African Journal of Biotechnology Vol. 11(31), pp. 7812-7818, 17 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.119
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Segregation and expression of transgenes in the progenies of *Bt* transgenic rice crossed to conventional rice varieties

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Accepted 20 February, 2012

β-Glucuronidase (GUS) activity bioassay, western blotting and polymerase chain reaction (PCR) analysis demonstrated that the *cry1Ab* gene was closely inherited and expressed with reporter gene *gus* in the progenies of *Bacillus thuringiensis* (*Bt*) transgenic rice (*Oryza sativa* L.) crossed to conventional rice varieties. Therefore, it is feasible using GUS-assisted-selection to preliminarily identify the *Bt* gene and study the inheritance of transgenes in breeding program. Mendelian segregation was observed in BC_1F_1, BC_1F_2 and F_2 populations derived from *Bt* rice crossed to japonica rice respectively which indicated that the *cry1Ab* gene was inherited as a single dominant locus. PCR, Southern blotting and Western dot blotting analysis confirmed that *cry1Ab* gene was transferred to the genome of conventional rice varieties and it was highly expressed in the different progenies of *Bt* rice crossed to conventional rice varieties. Among these lines, the highest *Bt* toxin protein content reached 2.88% of total soluble proteins, even though the amount of *Bt* toxin protein in leaves of some GUS positive plants was higher than that detected in the original *Bt* rice. Meanwhile, the variances in *Bt* toxin protein between crosses and its parents were significant at 0.05 or 0.01 levels, respectively. Therefore, foreign *cry1Ab* gene with native insect resistant trait can be easily transferred to other rice varieties with higher yield potential and good quality through classical breeding.

Key words: *Oryza sativa* L., transgenes, inheritance, expression.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crop plants worldwide, especially in Asia (IRRI, 1989). Currently, more than 200 million tons of rice is lost per year due to insect pests. The most destructive insects are the lepidopterous stem borers, which cause annual losses of an estimated 10 million tons (Herdt, 1991). An attractive method for protection is the production of proteins with insecticidal activity by the rice plant itself. The entomocidal spore-forming soil bacterium *Bacillus thuringiensis* (*Bt*) offers a promising variety of so-called *cry* genes that encode insect-specific δ-endotoxins. Since late 1980s, these *cry* genes have been transferred to higher plants including tobacco, tomato and cotton (Vaeck et al., 1987; Fischhoff et al., 1987; Perlak et al., 1990), resulting in insect-resistant plants.

In addition, monocotyledonous plants such as maize (Koziel et al., 1993; Armstrong et al., 1995) and rice (Fujimoto et al., 1993; Wunn et al., 1996; Ghareyazie et al., 1997; Nayak et al., 1997; Wu et al., 1997; Cheng et al., 1998; Tu et al., 1998, 2000; Maqbool and Christou, 1999; Zhu et al., 1999) have also been successfully transformed with these genes. The former Zhejiang Agricultural University has suc-ceeded in obtaining many transgenic rice plants via *Agrobacterium*-mediated transformation through close co-operation with the University of Ottawa, Canada in 1994. Biological assays by polymerase chain reaction (PCR) and Southern hybridization confirmed that *cry1Ab* gene is successfully integrated into the rice genome (Xiang et al., 1999;

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Abbreviations: *Bt*, *Bacillus thuringiensis*; **GUS,** β-glucuronidase; **PCR,** polymerase chain reaction.

Shu et al., 2000). Insect bioassays in both laboratory and field conditions showed that the transgenic plants were highly resistant to eight lepido-pteran rice pest species (Shu et al., 1998, 2000).

The introduction and expression of foreign genes in plants by genetic transformation is now routine for many species, including rice, cotton, maize, wheat and soybean (Christou, et al., 1989), but the segregation and expression of transgenes in rice hybrid plants have rarely been reported. Here, we report the inheritance and expression of transgenes in the progenies of *Bt* transgenic rice crossed to conventional rice varieties.

