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Pyramiding of insect- and disease-resistance genes into an elite *indica*, cytoplasm male sterile restorer line of rice, 'Minghui 63'

G. H. JIANG[1], C. G. XU[1], J. M. TU[1,2], X. H. LI[1], Y. Q. HE[1,3] and Q. F. ZHANG[1]

[1] National Key Laboratory of Crop Genetic Improvement, National Center of Crop Molecular Breeding, Huazhong Agricultural University, Wuhan, 430070, China; [2] Present address: Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong; [3] Corresponding author, E-mail: yqhe@mail.hzau.edu.cn

With 1 figure and 6 tables

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Abstract

The wild-rice-derived dominant gene *Xa21* conferring multi-race resistance to bacterial blight and a fused *Bt* gene *cry1Ab/cry1Ac* conferring resistance to lepidopteran insects were individually introduced into the same genetic background of an elite *indica* cytoplasm male sterile (CMS) restorer line 'Minghui 63'. The line showed the desirable insect- and disease-resistant phenotypes. To maximize the effect, the two genes were also pyramided into the same recipient plant of 'Minghui 63' by marker-assisted selection. After being subjected to natural infestation of leaf-folders and yellow stem borers and inoculation of *Xoo* strain mixtures, the pyramiding line and its derived hybrids showed high levels of resistance against both insect damage and disease. Furthermore, data from field trials demonstrated that the hybrids made by crossing this pyramiding line with the CMS lines 'Zhenshan 97A' and 'Maxie A' retained a similar level of yield under conditions without chemical spray, indicating that the pyramiding genes have a yield-stabilizing effect on the recipient line and its hybrids.

Key words: *Oryza sativa* — hybrid rice — gene pyramiding — marker-assisted selection — *Bt* — *Xa21*

Hybrid rice with a 20% yield advantage over inbred lines has played a key role in food availability in China for more than two decades (Yuan 1996). Hybrid rice has now became a commercial success in many Asian countries, and an estimated six million hectares of extra production would be required if hybrid rice had not been developed. The incorporation of resistance genes in a restorer line makes it widely applicable for development of resistant rice hybrids (Duvick 1999).

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive bacterial diseases of rice, affecting production in irrigated and rain-fed lowland ecosystems throughout the world (Mew 1987). In susceptible varieties, *Xoo* enters through the hydathodes, multiplies in the epithelium, and moves to the xylem vessels, where the infection becomes systemic so that chemical control is difficult (Ronald 1997). Rice yield losses caused by BB in some areas of Asia are reportedly up to 50% (Adhikari et al. 1995). The use of resistant cultivars is the most economical and effective method of controlling this disease (Ogawa 1993).

Infestation by stem borers is another serious problem for both inbred and hybrid rice. It can cause high annual yield losses, with occasional outbreaks of up to 95% (Herdt 1991, Pathak and Khan 1994, Teng and Revilla 1996). Chemical control is also difficult because stem borer larvae remain on the outer surface of the rice plant for only a short time before penetrating the stem (Teng and Revilla 1996).

In an effort to breed resistance to both disease and insect pests, a wild-rice-derived dominant gene *Xa21* conferring multi-race resistance against BB and a fused *Bt* gene *cry1Ab/cry1Ac* conferring resistance to lepidopteran insects have been individually introduced into the same genetic background of an elite *indica* cytoplasm male sterile (CMS) restorer line 'Minghui 63' by means of marker-assisted selection (MAS) (Chen et al. 2000) and by genetic engineering (Tu et al. 2000), respectively. The resulting lines and their hybrids showed high resistance to both disease and insect pests without reduced yield in field tests (Chen et al. 2000, Tu et al. 2000). These disease- and insect-resistant genes, however, were still not integrated into the same target plant of 'Minghui 63', thereby restricting their maximum utilization in rice production to some extent.

