Theor Appl Genet (2004) 108:1024–1032
DOI 10.1007/s00122-003-1528-1

ORIGINAL PAPER

J.-L. Wu · P. K. Sinha · M. Variar · K.-L. Zheng · J. E. Leach · B. Courtois · H. Leung

Association between molecular markers and blast resistance in an advanced backcross population of rice

Received: 13 June 2003 / Accepted: 27 October 2003 / Published online: 9 December 2003

Abstracts An advanced backcross population consisting of 80 BC_3F_3 lines derived from rice vars. *Vandana/Moroberekan* was analysed for blast resistance and genotyped with 50 candidate genes and 23 simple sequence repeat (SSR) markers. Six candidate defence response genes [thaumatin, three nucleotide-binding site-leucine-rich repeat sequences from maize and two resistance gene analogue (RGA) markers] and one SSR marker (RM21) were significantly associated with partial blast resistance in rice (P=0.01). These markers accounted for phenotypic variation ranging from 9.6% to 29.4% and contributed to 76% of the total variation of percentage diseased leaf area (DLA) observed under natural infection. Four candidate genes (oxalate oxidase, 14-3-3 protein and two RGA markers) and four SSR markers (RM21, RM168, RM215 and RM250) were significantly associated with resistance to a single pathogen isolate, PO6-6. Among these, two markers were for DLA, five for lesion number and one for lesion size. These markers accounted for 9.1–28.7% of the phenotypic variation. A moderate correlation (r=0.48, P<0.01) was found between the level of partial resistance measured in the greenhouse and that measured under natural conditions. Analysis of BC_3F_4 progeny using genotypes of BC_3F_3 confirmed the phenotypic contribution of these markers. Cluster analysis of DNA profiles showed that the BC_3 population was genetically similar (>85%) to the recurrent parent *Vandana*. Although no obvious relationship between DNA profiles and resistant phenotypes was observed, three lines (VM19, VM46 and VM76) in a cluster with high similarity to *Vandana* (89–96%) expressed a high level of partial blast resistance in the field. Analysis of disease progress in the field confirmed the performance of selected lines based on greenhouse and nursery analyses. The advanced backcross progeny with resistance phenotypes tagged by markers will be useful for accumulating blast resistance in upland rice.

Communicated by G. Wenzel

J.-L. Wu · K.-L. Zheng
National Center for Rice Improvement,
China National Rice Research Institute,
310006 Hangzhou, China

P. K. Sinha · M. Variar
Central Rainfed Upland Rice Research Station,
825 301 Hazaribag, Jharkhand, India

J. E. Leach
Department of Plant Pathology,
Kansas State University,
Manhattan, KS 66506-5502, USA

B. Courtois
CIRAD,
Biotrop,
Avenue Agropolis, 34398 Montpellier Cedex 5, France

J.-L. Wu · H. Leung (✉)
Entomology and Plant Pathology Division,
International Rice Research Institute,
DAPO P.O. Box 7777, Metro-Manila, Philippines
e-mail: h.leung@cgiar.org
Tel.: +63-2-8911236
Fax: +63-2-8451292

Introduction

Genes conferring resistance to diseases can be generally classified into two categories. The first category includes the major resistance (R) genes involved in specific recognition of a pathogen as the first step in initiating the resistant response. The second category includes defence response (DR) genes that are often downstream components of the defense pathways. Together these genes act in a coordinated manner to prevent or retard the invasion of pathogens. Structural and functional analyses of many major R genes have shown that they encode proteins with similar structural motifs—nucleotide binding site (NBS), kinase domains, leucine-rich repeats (LRR)—that are responsible for ligand recognition and signal transduction (Martin et al. 1993; Bent et al. 1996; Song et al. 1995; Cai et al. 1997; Wang et al. 1999; Bai et al. 2002; Meyers et al. 2003). A large number of defence-related genes have also been isolated from different plant

species that are correlated with the expression of resistance. For example, genes encoding chitinase and lipoxygenase are associated with resistance to plant diseases and insect pests (Huang et al. 1991; Schaffrath et al. 2000). Faris et al. (1999) reported a cluster of DR genes that included a catalase, a chitinase, thaumatin, and a 14-3-3 protein that had major effects on the resistance to leaf rust of wheat. They also identified two candidate genes associated with tan spot of wheat. Ramalingam et al. (2003) reported that oxalate oxidase was associated with bacterial blight resistance, while thaumatin and dihydrofolate reductase thymidylate synthase were associated with brown planthopper resistance in rice.

Resistance to rice blast caused by the fungal pathogen *Magnaporthe grisea* (Hebert) Barr can be controlled by a combination of qualitative and quantitative resistances. Wang et al. (1994) conducted the first comprehensive analysis of the quantitative resistance observed in *Moroberekan*, a traditional rice variety in Africa with a reputation for having durable resistance against blast. They used a mapping population of recombinant inbred lines (RILs) derived from *Moroberekan*/*CO39* (a highly susceptible rice variety) to map quantitative trait loci (QTLs). Two important observations were made in this study. First, durable resistance is contributed by a combination of major genes and QTLs. Second, some QTLs with strong phenotypic effects map at regions containing major blast resistance genes (*Pi*), suggesting that some QTLs are possibly a manifestation of major R genes. Since then, several mapping studies have been carried out for blast resistance but because the germplasm for genetic studies were chosen for polymorphism rather than the agronomic value favoured by farmers, no useful varieties have ever been generated from such mapping populations (Naqvi et al. 1995; D.H. Chen et al. 1997, 1999).

To address the problem of mapping experiments being disconnected from breeding objectives, Tanksley and Nelson (1996) advocated the use of advanced backcross (AB) populations to simultaneously analyse QTLs and to develop elite germplasm for commercial use. Following this strategy, we have developed an AB population with the objective of introgressing useful resistance from var. *Moroberekan* into a high-quality variety, *Vandana*, widely grown in eastern India. In the original approach of AB-QTL analysis, anonymous markers across the genome were used to determine the efficiency of the procedure. Unless markers are closely linked to the target gene, recombination between the flanking markers and the target gene often leads to a low predictability of linked markers for function. We here apply candidate genes involved in the defence pathways to analyse the backcross population. We have identified candidate genes contributing to a large degree of partial resistance. Our results indicate that the marker-tagged BC_3 lines are good material for increasing blast resistance in upland rice.

