Genetic and molecular dissection of blast resistance in rice using RFLP, simple sequence repeats and defense-related candidate gene markers

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Abstract
Blast, Pyricularia grisea (Cooke) Sacc., is one of the most destructive diseases of rice worldwide and can result in significant reductions in yield. The use of resistant cultivars is the most economical and effective way of controlling rice blast. A variety of DNA markers, including plant defense-related candidate gene markers are available for genetic characterization and molecular analysis of rice. A set of 161 recombinant inbred lines, RILs, from a cross between Nemat, an improved and high yielding cultivar, and Anbarboo, a traditional and aromatic rice, was used to identify defense-related candidate gene, RFLP and SSR markers linked to components of resistance to blast, i.e. infection type, lesion density, the percent of diseased leaf area, and lesion size in rice. The RILs were tested using two single blast isolates in greenhouse, and field population of blast in blast nursery in International Rice Research Institute, Philippines, in 2000–2001. Of the 86 defense-related candidate gene, 153 RFLP, and SSR markers 26 defense-related candidate gene, 66 RFLP, and 85 SSR markers were polymorphic in two parental lines. Results showed that a defense gene, b8, a NBS-LRR originated from barley, closely linked to different components of resistance to blast. The defense genes of r5, r7, PrP2, and ERS from rice, maize, and Arabidopsis, respectively, have had minor effects on different components of resistance to blast. The RFLP markers, i.e. RZ536, RG351, RZ76, RZ397 on chromosomes 7, 11 and 12, and the SSR markers including RM224, RM179, and RM277 on chromosomes 11 and 12 were tightly linked to components of resistance to blast. The linked markers can now be used for resistance gene pyramiding and marker-assisted selection in the breeding population. The results suggested the presence of race-specific resistance genes exhibiting strong differential pathogen-host interaction. We need to incorporate new sources of gene pool to make the genetic base broaden.
Keywords*: Blast resistance, RILs, RFLP, SSR, Defense related candidate gene, Rice*

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INTRODUCTION

Rice, after wheat, is the second most important cereal in Iran. Rice cultivation in Iran is over 600,000 hectares with yield of 4-5 ton/ha. Two provinces, Mazandaran and Guilan, account for >80% of Iran's rice production. Most rice cultivars grown in Iran are indica type and under irrigation (Maclean *et al*. 2002). However, productivity is limited by inadequate water, soil conditions, biotic and abiotic stresses (Maclean *et al*. 2002). Grain shape and aroma are two important quality factors for consumers in Iran. Between 1970 and 2000, more than 20 high-yielding rice cultivars were released to farmers but most of them have not gained popularity because of inadequate grain quality to match market needs. Local traditional varieties, e.g. Tarom mahali, Domsiah, Deylamani, Anbarboo, have good grain quality but they are susceptible to diseases (Annual report of Rice Research Institute of Iran, 2000).

Rice blast, caused by *Pyricularia grisea* (Cooke) Sacc., was reported for the first time in 1949 in Iran, but only recognized as an important disease causing economic losses in 1969 (Behdad, 1990). Currently,

blast is endemic throughout Iran, and in some early maturity varieties, yield loss up to 30% has been reported (Behdad, 1990). All stages of blast infection can be found in the field grown plant, seedling, collar, neck, panicle, and grain (Ou, 1985). Developing effective blast resistance in vegetative and reproductive stages of the plant is therefore a prerequisite for improving rice productivity in Iran.

The use of resistant cultivars is the most economical and effective way of controlling rice blast, devastating disease that occurs in most rice-growing area in the world (Ou, 1985). Several approaches have been used to study the complex nature of resistance to blast in rice. Genetic studies of resistance to rice blast were started when the differential system was established for races of *Pyricularia grisea* in Japan in the early 1960s (Ou, 1985). Since then, the inheritance of resistance to rice blast has been extensively studied (Wang *et al*. 1994). So far more than 24 major gene and ten quantitative trait loci (QTLs) associated with blast resistance have been localized using molecular marker technology (Wang *et al*. 1994; Yu *et al*. 1991, 1996; McCouch *et al*. 1988, 1994; Naqvi *et al*. 1995; Chen *et al*. 1999, 1997; Hittalmani *et al*. 2000; Temnykh *et al*. 2000). RFLP markers have been useful for constructing the foundation of rice genetic map (McCouch *et al*. 1988; Causse *et al*. 1995). Other DNA markers, such as microsattelite marker (or simple sequence repeats - SSR) (Wu and Tanksley 1993) based on the variation in the number of simple sequence repeats (SSRs), have become the markers of choice due to the effectiveness and the co-dominance nature of the markers (Jarne and Lagoda 1996; Powell *et al*. 1996).

More recently, progress in genome sequencing projects has provided a large amount of sequence information in plant species. Based on DNA sequence similarity, it is possible to use sequence information to infer gene functions and to identify candidate genes that are putatively associated with a particular biochemical pathway. Molecular polymorphism (or allelic states) of candidate genes can be more informative than anonymous DNA markers with respective to functional diversity (Ellis and Jones, 1998). Candidate gene approach is particularly relevant to assessing disease resistance because many resistance genes have been found to share sequence similarity in conserved domains (Ellis and Jones, 1998; Hulbert *et al*. 2001). In addition to the major resistance genes, many plant genes are known to be involved in disease pathways in both dicots and monocots (Pierpoint *et al*. 1990; Dixon and Harrison, 1990; Piffanelli *et al*. 1999; Dixon, 2001). Candidate genes have been used to associate resistance genes and QTL loci in mapping population (Faris *et al*. 1999; Leister *et al*. 1999, 1998; Pflieger *et al*. 1999, 2001; Toojinda *et al*. 2001).