Gene pyramiding is a very useful approach to maximize utilization of existing gene resources. Genes controlling different agronomic traits can be pyramided together to ensure that a variety may simultaneously acquire several traits, such as high iron, high lysine, and suitable amylose content. Furthermore, genes leading to different races or biotypes being resistant to a disease or insect pest can be also pyramided together to make a line with multi-race or multi-biotype resistances. Theoretically, these multi-race or multi-biotype resistances should be more durable than any single-race or single-biotype resistance. Gene pyramiding has been successfully applied in several crop breeding programmes, and many varieties and lines possessing multiple attributes have been produced (Huang et al. 1997, Porter et al. 2000, Wang et al. 2001, Samis et al. 2002). The joint expression of pyramided genes was even found to provide numerical increases or a broader spectrum of resistance over that conferred by single genes through gene interaction and quantitative complementation (Yoshimura et al. 1995, Singh et al. 2001).

In this study, gene pyramiding was used to combine the fused *Bt* insecticidal gene and the dominant BB resistance gene *Xa21* into the same target plant of an elite *indica* CMS restorer line 'Minghui 63' using MAS.

Materials and Methods

Plant materials: The lines of rice, *Oryza sativa* L., in this study were 'Minghui 63'/*Xa21*, and 'Minghui 63'/*Bt*. The former was bred from a recurrent backcross between 'IRBB21' as donor line and 'Minghui 63' as recurrent parent via MAS (Chen et al. 2000). The latter is a marker-free transgenic line obtained by biolistic transformation (Tu et al. 2000).

A cross between 'Minghui 63'/*Bt* and 'Minghui 63'/*Xa21* was performed in the spring of 1999 in Hainan. The F_1 seeds obtained were sown in the normal rice-growing season of the same year in the experimental farm at Huazhong Agricultural University, Wuhan, China. All F_3 families homozygous for both genes were selected on the basis of molecular analysis results and resistance phenotypes. During the spring of 2001 in Hainan, the best homozygous line was then used in crosses with the CMS lines 'Zhenshan 97A' and 'Maxie A' to produce double-resistant hybrids. The field trials of the lines homozygous for both genes and of their hybrids were carried out in the normal season of the same year in Wuhan. The tested lines and the hybrids were planted in a randomized complete block design with two replications. In each block, 24 plants for each line or hybrid were planted in a two-row plot with a 17 cm distance between plants within a row and 27 cm between rows. 'Minghui 63', 'Minghui 63'/*Bt*, and 'Minghui 63'/*Xa21* and their hybrids were used as susceptible or single-gene resistance controls. The central five plants in a row in each plot were examined for agronomic traits. Normal cultural practices for growing rice were followed during the course of the experiments, except that no chemical spray was applied after transplanting to allow for an objective evaluation of the resistance reaction of the test materials.

PCR analysis: Two pairs of primers were used for PCR analysis. Primers XAF 5′-AGACGCGGAAGGGTGGTTCCCGGA-3′ and XAR 5′-AGACGCGGTAATCGAAA GATGAAA-3′ were designed based on the published sequence (Chunwongse et al. 1993), and the amplified fragment cosegregating with the *Xa21* gene was used for detection of the *Xa21* gene. Primers BTF 5′-TTCAGCCTCGAG TGTTGCAG-3′ and BTR 5′-ATGGACAA CAACTGCAGG-3′ were designed according to sequence information and used for detection of the *Bt* gene. The amplification products derived from the former primer pair are codominant, whereas those from the latter pair are dominant. The PCR analysis was conducted according to the methods of Williams et al. (1996).

***Xoo* races, inoculum preparation, inoculation, and disease scoring:** A mixture of five *Xoo* strains from different races (see Table 1) was used to inoculate test materials. Each of these five strains had been confirmed to be highly virulent to the original 'Minghui 63' without *Xa21* (Chen et al. 2000). Four were collected in China, and another from the Philippines and provided by Dr T. Mew (EPPD, IRRI, Manila, Philippines). The methods of inoculum preparation and inoculation were the same as described previously by Lin et al. (1996). Disease scoring followed that used by Chen et al. (2000). Inoculation was conducted at the booting stage and the lesion length was scored 21 days later. Three leaves per plant and 10 plants per plot were scored.

