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Z. H. Yu · D. J. Mackill · J. M. Bonman
S. R. McCouch · E. Guiderdoni · J. L. Notteghem
S. D. Tanksley

Molecular mapping of genes for resistance to rice blast (*Pyricularia grisea* Sacc.)

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Abstract Two dominant genes conferring complete resistance to specific isolates of the rice blast fungus, *Pyricularia grisea* Sacc., were located on the molecular map of rice in this study. *Pi-1(t)* is a blast resistance gene derived from the cultivar 'LAC23'. Its map location was determined using a pair of nearly isogenic lines (NILs) and a B_6F_3 segregating population from which the isoline was derived. RFLP analysis showed that *Pi-1(t)* is located near the end of chromosome 11, linked to *RZ536* at a distance of 14.0±4.5 centiMorgans (cM). A second gene, derived from the cultivar 'Apura', was mapped using a rice doubled-haploid (DH) population. This gene was located on chromosome 12, flanked by *RG457* and *RG869*, at a distance of 13.5±4.3 cM and 17.7±4.5 cM, respectively. The newly mapped gene on chromosome 12 may be allelic or closely linked to *Pi*-ta (=*Pi-4(t)*), a gene derived from 'Tetep' that was previously reported to be linked to *RG869* at a distance of 15.4±4.7 cM. The usefulness of markers linked to blast resistance genes will be discussed in the context of breeding for durable blast resistance.

Key words Disease resistance · Rice (*Oryza sativa* L.) · Blast (*Pyricularia grisea* Sacc.) · Restriction fragment length polymorphism (RFLP) · Gene mapping

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Z. H. Yu[1] · S. R. McCouch (✉) · S. D. Tanksley
Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853, USA

D. J. Mackill
USDA-ARS, Department of Agronomy and Range Science, University of California, Davis, CA 95616, USA

J. M. Bonman
Du Pont Agricultural Products, Stire-Haskell Building 200, P.O. Box 30, Newark, DE 19714, USA

E Guiderdoni · J. L. Notteghem
Centre International de Recherches Agronomiques en cooperation pour le Developpement, BP 5035, F-34032 Montpellier Cedex, France

Present address:
[1] USDA-ARS-SCRL, F&B Road, College Station, TX 77845, USA

Introduction

Rice blast disease, caused by the fungal pathogen *Pyricularia grisea* Sacc. [formerly *Pyricularia oryzae* Cav. (Rossman et al. 1990)], constitutes one of the most serious disease problems in rice (*Oryza sativa* L.) worldwide. Efforts to control the disease based on the use of existing resistant varieties have been only partially successful, as the resistance is rapidly overcome in the field under conditions conducive to the pathogen (Kiyosawa 1982). Strategies aimed at breeding for durable rice blast resistance have recently focussed on the possibility of using molecular markers to help combine genes that confer complete and partial resistance (Wang et al. 1994) and on using a lineage exclusion strategy to target resistance gene combinations that are likely to provide an effective barrier to the fungus (Zeigler et al. 1995). At least 12 dominant genes conferring complete resistance to rice blast and 10 quantitative trait loci associated with partial resistance have been located via linkage to genetic markers (McCouch et al. 1994). At present, we do not know the gene products of blast resistance genes, nor are the relationships between resistance genes in the rice plant and avirulence (*avir*) genes in the fungal pathogen well established. However, several *avir* genes from the fungus have recently been cloned (Sweigard et al. 1995; Kang et al. 1995; Leong et al., University of Wisconsin, personal communication), and continued efforts to identify and isolate both resistance genes from the host and *avir* genes from the pathogen can be expected to help elucidate the molecular mechanisms involved in host-pathogen recognition. Knowing the chromosomal locations of blast resistance genes on the genetic map of rice provides the foundation for manipulating and cloning these important resistance genes.

We have previously reported the identification of restriction fragment length polymorphism (RFLP) markers linked to the blast resistance genes, *Pi-2(t)* and *Pi-4(t)*, in rice (Yu et al. 1991). In this paper, we report the molecular mapping of the blast resistance gene, *Pi-1(t)* on chromosome 11, and provide evidence for a closely linked gene, or possibly a new allele of *Pi-4(t)*, (allelic to *Pi*-ta (Inukai et al. 1994) located on chromosome 12.

Materials and methods

Nearly isogenic lines (NILs)

Pairs of nearly isogenic lines (NILs) were developed for several blast resistance genes by introgression of a single major dominant gene into the highly susceptible indica cultivar, 'CO39', via backcrossing (Mackill and Bonman 1992). The NIL used in this study contained the resistance gene, *Pi-1*, derived from the donor, 'LAC23', a japonica cultivar originating in Liberia. The NIL was a BC_6F_4 line and was designated C101LAC-4. Seeds of the parents and the NIL were grown in a greenhouse at Cornell University and fresh-leaf tissue was bulk harvested from several plants of each line for DNA extraction.