Materials and methods

Plant material and population development

The F_1 and the BC_1 generations of the cross between *Vandana*, an improved indica upland rice (*Oryza sativa* L.) variety popular in eastern India, and *Moroberekan*, a traditional japonica upland rice variety from Guinea, were developed in 1995 and 1996 in the Central Rainfed Upland Rice Research Station, Bihar, India. *Moroberekan* was used as the donor for blast resistance and *Vandana*, which is moderately susceptible to leaf blast and highly susceptible to neck blast, was used as the recurrent parent.

One hundred and fifty BC_1 seeds were sown in the greenhouse at IRRI in 1997, and 80 plants were crossed with the recurrent parent *Vandana*. One BC_2F_1 seed per plant was used to produce the next generation, which was backcrossed again to the recurrent parent in 1998. The BC_3F_1 seeds were planted in the greenhouse and the selfed seeds harvested. The BC_3F_2 generation was advanced to the BC_3F_3 generation under greenhouse conditions in 1999. All of the plants were bagged during the time it required to develop the greenhouse generations to prevent cross-pollination. The 80 lines each from the BC_3F_3 and BC_3F_4 generations were evaluated under field conditions at the IRRI farm in the Philippines. Some selection pressure at maturity for similarity to var. *Vandana* was applied by rouging plants differing too much from the average.

Performance in blast nursery

The BC_3F_3 and BC_3F_4 generations, parents and susceptible control CO39 were planted in the blast nursery at IRRI during the wet season in 1999 and dry season in 2000. Thirty to forty seeds were sown in a row of 10×3×1 cm. The experiment was a complete randomised block design with three replications. Border rows with a mixture of cultivars were set up around each replicate to sustain a diverse pathogen population. The scoring system of lesion type was based on Bonman et al. (1986). The percentage diseased leaf area (DLA) was estimated visually following the Standard Evaluation System (SES) for rice (IRRI 1996) once a week beginning 14 days after sowing; data collected at the 4th week were used for analysis.

Based on marker analysis and the performance of the BC_3F_3 in blast nursery, the five most resistant lines and five most susceptible lines from BC_3F_4, their parents and susceptible control CO39 were evaluated in the nursery in 2000 to observe their disease progress. The experimental design and scoring system were the same as above, but the DLA was recorded every 2 days from 2 weeks after sowing until the 4th week when the plants started recovery. Six observations were made to develop the disease progress curve for each line.

Greenhouse inoculation

An isolate, PO6-6, which is virulent to var. *Vandana* was chosen for inoculation of the progeny. Twenty lines with 12 seeds per line were sown in 35×28×11-cm plastic trays. A complete randomised block design with three replications was used. Plants were inoculated 14 days after sowing by spraying 20 ml inoculum per tray with a standard inoculum concentration (1×10^5 conidia/ml). Following inoculation, the plants were moved to cages covered with moist jute sacks overnight and then transferred the following day to a mist room maintained at 28°C. The plants were misted with water three to four times each day and then scored 6–7 days after inoculation based on the system of Bonman et al. (1986). Percentage DLA was recorded as described above from ten plants for each line per replication. The number of susceptible lesions was counted from five plants for each line per replication. Lesion size was measured on five plants per line per replication.

Table 1 Candidate defense gene clones used in this study[a]

Clone name	Code	Accession number or designation	Source	Clone name	Code	Accession number or designation	Source
NBS-LRR	r1	AF032688	Rice	Hypothetical proteinBH6-17	CG16	Y14202	Barley
NBS-LRR	r2	AF032689	Rice	14-3-3a protein	CG17	X62388	Barley
NBS-LRR	r3	AF032690	Rice	14-3-3b protein	CG18	X93170	Barley
NBS-LRR	r4	AF032691	Rice	14-3-3c protein	CG19	Y14200	Barley
NBS-LRR	r5	AF032692	Rice	Thionin	CG20	M19047	Potato
NBS-LRR	r6	AF032693	Rice	Cytochrome 450	CG21	U48434	Potato
NBS-LRR	r8	AF032695	Rice	PR-10	CG22	M2516	Potato
NBS-LRR	r9	AF032696	Rice	*S*-adenosy-L-homosystein	CG23	D16138	Tobacco
NBS-LRR	r10	AF032697	Rice	Hydrolase	CG24	D16139	Tobacco
NBS-LRR	r12	AF032699	Rice	Chalcone synthase	CG27	AB000801	Rice
NBS-LRR	r14	AF032701	Rice	CSU358	CG30	T27553	Maize
PR-1a	CG1	X74939	Barley	RGH	CG32	mRGH	Maize
PR-1b	CG2	X74940	Barley	PR-4-like protein	CG33	mRii17	Maize
β-1-3-glucanase	CG3	BH72.I1	Barley	hsp-70-like protein	CG35	mRRI11	Maize
PR-4	CG4	Y10814	Barley	PIC 20	CG36	AF056160	Maize
Chitinase 2a	CG5	X78671	Barley	PR-1	CG38	PR1	Maize
Chitinase 2b	CG6	X78672	Barley	5-33F	CG41	yac3	Maize
PR5 thaumatin	CG7	BH72-C6	Barley	Rp1	CG43	RP1–1700	Maize
Proline/glycine-rich protein	CG9	Y10813	Barley	Oxalate oxidase-like protein	CG11	X93171	Barley
Oxalate oxidase	CG10	Y14203	Barley	Flavonoid-7-*O*-methyltrans-ferase	CG12	X77467	Barley
Chalcone synthase	CG13	Y09233	Barley				
GRP94 homologue	CG14	X67960	Barley	RIP	CG46	T18690	Maize
BH6-12	CG15	Y14201	Barley	Wound inducible protein	CG47	T15276	Maize
PPO	CG49	T70649	Maize	Lipoxygenase L-2	CG51	T18420	Maize
7-4F	CG52	AF392837	Maize	PIC 19	CG54	AF056160	Maize

