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Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar PR106

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Abstract Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major disease of rice in several countries. Three BB resistance genes, *xa5*, *xa13* and *Xa21*, were pyramided into cv. PR106, which is widely grown in Punjab, India, using marker-assisted selection. Lines of PR106 with pyramided genes were evaluated after inoculation with 17 isolates of the pathogen from the Punjab and six races of *Xoo* from the Philippines. Genes in combinations were found to provide high levels of resistance to the predominant *Xoo* isolates from the Punjab and six races from the Philippines. Lines of PR106 with two and three BB resistance genes were also evaluated under natural conditions at 31 sites in commercial fields. The combination of genes provided a wider spectrum of resistance to the pathogen population prevalent in the region; *Xa21* was the most effective, followed by *xa*5. Resistance gene *xa13* was the least effective against *Xoo*. Only 1 of the BB isolates, PX04, was virulent on the line carrying *Xa21* but avirulent on the lines having *xa5* and *xa13* genes in combination with *Xa21*.

Keywords Rice · Resistance gene · Bacterial blight · *Xanthomonas oryzae* pv. *oryzae* · MAS · Pyramiding

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Introduction

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases active in the major rice growing countries of Asia. In the northern plains of India, including the State of Punjab, the disease is a serious problem, as rice is grown under irrigated and high fertilizer input conditions that are conducive to disease development. In severe epidemics, yield losses ranging from 20% to 40% have been reported (Sonti 1998). Breeding for disease resistance is the most effective and economical method for control of BB that has a neutral impact on the environment. Several germplasm donors carrying diverse genes for BB-resistance have been used to develop BB resistant varieties (Khush et al. 1989). So far, 14 dominant [*Xa1*, *Xa2*, *Xa3*, *Xa4*, *Xa7*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa16*, *Xa17*, *Xa18*, *Xa21* and *Xa22* (t)] and six recessive (*xa5*, *xa8*, *xa13*, *xa15*, *xa19* and *xa20*) resistance genes for BB have been identified (Khush and Kinoshita 1991; Kinoshita 1995; Lin et al. 1996).

Large-scale and long-term cultivation of varieties with single genes may enable the pathogen to overcome BB resistance. However, this can be delayed by pyramiding multiple resistance genes into rice cultivars. The probability of simultaneous pathogen mutation for virulence to 2 or more effective genes is much lower than for a single gene. Gene pyramiding is difficult using conventional breeding methods due to the dominance and epistasis effects of genes governing disease resistance. Moreover, genes with similar reactions to 2 or more races are difficult to identify and transfer through conventional approaches. However, the availability of molecular markers closely linked with each of the resistance genes makes the identification of plants with 2 and three genes possible.

The dominant expression of molecular markers linked to the resistance genes can be used to follow all the possible resistance genes in any breeding program. Marker-assisted selection (MAS) offers unique advantages to

overcome some of these limitations. Of the 20 resistance genes, sequence tagged site (STS) markers are available for *xa5*, *xa13* and *Xa21* (Huang et al. 1997; Chunwongse et al. 1993).

We report here the successful pyramiding of 3 BB resistance genes, *xa5*, *xa13* and *Xa21*, through MAS in a high-yielding but BB-susceptible rice cultivar, PR106. The results demonstrate on increased and wide-spectrum resistance to the pathogen populations from Punjab and to the Philippine races of *Xoo* in PR106 lines having pyramided genes.

Materials and methods

Plant materials

The materials comprised of three isogenic lines, IRBB5 (*xa5*), IRBB13 (*xa13*), IRBB21 (*Xa21*), and a line IRBB62 with all three genes in the background of IR24. These lines, including PR106, were grown at the Punjab Agricultural University (PAU), Ludhiana, India and the International Rice Research Institute (IRRI), Philippines. Crosses were made between PR106, (a high-yielding, BB-susceptible cultivar) and IRBB62, which carries 3 BB resistance genes. F_1 plants were backcrossed with PR106. Starting from the BC_1F_1 onward, polymerase chain reaction (PCR)-based molecular markers linked to *xa5*, *xa13* and *Xa21* were used to select plants with resistance alleles. A similar strategy was used in the BC_2F_1 to obtain BC_2F_2 populations from which lines with pyramided genes were selected. The BC_2F_2 plants were selfed and backcrossed again with PR106. The BC_2F_3 lines having 2 and 3 homozygous genes for resistance to BB were identified on the basis of molecular marker analysis and inoculated with BB isolates/races to determine disease reaction.