MATERIALS AND METHODS

Two homozygous transgenic lines, KMD1 and KMD2, derived from a commercial Chinese japonica rice variety (Xiushui 11) transformed by *Agrobacterium* infection (Cheng et al., 1998; Xiang et al., 1999) were provided by Dr. Dianxing Wu from Zhejiang University. They contained a synthetic *cry1Ab* gene from *Bt* under the control of a maize *ubiquitin* promoter, and linked in tandem with *gusA* and *hpt* genes (Xiang et al., 1999). KMD1 comes from primary R_0 transformant TR30 and KMD2 from GS5, which is independent on TR30 (Shu et al., 1998; 2000). Although KMD1 and KMD2 were only at R_3 and R_2 generation, respectivel when the crosses were made with conventional varieties, they were already homozygous for *cry1Ab* according to the results of PCR analysis (Shu et al., 1998, 2000).

Representative commercial rice materials susceptible to lepidopteran were selected for this research. The first group comprises 3 conventional indica rice varieties: Zhe 733, Yongxian 57 and Jiayu 293, which are cultivated as early season crop varieties in Zhejiang and neighboring provinces of China. The second group includes 3 conventional japonica varieties: Zhejing 22, Yongjing 18 and Ning 04-81; these are widely planted as single or late season crop varieties in Zhejiang and Jiangsu Province of China. While the third group is represented by one indica cytoplasmic male sterility (CMS) restorer lines, Miyang 46, and one indica CMS maintainer lines, Longtefu B. Miyang 46 was developed by breeders of Republic of Korea and has been widely used as a commercial indica restorer line in hybrid rice production in China since late 1980s, while Longtefu B has been used as a commercial maintainer line in southern China since the early 1990s. Seeds of the above mentioned rice varieties were kindly provided by Ms. Juanying Han at the Seeds Administration Station of Yuyao County, Zhejiang Province, China.

In the autumn of 2009, KMD1 and KMD2 lines were crossed to the above non-transgenic susceptible rice varieties or lines in the paddy field of Zhejiang Wanli University. In all the crosses, transgenic parents were used as pollen donor. A part of the F_1 seeds were sent to Hainnan Province, China, and true F_1 seedlings identified by β-glucuronidase (GUS) assay were transplanted to rice fields together with non-transgenic parents in our winter breeding nursery in the winter of 2009. In the spring of 2010, back-crosses were made by using non-transgenic parents as pollen donor. Meanwhile, F_2 seeds were obtained from F_1 plants.

GUS staining assay

Histochemical GUS assays were performed as described by Rueb and Hensgens (1989) with some modifications. Leaf tissue of rice plants were incubated in X-Gluc staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5% Triton X-100, 20% methanol and 100 mM phosphate buffer (pH 7.0). The leaves were pretreated in 100 mM phosphate buffer (pH 7.0) at 37°C for 1 to 2 h, and then incubated in X-Gluc staining solution at 37°C for more than 6 h or overnight. Finally, the leaves were washed with ddH_2O and put in 95% ethanol for 2 to 3 h. The leaf with GUS expression displays blue color.

PCR analysis

Genomic DNAs were mini-prepared following the method of Steiner et al. (1995). For identifying *cry1Ab* gene in the progenies of *Bt* rice crossed to conventional rice varieties, one pair of specific primers were designed and synthesized. The sequences of the primers were as follows: forward primer 5′-TTCCTTGGACGAAATCCCACC-3′, reverse primer 5’-GCCAGAATTGAACACATGAGCGC-3′. The putative PCR product for *cry1Ab* gene was 559 base pairs (bp). First, DNA was subjected to 1 cycle with 94°C for 4 min (initial denaturation), then 35 cycles of three steps each (94°C, 30 s; 54°C, 1 min; 72°C, 90 s) in 25 µL of PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.0 mM $MgCl_2$) containing 0.1 mM of each dNTPs, 50 to 100 ng primers and 1 U of Taq polymerase, and finally to 1 cycle with 72°C for 7 min (maintaining temperature). PCR products were then analyzed by agarose gel electrophoresis.

Southern blot

Leaves of 8 rice plants from KMD1, F_2 GUS positive plants of KMD1 crossed to Zhefu 504, together with Zhefu 504 control, were randomly chosen at the tillering stage respectively and were ground to fine powder using liquid nitrogen. DNA isolation was performed as described by Lu and Zheng (1992). About 15 µg of rice genomic DNAs was digested with Hind III, the DNA fragments were subsequently separated overnight on a 0.8% agarose gel and transferred to Hybond N^+ nylon membrane following the method described by Sambrook et al. (1989). The 559 bp DNA fragment amplified with one pair of primers specific for the *cry1Ab* gene was used as hybridization probe. Probe labeling, membrane prehybridization and hybridization were carried out according to the hybridization kit instruction supplied by Amersham Pharmacia Biotech Company. After hybridization, the membrane was seal with a plastic sheet and exposed to X-ray film.