Table 2 A list of the simple sequence repeat (SSR) markers used in this study

Marker[a]	Chromosome	Reference	Marker	Chromosome	Reference
RM23	1	X. Chen et al. 1997	RM214	7	X. Chen et al. 1997
RM220	1	X. Chen et al. 1997	RM210	8	X. Chen et al. 1997
RM48	2	X. Chen et al. 1997	RM201	9	X. Chen et al. 1997
RM213	2	X. Chen et al. 1997	RM215	9	X. Chen et al. 1997
RM250	2	X. Chen et al. 1997	RM222	10	X. Chen et al. 1997
RM60	3	X. Chen et al. 1997	RM258	10	X. Chen et al. 1997
RM168	3	X. Chen et al. 1997	RM209	11	Panaud et al. 1996
RM241	4	X. Chen et al. 1997	RM21	11	X. Chen et al. 1997
RM261	4	X. Chen et al. 1997	RM224	11	X. Chen et al. 1997
RM26	5	X. Chen et al. 1997	RM4	12	Panaud et al. 1996
RM249	5	X. Chen et al. 1997	RM17	12	Panaud et al. 1996
RM204	6	X. Chen et al. 1997			

[a] A total of 25 alleles were amplified with these 23 primers

DNA extraction and Southern blotting

Following scoring, the diseased leaves of BC_3F_3 plants were removed and a handful of urea was applied to the plants for regeneration of new leaves for DNA extraction. A 5-g sample of fresh leaves from ten plants was bulked for DNA extraction using the CTAB method (Murray and Thompson 1980). Seven restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I and *Bgl*II) were used for parental survey of polymorphism. Southern blotting with Hybond-N+ membrane (Amersham Phamacia Biotech, Piscataway, N.J.) was performed according to the standard procedure (Sambrook et. al. 1989). Candidate gene probes were amplified using the following profile: pre-denaturation at 94°C for 4 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; a final extension at 72°C for 5 min. Blots were detected using ECL kits according to the manufacturers' recommendation (Amersham Phamacia Biotech). A list of candidate genes used in this experiment is shown in Table 1.

PCR marker genotyping

In addition to the candidate gene markers, 23 simple sequence repeat (SSR) markers were used to determine genetic relationships among the closely related lines (Table 2). PCR amplification of SSR primers (Research Genetics) was conducted as described by X. Chen et al. (1997). The products were detected using a 2.5% agarose gel or a 4% denaturing polyacrylamide gel with silver staining. Five resistance gene analogue (RGA) markers (PCR fragments amplified by primers designed from the conserved region of resistance genes) were used in the analysis (Chen et al. 1998). It has been shown that some of these markers map to chromosomal regions containing major resistance genes as well as QTLs for quantitative resistance (Shen et al. 1998; Zhuang et al. 1998; Toojinda et al. 2000; Shi et al. 2001). The five RGA primers were: RGA1F—CCG TTG GAC AGGAAG GAG; RGA1R—CCC ATA GAC CGG ACT GTT; RGA4F—TAG GGC CTC TTG CAT CGT; RGA4R—TAT AAA AAG TGC CGG ACT; RGA6F—GCA TTG GAA CAA GGT GAA; RGA6R—AGG GGG ACC ACC ACG TAG; RGA8F—ARI GCT ARI GGI ARI CC; RGA8R—GGI GGI

GTI GGI AAI ACI AC; RGA14F—TTG TCA GGC CAG ATA CCC; RGA14R—GAG GAA GGA CAG GTT GCC. These were amplified based on the method described by Chen et al. (1998) with the following modifications: initial denaturation at 94°C for 4 min; 40 cycles consisting of 1 min at 94°C, 1 min at 42°C and 1.5 min at 72°C; a final extension of 8 min at 72°C. The PCR products were separated on a 4% polyacrylamide gel at 1,800 V for 2 h after a pre-run at 100 W for 1 h to reach 45–50°C before sample loading. Silver staining for the detection of DNA band patterns was performed using the method recommended by the manufacturer (Promega, Madison, Wis.). All PCR amplifications were run on a PTC-100 thermal controller (MJ Research, Waltham, Mass.).

Statistical analysis

To determine the genetic relationship among the lines, the numerical taxonomy and multivariate analysis system (NTSYS-pc), version 1.80 (Rohlf 1993) was used for cluster analysis of BC_3F_3 genotypic data using simple matching coefficient and unweighted pair-group method, arithmetic mean (UPGMA). Simple and multiple regression analysis were conducted using the General Linear Model (GLM) procedure in SAS (SAS Institute 1989) for test of association between candidate gene markers and phenotypes at the 0.05 and 0.01 levels of significance. Epistatic interaction between markers with significant effects was performed using the GLM procedure, and a higher threshold of P=0.001 was used to declare a significant interaction**.** Since data without normality affects the sensitivity of QTL detection, non-parametric one-way analysis of variance (PROC NARIWAY) in SAS was also conducted on this type of data. A similar treatment of non-normal data has been reported by others (Doebley et al. 1990; Veldboom et al. 1994; Byrne et al. 1996). The PROC CORR procedure was used for estimating the Pearson correlation coefficient. A permutation test for simple regression analysis using SAS (version 8.2) was performed to determine the association between phenotypes and marker genotypes. Permutation was run 100 times and the topmost R^2 value (P=0.01) and fifth R^2 value (P=0.05) were compared with the original R^2 value to determine the significance of association between marker genotype and percentage DLA in the blast nursery, percentage DLA, lesion number and lesion size measured under controlled inoculation. If the threshold values for each trait were less than the original R^2 value, then the association between marker genotypes and the trait was not significant at P=0.05 or 0.01 levels.