Bacterial cultures

Seventeen isolates of *Xoo*, prevalent in Punjab and six races from the Philippines were isolated, multiplied and maintained on peptone sucrose agar (PSA) at 28°C. These isolates were preserved in glycerol at –70°C.

Pathotype analysis

Plants selected on the basis of molecular marker analysis from the BC_1F_2, BC_2F_2 and BC_2F_3 generations were inoculated with 4 of the most predominant *Xoo* isolates from Punjab and six races of the pathogen from the Philippines. Disease reaction was scored 16 days after inoculation by measuring lesion length (Kauffman et al. 1973).

Forty-day-old plants of the PR106 carrying resistance genes (*xa5*, *xa13* and *Xa21*) individually and in combinations, along with the control, were inoculated with 17 isolates of *Xoo* prevalent in Punjab State using a bacterial suspension of 10^9 cells/ml (Kauffman et al. 1973). An average of five leaves per plant were inoculated with each isolate-genotype combination and replicated three times.

DNA isolation and PCR amplification

Miniscale DNA isolation for PCR analysis of the parents and backcross progenies was carried out following the procedure described by Dellaporta et al. (1993). Three STS markers, RG556, RG136 and pTA248, tightly linked to resistance genes *xa5*, *xa13* and *Xa21*, respectively, were used to confirm the presence of each gene and the different combinations.

The PCR reaction mixture contained 50 ng template DNA, 50 ng of each primer, 0.05 m*M* dNTPs, 1× PCR buffer (10 m*M* Tris, pH 8.4, 50 m*M* KCl, 1.8 m*M* MgCI2 and 0.01 mg/ml gelatin) and 1 U *Taq* DNA polymerase in a volume of 20 µl. Template DNA was initially denatured at 94°C for 5 min followed by 30 cycles of PCR amplification with the following parameters: a 30-s denaturation at 94°C, a 30-s primer annealing at 55°C and 1 min of primer extension at 72°C. The amplified product was electrophoretically resolved on a 1% agarose gel in 1× TAE buffer. Initially, 5 µl of PCR product was used for gel electrophoresis to determine whether PCR amplification was successful. The remaining PCR product was used for restriction digests. The reaction mixture consisted of 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10×), 0.3 µl restriction enzyme (10 units/µl) and 15 µl of PCR product. Incubation of the reaction mixture varied from 2 h to overnight at 37°C. The DNA fragments produced by restriction were separated by gel electrophoresis (1.5% agarose) and visualized under UV light after staining with ethidium bromide.

Backcross progenies were analyzed and classified for markers representing one, two and three gene combinations. The plants homozygous for all 3 genes, each of the individual genes and various combinations of two were identified and inoculated with the isolates from the Punjab and the Philippines races of BB pathogen.

Field evaluation of pyramided lines of PR106

Homozygous lines with a single resistance gene and the different combinations were evaluated in commercial fields during 1999. These lines and susceptible parent PR106 were planted in four rows in nine districts of the Punjab State covering 31 sites. The presence of BB was observed in 8 sites, and disease incidence was recorded under natural field conditions during the crop season following the Kauffman et al. (1973) technique.

Results and discussion

Pyramiding of BB resistance genes through MAS

Twelve F_1 plants were obtained from the cross of cv. PR106 with IRBB62 having 3 BB resistance genes, *xa5*, *xa13* and *Xa21*. When tested against *Xoo* isolates prevalent in the Punjab, lesion length in these F_1 individuals ranged from 0.2 to 1.0 cm compared with 20–28 cm in the recurrent parent PR106. Molecular markers linked to resistance genes were confirmed in the F_1 and 6 plants were backcrossed with PR106, which resulted in 84 BC_1F_1 seeds. Fifty-one BC_1F_1 plants were analyzed with three STS markers. Twenty-one plants were identified as having an allele of 1 or more of the 3 resistance genes on the basis of molecular analysis. Three plants having all 3 resistance gene loci were identified and selfed. A total of 180 BC_1F_2 plants were screened for resistance gene alleles through molecular marker analysis. Five plants with alleles of all 3-resistance genes were backcrossed with PR106.