Assay for Cry1Ab protein

Leaves of GUS positive plants at tillering stage were collected and ground in liquid nitrogen. About 0.4 g of above powder was transferred to 1.5 ml Eppendorf tubes with 400 to 600 µL extraction buffer (50 mM Na_2CO_3, 10 mM DTT). After homogenizing, the tubes were kept at 4°C for 2 to 3 h and then 1 µL crude extract was applied to nitrocellulose membrane and subjected to Western dot blotting by using a polyclonal goat antibody specific for Cry1Ab essentially as described by Sardana et al. (1996). Total protein concentration was measured by using bicinchoninic acid (BCA) protein assay reagents.

RESULTS

Linkage analysis of reporter gene (gus) and cry1Ab gene

Previous studies have proved that the reporter gene (*gus*) was linked closely with *cry1Ab* gene in T-DNA of *Bt*

Table 1. *cry1Ab* expression in GUS positive plants in the progenies of *Bt* transgenic rice crossed to conventional rice varieties.

Generation	Number of combination	Number of GUS positive plant	Number of *cry1Ab* gene expressing
F_1	4	74	74 (100%)
F_2	7	166	164 (98.8%)
BC_1F_1	10	86	86 (100%)
BC_2	7	68	68 (100%)
Total	28	394	392 (99.5%)

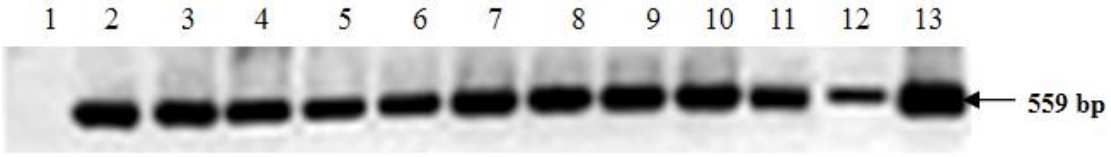

Figure 1. Identification of the *cry1Ab* gene in F_2 GUS positive plants derived from *Bt* transgenic line KMD1 crossed to indica rice variety Zhe 733. The DNA templates for PCR were the parental lines Zhe 733 (Lane 1), KMD1 (Lane 2) and F_2 plants (Lanes 3 to 13). The size of fragment is shown at the right.

transgenic rice (Shu et al., 1998; Xiang et al., 1999; Wu et al., 2000). After *Bt* transgenic rice was crossed or backcrossed to conventional rice varieties, it was found that this relationship still stayed (Table 1). The result demonstrates that the *cry1Ab* gene was closely inherited and co-expressed with the reporter gene *gus*. To identify the presence of *cry1Ab* gene in the GUS positive plants of the progenies of *Bt* transgenic rice crossed to conventional rice varieties, PCR analysis was conducted with specific DNA primers. The expected 559 bp size fragment was amplified from all random F_2 positive plants derived from Zhe 733×KMD1 (Figure 1). Southern blot of Hind III digested DNA randomly chosen from F_2 GUS positive plants of KMD1 crossed to Zhe 733 were performed. The expected 4.1 kb DNA fragment consisting of the *ubiquitin* promoter, the *cry1Ab* gene and the *nos* terminator was detected. The result demonstrates that the *cry1Ab* gene was indeed transferred to the progenies via sexual reproduction and the expression unit was kept intact in the successive generations (Figure 2).

The results obtained herein confirmed that *cry1Ab* gene was evidently presented in the GUS-positive progenies of *Bt* transgenic rice crossed to conventional rice varieties. Meanwhile, any issue related with the ecological risks and food bio-safety of *gus* gene and its expression product in transgenic modified plants were not found (Wang and Xia, 2000). Therefore, it is practicable using GUS-assisted-selection to preliminarily identify the *Bt* gene and study the inheritance of transgenes in back-cross breeding program.