In the present study, the RIL population was derived from a cross between two parental genotypes. The female parent, Nemat, was resistant to blast races isolates in Iran but susceptible to races of the blast pathogen in the Philippines. Anbarboo, a traditional cultivar, had different reaction to blast isolates in two mentioned conditions. Thus, this population provided a unique opportunity to study the genetics of resistance to blast, to identify molecular markers and plant defense-related candidate genes associated with components of resistance in Iranian rice germplasm.

MATERIALS AND METHODS

Plant material: The plant material consists of 161 recombinant inbred (RI) lines developed from a cross between a resistant rice cultivar to blast pathogen, Anbarboo, and a susceptible rice cultivar, Nemat, as described by Wang *et al*. (1994) in International Rice Research Institute (IRRI), Manila, The Philippines (Fig. 1).

DNA manipulation: To identify probe-enzyme combinations revealing polymorphism between Anbarboo and Nemat, DNA was extracted from the leaves of the two parents by CTAB (Cethyl Trimethyl Ammonium Bromide) method as described by Doyle and Doyle (1990) with a few modifications intended to improve the quality of DNA; two consequence extractions with Chloroform-isoamyl alcohol (24:1) were performed and digested with the restriction enzymes *Sca*I, *Hin*dIII, *Dra*I, *Eco*RI, *Bam*HI, *Xba*I, *Bgl*II and

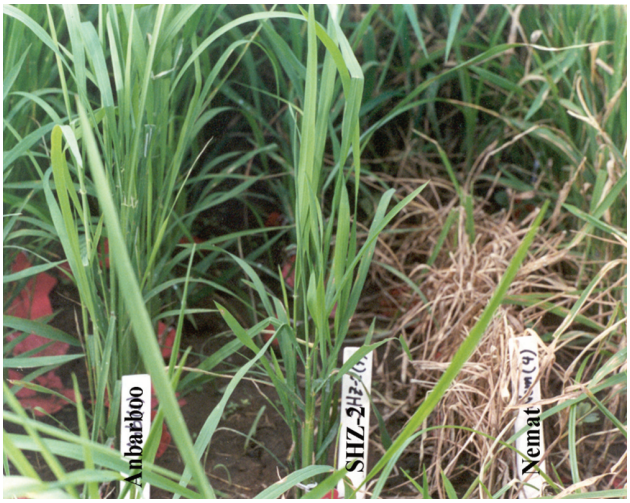

Figure 1. Performance of two parental lines of RIL population, Nemat (Susceptible) and Anbarboo (Resistant), against rice blast isolates (*Pyricularia grisea*) in the field nursery in comparison with SHZ-2, a durably resistant line.

*Eco*RV (Fig. 2). For Southern hybridization, approximately 5 µg genomic DNA was digested with the endonuclease, electrophoresed on a 0.9% agarose gel. The gels were blotted onto a positively charged nylon membrane (Hybond N+ membranes, Amersham, City, UK) by capillary transfer in alkali transfer solution, 0.25 M NaOH and 1.5 M NaCl for 12–16 h and the membrane was washed in 2x SSC for 5 min, and air-dried. All probes were amplified by PCR and labeled with HRP (horse radish peroxidase) according to the protocol of ECL direct nucleic acid labeling and detection system (Amersham, Pharmaciabiotech, UK). Hybridized filters were detected by enhanced chemi-luminescences on Amersham high performance autoradiography X-ray film for 1 to 2h.

Genetic markers: Three sets of DNA markers, including 86 defense-related candidate genes, 153 RFLP and 155 SSR markers distributed over 12 chromosomes were used to characterize the population. For SSR analysis the PCR amplification mixture of 15 µl contained 20–40 ng of genomic DNA, 1.5 µl of 10X PCR buffer solution, 0.9 µl of 25 mM $MgCl_2$, 0.75 µl of 2 mM dNTPs, 0.75 µl of forward and reverse primers (Temnykh *et al*. 2000), 0.5– 1.0 unit

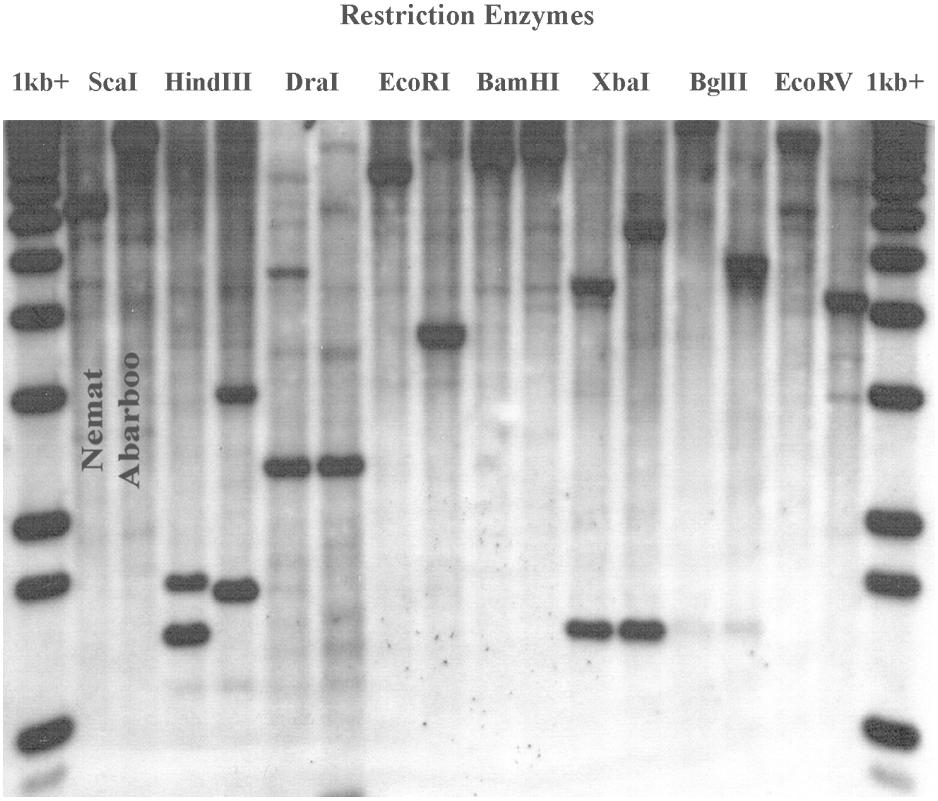

Figure 2. Polymorphism survey of two parental lines, Nemat and Anbarboo, Eight different restriction enzymes were used for RFLP and Candidate gene markers.