Three hundred and twenty-three BC_2F_1 progenies obtained from 5 different plants were analyzed with STS markers; 156 plants heterozygous for alleles (*Xa21xa21+Xa13xa13+Xa5xa5*) of the 3 resistance genes were identified. Plants with alleles of all 3 genes that were similar to PR106 were used for further backcrossing and selfing. Two hundred twenty-five BC_2F_2 plants were analyzed with STS markers, and plants homozygous for the alleles

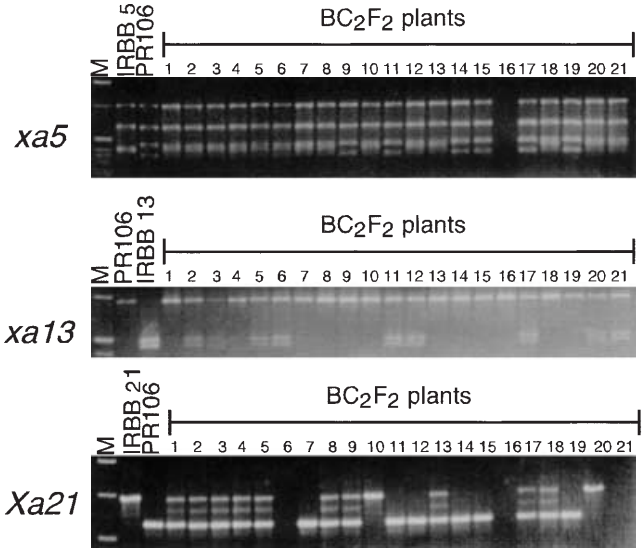

Fig. 1 PCR analysis of the parental lines and BC_2F_2 plants. DNA amplified with primers RG556 (digested with *Dra*I) and RG136 (digested with *Hinf*I) were linked with resistance genes *xa5* and *xa13*, respectively. DNA amplified using the pTA248 primers were linked with resistance gene *Xa21*. For *xa5*, BC_2F_2 plants with the resistance gene are shown in *lanes 1–8, 10, 12, 13, 18, 20* and *21*; for *xa13*, resistant plants are shown in *lanes 2, 3, 5, 6, 11, 12, 17, 20* and *21*; for *Xa21*, the resistant plants are shown in *lanes 1–5, 8–10, 13, 17, 18* and *20*. *Lane M* DNA marker. Lanes without bands did not amplify PCR product

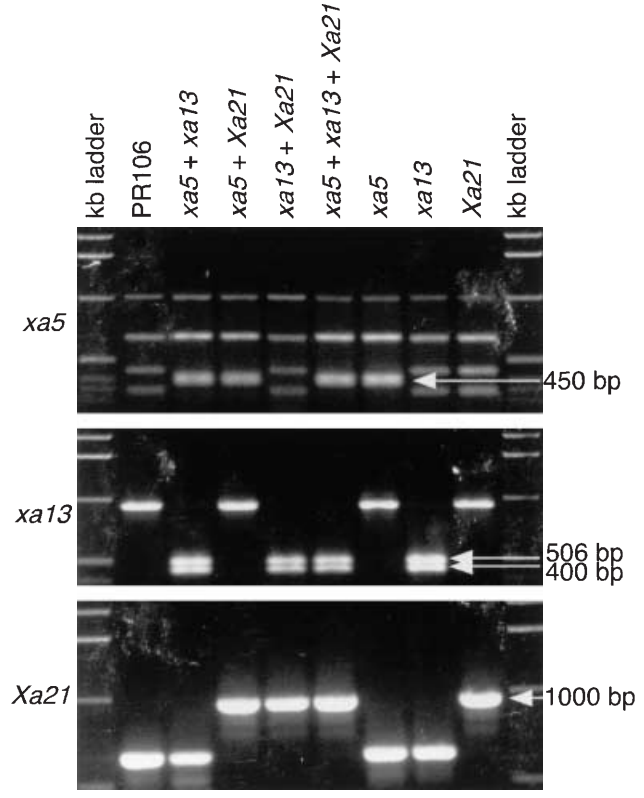

Fig. 2 PR106 lines, homozygous for BB resistance genes *xa5*, *xa13* and *Xa21* individually and in combinations were confirmed in BC_2F_3 progenies. *Arrows* indicate polymorphic bands that are linked to each respective resistance gene

of all resistance genes, each of the individual genes and various combinations of two were identified. One plant homozygous for the alleles of all resistance genes was detected. Fifty plants with homozygous resistance for *xa5xa5* and 44 for *xa13xa13* were identified, whereas 60 were homozygous resistant for *Xa21Xa21*. Figure 1 shows plant numbers 2, 3, 5 and 20 having alleles for resistance genes *xa5*, *xa13* and *Xa21*. Plant numbers 6, 12, and 21 carry resistance alleles for *xa5* and *xa13*, whereas plants 1, 4, 8, 10, 13 and 18 carry alleles for resistance genes *xa5* and *Xa21*. Plant number 17 has resistance alleles for genes *xa13* and *Xa21*.