Results

Polymorphism of candidate gene markers

A total of 50 candidate gene probes (Table 1) was surveyed for restriction fragment length polymorphism (RFLP) between vars. *Vandana* and *Moroberekan* using seven restriction enzymes. Of the 50 probes, 11 were NBS-LRRs from rice and all of these showed polymorphism between the two parents. The level of polymorphism was lower with probes of defence genes. Seven probes from other cereals, including maize and barley, gave only faint bands, thus they were not included in the progeny analysis. In total, 25 selected polymorphic probes were used for analysing the entire BC_3F_3 population. The candidate genes showed multiple loci, with a total of 70 bands detected by these 25 probes.

The RGA markers showed a higher degree of polymorphism between the parents than that revealed by RFLP probing with the candidate genes. All five RGA primers amplifying 60 bands showed polymorphism between the two parents. As expected, SSR markers were highly polymorphic between subspecies: 47 of the 53 SSR primers surveyed (88.7%) showed polymorphism between vars. *Moroberekan* and *Vandana*. Twenty-five markers amplified by the 23 primers listed in Table 2 were used for further analysis in this study.

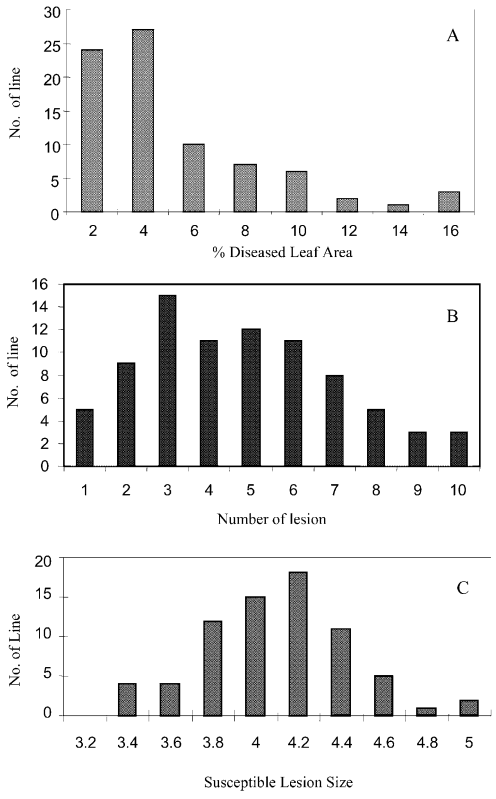

Fig. 1 Distribution of percentage diseased leaf area (**A**), lesion number (**B**) and lesion size (**C**) in the backcross lines inoculated with rice blast isolate PO6-6

Association of markers to partial resistance

We evaluated partial resistance in the BC_3F_3 lines by measuring percentage DLA, lesion number and lesion size against a single isolate of rice blast, PO6-6. *Moroberekan* showed complete resistance to PO6-6, while *Vandana* was moderately susceptible. Of the 80 lines tested, eight (VM14, -15, -19, -42, -46, -53, -57, -60) showed complete resistance (lesion types 1–3, Bonman et al. 1986). Distributions of percentage DLA, lesion number and lesion size are shown in Fig. 1. Among these traits, lesion number per leaf (7.79±4.11) showed the widest range of variation, followed by percentage DLA (4.60±3.98) and lesion size (3.78±1.04, in millimetres). Significant differences in DLA, lesion number and lesion size were observed among the lines. The permutation test also showed significant association between all three traits and marker genotypes at the 0.01 level of significance. In general, DLA was correlated significantly with

Table 3 Molecular markers associated with partial blast resistance to PO 6-6 in the BC_3F_3 lines

Trait	Marker	Source	R^2 (%)	*F*	*P*
DLA[a]	RGA8-4	Flax	11.79	9.36	0.003
	RM215	Rice	9.19	7.09	0.009
Lesion number	Oxalate oxidase	Barley	28.65	27.71	0.0001
	14-3-3 protein	Barley	14.07	11.30	0.001
	RGA1-10	Rice	9.39	7.25	0.009
	RM21	Rice	9.09	6.90	0.01
	RM168	Rice	10.73	8.42	0.005
Lesion size	RM250	Rice	9.55	7.39	0.008

[a] DLA, Diseased leaf area

lesion number (r=0.75) but less correlated with lesion size (r=0.48). Lesion number and lesion size were moderately correlated (r=0.56).

Since resistance often results from the interaction between a resistance gene and an incompatible isolate, the effects of minor genes on quantitative resistance would be masked in the presence of major genes. To examine the contribution of candidate gene markers to partial resistance, we removed the eight lines that had been determined to be resistant to PO6-6 in the greenhouse experiment and analysed the remaining 72 lines for partial susceptibility to PO6-6.

Our analysis of DLA for BC_3F_3 progenies inoculated with PO6-6 suggested that four markers were significantly associated with DLA at P<0.05, of which two of these, RGA8-4 and RM215, were significant at P<0.01. Since the distribution of DLA was skewed, a non-parametric one-way analysis of variance was adopted for analysis, and this confirmed the significance of these two markers (see Materials and methods). RGA8-4 explained 11.8% and RM215 explained 9.2% of the phenotypic variation (Table 3). No epistatic interaction between these markers was observed for this trait.

Nine markers contributed to the variation for lesion number (P<0.05). Among these, five markers, including two candidate genes (oxalate oxidase and 14-3-3 protein), two SSRs (RM21 and RM168) and one RGA marker, were highly significant (Table 3). These five markers explained from 9.1% to 28.7% of the phenotypic variation. Together, they contributed 56.4% of the total variation in the model. No epistatic interaction was found among these markers contributing to lesion number.

Seven markers were significantly associated with a reduction in lesion size at the P=0.05 level, while only RM250 was highly significant at P=0.01 (Table 3). The best-fit model for these seven markers explained 34.5% of the total variation. RM250 accounted for 9.6% of the phenotypic variation.