of *Taq* DNA polymerase. Amplification was performed in a PTC 100 thermal controller (MJ Research Inc., USA) as described by Chen *et al*. (1997). The basic profile was: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 7 min at 72°C for final extension. Two different annealing temperatures, 61°C and 67°C, were used to amplify specific microsatellite primer sets. PCR products were separated on 4% polyacrylamide denaturing gels and marker bands were revealed using the silver staining protocol as described by Panaud *et al*. (1996).

The candidate gene markers (RFLP) used to characterize the RILs population consisted of sequences carrying the conserved domains of nucleotide binding site–leucine rich repeat (NBS-LRR) and defense-related genes isolated from different studies. Details of these clones are described in web site http://www.ksu.edu/ksudgc maintained by Jan Leach, Department of Plant Pathology at Kansas State University, USA. Although the specific roles of these candidate resistance genes differ (e.g., the NBS-LRR sequences likely represent putative major resistance genes, and the defense-related sequences most likely correspond to quantitative resistance loci), we have called them collectively candidate defense genes as they have putative function to defend the plants against pathogen invasion.

Inoculation tests and disease screening: All RILs were evaluated for resistance to blast by artificial inoculation in the greenhouse at IRRI using two blast fungal isolates that represented the diversity of virulence in the Philippines [M64-1-3-9-1 and Po6-6 (clonal lineage 4)]. Standard procedures for spore preparation, inoculation and disease scoring were followed according to Bonman *et al*. (1986). The RILs were inoculated in 3 replicates (~12 plants per replicate) for each inoculation experiment. The same set of RILs was also tested for disease resistance in the Blast Nursery at IRRI experimental farm. In the disease nursery, the plants were exposed to a diverse pathogen population maintained by planting different susceptible cultivars as spreader rows. Seeds were planted in rows in high density (~ 3 g seeds per 60 cm row) and two rows per RIL were planted as replicates in a completely randomized design. Disease reaction of each line was scored for percent diseased leaf area (DLA%) at 31 days after sowing (Marchetti, 1983; Bonman *et al*. 1986).

Data analysis: The General Linear Model (GLM) procedure in SAS (SAS Institute, V 6.12, 1996) was used for analysis of variance and for test of association between different marker loci; e.g. RFLP, SSR and candidate gene markers; and phenotypes at significant level of P= 0.0001. Since data without normality affects the sensitivity of gene or QTL detection, therefore non-parametric one way analysis of variance (PROC NPAR1WAY) in SAS was also conducted on this type of data. Similar procedure for non-normal data has been reported by other authors (Byrne *et al*. 1996; Doebley *et al*. 1990; Veldboom *et al*. 1994). Single-marker analysis was used to detect associations between marker (genotype) classes and their respective phenotypic values. Interval mapping method using LOD score according to Lander and Botstein (1989), was applied through the use of MAPMAKER/EXP V3.0b and QTL V1.1b software to locate the candidate genes and other markers on the rice map. The PROC CORR in SAS was used for estimation of Pearson and Spearman correlation coefficients.

RESULTS

Parental polymorphism survey: From the 86 defense candidate genes, 153 RFLP, and 155 SSR markers 26 defense candidate genes, 66 RFLP, and 85 SSR markers were polymorphic between Nemat and Anbarboo with one or more enzymes out of the eight used. The level of polymorphism revealed by each enzyme ranged from 11.5 (*Bgl*II) to 69% (*Dra*I), and from 11.1 (*Bgl*II) to 27.5% (*Hin*dIII) for defense candidate gene and RFLP markers, respectively (Table 1). The overall level of polymorphism detected by RFLP and candidate gene markers between two parental lines were 41.94 and 43.14%, respectively (Table 2). Level of polymorphism using rice genomic (RG), rice cDNA (RZ) and oat cDNA (CDO) clones were 61.2, 36.1 and 2.67%, respectively.

Table 1. Frequency of polymorphic markers for different restriction enzymes.

	Restriction enzyme[a]							
Marker	*Sca*I	*Hin*dIII	*Dra*I	*Eco*RI	*Bam*HI	*Xba*I	*Bgl*II	*Eco*RV
Candidate gene	28	28	69	46	53	23	11.5	34.6
RFLP	24.2	27.5	23.5	19.6	17.7	20.9	11.1	27.5

a= Values are in percent (%)

Table 2. Molecular markers used for screening of parental lines and RIL population.

Marker	number of markers	number of polymorphic markers	percent of polymorphic markers
Candidate genes	86	26	41.94
RFLP	153	66	43.14
SSR	155	85	54.84

Table 3. Performance of RIL population of rice against different blast isolates under greenhouse and field conditions (IRRI, Philippines).

	Single isolate (greenhouse test)						blast nursery		
	Po6-6			M64-1-3-9-1					
Trait	mean	Std	Var	mean	Std	Var	mean	Std	Var
LD	40.33	51.11	2612.5	138.01	150.24	22572.48			
DLA	4.33	6.10	37.22	15.49	17.56	308.36	11.63	18.70	349.87
LS	1.30	1.67	2.87	1.89	1.98	3.92			

Std= Standard deviation; Var= Variance, LD= Lesion density (per 100 cm^2); DLA=Diseased leaf area (%); LS=lesion size (mm^2).