Reaction to the bacterial blight pathogen

The PR106 lines homozygous for each of the individual genes and those having different combinations were confirmed in the BC_2F_3 progenies (Fig. 2) using STS markers linked to resistance genes and inoculation with the 17 isolates of *Xoo* prevalent in Punjab. The results indicated that the genes in combinations were more effective against the pathogen than a single gene (Table 1). Resistance gene *Xa21* was effective against 16 of the isolates from Punjab used in this study. However, isolate PXo4 produced a lesion length of 7.50 cm on lines carrying the *Xa21* resistance gene in a PR106 genetic background (Table 1). Resistance gene *xa13* was effective against 6 isolates (PXo7, PXo8, PXo11, PXo14, PXo15 and PXo16), whereas a line with gene *xa5* was resistant to 14 isolates. Based on our results, we can infer that, taken individually, *Xa21* was the most effective gene, followed by *xa5* and that gene *xa13* was the least effective. Isolate PXo4 was avirulent on lines carrying *Xa21* in combination with resistance genes *xa5* and *xa13*, on which it is otherwise virulent. All four combinations of resistance genes (*xa5+xa13*, *xa5+Xa21*, *xa13+Xa21* and *xa5+xa13+Xa21*) provided resistance to all *Xoo* isolates from the Punjab (Table 1).

Homozygous PR106 lines carrying genes *xa5*, *xa13*, and *Xa21* individually and in combinations were also inoculated with 4 isolates of *Xoo* widely prevalent in the Punjab and six races of the pathogen from the Philippines (Table 2). All combinations of genes were effective against the *Xoo* isolates of the Punjab and all six races from the Philippines. Resistance gene *Xa21* was effective against predominant BB isolates from the Punjab and *Xoo* races of the Philippines used in this study. Gene *xa13* was effective against race 6 of the Philippines and isolates 3 and 4 from the Punjab, whereas the line with gene *xa5* was resistant to five BB races (1–5) of the Philippines and 2 isolates (1 and 2) prevalent in the Punjab.

When different genes are combined, the resistance appears to be more durable. For example, combining the resistance genes *Xa4* and *xa5* or *xa5* and *Xa10* provided a higher level of resistance to *Xoo* than is found in the sum of the individual genes at the parental level (Yoshimura et al. 1995). Similarly, the combination of resistance genes *Lr16* and *Lr13* are reported to give

Table 1 Lesion length[a] in centimeters on PR106 lines carrying gene(s) individually and in combinations, after inoculation with isolates of *Xanthomonas oryzae* pv. *oryzae* from the Punjab, India