Insect infestation: Plants were naturally infested with leaf-folders and yellow stem borers. Plant reaction to the natural infestation of leaf-folder was scored 5–7 days after peak damage appeared. Leaves were scored as damaged (visible scrapes). Similarly, plant reaction to the natural infestation of yellow stem borers was scored 7–10 days after dead-heart or white-head symptoms appeared. A plant with one dead-heart or white-head was scored as damaged. Finally, theses scores were used to calculate the percentages of the plants damaged.

Table 1: The sources of five *Xoo* races used in the experiment

Strain	Source	Race
HB17	Heibei, China	2
LN44	Liaoning, China	1
ZJ173	Zhejiang, China	4
PXO99	Philippines	6
GD1358	Guangdong, China	5

Results

Production of a pyramiding line homozygous for both *Bt* and *Xa21* genes

Fourteen F_1 and 12 'Minghui 63' control plants were challenged with manual inoculation of *Xoo* strain mixtures and natural infestation of yellow stem borers. The average lesion length in the F_1 plants was < 6.0 cm with no white-head symptoms, whereas that in the control plants was four times longer with at least one tiller showing white-head (Table 2). These results indicate that the pyramiding genes in the background of the recipient 'Minghui 63' genome functioned as expected.

In the F_2 generation, 473 plants were grown and the two pyramiding genes were traced by PCR analysis. Of them, 106 plants were identified as homozygous for the *Xa21* gene, as the codominant fragment marking its recessive allele was not detected in these plants (Fig. 1a). A chi-square test confirmed that the segregation of *Xa21* homozygotes (106), heterozygotes (234) and the non-*Xa21* homozygotes (133) fitted the expected 1 : 2 : 1 Mendelian ratio ($\chi^2 = 2.9017$, P = 0.2085). These 106 plants were further analysed by *Bt*-primer, and 69 of them were found to be positive for the target gene. The homozygous status, however, could not be confirmed in this F_2 generation because there is no detectable codominant allele present in the recipient genome of 'Minghui 63'.

To obtain the line homozygous for both pyramiding genes, F_3 families from each of the 69 selected F_2 plants were grown; the families homozygous for the pyramided *Bt* gene were identified using both PCR analysis and phenotyping. Twenty-one of the 69 F_3 families were positive for both *Bt* and *Xa21* genes without any segregation (Fig. 1b), thus indicating that the expected homozygous lines had been obtained. For further tests, the seeds from one of these 21 lines with the best agronomic performance were then harvested and plants from these seeds were used to produce the hybrids by crossing them with two CMS lines, 'Zhenshan 97A' and 'Maxie A', for further analyses.

Field evaluation

The pyramided line 'Minghui 63'/*Bt & Xa21* and its derived hybrids were exposed to natural insect infestation and artificial inoculation of *Xoo* strain mixtures. The symptoms caused by either stem borers or leaf-folders were not observed on the pyramiding line 'Minghui 63'/*Bt & Xa21* in both the testing years of 2000 and 2001. Similarly, the *Bt* donor line 'Minghui 63'/*Bt* had a percentage of white-head and foliage-folded plants of $< 3.1\%$ (Table 3). The resistance performance observed in the non-*Bt* donor line 'Minghui 63'/*Xa21* and negative control 'Minghui 63', however, were much poorer, with each line showing white-head symptoms or folded leaves in at least 77.1% of the plants (Table 3).

The lesion lengths of BB in the pyramiding line 'Minghui 63'/*Bt & Xa21* and in 'Minghui 63'/*Xa21* were all < 5.7 cm in

Table 2: The resistance of F_1 *Bt*-'Minghui 63'/*Xa21*-'Minghui 63' plants to strain mixtures of PXO99 and ZJ173

	No. of plants	Lesion length (cm)[1]		
		Average	Min	Max
Bt-'Minghui 63'/*Xa21*-'Minghui 63'	14	5.8 ± 0.2	4.7	7.3
'Minghui 63' (control)	12	21.4 ± 0.6	18.7	25.7

[1] Lesion length was measured on three leaves 21 days after inoculation.