Disease reaction in the blast nursery

To test whether the performance of the lines against a single isolate could be correlated with that in the field, both the BC_3F_3 and BC_3F_4 populations were evaluated in IRRI blast nursery where a diverse pathogen population was maintained by planting different susceptible rice genotypes in the spreader rows. In the blast nursery var. *Vandana* was moderately susceptible with 28.3% DLA and var. *Moroberekan* was highly resistant with only a few small brown spots (type I lesion). The DLA of *Moroberekan* was estimated to be 0.3% due to the accumulation of small lesions (see Material and methods). The DLA of 80 lines from BC_3F_3 and BC_3F_4 showed a normal distribution, ranging from 0.3% to 78.3% (Fig. 2). Transgressive segregation was observed in both the BC_3F_3 and BC_3F_4 generations where families exhibited a higher DLA than that of the susceptible parent *Vandana*. For example, two lines, VM34 and VM37, were the most susceptible among the 80 lines, whereas two other lines, VM14 and VM15, exhibited complete resistance. Data collected from the first 4 weeks of the experiment were used for analysis as the plants began to recover approximately 4 weeks after sowing. A significant correlation (r=0.81, P<0.01) was found between the DLAs of the BC_3F_3 and BC_3F_4 lines. On average, the BC_3F_4 families showed higher DLA than that of BC_3F_3, suggesting fixation of variation of DLA as the lines approached homozygosity.

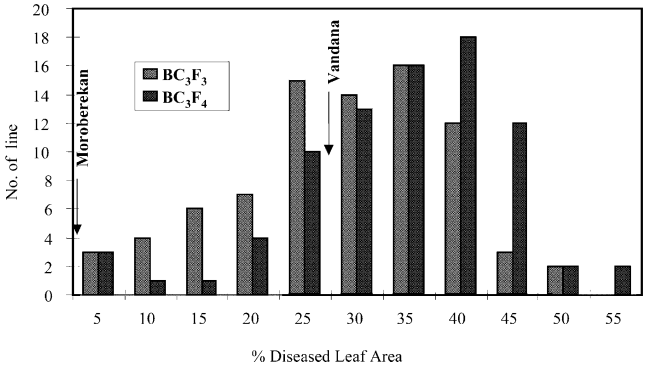

Fig. 2 Distribution of percentage diseased leaf area in the BC_3F_3 and BC_3F_4 populations derived from vars. *Vandana*/*Moroberekan* in the blast nursery, IRRI. A significant correlation between the diseased leaf areas was observed in two populations (r=0.81, P=0.0001)

DLA in the greenhouse and blast nursery was moderately correlated (r=0.48, P<0.01). Both VM14 and VM15 were completely resistant in both the greenhouse and blast nursery. VM34 and VM37 were highly susceptible in the blast nursery but were moderately susceptible to PO6-6 in the greenhouse test. VM2, VM8 and VM40 were judged to be highly susceptible in tests conducted in the greenhouse and in the blast nursery.

Association of markers to DLA in blast nursery

We identified seven markers—PIC19, PIC20, 7-4F (NBS-LRR sequences from maize), PR5 (encoding a putative

Table 4 Markers associated with percentage disease leaf area (DLA) in the BC_3F_3 and BC_3F_4 lines at the blast nursery, IRRI

Marker	Gene type	BC_3F_3		BC_3F_4 [a]	
		R^2 (%)	*P*	R^2 (%)	*P*
PIC19	NBS-LRR	29.64	0.0001	33.37	0.0001
PIC20	NBS-LRR	22.64	0.0001	16.11	0.0002
7-4F	NBS-LRR	21.09	0.0001	15.59	0.0016
PR5 thaumatin	PR protein	13.44	0.0011	8.44	0.0109
RM21	SSR	13.26	0.0010	10.15	0.0045
RGA1–8	Resistance gene analog (LRR)	11.21	0.0026	15.56	0.0003
RGA6-7	Resistance gene analog (kinase)	9.60	0.0054	4.35	0.065

[a] Values of BC_3F_4 were inferred from the genotypes of BC_3F_3

thaumatin), RM21, RGA1-8 and RGA6-7—that contributed to partial resistance under conditions of natural infection in the blast nursery (Table 4). Analysis of variance suggested that these seven markers individually contributed from 9.6% to 29.6% of the variation in DLA observed in BC_3F_3 plants, while together they accounted for 76.0% of the total variation. Epistatic interaction among markers with significant effects was tested using GLM (SAS Institute). Three pairs of digenic interaction were found between PIC19/PIC20, 7-4F/PIC20 and RGA1-8/PIC20 at the P=0.001 level of significance.

Of all the 155 markers analysed, including candidate genes, RGAs and SSR markers, four candidate genes (PIC19, PIC20, 7-4F, PR5) were highly significant and ranked among the top contributors to the resistance phenotype. A single SSR marker (RM21) was found to contribute to lesion number in the greenhouse and to DLA in the blast nursery (Tables 3, 4). RGA1-10 contributed to lesion number in the greenhouse, and RGA1-8 contributed to percentage DLA in the blast nursery. These two markers were amplified from the same primers, but they apparently represent two different loci, contributing to resistance differentially under different stress conditions.

To determine whether significant marker effects detected in the BC_3F_3 generation were also detected in BC_3F_4 plants, we analysed DLA of BC_3F_4 plants from the blast nursery with the genotypes of the BC_3F_3 generation (Table 4). Markers with high effects in BC_3F_3 plants (PIC19, 7-4F, PIC20, PR5, RGA1-8 and RM21) were also detected in BC_3F_4 plants, with the effects of PIC19 and RGA1-8 increasing by 3.7% and 3.3%, respectively, while those of the other markers were reduced by 3.1% (RM21) and 6.5% (PIC20). The only marker that was not detected in BC_3F_4 plants was RGA6-7, which had a relatively small effect in BC_3F_3 generation. The data from BC_3F_3 and BC_3F_4 together indicated a strong association between specific candidate genes and DLA observed in the blast nursery.