Segregation of transgenes

Segregation ratio of GUS positive and negative plants in the progenies of *Bt* transgenic rice crossed to conventional rice varieties was investigated by GUS histochemical assay. Results indicate that the segregation ratio of reporter gene *gus* also showed the inheritance pattern of *cry1Ab* gene. Mendelian segregation of transgenes was observed in BC_1F_1 and BC_1F_2 populations of *Bt* transgenic rice backcrossed to conventional rice varieties respectively (Table 2). The result suggested that the *cry1Ab* gene of *Bt* transgenic rice was inherited as a single, dominant locus. However, it was found that segregation ratio in the F_2 population of *Bt* transgenic rice crossed to early season indica rice varieties deviated from the expected 3:1 ratio (χ^2 >3.84) (Table 2).

Cry1Ab protein assay

Cry1Ab insecticidal protein expression level in the different progenies of *Bt* transgenic rice crosses to conventional rice varieties was analyzed by Western dot blotting. The samples derived from GUS positive plants of *Bt* transgenic rice crossed to conventional rice varieties were found to produce a higher level of toxin protein, but with greater range of variation. The maximum level is up to 2.88% SP (Table 3). As seen in Table 3, Cry1Ab toxin protein in all hybrid lines was expressed higher than in *Bt* transgenic rice lines KMD1 and KMD2. In addition, it was also found that the variation of *Bt* toxin protein between F_1 plants derived Zhe 733×KMD1 or Zhejing 22×KMD1 and *Bt* transgenic rice control is highly significant at 0.01 level.

DISCUSSION

Typically, transgenes with a single copy usually segregate

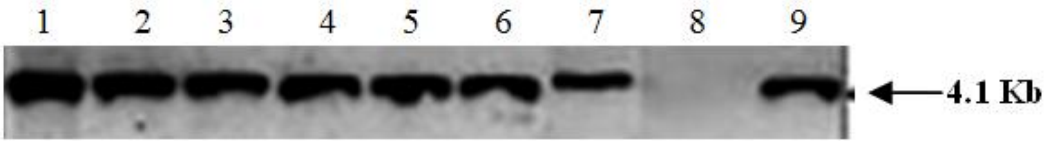

Figure 2. Southern blot of Hind III-digested DNA from *Bt* transgenic rice line KMD1 and F_2 GUS positive plants of KMD1 crossed to indica rice variety Zhe 733. Zhe 733 (Lane 8); *Bt* transgenic rice KMD1 (Lane 9); F_2 plants (Lanes 1 to 7). The size of fragment was estimated using marker ladder as shown on the left.

Table 2. Segregation ratio of GUS positive and negative plants in the progenies of *Bt* transgenic rice crossed to conventional rice varieties.

Combination	Generation	Tested Plant	GUS positive plant	GUS negative plant	GUS^+/GUS^-	χ^{2*}
Z733/KMD1	BC_1F_1	116	57	59	0.97:1	0.008 (1:1)
	F_2	85	53	32	1.66:1	6.251 (3:1)
	BC_1F_2	105	79	26	3.04:1	0.003 (3:1)
YX57/KMD1	BC_1F_1	6	3	3	1:1	0.166 (1:1)
	F_2	176	105	71	1.48:1	17.212 (3:1)
JY293/KMD1	BC_1F_1	23	11	12	0.92:1	0.000 (1:1)
	F_2	133	67	66	1.01:1	42.040 (3:1)
	BC_1F_2	63	47	16	2.71:1	0.005 (3:1)
LTH/KMD1	BC_1F_1	41	21	20	1.05:1	0.000 (1:1)
MY46/KMD1	BC_1F_1	43	21	22	0.95:1	0.000 (1:1)
ZJ22/KMD1	F_2	190	135	55	2.45:1	1.718 (3:1)
Z733/KMD2	BC_1F_1	112	56	56	1:1	0.009 (1:1)
	F_2	125	77	48	1.60:1	8.838 (3:1)
	BC_1F_2	80	59	21	2.81:1	0.067 (3:1)
JY293/KMD2	F_2	146	90	56	1.61:1	13.891 (3:1)
YJ18/KMD2	BC_1F_1	17	8	9	0.89:1	0.000 (1:1)
	F_2	140	94	46	2.04:1	4.609 (3:1)
N0481/KMD2	F_2	185	139	46	3.02:1	0.001 (3:1)