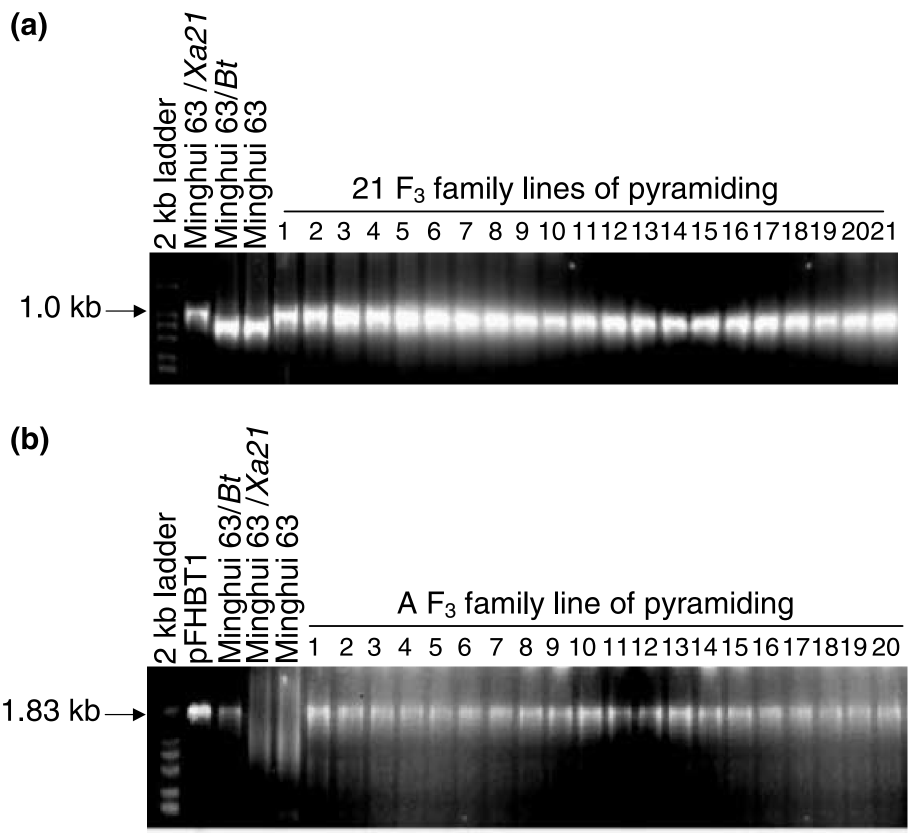

Fig. 1: PCR analysis of ‘Minghui 63’ and its improved versions with one or both genes, *Xa21* (a) and *Bt* (b). In (a), the sample DNA is a mixture of 10 individuals per line from each of the 21 lines ($F_{2:3}$ families) protecting 100% against insect infestation. In (b), the DNA samples each come from individuals of a representative pyramided line ($F_{2:3}$ family) with no insect infestation in the field. pFHBT1 contains *Bt* coding sequence fragments. Results in (a) and (b) confirm the homozygosity of *Xa21* and *Bt* genes in the pyramided lines

Table 3: The resistance reaction of ‘Minghui 63’ (1), ‘Minghui 63’/*Bt* (2), ‘Minghui 63’/*Xa21* (3), and ‘Minghui 63’/*Bt & Xa21* (4) against natural infestation of stem borers and manual inoculation of *Xoo* stain mixtures in 2000 and 2001 (Wuhan)

Symptoms	1	2	3	4
2000				
Percentage of plants with WH	81.2	3.1	77.1	0.0
Percentage of plants with DL	100.0	3.0	100.0	0.0
Lesion length (cm) of BB	23.1	18.4	5.7	4.0
2001				
Percentage of plants with WH	100.0	0.0	100.0	0.0
Percentage of plants with DL	100.0	0.0	100.0	0.0
Lesion length (cm) of BB	18.7 ± 2.5**	17.7 ± 2.1**	6.2 ± 1.2	4.4 ± 1.2

WH, white-head; DL, damaged leaves; BB, bacterial blight.

** Significantly different at $P = 0.01$ from the improved line ‘Minghui 63’/*Bt & Xa21*.

2000 and 6.2 cm in 2001, whereas those in ‘Minghui 63’/*Bt* and the negative control ‘Minghui 63’ had lesion lengths of at least 18.4 cm in 2000 and 17.7 cm in 2001. Thus, these data further confirmed the results observed in the previous generations.