BC_6F_3 segregating populations

A population of BC_6F_3 families, from which NIL (C101LAC-4) was developed, were scored for blast reaction (resistant=no sporulating lesions, coded "R"; susceptible=full sporulating lesions, "S"; and Segregating, "Seg") at the International Rice Research Institute (IRRI) in the Philippines. Remnant seeds from the same BC_6F_3's were grown in a greenhouse at Cornell University, and DNA was extracted from fresh leaf tissue. Thirty-six individual BC_6F_3 lines (bulked DNA samples from 10–15 individuals per line) with known R/S phenotypes were used for segregation analysis and to estimate the recombination value between molecular marker loci and *Pi-1(t)*.

Double haploid (DH) mapping population

Seventy-five double haploid (DH) individuals derived from a cross between the blast-resistant indica cultivar, 'Apura', and the blast-susceptible japonica cultivar, 'IRAT177' (Guiderdoni et al. 1989), were scored for blast resistance and molecular markers. Screening with *P. grisea* was performed at the Centre International de Recherche Agronomique pour le Developpement (CIRAD-CA) in France, while molecular marker analysis on the same genotypes was performed at Cornell University.

Fungal inoculation and disease evaluations

During development of the C101LAC-4 NIL, resistance conferred by *Pi-1(t)* was detected following inoculation with *P. grisea* isolate IK81-3 (designated 101) from the Philippines. This isolate belongs to the international race designated IA-125 and to MGR Lineage 19 (Levy et al. 1993; R. Nelson, IRRI, Philippines, personal communication). It is genetically stable and routinely used for genetic studies at IRRI. Isolate 101 was also used to inoculate the BC_6F_3 segregating population from which C101LAC-4 was derived. Inoculation and disease evaluation of the NILs and derived BC_6F_3 populations were conducted at IRRI according to Yu et al. (1991).

Inoculation and disease evaluation of the 'Apura'/'IRAT177' DH population were done at CIRAD-CA in France using *P. grisea* isolates BR14 and BR26. These isolates were respectively collected at Goias and Mato Grosso in Brazil. Unlike the inoculation procedure at IRRI, which involved spraying Isolate 101 inocula onto rice seedlings, BR14 and BR26 were injected into the sheaths of greenhouse-grown rice plants (fourth or fifth leaf stage), using spore suspensions of 2.5×10^4 conidia/ml according to the procedures detailed in Silue et al. (1992).

DNA clone selections

Molecular maps of the rice genome have been constructed at Cornell University (McCouch et al. 1988, Causse et al. 1994). The recent published version of the rice RFLP map consists of over 700 DNA markers (Causse et al. 1994), including both genomic and cDNA clones. As nothing was known about the chromosomal location of the genes we were investigating when we initiated this study, 197 markers distributed throughout the entire rice genome were surveyed to establish the map position of our target genes.

Southern analysis

For analysis of the *Pi-1(t)* -NILs, preparation of rice genomic DNA, restriction endonuclease digestion, electrophoresis, and Southern analysis were according to Yu et al. (1991). For the DH population, the same procedures were followed except that parental DNA of 'Apura' and 'IRAT177' was digested with nine restriction enzymes: *Eco*RI, *Eco*RV, *Dra*I, *Hin*dII, *Xba*I, *Bam*HI, *Bgl*II, *Pst*I, and *Sca*I to increase the probability of detecting polymorphic probe/enzyme combinations for mapping.

Linkage analysis

Linkage analysis was performed using MAPMAKER software (Lander et al. 1987) on the segregation data obtained from RFLP and blast resistance scoring of the BC_6F_3 and DH populations. Distances between markers were presented in Kosambi centiMorgans (cM) (Kosambi 1944). The distances expressed in mean±standard deviation were estimated by Allard's maximum likelihood equation (Allard 1956).

Results and discussion

Polymorphism between parents

There was a difference in the percentage of clones detecting polymorphism between the parents used in NIL development and those used to construct the DH population, despite the fact that both were considered indica×japonica crosses. A higher level of polymorphism was detected between 'CO39' and 'LAC23', the donor of *Pi-1(t)* (64%) than between 'Apura' and 'IRAT177' (40%).