Disease reaction to *Xoo* isolates from Punjab (cm)								
Isolate	PR106	*xa5*	*xa13*	*Xa21*	*xa5+xa13*	*xa5+Xa21*	*xa13+Xa21*	*xa5+xa13+Xa21*
PXo1	15.0±0.76	4.50±0.30	15.00±0.11	2.67±0.25	3.83±0.82	1.47±0.00	2.00±0.50	0.60±0.20
PXo2	18.66±0.20	3.53±0.0	11.00±0.25	0.97±0.45	2.83±0.55	1.00±0.10	0.62±0.24	0.20±0.80
PXo3	22.5±0.50	4.65±0.38	11.67±0.50	2.5±0.26	3.52±0.76	2.55±0.90	1.25±0.82	1.50±0.29
PXo4	24.7±0.43	14.57±0.25	20.28±0.45	7.50±0.78	4.22±0.00	2.25±0.55	3.45±0.00	1.67±0.52
PXo5	23.83±0.56	3.90±0.10	7.95±0.46	1.86±0.00	3.50±0.00	1.25±0.45	1.52±0.29	0.10±0.22
PXo6	18.2±0.20	8.00±0.60	18.25±0.12	1.55±0.00	4.60±0.85	1.86±0.95	0.32±0.96	0.70±0.75
PXo7	21.8±0.44	0.00±0.10	2.98±0.55	1.45±0.28	3.15±0.13	1.12±0.76	1.50±0.25	0.25±0.09
PXo8	11.33±0.66	18.00±0.25	4.98±0.06	2.65±0.12	4.27±0.12	2.56±0.62	1.97±0.00	1.24±0.20
PXo9	15.13±0.24	3.43±0.00	12.00±0.23	0.40±0.95	3.75±0.29	0.67±1.04	1.60±0.85	0.52±0.45
PXo10	12.33±0.17	4.83±0.15	26.00±0.23	1.25±0.41	4.00±0.65	0.70±0.25	1.00±0.12	0.67±0.08
PXo11	15.0±0.0	3.98±0.22	2.55±0.05	0.20±0.80	3.00±0.98	0.10±0.53	0.88±0.25	0.10±0.65
PXo12	15.6±0.60	3.55±0.15	8.55±0.00	1.27±0.20	3.24±0.56	1.47±0.00	1.10±0.11	0.37±0.28
PXo13	15.46±0.24	3.17±0.25	18.67±0.07	0.67±0.64	3.88±0.34	1.60±0.30	0.25±0.10	0.35±0.64
PXo14	26.33±0.88	4.89±0.12	3.00±0.40	1.50±0.08	2.85±0.92	0.84±0.78	0.15±0.66	0.25±0.98
PXo15	15.36±0.18	3.75±0.00	4.75±0.24	0.88±0.95	3.98±0.54	0.55±0.00	1.00±0.00	0.20±0.12
PXo16	14.63±0.31	4.33±0.00	2.57±0.34	0.15±0.76	2.35±0.00	0.89±0.17	0.45±0.54	0.33±0.00
PXo17	21.13±0.85	8.03±0.21	6.67±0.45	1.40±0.10	4.33±0.66	1.50±1.90	1.23±0.11	0.55±0.80

[a] Mean±standard error

Table 2 Lesion length in centimeters on PR106 lines carrying the *xa5*, *xa13, and Xa21* genes singly and in combinations after inoculation with bacterial blight isolates from the Punjab and Philippines

Lesion length (cm)[a]										
BB resistant gene(s)	*Xoo* isolates (Punjab)				*Xoo* races (Philippines)					
	1	2	3	4	1	2	3	4	5	6
PR106+*xa5*	3.5	4.8	12.8	7.5	2.2	3.0	3.5	4.0	3.0	8.5
PR106+*xa13*	5.5	14.0	2.0	4.0	9.5	13.3	8.8	13.6	6.5	2.1
PR106+*Xa21*	0.5	2.5	2.5	1.0	0.2	0.8	0.4	0.9	0.7	1.2
PR106+*xa5+xa13*	2.5	4.0	4.0	3.0	1.2	1.5	1.6	6.0	4.0	2.0
PR106+*xa5+Xa21*	0.4	1.5	2.0	0.8	0.2	0.6	0.4	0.5	0.6	1.4
PR106+*xa13+Xa21*	0.5	2.6	0.3	0.5	0.3	1.6	1.6	0.8	0.6	0.4
PR106+*xa5+xa13+Xa21*	0.2	0.4	0.5	0.2	0.1	0.4	0.4	0.6	0.2	1.3
PR106	10.5	25.0	21.0	16.0	22.5	18.2	20.0	21.0	14.0	24.0

[a] Lesion length is an average of three replications

Table 3 Disease incidence of bacterial blight on PR106 lines carrying the *xa*5, *xa*13, and *Xa*21 resistance gene(s) in commercial fields during 1999

BB score[a] at different sites[b]								
Line	Ludhiana				Jallandhar		Ferozepur	Sangrur
	I	II	III	IV	I	II	I	I
PR106	6	5	6	7	5	4	6	3
PR106+*xa5*	5	6	6	7	5	4	6	3
PR106+*xa13*	3	3	6	6	4	4	5	0
PR106+*Xa21*	2	0	3	3	0	2	3	0
PR106+*xa5+xa13*	0	0	3	4	3	0	5	0
PR106+*xa5+Xa21*	0	0	1	0	0	0	1	0
PR106+*xa13+Xa21*	0	0	1	0	0	0	0	0
PR106+*xa5+xa13+Xa21*	0	0	1	0	3	0	0	0

[a] 0, Immune; 1–3, resistant; more than 3, susceptible
[b] Numeral indicates sites; i.e. BB was observed at four sites in the Ludhiana, 2 in Jallandhar and one each in Ferozepur and Sangrur

reliable control for the leaf rust pathogen in wheat (Samborski and Dick 1982; Long et al. 1993).