As with the data observed on the pyramiding line, the high resistances against natural infestation of stem borers and manual inoculation of mixture of *Xoo* strains in hybrids produced by crossing it with ‘Zhenshan 97A’ and ‘Maxie A’ were observed. The percentage of plants with white-head or folded leaves was 0.0% in hybrids derived from the pyramiding line, < 4.2% in hybrids derived from the *Bt* donor line, but 100% in the hybrids derived from ‘Minghui 63’/*Xa21* and the negative control (Table 4). Similar results were found in BB resistance. The lesion lengths detected on hybrids derived from the pyramiding line and from the *Xa21* donor line were similar, all < 8.8 ± 1.0 cm (Table 4). The lesion lengths on hybrids derived from ‘Minghui 63’/*Bt* and the negative control, however, were at least 19.4 cm, significantly longer than those observed on the pyramiding line hybrids (Table 4). These results therefore demonstrated that the pyramiding line and its derived hybrids inherit the high levels of both stem borer and BB resistance from their *Bt* and *Xa21* donor lines, respectively.

Yield and agronomic traits of the pyramided line ‘Minghui 63’/*Bt & Xa21* and its hybrids

The agronomic performance of the pyramided line ‘Minghui 63’/*Bt & Xa21* and its hybrids produced by crossing it with two different CMS lines, ‘Zhenshan 97A’ and ‘Maxie A’, was also examined in the same plots in 2001. The original line ‘Minghui 63’ and its hybrids with these same CMS lines, with or without chemical spray, were used as controls for trait and yield comparisons. The data showed that without chemical sprays the pyramiding line ‘Minghui 63’/*Bt & Xa21*

Table 4: The resistance of the hybrids of 'Minghui 63' (1), 'Minghui 63'/*Bt* (2), 'Minghui 63'/*Xa21* (3), and 'Minghui 63'/*Bt & Xa21* (4) with 'Zhenshan 97A' and 'Maxie A' in 2001

	With Zhenshan 97 A				With Maxie A			
Symptoms	1	2	3	4	1	2	3	4
Percentage of plants with WH	100.0	4.2	100.0	0.0	100.0	2.1	100.0	0.0
Percentage of plants with DL	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
Lesion length (cm) of BB	21.4 ± 2.1**	19.4 ± 2.0**	8.7 ± 0.9	8.0 ± 1.1	24.4 ± 2.5**	22.2 ± 2.6**	8.8 ± 1.0	8.1 ± 1.1

WH, white-head; DL, damaged leaves; BB, bacterial blight.
** Significant difference from the hybrid with the improved line 'Minghui 63'/*Bt & Xa21* at P = 0.01.

Table 5: The agronomic traits and single-plant yield performance of the pyramiding line 'Minghui 63'/*Bt & Xa21* and negative control 'Minghui 63' in 2001

Lines	Plant height (cm)	No. of tillers per plant	Seed-setting tillers (%)	No. of grains per panicle	Spikelet fertility (%)	1000-grain weight (g)	Single-plant yield (g)
'Minghui 63'	108.4 ± 4.2	15.8 ± 0.8	41.6 ± 20.9**	69.7 ± 20.0**	70.7 ± 12.7**	28.7 ± 2.1**	14.4 ± 10.3**
'Minghui 63' (sprayed)	106.5 ± 2.7	15.9 ± 1.1	100.0 ± 0.0	103.2 ± 5.3	93.2 ± 2.2*	28.9 ± 0.4**	47.4 ± 4.7
'Minghui 63'/*Bt & Xa21*	107.0 ± 3.7	16.9 ± 1.6	100.0 ± 0.0	102.5 ± 6.4	83.7 ± 1.9	26.0 ± 0.5	44.9 ± 3.4

Significantly different from the performance of 'Minghui 63'/*Bt & Xa21* at * P = 0.05 and ** P = 0.01.