Segregation of polymorphism in RI lines: Twenty-six defense candidate gene, 66 RFLP, and 20 SSR markers showing polymorphism between parental lines were chosen for molecular screening and association test in the RI lines. Most of the defense candidate gene markers showed multiple hybridizing bands (up to four), but only four RFLP clones with four bands have been detected. Because allelism could not be assumed for multiple copy probe bands, scoring was dominant /recessive for individual bands in these cases.

Disease reactions of RI lines under field conditions: All the RILs were evaluated for resistance to blast in the Blast Nursery at IRRI experimental farm. Two parental lines, Nemat and Anbarboo, had different reactions against blast isolates in field conditions. Anbarboo showed no disease only with a few lesion type 1 and 2, and therefore it was inferred to contain qualitative resistance gene(s), but Nemat was susceptible with an infection type (IT) 4 or greater and diseased leaf area (DLA) 25.5%. The DLAs of 144 RI lines ranged from 0.1 – 95%. Sixty lines had DLA (1%) or lesser and 14 lines with DLA greater than 30%. Table 3 shows the performance of RI lines in greenhouse and field conditions for components of resistance to blast pathogen.

Performance of RI lines under greenhouse conditions: The RILs were evaluated for resistance to blast by artificial inoculation in the greenhouse at IRRI using two blast fungal isolates that represented the diversity of virulence in the Philippines [M64-1-3-9 and Po6-6 (lineage 4)]. Studies on phenotypic

Table 4. Correlation analysis between components of resistance to blast under greenhouse and field conditions in RIL population of rice.

		Blast isolates (greenhouse tests)								
		Po6-6				M64-1-3-9-1				
Trait		IT (1)	LD (2)	DLA (3)	LS (4)	IT (5)	LD (6)	DLA (7)	LS (8)	DLA (9)
LD(2)	P	0.89**	1.00							
	S	0.88**	1.00							
DLA(3)	P	0.84**	0.90**	1.00						
	S	0.94**	0.91**	1.00						
LS(4)	P	0.92**	0.88**	0.94**	1.00					
	S	0.90**	0.96**	0.92**	1.00					
IT(5)	P	0.007^{ns}	-0.002^{ns}	0.06^{ns}	0.05^{ns}	1.00				
	S	0.03^{ns}	0.0007^{ns}	0.03^{ns}	0.01^{ns}	1.00				
LD(6)	P	0.07^{ns}	0.08^{ns}	0.14^{ns}	0.13^{ns}	0.91**	1.00			
	S	0.06^{ns}	0.07^{ns}	0.08^{ns}	0.09^{ns}	0.92**	1.00			
DLA(7)	P	0.04^{ns}	0.02^{ns}	0.10^{ns}	0.11^{ns}	0.90**	0.94**	1.00		
	S	0.02^{ns}	0.007^{ns}	0.02^{ns}	0.03^{ns}	0.96**	0.93**	1.00		
LS(8)	P	0.02^{ns}	0.003^{ns}	0.08^{ns}	0.09^{ns}	0.94**	0.93**	0.94**	1.00	
	S	0.03^{ns}	0.03^{ns}	0.04^{ns}	0.06^{ns}	0.92**	0.95**	0.93**	1.00	
DLA(9)	P	0.68**	0.68**	0.71**	0.67**	0.25^{ns}	0.35*	0.27*	0.28*	1.00
	S	0.82**	0.81**	0.81**	0.81**	0.17^{ns}	0.21^{ns}	0.16^{ns}	0.19^{ns}	1.00

P= Pearson correlation coefficient; S=Spearman correlation coefficient; IT= infection type; LD= lesion density (per 100 cm^2); DLA= diseased leaf area (%); LS= lesion size (mm^2); ns= nonsigificant; * = significance at α= 5%; **= significance at α=1%

reaction to both major Philippines blast isolates would provide insight into possible genetic control of blast resistance in this population. Anbarboo was resistant to both isolates while Nemat was susceptible. Ninety-four lines (60%) had resistant infection type (0-2) to Po6-6, which is significantly different from ratio 1:1 (χ^2=7.02) while 52% of RI lines were resistant to M64-1-3-9-1 (χ^2=1.36). This means that at least resistance to each blast isolate is controlled by one major blast resistance gene.

Correlation analysis of resistance to blast under field and greenhouse conditions: Results of correlation analysis (Pearson and Spearman) showed that there was a highly significant correlation between all components of resistance, e.g. infection type (IT), Diseased leaf area (DLA), lesion density (LD) and lesion size (LS) in each test and between components of resistance to Po6-6 and field test (Table 4). It was not significant with results from two single isolates and field test vs. M64-1-3-9. It shows that the genes controlling resistance to these two blast isolates are different.

Association of molecular markers with phenotypic data and preliminary genetic linkage map: Candidate gene markers for two parental lines had a 1:1 segregation ratio. Only a few markers, nine, displayed a skewed segregation tested by a χ^2 test at P< 0.01. Analysis of variance using PROC GLM and NPAR1WAY Model in SAS (SAS Institute, Cary, USA) for RI lines inoculated with Po6-6 suggested that a defense candidate gene, b8, a marker consisted of sequences carrying the conserved domains of nucleotide binding site – leucine rich repeat (NBS-LRR) was significantly (P<0.0001) associated with all components of resistance, IT, LD, DLA, and LS. This candidate gene explained the phenotypic variation from 82% for IT to 45% for DLA. Candidate genes r5, a NBS-LRR originated from rice had minor effects on resistance components with R^2 from 10.4 to 6.43% for IT and DLA, respectively (Table 5). These two candidate gene were mapped to chromosome 11, to a region having many resistance gene clusters, including *Pi-1* and *Pi-44(t)* (Fig. 3). Two RFLP and SSR markers RZ536 and RM224 previously mapped to genes and QTLs for resistance, *Pi-*

