leaffolders; *right*positive: no single affected leaf was observed. **D** The field resistance of T1c-19 plants against stemborers at the flowering stage. *Left* Control: many white heads were observed; *right* positive: in T1c-19 plants, not one single whitehead was observed

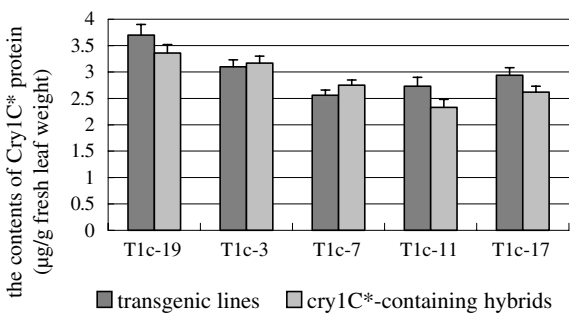

Fig. 4 Protein assay for transgenic lines and their hybrids. Cry1C* protein contents varied considerably from one line to another. T1c-19 and its hybrid had higher Cry1C* protein contents than the other lines and the corresponding hybrids

striped stemborer attack rice plants from the tillering up to the reproductive stage, resulting in dead hearts at the vegetative stage and white heads at the reproductive stage. Leaffolders damage rice plants by eating and folding leaves throughout the entire life cycle of the plant (Pathak 1977).

T1c-19 and its hybrid were field-tested for resistance to natural infestation by leaffolders and stemborers and also evaluated for agronomic performance. The results of natural infestation under field conditions are given in Table 1, from which it can be seen that all tillers of the wild-type Minghui 63 were damaged to various degrees by leaffolders, with an average of 3.7 leaves affected per tiller, while no damage was observed on T1c-19 plants. It was also clear from Table 1 that Minghui 63 was severely damaged by stemborers, with 17.3% and 5.6% of the plants having dead hearts and white heads, respectively, while again no damage to T1c-19 was observed.

Likewise, the *cry1C**-containing hybrid, T1c-19-Zh was highly resistant to both leaffolders and stemborers: no damage due to infestations by these pests was observed. In contrast, the control hybrid Shanyou 63 was seriously damaged, indicating that the *cry1C** gene was effective in providing insect resistance in both the homozygous and hemizygous states. This is consistent with the results of the analysis of Cry1C* protein contents in the parents and hybrids (Fig. 4).

Table 1 Resistance of the transgenic line T1c-19 and its derived hybrid against natural infestations of stemborers and leaffolders under field conditions (2004, Wuhan, China)[a]

Rice genotype	Damage by leaffolders		Damage by stemborers	
	Number of tillers affected (%)	Number of damaged leaves per tiller	Dead hearts (%)	White heads (%)
T1c-19	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Minghui 63	100.0±0.0	3.8±0.3	17.3±1.1	5.6±0.6
Hy-19-Zh	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Shanyou 63	100.0±0.0	1.2±0.1	13.6±1.3	14.8±1.8

[a]Data in each cell of the table are expressed as the mean ± standard deviation

Table 2 Comparison of agronomic traits of T1c-19 and its derived hybrid Hy-19-Zh under field conditions (Wuhan, China, 2004)

Rice genotype	Plant height (cm)	Panicle length (cm)	Tillers/plant	Grains/panicle	1000 grain weight (g)	Yield/plant (g)
T1c-19	96.9a[a]	23.8A	10.3A	96.0A	26.9A	26.5A
Negative segregant	93.3b	23.0A	9.2B	87.7B	26.8A	21.6B
Minghui 63	93.7b	23.7A	9.3B	88.4B	26.6A	21.9B
Hy-19-Zh	114.3a	24.9A	17.2A	139.4A	27.0A	64.5A
Hy-0-Zh	111.2b	24.4A	15.8B	124.6B	26.8A	52.7B
Shanyou 63	110.9b	24.0A	15.5B	127.0B	26.9A	52.9B

[a]a, b and A, B: ranks resolved by Duncan's multiple range test at the 0.05 and 0.01 probability levels, respectively

Agronomic performance of T1c-19 and its hybrid Hy-19-Zh

ANOVA of the data collected from the field experiment for each of the traits showed that there was no significant difference in panicle length and grain weight among T1c-19, the negative segregant and the wild type Minghui 63, as was also the case for the differences among Hy-19-Zh, Hy-0-Zh, and the control hybrid Shanyou 63 (data not shown). T1c-19 differed at $p < 0.05$ from the negative segregant and Minghui 63 with respect to plant height, and at $p < 0.01$ for number of tillers per plant, number of grains per panicle, and grain yield per plant. The same is true for the differences between Hy-19-Zh and Hy-0-Zh and the control hybrid Shanyou 63.