Z733, Zhe 733; YX57, Yongxian 57; JY293, Jiayu 293; LTH, Lontefu B; MY46, Miyang 46; ZJ22, Zhejing 22; YJ18, Yongjing 18; N0481, Ning 04-81; $*\chi^2_{(0.05)}=3.84$

with 3:1 ratio in the self- population (Christous et al., 1989; Datta et al., 1990; Ulian et al., 1994; Peng et al., 1995), and 1:1 in the backcross population (Hiei et al., 1994; Casas et al., 1995; Peng et al., 1995; Dillen et al., 1997). In the present study, Mendelian segregation of reporter gene was also observed in F_2, BC_1F_1 and BC_1F_2 populations derived from *Bt* transgenic rice lines KMD1 and KMD2 crossed to conventional rice varieties, which indicates that the *cry1Ab* gene was inherited as a single, dominant trait. On the other hand, it also shows that T-DNA was integrated as a single locus into rice genome.

However, non-Mendelian segregation of transgenes has been reported in previous studies on transgenic rice (Datta et al., 1990; Peng et al., 1995) and other transgenic crops such as soybean (Christou et al., 1989), wheat (Srivastava et al., 1996) and cotton (Sachs et al., 1998). Some mechanisms responsible for this phenol-menon, including the lower viability of transgenic pollen, lower fertilization ability (Zhang et al., 1996), transgene inactivation (Spencer et al., 1992; Walters et al., 1992),

Table 3. Cry1Ab insecticidal protein concentration [as percent of total soluble protein (%SP)] in young terminal leaves of different hybrid lines derived from *Bt* transgenic rice crossed or backcrossed to conventional rice varieties at tillering stage.

Combination	Generation	Plant tested	Bt protein (%SP)*	The difference between hybrid lines and Bt rice
Z733/KMD1	F_1	30	1.04 ± 0.54 (0.48-2.52)	0.53***
	F_2	12	1.29 ± 0.55 (0.41-2.05)	0.78***
	BC_1F_1	10	0.98 ± 0.31 (0.51-1.57)	0.47***
YX57/KMD1	F_2	50	0.64 ± 0.60 (0.12-2.88)	0.13
	BC_1F_1	3	1.07 ± 0.08 (1.02-1.17)	0.56***
JY293/KMD1	BC_1F_1	4	0.70 ± 0.24 (0.52-1.04)	0.19
LTH/KMD1	BC_1F_1	11	0.90 ± 0.27 (0.52-1.37)	0.39**
MY46/KMD1	BC_1F_1	21	0.99 ± 0.45 (0.20-1.80)	0.48***
ZJ22/KMD1	F_1	24	0.85 ± 0.40 (0.34-1.48)	0.34**
Z733/KMD2	F_2	10	1.32 ± 0.30 (0.69-1.85)	0.91***
	BC_1F_1	9	0.64 ± 0.20 (0.36-0.99)	0.23
JY293/KMD2	BC_1F_1	5	1.38 ± 0.44 (1.02-2.04)	0.97***
N0481/KMD2	F_2	46	0.80 ± 0.38 (0.15-2.35)	0.39**
KMD1	R_3	30	0.51 ± 0.29 (0.20-1.48)	-
KMD2	R_2	10	0.41 ± 0.19 (0.11-1.09)	-
Z733	-	10	0.00	-
YX57	-	10	0.00	-
JY293	-	10	0.00	-
LTH	-	10	0.00	-
MY46	-	10	0.00	-
ZJ22	-	10	0.00	-
YJ18	-	10	0.00	-
N0481	-	10	0.00	-