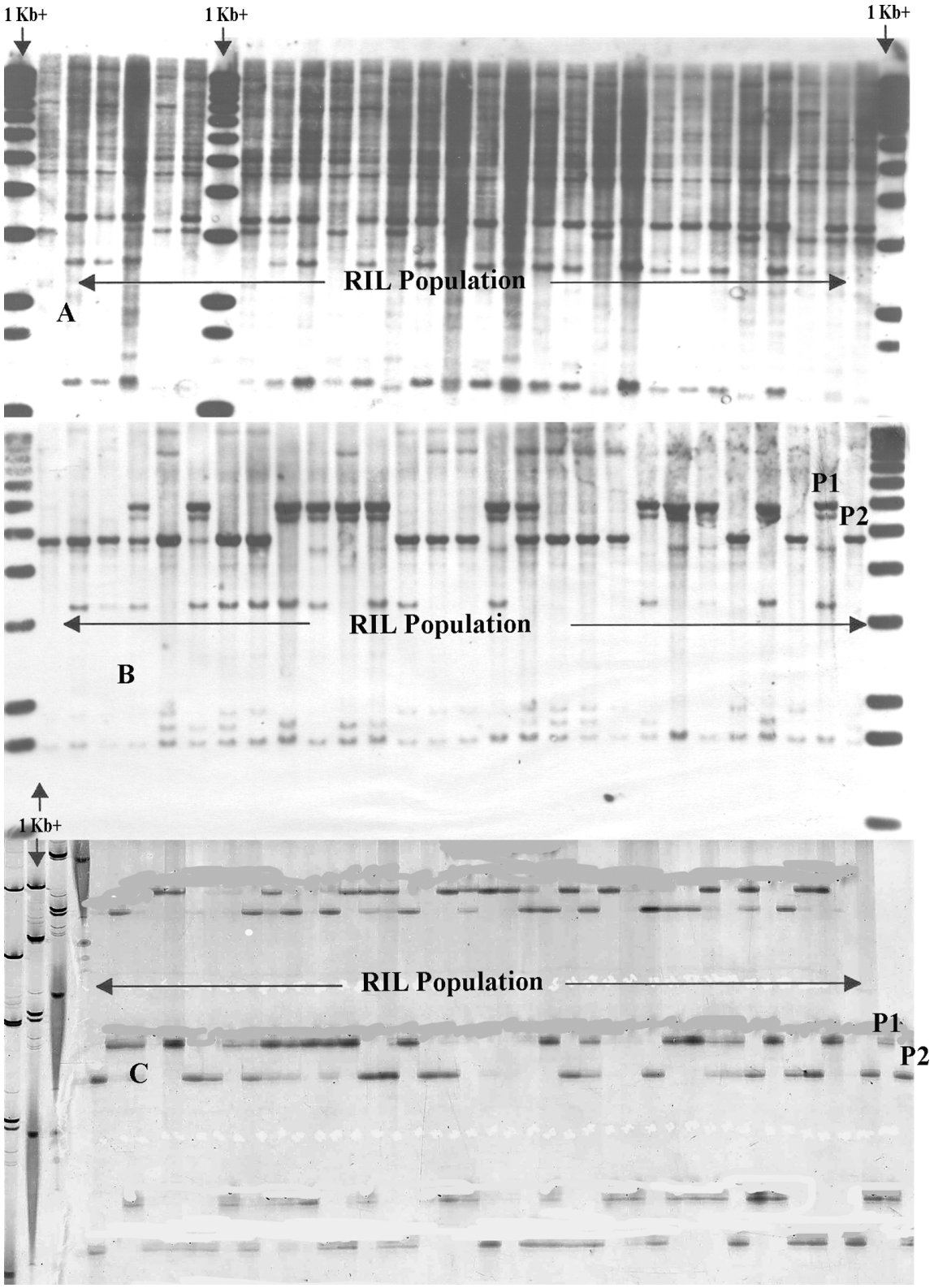

Figure 3. Molecular markers associated with components of resistance and resistance genes to blast in a RILs population of rice. A) Candidate defense gene marker, b8; B) RFLP marker, RZ76; C) SSR marker, RM224; P1= Nemat, P2= Anbarboo.

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Table 5. Candidate genes associated with components of resistance to blast under greenhouse and field conditions in RIL population of rice.

Isolate	trait	marker	gene name	source	R^2(%)	F	P	χ^2(%)	P
Po6-6									
	IT	b8	NBS-LRR	barley	82.18	622.74	0.0001	98.41	0.0001
	IT	r5	NBS-LRR	rice	10.40	17.99	0.0001	16.53	0.0001
	LD	b8	NBS-LRR	barley	61.97	219.97	0.0001	103.43	0.0001
	LD	r5	NBS-LRR	rice	8.13	13.72	0.0001	14.65	0.0001
	DLA	b8	NBS-LRR	barley	45.60	113.32	0.0001	88.42	0.0001
	LS	b8	NBS-LRR	barley	57.42	182.07	0.0001	100.37	0.0001
	LS	r5	NBS-LRR	rice	8.61	14.61	0.0001	15.34	0.0001
M64-1-3-9-1									
	IT	CG28	PrP2	maize	5.22	6.77	0.010	6.51	0.0100
	LD	CG69	ERS	*Arabidopsis*	5.29	6.87	0.0099	6.68	0.0097
	LS	r7	NBS-LRR	rice	5.56	7.24	0.0081	9.38	0.0022
Field nursery									
	DLA	b8	NBS-LRR	barley	40.61	85.48	0.0001	85.47	0.0001

IT= infection type; LD= lesion density (per 100 cm^2); DLA= diseased leaf area (%); LS= lesion size (mm^2).

Table 6. Microsatellites and RFLP markers closely associated with components of resistance to blast in a RIL population of rice.