Cluster analysis of selected lines and their resistance performance

Based on the DNA profiles of 155 bands derived from fingerprinting with candidate genes, RGAs and SSRs, all of the lines were genetically distant (less than 55% similarity) from the donor parent *Moroberekan*. The lines showed an average similarity of 91±8% to *Vandana*, as was expected in the BC_3 generation. A single line, VM66 which had all of its alleles from *Vandana* and showed 35% DLA, was probably a selfed progeny of *Vandana*. Contrary to our expectation, there was no clear relationship between genotypic groups and phenotypes in the whole population (data not shown). Figure 3 shows the genetic relationships of the five most resistant and five most susceptible lines (5% DLA in the left tail and the right tail of Fig. 1) and their parents in the dendrogram. At 80% similarity level, there are four distinct groups. VM14, VM15 and var. *Moroberekan*, which were all highly resistant, appear as a singleton in three separate branches. Among the five resistant lines, VM15 showed complete resistance with mostly hypersensitive brown spots and resembled *Moroberekan* with respect to leaf morphology and plant type, whereas VM14 was resistant but genetically more similar to var. *Vandana* (66% similarity). The main cluster contains both partial resistance and susceptible lines, all of them resembling *Vandana*. VM76, VM46 and VM19 resembled *Vandana* with a similarity of 89%, 95% and 96%, respectively, but with an improved level of partial blast resistance in the field (Fig. 3). The five most susceptible lines (VM2,

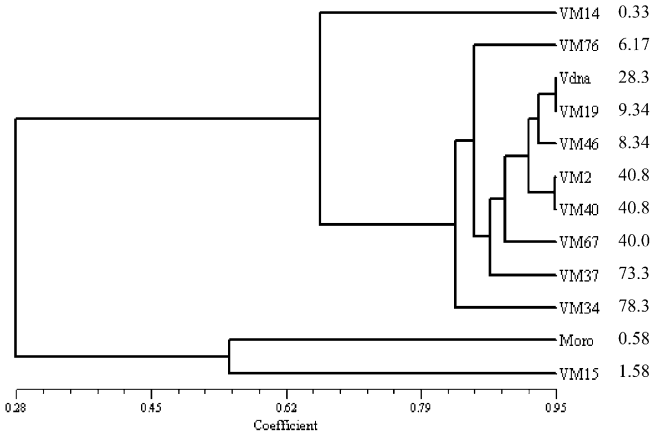

Fig. 3 Dendrogram of five most resistant (*VM14*, *-15*, *19*, *-46*, *-76*) and five most susceptible lines (*VM2*, *-34*, *-37*, *-40*, *-67*) based on 155 molecular markers. *Moro Moroberekan*, *Vdna Vandana*. *Numbers* to the *right* of each *line* indicate percentage diseased leaf area

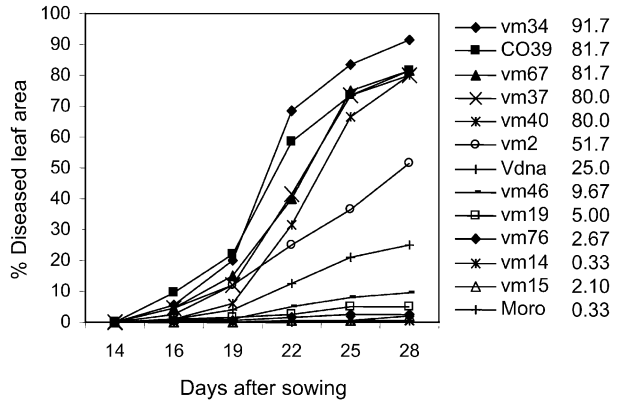

Fig. 4 Disease progress in the five most resistant and five most susceptible lines (see Fig. 3) in the blast nursery. *Moro Morobereken*, *Vdna Vandana*, *CO39* susceptible control. *Numbers* to the *right* indicate percentage diseased leaf area at 28 days after sowing

VM40, VM67, VM34 and VM37) were genetically similar (85–95%) to *Vandana*.

The performance of the selected lines under natural infection was determined by planting ten representative lines from the BC_3F_3 generation—completely resistant lines (VM14 and VM15), partially resistant lines (VM19, VM46 and VM76), susceptible lines (VM2, VM34, VM37, VM40 and VM67), the two parents and susceptible check CO39—in the blast nursery. Disease progress curves observed over a 4-week period showed that the partially resistant lines had a low DLA and relatively slow disease development (Fig. 4). At 4 weeks after sowing, resistant lines VM14 and VM15 remained highly resistant with no susceptible lesions; the partially resistant lines had a DLA ranging from 2.7% to 9.7%, while susceptible lines had a DLA ranging from 51.7% to 91.7% and CO39 had a DLA of 81.7% (Fig. 4). The performance of these lines between the BC_3F_3 and BC_3F_4 generations was also highly correlated (r=0.93, P<0.01).

Discussion

To improve the resistance of upland variety *Vandana*, a set of backcross lines was generated by introgressing resistance from a traditional variety, *Moroberekan*, that is known to have blast resistance (Wang et al. 1994). We applied a panel of candidate defence genes to characterise the backcross population. Markers were identified to be associated with blast resistance following artificial inoculation of the plants with a single pathogen isolate and following exposure to diverse pathogen populations in the field. Using both control and field conditions to evaluate blast resistance has the advantage of not only gaining insights into the genetic control but also revealing the agronomic performance in advanced backcross progeny. In the present study, a total of eight putative QTLs, including five candidate genes, were identified to be associated with blast partial resistance against a single rice blast isolate, PO6-6. The number of QTLs detected was proportional to the variation of the trait: four for lesion number, three for DLA and one for lesion size. A similar phenomenon was observed by Wang et al. (1994).