Z733, Zhe 733; YX57, Yongxian 57; JY293, Jiayu 293; LTH, Lontefu B; MY46, Miyang 46; ZJ22, Zhejing 22; YJ18, Yongjing 18; N0481, Ning 04-81.
*Percentage of Bt protein to total soluble protein, Mean ± SE (change range); ***significant at 0.05 level; *****significant at the 0.01 level

or the recessive lethal (Scott et al., 1998) were proposed in recent years. Christou et al. (1989) and Srivastava et al. (1996) proposed that the failure of passing the transgene to the next generation through pollen (pollen lethality) is the reason for abnormal segregation. Moreover, Sachs et al. (1998) attributed abnormal phenotypic ratios in F_2 progeny derived from MON 249 to poor germination and survival rate resultant from foreign gene insertion and/or somaclonal effects. In this experiment, it was found that the segregation ratio of F_2 population derived from *Bt* transgenic rice crossed to early season indica rice varieties deviated from Mendelian law (Table 2). The irregular ratios in F_2 population of indica/japonica crosses may result from pollen sterility of F_1 hybrid plants. High pollen sterility of F_1 plants is a common phenomenon in interspecies crosses between indica and japonica rice, and abnormal segregation has been observed for molecular markers in resultant F_2 populations (Xu et al., 1995, 1997; Zhuang et al., 1999). This was also supported by Zhuang et al. (1999) who indicated that the high sterility of pollens is the main factor leading to the distortion of molecular markers in F_2 population of indica-japonica crosses.

Previous reports on the expression of *Bt* gene in rice only showed original transgenic plants (Fujimoto et al., 1993; Wunn et al., 1996; Ghareyazie et al., 1997; Nayak et al., 1997; Cheng et al., 1998; Tu et al., 1998, 2000; Maqbool and Christou, 1999; Xiang et al., 1999; Wu et al., 2002). In this study, it was found that *Bt* gene can also be highly expressed in hybrid rice plants derived from transgenic rice crossed to conventional rice varieties. It was also found that Cry1Ab toxin protein concentration (%SP) in F_1 populations derived from *Bt* transgenic rice crossed to early season indica variety or japonica variety was higher significantly than in original *Bt* transgenic rice ($P< 0.01$) (Table 3). This might be related to hybrid vigor

of plants. Previous studies showed genetic background affected the expression of transgenes. Sachs et al. (1998) reported that genetic background greatly impacted the content of Cry1Ab toxin protein in F_2 population derived from *Bt* transgenic cotton crossed to conventional insect-resistant cotton isoline. The Cry1Ab protein concentration (%SP) was 19% lower in the C312×ST213 background than that in the C312×CAMD-E and C312×DP61 background. In the present experiment, it was found that the Cry1Ab protein concentration (%SP) was 22.3% higher in the Zhe 733×KMD1 than in the Zhejing 22×KMD1 background.

In addition, our results indicated that the average Cry1Ab content of F_1, GUS positive BC_1F_1 and F_2 plants is higher than that of KMD1 and KMD2 in most crosses, although difference among crosses exists. Plant heterosis and genotypic effects may in part explain the Cry1Ab content increase and difference among crosses; other mechanisms for this phenomenon may also exist. Based on these results, it is reasonable to conclude that KMD1 and KMD2 could be used as an exploitable source of striped stem borer (SSB) resistance in various rice breeding programs, including japonica, indica and hybrid rice. When establishing a breeding program using KMD1 or KMD2 as SSB resistance donor, one should consider the segregation difference of *cry1Ab* gene.

Genetic engineering is a powerful tool for crops improvement, and enables plant breeders to utilize genes from other species, which otherwise is impossible. Once a gene is introduced into rice plants, conventional breeding techniques should be as effective as for other rice own genes. Therefore, genetic transformation is not always necessary, and sometimes not always capable or available in developing countries to introduce genes into each variety of crops. Plant breeders should be able to develop new varieties by using transgenic plants as donors. In this case, studies on phenotypic performance of progenies from crosses between transgenic and non-transgenic plants are of importance in breeding programs. A previous study showed that no significant differences in main agronomic traits, such as plant height, panicle length, the number of tillers per plant, days to heading and 1000-grain weight, were found between the two classes of the GUS-positive plant and the GUS-negative plant in the progenies of *Bt* transgenic rice lines KMD1 and KMD2 crossed to conventional rice varieties (Cui et al., 2001).

In conclusion, therefore, the foreign *cry1Ab* gene with native insect resistant trait in a single genetic background can be successfully accomplished by a traditional cross or backcross breeding program.

ACKNOWLEDGEMENTS

A part of this research was financially supported by grants from Ministry of Science and Technology, China, and the Science and Technology Department, Zhejiang Province, China. For critical reading we thank Dr. Yulin Jia of Dale Bumpers National Rice Research Center, USDA-ARS, United States of America.

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