Accumulating major genes for resistance in an elite genotype by conventional breeding is laborious and time-consuming when one or more of the genes are effective against all known isolates of the pathogen. We observed that two- and three-gene combinations provided resistance against isolates of *Xoo* prevalent in the Punjab. Isolated lines carrying different genes in a homozygous condition in a PR106 background have been advanced for multi-location evaluation. Advanced lines with resistance gene combinations have practical breeding value by providing a wider spectrum of resistance against most of the existing isolates of BB in the region and will have a high impact on yield stability and sustainability of rice crop in the region.

Evaluation of PR106 lines with pyramided genes for resistance to the BB pathogen in commercial fields

BC_2F_4 progenies were evaluated in commercial fields against the pathogen populations prevalent in the region during 1999. PR106 lines carrying individual genes and a pyramid combination of the genes were grown in four rows in nine districts of Punjab State covering 31 sites. Disease was observed at 8 of the sites covering the districts of Ludhiana, Sangrur, Jallandhar and Ferozpur (Table 3). Gene *Xa21* was the most effective single gene against the naturally prevalent pathogen population in the region. The BB score at different sites on PR106 lines having different resistance gene(s) combinations varied, indicating the diversity of the *Xoo* population in the state (Table 3).

Bacterial blight was observed in only one of the PR106 lines carrying the *xa5+xa13* genes in the Ludhiana and Ferozpur districts, which indicates the prevalence of a highly virulent isolate (Table 3). However, the combination of *Xa21* with other genes will be more desirable for effective control of BB. Multi-location testing of different gene combinations and monitoring the population of the pathogen over several crop cycles will be useful in identifying durable resistance in rice against the BB pathogen.

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References

Chunwongse J, Martin GB, Tanksley SD (1993) Pregermination genotypic screening using PCR amplification of half seeds. Theor Appl Genet 86:694–698

Dellaporta SL, Woo J, Hicks JB (1993) A plant DNA minipreparation:version II. Plant Mol Biol Rep 1:19–21

Huang N, Angeles ER, Domingo J, Magpantay G, Singh S, Zhang G, Kumaravadivel N, Bennett J, Khush GS (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor Appl Genet 95:313–320

Kauffman HE, Reddy APK, Hsien SPY, Merca SD (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep 57:537–541

Khush GS, MacKill DJ, Sidhu GS (1989) Breeding rice for resistance to bacterial blight. In: Bacterial blight of rice, Proc. Int. Workshop Bact. Blight Rice. IRRI, Manila, the Philippines, pp 207–217

Khush GS, Kinoshita T (1991) Rice karotype, marker gene and linkage groups. In: Khush GS, Toenniessen GH (eds) Rice biotechnology. CABI Int, Wallingford, UK, pp 83–108

Kinoshita T (1995) Report of committee on gene symbolization, nomenclature and linkage groups. Rice Genet Newsl 12:9–153

Lin XH, Zhang DP, Xie YF, Gao HP, Zhang Q (1996) Identifying and mapping a new gene for bacterial blight resistance in rice based on RFLP markers. Phytopathology 86:1156–1159

Long DL, Roelfs AP, Leonard KJ (1993) Virulence and diversity of *Puccinia recondita f. sp. tritici* in the United States in 1991. Plant Dis 77:786–791

Samborski DJ, Dyck PL (1982) Enhancement of resistance to *Puccinia recondita* by interactions of resistance genes in wheat. Can J Plant Pathol 4:152–156

Sonti RV (1998) Bacterial leaf blight of rice: new insights from molecular genetics. Curr Sci 74:206–212

Yoshimura SA, Yishimura N, Iwata McCouch SR, Abenes ML, Baraoidian MR, Mew TW, Nelson RJ (1995) Tagging and combining bacterial blight resistance genes in rice using RAPD and RFLP markers. Mol Breed 1:375–387