Very similar results were revealed by Duncan's multiple range tests (Table 2), in which the transgenic line and its hybrid had a significantly higher yield than the respective negative segregant and the wild-type controls. It was also clear from Table 2 that the higher yields were primarily contributed by two of the component traits – number of tillers per plant and number of grains per panicle.

Discussion

The results of this study clearly demonstrate that the synthetic gene *cry1C** is effective in developing a transgenic rice line resistant to leaffolders and stemborers, as shown by bioassay experiments and natural infestation trials under field conditions. Consequently, this gene may provide a useful resistance resource for developing transgenic rice against lepidopteran pests as well as an alternative to the Cry1A toxins. It should be mentioned that a bioassay of the Cry1C* toxin against the cotton ball-worm (*Heliothis armigera*)also showed that this protein is also highly toxic to this insect (Zhang et al. unpublished data). We therefore conclude that this gene may have a broad utility in developing insect-resistant transgenic crops against lepidopteran pests.

Another desirable feature revealed by the results of our investigation is the complete

dominance of the expression level of the *Bt* toxin in the *cry1C** transgenic plants. Although different transgenic events appeared to express different levels of the transgene, the hybrid between a homozygous transgenic plant and a non-transgenic plant (Zhenshan 97A) expressed approximately the same amount of the toxin protein as the transgenic parent. Furthermore, the levels of expression of *cry1C** in the hybrids were highly correlated with the expression levels in the transgenic parents. This is extremely important for the development of hybrid rice, as resistance is critically dependent on the concentration of the toxin protein.

A large number of studies have been reported on the development of insect-resistant transgenic rice (Nayak et al. 1996; Ghareyazie et al. 1997; Wu et al 1997; Cheng et al. 1998; Maqbool et al. 1998; Tu et al. 2000; Maqbool et al. 2001;Ye et al. 2001; Bashir et al. 2004; Ramesh et al. 2004; Chen et al. 2005). Although there has not been a single case of commercial utilization of transgenic rice, serious concerns have already been expressed for the potential emergence of insect resistance to the *Bt* toxins following large-scale commercial cultivation (Roush 1998; Frutos et al. 1999; Ferré and Van Rie 2002; Shelton et al. 2002; Bates et al. 2005). These concerns are based on laboratory selection experiments in which strains of more than a dozen species of insects have evolved resistance to *Bt* toxins (Ferré and Van Rie 2002). However, to date the evolution of insect resistance to *Bt* crops in the field has not been reported (Tabashnik et al. 2003), and the only documented case of resistance occurring in the field was the result of the heavy use of *Bt* spray against the diamondback moth (Ferré et al. 1991; Hama et al. 1992). It has been suggested that the cultivation of transgenic crops with two or more different forms of resistance would prevent or significantly delay the development of insect resistance (Zhao et al. 2003). Moreover, there has been evidence suggesting that transgenic cotton plants with two different *Bt* genes has a stronger resistance to insects than those having a single *Bt* gene (Greenplate et al. 2000; Stewart et al. 2001). Thus, it would appear to be advantageous in several ways to have pyramids of two or more genes rather than a single insect resistance gene.

Based on an assay of δ-endotoxin binding to brush border membrane vesicles of rice stemborers, Alcantara et al. (2004) proposed that *cry1Ab* or *cry1Ac* could be combined with *cry1C*, *cry2A*, or *cry9C* for a more durable resistance in transgenic rice. We recently developed transgenic rice harboring the *cry2A* gene that showed strong field resistance to natural infestation of leaffolders and stemborers (Chen et al. 2005). A major objective of this study for testing the Cry1C* toxin in transgenic rice was to identify a second gene that could be used in combination with the Cry1A toxins for developing transgenic rice with two different forms of resistances so as to realize the gene stacking strategy for resistance management. An earlier developed transgenic line harboring a *cry1Ab/cry1Ac* fusion gene in the same rice genotype Minghui 63 (Tu et al. 2000) has now completed the production demonstration stage trials according to regulatory procedures of the Chinese Government. Thus, the high field resistance of the transgenic lines harboring the *cry1C** gene with normal agronomic performance developed in this study has provided potential plant materials for developing rice lines with various combinations of multiple resistances by gene stacking.

It may also be worth commenting on the strategies for pyramiding the *Bt* genes. Our next step in developing lines of multiple resistances is to combine the genes by sexual crossing – an approach similar to the one used by Cao et al. (2002) in broccoli – to achieve the stacking of two *Bt* genes. Compared with the strategies of having the two genes in the same transformation construct (Tian et al. 2000) or the co-transformation of two constructs (Hua et al. 1993), this strategy may have certain advantages. First, technically speaking, it is easier to obtain large numbers of transgenic plants carrying constructs of single genes than large numbers of those carrying constructs of multiple ones, or co-transformation, thereby providing more opportunities for phenotypic selection. Second, the performance of the genes can be individually evaluated to assure the appropriate functioning of each gene in the transgenic plants; conversely, it is difficult to evaluate the resistance conferred by individual genes in plants simultaneously transformed with

multiple genes. More importantly, this strategy of a single-gene construct offers the flexibility to utilize the gene in various ways, either individually or in any of a number of desired combinations.