Our results are consistent with those of previous reports which indicated that some candidate genes, such as oxalate oxidase and 14-3-3 protein, might play important roles in disease response. Faris et al. (1999) reported that both oxalate oxidase and 14-3-3 protein are significantly associated with leaf rust and tan spot in wheat. These two proteins have also been associated with quantitative blast resistance in rice variety *Shanhuangzhan 2* from southern China (Liu et al., personal communication). Oxalate oxidase is involved in the production of active oxygen species that attack and inhibit pathogens (Lamb and Dixon 1997) and can be induced in response to the attack of the powdery mildew fungus in barley (Zhang et al. 1995). Therefore, this enzyme is likely to be involved in resistance to various diseases in different plant species. Interestingly, both of these two candidate genes were not detected for DLA and lesion size, even though the three traits were moderately correlated. Under the natural condition in the blast nursery, six candidate genes including, four NBS-LRR sequences (PIC19, PIC 20, 7-4F and RGA1-8), a kinase RGA (Pto kinase) and PR5 protein were identified as having strong effects on DLA. RM21, a microsatellite on chromosome 11 linked to *Xa21,* was also associated with DLA variation. RM21 was located about 9 cM from the *Xa21* gene (X. Chen et al. 1997) and RGA1-8 was a fragment amplified by the primers designed from *Xa21*. It would be interesting to further investigate what gene(s) nearby *Xa21* may act as a QTL for blast resistance.

Evidence of an association between a QTL and a member of a multigene family has been reported by Wang et al. (1998). *Xa21D*, a member of the *Xa21* family, encoded a receptor-like protein with a LRR domain and conferred partial resistance to bacterial blight. The major gene, *Xa4*, was reported to behave as a recessive QTL against strain CR6 (Li et al. 1999). Results from mapping studies also suggested that some QTLs are located near major genes. In rice blast, a QTL for partial resistance was found near the major gene *Pi7*(t) (Wang et al.1994). QTLs for percentage DLA and lesion number were located near *Pi11*(t) on chromosome 11 in a RIL population derived from Zhong 156/ Gumei 2 (Zhuang et al. 2002a). It is interesting that *Xa21* and *Pi7*(t) are tightly linked based on map position (Causse et al. 1994). There is also circumstantial evidence suggesting co-localisation of QTLs for multiple diseases. QTLs for blast resistance (Zhong 156/Gumei 2) and bacterial blight resistance (Ramalingam et al. 2003) were identified near PIC20 on chromosome 1. This might provide an opportunity to study relationships between QTLs conferring resistance to bacterial blight or blast in rice.

Although we considered var. *Moroberekan* to be the resistance gene donor in this experiment, the candidate defence gene markers showing significant effects are largely var. *Vandana* alleles. At least two possible

explanations may account for this seemingly anomalous result. First, even though *Vandana* was used as the susceptible recurrent parent in the backcrossing scheme, it was originally released as a variety with moderate resistance to leaf blast in eastern India. *Vandana* was a popular upland variety until it succumbed to neck blast. Thus, it is not surprising that beneficial alleles for resistance are present in *Vandana*. Second, only a relatively small number of candidate gene markers were used in this study. It is likely that most of the positive *Moroberekan* alleles were not detected using our limited set of probes.

Epistatic interaction of QTLs from a donor parent is considered to be difficult to detect, especially in advanced backcross generations, since they do not have enough donor genetic background (Tanksley and Nelson 1996). Nevertheless, backcross lines allow dissection of introgressed QTLs. It is interesting that PIC19 was the only candidate gene involved in all three pairs of digenic interaction among the seven markers with significant effects. It is a well-established fact that incompatible interaction between major resistance genes and avirulent pathogen strains is epistatic to compatible interaction. PIC19, being an NBS-LRR sequence, may have a role in the recognition of the invading pathogen, triggering the signal tranduction pathway to activate a downstream defence response. It is possible that diverse pathogen isolates in the field may trigger strong epistatic interaction. This interpretation seems to be consistent with the observation that we did not detect any interaction in experiments in which a single isolate was used. Since there is a diversity of pathogen strains in the field, it is likely that epistasis could play an important role in resistance to blast under natural conditions. The importance of epistasis has been reported in complex traits such as grain yield and its components (Li et al. 1997; Yu et al. 1997; Zhuang et al. 2002b).

Field performance of the lines selected from the BC_3F_3 population indicated that the major QTLs have been captured in the BC lines. The selected lines exhibited partial blast resistance with a coefficient r=0.93 between BC_3F_3 and BC_3F_4 in the blast nursery. The variability of the BC_3F_4 population is slightly less than that of the BC_3F_3 population. Although a small proportion of alleles were from *Moroberekan*, these partial resistant lines will be useful for improving blast resistance in a breeding programme. Our current work is directed towards extending the phenotypic marker analysis to correlate seedling resistance with neck blast resistance under high disease pressure in upland environments.

Acknowledgements We thank many colleagues who provided the defence genes; Nollie Vera Cruz for helpful discussion, Noel Salac and Abe Ona for technical assistance, and Violeta Bartolome for help on statistical analysis. This work was supported in part by an IRRI research scholarship (J. Wu) and by the Asian Rice Biotechnology Network sponsored by the Asian Development Bank.

References

Bai J, Pennill LA, Ning J, Lee SW, Ramalingam J, Webb CA, Zhao B, Sun Q, Nelson JC, Leach JE, Hulbert SH (2002) Diversity in nucleotide binding site-leucine-rich repeat genes in cereals. Genome Res 12:1871–1884

Bent AF (1996) Plant disease resistance genes: function meets structure. Plant Cell 8:1757–1771

Bonman JM, Vergael de Dios TI, Khin MM (1986) Physiological specialization of *Pyricularia oryzae* in the Philippines. Plant Dis 70:767–769

Byrne PF, McMullen MD, Snook ME, Musket TA, Theuri JM, Widstrom NW, Wiseman BR, Coe EH (1996) Quantitative trait loci and metabolic pathways: genetic control of the concentration of maysin, a corn earworm resistance factor, in maize silks. Proc Natl Acad Sci USA 93:8820–8825

Cai D, Kleine M, Kifle S, Harloff H-J, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMA, Lange W, Stiekema WJ, Wyss U, Grundler FMW, Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275:832–834

Causse MA, Fulton TM, Cho YG, Ahn SN, Chunwongse J, Wu K, Xiao J, Yu Z, Ronald PC, Harrington SE, Second G, McCouch SR, Tanksley D (1994) Saturated molecular map of the rice genome based on an interspecific backcross population. Genetics 138:1251–1274