Isolate	trait	marker	chromosome	R^2(%)	F	P	χ^2(%)	P
Po6-6	IT	RZ536	11	71.08	324.51	0.0001	78.16	0.0001
	IT	RM224	11	59.64	143.24	0.0001	75.89	0.0001
	LD	RZ536	11	58.19	183.68	0.0001	92.44	0.0001
	LD	RM224	11	39.04	83.91	0.0001	66.67	0.0001
	DLA	RZ536	11	46.58	114.91	0.0001	73.19	0.0001
	DLA	RM224	11	30.46	57.37	0.0001	63.84	0.0001
	LS	RZ536	11	54.16	155.99	0.0001	89.71	0.0001
	LS	RM224	11	39.83	86.73	0.0001	68.56	0.0001
M64-1-3-9-1								
	IT	RG351	7	52.89	160.52	0.0001	72.52	0.0001
	IT	RZ397	12	36.60	72.75	0.0001	43.89	0.0001
	IT	RZ76	12	42.82	92.87	0.0001	49.82	0.0001
	IT	RG958	12	9.89	13.92	0.0003	13.40	0.0001
	IT	RG463	12	11.88	12.94	0.0001	15.10	0.0001
	IT	RM179	12	53.84	153.99	0.0001	67.55	0.0001
	IT	RM277	12	53.20	143.13	0.0001	56.84	0.0001
	LD	RG351	12	45.78	120.73	0.0001	69.85	0.0001
	LD	RZ397	12	31.04	56.71	0.0001	40.74	0.0001
	LD	RZ76	12	38.80	78.62	0.0001	49.05	0.0001
	LD	RM179	12	55.69	165.90	0.0001	72.92	0.0001
	LD	RM277	12	49.70	116.59	0.0001	61.58	0.0001
	DLA	RG351	7	43.97	112.21	0.0001	74.19	0.0001
	DLA	RZ397	12	34.76	67.13	0.0001	79.67	0.0001
	DLA	RZ76	12	36.12	70.13	0.0001	47.57	0.0001
	DLA	RM179	12	54.54	158.33	0.0001	68.41	0.0001
	DLA	RM277	12	48.05	109.13	0.0001	59.98	0.0001
	LS	RG351	7	58.27	199.71	0.0001	82.58	0.0001
	LS	RZ397	12	34.81	67.29	0.0001	42.35	0.0001
	LS	RZ76	12	39.44	80.75	0.0001	30.09	0.0001
	LS	RM179	12	54.49	157.98	0.0001	68.61	0.0001
	LS	RM277	12	49.38	117.46	0.0001	59.15	0.0001
Field Nursery								
	DLA	RZ536	11	36.56	69.74	0.0001	78.95	0.0001
	DLA	RM224	11	27.67	46.69	0.0001	69.71	0.0001

IT= infection type; LD= lesion density (per 100 cm^2); DLA= diseased leaf area (%); LS= lesion size (mm^2).

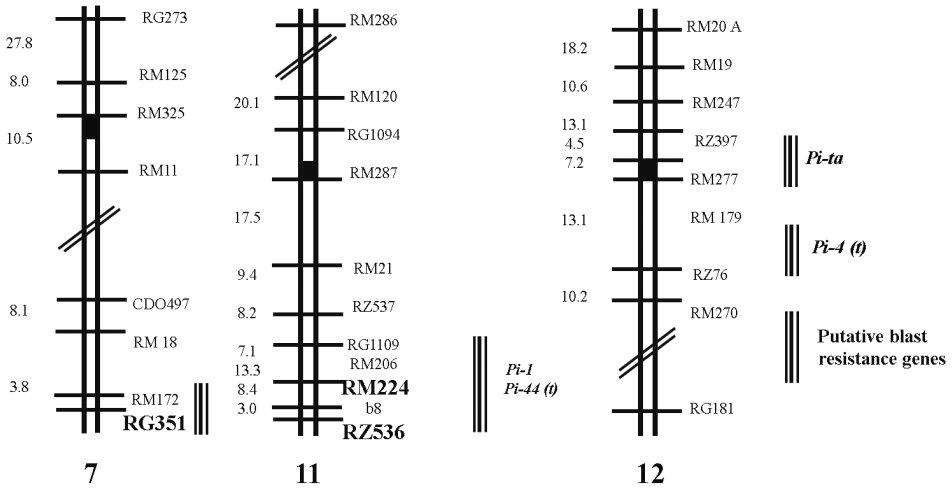

Figure 4. Combined RFLP and SSR linkage map of rice and location of candidate gene. RFLP and SSR markers linked to different blast resistance genes in a RIL population derived from a cross between Nemat and Anbarboo (Original map from Temnykh et al 2000).

1 and *Pi-44(t)* (Mew *et al*. 1994; Hittalmani *et al*. 2000; Chen *et al*. 1999) on chromosome 11 were also associated with b8 and components of resistance in that region (Table 6). Molecular analysis using Mapmaker/Exp showed that the candidate gene b8 is closely linked to a single-copy DNA clone RZ536, with a distance 3.0 cM, and microsatellite marker RM224, on 11, with a distance 8.4 cM (Fig. 4).

Results from candidate gene analysis on resistance components for RI population with second blast isolate, M64-1-3-9-1, showed that no candidate genes with major effects on components of resistance could be detected in this population. Three defense candidate genes, PrP2 from maize, ERS from *Arabidopsis* and r7 from rice with minor effects (R^2=5.22 ~ 5.56) were significantly associated (Table 5). Molecular analysis using anonymous RFLP and SSR markers showed that RFLP clones RZ76 and RZ397, SSR markers RM179 and RM277 mapped on chromosome 12, and RG351 on 7 were tightly linked to resistance to blast pathogen in this population.

Molecular analysis of resistance under field conditions: The DLA data from field conditions was used to detect candidate gene markers affecting the field disease reactions of RI lines using SAS/GLM, NPAR1WAY and Mapmaker/Exp. A candidate gene marker, b8, was also found to be correlated with DLA under field nursery (Table 5). Constructing preliminary linkage map using Mapmaker/Exp suggest that b8 falls in an 11.4 cM interval bracketed by RZ536 and RM224 (Fig. 4).