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References

Alam M, Datta K, Abrigo E, Oliva N, Tu J, Virmani SS, Datta SK (1999) Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice. Plant Cell Rep 18:7–8

Alcantara EP, Aguda RM, Curtiss A, Dean DH, Cohen MB (2004) *Bacillus thuringiensis* δ-endotoxin binding to brush border membrane vesicles of rice stem borers. Arch Insect Biochem Physiol 55:169–177

Ballester V, Granero F, Tabashnik BE, Malvar T, Ferré J (1999) Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). Appl Environ Microbiol 65:1413–1419

Barton KA, Whiteley HR, Yang NS (1987) *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol 85:1103–1109

Bashir K, Husnain T, Fatima T, Latif Z, Mehdi SA, Riazuddin S (2004) Field evaluation and risk assessment of transgenic indica basmati rice. Mol Breed 13:301–312

Bates SL, Zhao JZ, Roush RT, Shelton AM (2005) Insect resistance management in GM crops: past, present and future. Nat Biotechnol 23:57–62

Block MD, Botterman J, Vandewiele M, Dockx JT, Gosselé V, Movva NR, Thompson C, Montagu MV, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518

Cao J, Tang JD, Strizhov N, Shelton AM, Earle ED (1999) Transgenic broccoli with high levels of *Bacillus thuringiensis* Cry1C protein control diamondback moth larvae resistant to Cry1A or Cry1C. Mol Breed 5:131–141

Cao J, Zhao JZ, Tang JD, Shelton AM, Earle ED (2002) Broccoli plants with pyramided *cry1Ac* and *cry1C Bt* genes control diamondback moths resistant to Cry1A and Cry1C proteins. Theor Appl Genet 105:258–264

Chen H, Tang W, Xu CG, Li XH, Lin YJ, Zhang Q (2005) Transgenic indica rice plants harboring a synthetic *cry2A** gene of *Bacillus thuringiensis* exhibit enhanced resistance against rice lepidopteran pests. Theor Appl Genet 111:1330–1337

Cheng X, Sardana R, Kaplan H, Altosaar I (1998) *Agrobacterium*-transformed rice plants expressing synthetic *cry1Ab* and *cry1Ac* genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci USA 95:2767–2772

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue specific differential expression of the gene in transgenic rice plants conferring resistance to rice insect pest. Theor Appl Genet 97:20–30

Escriche B, Ferré J, Silva FJ (1997) Occurrence of a common binding site in *Mamestra brassicae*, *Phthorimaea operculella* and *Spodoptera exigua* for insecticidal crystal proteins Cry1A from *Bacillus thuringiensis*. Insect Biochem Mol Biol 27:651–656

Ferré J, Van Rie J (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 47:501–533

Ferré J, Real MD, Van Rie J, Jansens S, Peferoen M (1991) Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc Natl Acad Sci USA 88:5119–5123

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCoormick SM, Niedermeyer JG, Dean DA, Kusano KK, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. Biotechnology 5:807–813

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*. Biotechnology 11:1151–1155

Ghareyazie B, Alinia F, Menguito CA, Rubia LG, Palma JM, Liwanag EA, Cohen MB, Khush GS, Bennett J (1997) Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cry1A(b)* gene. Mol Breed 3:401–414

Gould F, Anderson A, Reynolds A, Bumgarner L, Moar W (1995) Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: *Noctuidae*) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J Econ Entomol 88:1545–1559

Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726

Greenplate JT, Penn SR, Shappley Z, Oppenhizen M, Mann J, Reich B, Osborn J (2000) Bollgard II efficacy: quantification of total lepidopteran activity in a two-gene product. Proc Beltwide Cotton Conf 2:1041–1043

Hama H, Suzuki K, Tanaka H (1992) Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: *Yponomeutidae*). Appl Entomol Zool 27:355–362

Hua X, Ma W, Fan YL (1993) Transgenic tobacco by cotransformation of proteinase inhibitor II and δ-endotoxin. Chinese Sci Bull 38:747–751

James C (2005) Global status of commercialized biotech/GM crops: 2004. ISAAA, Ithaca, N.Y.