Chen DH, Wang GL, Ronald PC (1997) Location of the rice resistance locus *Pi5* (t) in Moroberekan by AFLP bulk segregation analysis. Rice Genet Newsl 14:95–98

Chen DH, Dela Vina M, Inulai T, Mackill DJ, Ronald PC, Nelson RJ (1999) Molecular mapping of the blast resistance gene, *Pi44*(t), in a line derived from a durably resistant rice cultivar. Theor Appl Genet 98:1046–1053

Chen X, Temnykh S, Xu Y, Cho YG, McCouch SR (1997) Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). Theor Appl Genet 95:553–567

Chen XM, Line RF, Leung H (1998) Genome scanning for conserve motifs of disease resistance genes in rice, barley, and wheat using high-resolution electrophoresis. Theor Appl Genet 97:345–355

Doebley J, Stec A, Wendel J, Edwards M (1990) Genetic and morphological analysis of a maize-teosinte F_2 population: Implications for the origin of maize. Proc Natl Acad Sci USA 87:9888-9892

Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98:219–225

Huang JK, Wen L, Swegle M, Tran HC, Thin TH, Naylor HM, Muthukrishnan S, Reeck GR (1991) Nucleotide sequence of a rice genomic clone that encodes a class I endochitinase. Plant Mol Biol 16:479–480

International Rice Research Institute (1996) Standard evaluation system for rice, 4th edn. International Rice Research Institute, Manila, Philippines

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275

Li ZK, Pinson SRM, Park WD, Paterson AH, Stansel JW (1997) Epistasis for three grain yield components in rice (*Oryza sativa* L.). Genetics 145:453–465

Li ZK, Luo LJ, Mei HW, Paterson AH, Zhao XH, Zhong DB, Wang YP, Yu XQ, Zhu L, Tabien A, Stansel JW, Ying CS (1999) A "defeated" rice resistance gene acts as a QTL against a virulent strain of *Xanthomonas oryzae* pv. *oryzae*. Mol Gen Genet 261:58–63

Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432–1435

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15:809–834

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Naqvi NI, Bonman JM, Mackill DJ, Nelson RJ, Chatto BB (1995) Identification of RAPD markers linked to a major blast resistance gene in rice. Mol Breed 1:341–348

Panaud O, Chen X, McCouch SR (1996) Development of microsatellite markers and characterization of simple sequence length polymorphism (SSR) in rice (*Oryza sativa* L.). Mol Gen Genet 252:597–607

Ramalingam J, Vera Cruz CM, Kukreja K, Chittoor JM, Wu J-L, Lee SW, Baraoidan MR, George ML, Cohen M, Hulbert S, Leach JE, Leung H (2003) Candidate resistance genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. Mol Plant Microbe Interact 16:14–24

Rohlf FJ (1993) NTSYS-pc: numerical taxonomy and multivariate analysis system, version 1.80. Exater Software, New York

Sambrook J, Fritch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

SAS Institute (1989) SAS/STAT user's guide, version 6, 4th edn, vol 1 and 2. SAS Institute, Raleigh, N.C.

Schaffrath U, Zabbai F, Dudler R (2000) Characterization of RCI-1, a chloroplastic rice lipoxygenase whose synthesis is induced by chemical plant resistance activators. Eur J Biochem 267:5935–5942

Shen KA, Meyers BC, Islam-Faridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant Microbe Interact 11:815–823

Shi ZX, Chen XM, Line RF, Leung H, Wellings CR (2001) Development of resistance gene analog polymorphism markers for the *Yr9* gene resistance to wheat stripe rust. Genome 44:509–516

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang J, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804–1806

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Toojinda T, Broers LH, Chen XM, Hayes PM, Kleinhofs A, Korte J, Kudrna D, Leung H, Line RF, Powell W, Ramsay L, Vivar H, Waugh R (2000) Mapping quantitative and qualitative disease resistance genes in a doubled haploid population of barley (*Hordeum vulgare*). Theor Appl Genet 101:580–589

Veldboom LR, Lee M, Woodman WL (1994) Molecular marker-facilitated studies in an elite maize population: I. Linkage analysis and determination of QTL for morphological traits. Theor Appl Genet 88:7–16

Wang G-L, Mackill DJ, Bonman JM, McCouch SR, Champoux MC, Nelson RJ (1994) RFLP mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. Genetics 136:1421–1434

Wang G-L, Ruan D-L, Song W-Y, Sideris S, Chen L, Pi L-Y, Zhang S, Zhang Z, Fauquet C, Gaut BS, Whalen MC, Ronald PC (1998) *Xa21*D encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. Plant Cell 10:765–779

Wang Z-X, Yano M, Yamanouch U, Iwamoto M, Monna L, Hayasaka H, Katayose Y, Sasaki T (1999) The *Pib* gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. Plant J 19:55–64

Yu SB, Li JX, Tan FY, Gao YJ, Li XH, Zhang QF, Saghai Maroof MA (1997) Importance of epstasis as the genetic basis of heterosis in an elite rice hybrid. Proc Natl Acad Sci USA 94:9226–9231

Zhang Z, Collings DB, Thordal-Christensen H (1995) Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. Plant J 8:139–145

Zhuang J-Y, Wu J-L, Chai R-Y, Fan Y-Y, Jin M-Z, Leung H, Zheng K-L (1998) Rapid identification of resistance gene analogs linked to two blast resistance genes in rice. Rice Genet Newsl 15:125–127

Zhuang J-Y, Ma W-B, WuJ-L, Chai R-Y, Lu J, Fan Y-Y, Jin M-Z, Leung H, Zheng K-L (2002a) Mapping of leaf and neck blast resistance genes with resistance gene analog, RAPD and RFLP in rice. Euphytica 128:363–370

Zhuang J-Y, Fan Y-Y, Rao Z-M, Wu J-L, Xia Y-W, Zheng K-L (2002b) Analysis on additive effects and additive-by-additive epistatic effects of QTLs for yield traits in a recombinant inbred line population of rice. Theor Appl Genet 105:1137–1145