Table 6: The agronomic traits and single-plant yield of hybrids with and without pyramiding genes in 2001

Hybrids								
Restorer line	CMS line	Plant height (cm)	No. of tillers per plant	Seed-setting tillers (%)	No. of grains per panicle	Spikelet fertility (%)	1000-grain weight (g)	Single-plant yield (g)
'Minghui 63'	'Maxie A'	119.3 ± 2.7	16.0 ± 3.4	50.1 ± 11.9**	102.3 ± 27.4**	67.6 ± 11.3**	27.9 ± 1.6	23.1 ± 8.0**
'Minghui 63' (sprayed)	'Maxie A'	122.5 ± 2.5	17.3 ± 1.1	100.0 ± 0.0	137.4 ± 6.2	85.0 ± 4.2	28.6 ± 0.5	67.6 ± 6.1
'Minghui 63'/*Bt & Xa21*	'Maxie A'	123.4 ± 4.0	17.2 ± 1.1	100.0 ± 0.0	134.3 ± 6.2	84.8 ± 3.1	28.8 ± 0.5	66.6 ± 5.8
'Minghui 63'	'Zhenshan 97 A'	123.9 ± 4.7	17.7 ± 2.0	36.1 ± 8.1**	100.9 ± 23.6**	72.1 ± 14.1**	26.3 ± 1.6**	17.2 ± 6.7**
'Minghui 63' (sprayed)	'Zhenshan 97 A'	123.9 ± 5.6	18.1 ± 1.8	100.0 ± 0.0	148.2 ± 11.3	88.5 ± 4.7	27.7 ± 1.8	73.9 ± 4.0
'Minghui 63'/*Bt & Xa21*	'Zhenshan 97 A'	125.1 ± 3.7	18.2 ± 1.4	100.0 ± 0.0	148.8 ± 11.6	85.7 ± 0.7	28.1 ± 0.4	75.4 ± 3.5

** Significantly different at P = 0.01 from the performance of the hybrid with 'Minghui 63'/*Bt & Xa21* as the restorer line.

performed as well as the control 'Minghui 63' did under chemical control for most traits except spikelet fertility and 1000-grain weight (Table 5). These findings imply that integrating the two foreign genes *Bt* and *Xa21* into a 'Minghui 63' genetic background might, to some extent, disrupt the seed-filling process. However, in the hybrids of 'Minghui 63'/*Bt & Xa21* with 'Zhenshan 97A' and 'Minghui 63'/*Bt & Xa21* with 'Maxie A', such disruption was inhibited. For all the traits investigated, no significant performance differences could be found between these improved hybrids under natural conditions and their corresponding control hybrids that had received chemical sprays (Table 6). Under unsprayed conditions, yield per plant was a great deal better in the pyramiding line 'Minghui 63'/*Bt & Xa21* and its hybrids with CMS lines 'Zhenshan 97A' and 'Maxie A' than in the corresponding controls lacking either the *Bt* or *Xa21* genes. Further analysis showed that these differences were primarily because of significant discrepancies among the traits of spikelet fertility, number of seed-setting panicles, and number of grains per panicle between the improved and unimproved lines (Tables 5 and 6). These results are understandable: in plants that do not have the resistance genes, stem borers destroy panicles and thus reduce the number of effective panicles, and infestation by leaf-folders and by *Xoo* damages leaf function so as to greatly reduce photosynthetic competence. These results accord well with reports made by Chen et al. (2000) and Tu et al. (2000).

Discussion

'Minghui 63' is the restorer line for a number of widely used hybrids in China, including 'Shanyou 63', the most widely cultivated hybrid in China for the last two decades. The results presented in this paper clearly showed that the target genes of *Bt* and *Xa21* were successfully pyramided into the same plant of the elite *indica* CMS restorer line 'Minghui 63' by means of MAS. The resulting plants and their derived hybrids showed high levels of resistance to natural infestations of leaf-folders and yellow stem borers and manual inoculation of five *Xoo* strain mixtures. The levels of resistance are identical to the single-gene performances as reported previously (Chen et al. 2000, Tu et al. 2000). Hybrids derived from the pyramided line produced the same grain yield as hybrid controls treated with chemicals; however, under natural conditions the pyramiding hybrids yielded two to three times more than the controls. These results indicated that the pyramided insect- and disease-resistance genes in the hybrid plants functioned normally without reducing the yield potential. The pyramided line should be immediately useful for improving the hybrid series in rice production.

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