DISCUSSION

This study provides an overview of the genetics of resistance in Iranian rice germplasm. We focused on a RIL population representing the core breeding materials for improving grain quality and blast resistance. So far, we have been using anonymous markers, including RFLP, RAPD, SSR and so on in our breeding programs to find a marker loci linked to a target trait. For analysis we have to apply many markers distributed over all chromosomes. A new approach to study of genetic and population fingerprint is “Candidate gene approach”. A correlation between the trait under study and allelic polymorphism of the candidate, regardless of the genetic background, is a strong argument in favor of candidate. This approach is largely used in plants and human genetics (Bryne et al. 1996; Prioul et al. 1999). In this study, we observed the RI lines and their parents fall into two group that were either uni-

formly resistant or susceptible, with little quantitative variation or differential disease response. We found that Anbarboo, an Iranian local cultivar, was resistant to Philippines blast isolates whereas Nemat, an improved cultivar in Iran, was mostly susceptible. In contrast, when these cultivars were tested with local isolates in Iran, Anbarboo was found susceptible while Nemat was resistant. The results suggested the presence of race-specific resistance genes exhibiting strong differential pathogen-host interaction. Nikkhah et al., (2002) showed that blast pathogen isolates fromIran and Philippines belong to different races and their DNA banding pattern (Clonal l-in eage) were quite different.

We observed that level of polymorphism revealed by RFLP, SSR and candidate gene markers were high account for 43, 54 and 42%, respectively. Wang et al. (1994) reported high level of polymorphism in their study. The results of this study were in agreement with Chen et al. (1997). We found that the level of polymorphism using rice genomic (RG), rice cDNA (RZ) and oat cDNA (CDO) clones were 61.2, 36.1 and 2.67%, respectively. This is slightly less than 68.3 and 48.3% previously reported for indica/japonica crosses (Wang et al. 1994). Levels of polymorphism using the rice cDNA (RZ) with EcoRV having 18.9% and oat cDNA (CDO) clones with EcoRV and HindIII having 28.5% were the highest level of polymorphism (Table 1). These results were good consistency with those from other experiments and suggest that the RG clones are more efficient at detecting polymorphism in rice crosses. The overall level of polymorphism showed by rice microsatelitte markers (SSR) was 54 .84% compared to 43.14 and 41.94%, with RFLP and defense candidate gene clones, respectively. The same results were reported by Chen et al. (1997).

Small differences in rate of polymorphism in different study could be resulted from different number of markers used. Segregation ratio 1:1 was also good consistency with the ratio 1:1 reported for RI population by Wang et al. (1994) and McClean (http://www.cc.ndsu.nodak.edu). According to results, we suggest that at least one locus on chromosome 11, 12, and on 7, respectively, are involved in conferring complete resistance to blast isolates tested in this study. The loci on chromosome 11 and 12 were tentatively named Pi-1(t), Pi-4(t) and Pi-ta, by Mew et al. (1994), Hittalmani et al. (2000), Yu et al. (1991), Bryan et al. (2000) cloned Pi-ta, which is linked to the centromer of chromosome 12. These results were confirmed by phenotypic data and molecular analysis, specifically candidate gene. Leister et al. (1998) reported the candidate gene b8 and r5 located on choromosome 11 and falls in a 20 cM interval and closely linked to Pi-1(t), Pi-k and Pi-f. Hittalmani et al. (2000) confirmed that RZ397 is linked to Pi-ta gene on chromosome 12 at 3.3 cM, and RZ536 to Pi-1 gene on 11 at 7.9 cM. Chen et al. (1999) reported that the multi-allele locus Pi-k is also present on chromosome 11 near Pi-1. Bryan et al. (2000) cloned Pi-ta gene on centromeric region of chromosome 12. The results of this experiment were good consistency with them. In this experiment, we found that the RFLP clone RG351 on chromosome 7 is linked to components of resistance to blast. Therefore at least one locus controls resistance to blast in that chromosomal region. Wang et al. (1994) reported that this RFLP clone, RG351, is already linked to lesion size. In this study we found that the genetic base of Iran cultivars and germplasm is rather narrow. The results suggest a need to broaden the genetic base through introduction of more distinct germplasm, because rice varieties carrying one or few major resistance genes have a tendency to break down as unpredictable changes occur in the race composition of the pathogen population (Ahn & Ou, 1982). Efforts have been made to develop partial resistance via the accumulation of putative quantitative trait loci (QTLs) (Bonman et al. 1992). We observed that using low number of candidate gene markers with putative functions is potentially more informative than anonymous markers.

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References

Ahn SW and Ou SH. (1982) Quantitative resistance of rice to blast disease. *Phytopathology* 72, 279-282.

Behdad E. (1990) *The Diseases of Field Crops*. (Sepehr Pub. Co. Press, Tehran, Iran). (In Farsi).

Bonman JM, Khush GS and Nelson RJ. (1992) Breeding rice for resistance to pest. *Annu. Rev. Phytopathol.* 30: 507-528.

Bonman JM, Vergel De Dios TI and Khin MM. (1986) Physiologic specialization of *Pyricularia oryzae* in the Philippines. *Plant Dis*. 70: 767- 769.

Bryan GT, Wu KS, Farrall L, Jia Y, Hershey HP, McAdams SA, Faulk N, Donaldson GK, Tarchini T, and Valnet B. (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. *The Plant Cell* 12: 2033-2045.

Byrne PF, McMullen MD, Snook ME, Musket TA, Theuri JM, Widstrom NW, Wiseman BR, and 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.* 93: 8820-8825.

Causse M, Rocher JP, Henry AM, Charcosset A, Prioul JL and Vienne D. (1995) Genetic dissection of the relationship between carbon methabolism and early growth in maize, with emphasis on the key-enzyme lock. *Mol. Breed*. 1: 254-272.