Kain WC, Zhao JZ, Janmaat AF, Myers J, Shelton AM, Wang P (2004) Inheritance of resistance to *Bacillus thuringiensis* Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (Lepidoptera: *Noctuidae*). J Econ Entomol 97:2073–2078

Karim S, Dean DH (2000) Toxicity and receptor binding properties of *Bacillus thuringiensis* delta-endotoxins to the midgut brush border membrane vesicles of the rice leaf folders, *Cnaphalocrocis medinalis* and *Marasmia patnalis*. Curr Microbiol 41:276–283

Lin YJ, Zhang Q (2005) Optimizing the tissue culture conditions for high efficiency transformation of indica rice. Plant Cell Rep 23:540–547

Liu KD, Wang J, Li HB, Xu CG, Zhang QF (1997) A genome-wide analysis of wide-compatibility in rice and the precise location of the S_5 locus in the molecular map. Theor Appl Genet 95:809–814

Maqbool SB, Husnain T, Raizuddin S, Christou P (1998) Effective control of yellow rice stem borer and rice leaf folder in transgenic rice indica varieties Basmati 370 and M 7 using novel δ-endotoxin cry2A *Bacillus thuringiensis* gene. Mol Breed 4:501–507

Maqbool SB, Raizuddin S, Loc TN, Gatehouse AMR, Gatehouse JA, Christou P (2001) Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. Mol Breed 7:85–93

McGaughey WH (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229:193–195

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1996) Transgenic elite *indica* plants expressing *cryA(c)* δ-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc Natl Acad Sci USA 94:2111–2116

Pathak MD (1977) Insect pests of rice. The International Rice Research Institute, Manila, Philippines, pp 1–15

Ramesh S, Nagadhara D, Pasalu IC, Kumari AP, Sarma NP, Reddy VD, Rao KV (2004) Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. J Biotechnol 111:131–141

Roush RT (1998) Two-toxin strategies for management of insect resistant transgenic crops: can pyramiding succeed where pesticide mixtures have not?. Philos Trans Proc R Soc London Ser B 353:1777–1786

Sayyed AH, Haward R, Herrero S, Ferré J, Wright DJ (2000) Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl Environ Microbiol 66:1509–1516

Shelton AM, Zhao JZ, Roush RT (2002) Economic, ecological, food, safety and social consequence of the deployment of Bt transgenic plants. Annu Rev Entomol 47:845–881

Stewart SD, Adamczyk JJ Jr, Knighten KS, Advis FM (2001) Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of noctuid (Lepidoptera) larvae. J Econ Entomol 94:752–760

Strizhov N, Keller M, Mathr J, Koncz KZ, Bosch D, Prdovsky E, Schell J, Sneh B, Koncz C, Zilberstein A (1996) A synthetic *cryIC* gene, encoding a *Bacillus thuringiensis* delta-endotoxin, confers Spodoptera resistance in alfalfa and tobacco. Proc Natl Acad Sci USA 93:15012–15017

Tabashnik BE, Finson N, Groeters FR, Moar WJ, Johnson MW, Luo K, Adang MJ (1994) Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc Natl Acad Sci USA 91:4120–4124

Tabashnik BE, Carri Y, Dennehy TJ, Morin S, Sisterson MS, Roush RT, Shelton AM, Zhao JZ (2003) Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J Econ Entomol 96:1031–1038

Thompson CJ, Movva NR, Crameri R, Davvies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *Bar* from *streptomyces hygroscopicus*. EMBO J 6:2519–2523

Tian YC, Zheng JB, Yu HM, Liang HY, Li CQ, Wang JM (2000) Studies of transgenic hybrid poplar 741 carrying two insect-resistant genes. Acta Bot Sinica 42:263–268

Tu JM, Zhang GA, Data K, Xu CG, He YQ, Zhang QF, Khush GS, Datta SK (2000) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. Nat Biotechnol 18:1101–1104

Vaeck M, Reynaerts A, Höfte H, Jansens S, Beukeleer MD, Dean C, Zabeau M, Montagu MV, Leemans J (1987) Transgenic plants protected from insect attack. Nature 328:33–37

Wang Z, Shu Q, Ye G, Cui H, Wu D, Altosaar I, Xia Y (2002) Genetic analysis of resistance of Bt rice to stripe stem borer (*Chilo suppressalis*). Euphytica 123:379–386

Wu C, Fan Y, Zhang C, Oliva N, Datta SK (1997) Transgenic fertile *japonica* rice plants expressing a modified *cry1A(b)* gene resistant to yellow stem borer. Plant Cell Rep 17:129–132

Wünn J, Kloti A, Burkhardt PK, Biswas GCG, Launis K, Iglesias VA, Potrykus I (1996) Transgenic indica rice breeding line IR58 expressing a synthetic *cryA(b)* gene from *Bacillus thuringiensis* provides effective insect pest control. Biotechnology 14:171–176

Ye GY, Sh QY, Yao HW, Cui HR, Cheng XY, Hu C, Xia YW, Gao MW, Altosaar I (2001) Field evaluation of resistance of transgenic rice containing a synthetic *cry1Ab* gene from *Bacillus thuringiensis* Berliner to two stem borers. J Econ Entomol 94:271–276

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21:1493–1487