Chen DH, dela Vina M, Inukai T, Mackill DJ, Ronald PC and Nelson RJ. (1999) Molecular mapping of the blast resistance gene, Pi 44(t), in a line derived from durably resistant rice cultivar. *Theor. Appl. Genet*. 98: 1046-1053.

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

Dixon R. (2001) Natural products and plant disease resistance. *Nature* 411,843-847.

Dixon RA and Harrison MJ. (1990) Activation, structure and organization of genes involved in microbial defense in plants. *Adv. Genet.* 28: 165-234.

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

Doyle JJ and Doyle JL. (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Ellis J and Jones D. (1998) Structure and function of proteins controlling strain-specific pathogen resistance in plants. *Curr. Opin. Plant Biol.* 1, 288-293.

Faris JD, Li WL, Liu DJ, Chen PD and Gill BS. (1999) Cadidate gene analysis of quantitative disease recistance in wheat. *Theor. Appl. Genet.* 98: 219-225.

Hittalmani S, Parco A, Mew TW, Zeigler RS and Huang N. (2000) Fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. *Theor. Appl. Genet*. 100: 1121-1128.

Hulbert SH, Webb CA, Smith SM and Sun Q. (2001) Resistance gene complexes: evolution and utilization. Annu. Rev. Phytopathol. 39, 285-312.

Jarne P and Lagoda PJL. (1996) Microsatellites from molecules to populations and back. *Trends Ecol. Evol.* 11: 424-429.

Lander ES and Botstein D. (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* 121: 185-199.

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Gardner A and Schulze-Lefert P. (1998) Rapid reorganization of resistance gene homologues in cereal genomes. *Proc Natl. Acad. Sci*. 95: 370-375.

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Gardner A and Schulze-Lefert P. (1999) RFLP-and physical mapping of resistance gene homologues in rice (*O.sativa*) and Barley (*H.Vulgare*). *Theor. Appl. Genet*. 98: 509-520.

Maclean JL, Dawe DC, Hardy B and Hettel GP. (2002) *Rice Almanac, Source Book for the Most Important Economic Activity on Earth*. (Intrernational Rice Research Institute Pub., Manila, The Philippines).

Marchetti MA. (1983) Dilatory resistance to rice blast in USA rice. *Phytopathology* 73: 645-649.

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR and Tanksley, S.D. (1988) Molecular mapping of rice chromosomes. *Theor. Appl. Genet.* 76: 815-829.

McCouch SR, Nelson RJ, Tohme J and Zeigler RS. (1994) Mapping of blast resistance genes in rice. In: Rice blast disease, RS, Zeigler, S.A., Leong, and P.S., Teng, ed. (CABI and IRRI @ CABI press), pp. 167-186.

Mew TW, Parco AR, Hittalmani S, Inukai T, Nelson R, Zeigler RS and Huang N. (1994) Fine mapping of major genes for blast resistance in rice. *Rice Genet. Newsl*. 11: 126-128.

Naqvi NI, Bonman JM, Mackill DJ, Nelson RJ and Chattoo BB. (1995) Identification of RAPD markers linked to a major blast resistance gene in rice. *Molecular Breeding* 1: 341-348.

Nikkhah MJ, Hedjaroude GH, Sharifi-Tehrani A, Okhovvat SM and Mohammadi M. (2002) Investigation on genetic diversity of populations of *Maganaporthe grisea* (Hebert) Barr, the rice blast fungus, using molecular, pathogenecity and vegetative compatibility characters in Guilan provience, Iran.

Ph.D. thesis, Plant Pathology, University of Tehran.

Ou SH. (1985) *Rice Diseases* (Commonwealth Agriculrural Bureaux, wallingford, UK).

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

Pflieger S, Lefebvre V and Causse M. (2001) The candidate gene approach in plant genomics: a review. *Molecular Breeding* 7, 275-291.

Pflieger S, Lefebvre V, Caranta C, Blattes A, Goffinet B and Palloix A. (1999). Disease resistance gene analog as candidates for QTLs involved in pepper-pathogen interactions. *Genome* 42: 1100-1110.

Pierpoint WS, Jackson PJ and Evans RM. (1990) The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum turberosum*). *Physiol. Mol. Plant Pathol*. 36, 325-338.

Piffanelli P, Devoto A and Schulze-Lefert P. (1999) Defence signaling pathways in cereals. *Curr. Opinion in Plant Biology* 2: 295-300.

Powell W, Machray GC and Provan J. (1996) Polymorphism revealed by simple sequence repeats. *Trends Plant Sci*. 1, 215-222.

Prioul JL Pelleschi S, Sene M, Thevenot C, Causse M, de Vienne D and Leonard A. (1999) From QTLs for enzyme activity to candidate genes in maize. Journal of Experimental Botany 50: 1281-1288.

Temnykh S, Park WD, Ayres N, Cartinhour S, Hauck N, Lipovich L, Cho YG, Ishii T and McCouch, S.R. (2000) Mapping and genome organization of microsatellite sequences in rice. *Theor. Appl. Genet*. 100: 697-712.

Toojinda T, Broers L.H, Chen X.M, Hayes PM, Kleinhofs A, Korte J, Kudma D, Leung H, Line R, Powell W, Ramsey L, Vivar H and Waugh R. (2001) 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 and 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 GL, Mackill DJ, Bonman M, McCouch SR, Champoux MC and Nelson RJ. (1994) RFLP mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. *Genetics* 136: 1421-1434.

Wu KS and Tanksley SD. (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol. Gen. Genet*. 241: 225-235.

Yu ZH, Mackill DJ, Bomman JM and Tanksley SD. (1991) Tagging genes for blast resistance in rice via-linkage to RFLP markers. *Theor. Appl. Genet.* 81: 471-476.

Yu ZH, Mackill DJ, Bonman JM, McCouch SR, Guiderdoni E, Notleghem JL and Tanksley SD. (1996) Molecular mapping of genes for resistance to rice blast (*Pyricularia grisea* Sacc). *Theor. Appl. Genet.* 93: 854-863.