NILs analysis

Introgression of more than a dozen segments of the 'LAC23' donor genome located on 8 different chromosomes was detected in the NIL, C101LAC-4. Markers detecting introgressed segments showed an identical molecular weight (mw) restriction fragment in both C101LAC-4 and 'LAC23', and a different mw fragment in the susceptible recurrent parent, 'CO39'. At some loci, a resistant NIL received alleles from both parents (Fig. 1). This may be due to either residual heterozygosity (Hanson 1959), or heterogeneity of fixed NILs in the BC_6F_4 generation. Though individuals were progeny tested in each generation of NIL development and only those homozygous for blast resistance were selected for future selfing, the fact that the DNA used in this study was extracted from multiple plants means that it is possible that NILs were homozygous for blast resistance, but that in some of the lines, a crossover event had separated a linked RFLP marker from the resistance gene. This would lead to the appearance of heterozygosity or heterogeneity at the marker locus, despite a consistent blast reaction.

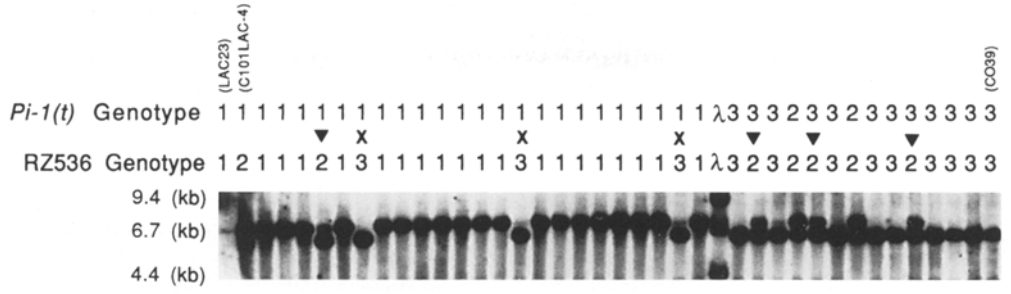

Fig. 1 Cosegregation of *RZ536* and Pi-1(t) in the BC_6F_3 family derived from the NIL, C101LAC-4. The filter consisted of *Dra*I-digested DNA mainly from homozygous-resistant (*left* to lambda) and homozygous-susceptible BC_6F_3 lines (*right* to lambda). Two lines in the susceptible group were segregating for blast resistance and were included in the filter accidentally. A total of ten crossovers were observed among 72 F_2 gametes. C101LAC-4 has two alleles due to heterogeneity in this NIL. The *arrows* mark the occurence of putative crossover events between the *RZ536* locus and the *Pi-1(t)* gene. *X* marks the occurrence of crossovers in both gametes of the F_2 generating the line. *Genotype 1* 'LAC23'/'LAC23', *2* 'LAC23'/'CO39', *3* 'CO39'/'CO39'

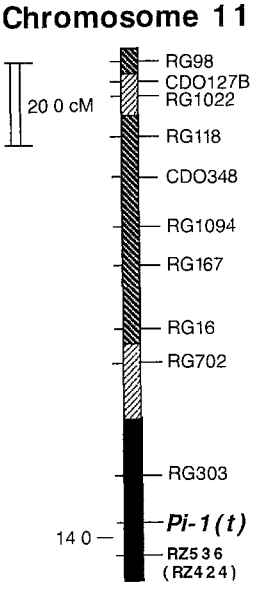

Fig. 2 RFLP map of rice chromosome 11 [from Causse et al. (1994) but in reverse orientation with short arm up (Singh et al. 1996)] showing the region near *Pi-1(t)*. The black area represents the putative introgressed segment from 'LAC23' (resistant donor) in the 'CO39' NIL (C101LAC-4) and is defined by 'LAC23' alleles at *RZ424*, *RZ536*, and *RG303*. Linkage between *Pi-1(t)* and both *RZ536* and *RZ424* was confirmed, at 14.0±4.5 cM and 19.6±5.3 cM, respectively. Linkage between *Pi-1(t)* and *RG303* could not be confirmed on the BC_6F_3 verification filter (see text for details) The *parenthesis* around *RZ424* indicates that this marker has been placed on the map with a LOD<2.5. The *dark, diagonally hatched* area represents a region containing only recurrent parent ('CO39') DNA. The origin of alleles at *RG1022*, CDO127B, and RG702 (*light, diagonally hatched* area) could not be determined because of monomorphism between 'CO39' and 'LAC23'

RFLP mapping of *Pi*-1(t)

To confirm whether *Pi-1(t)* was located in any of the regions of introgression identified during NIL analysis, we scored markers detecting 'LAC23' alleles in each of the introgressions in the NIL on BC_6F_3 populations segregating for blast resistance. Results confirmed that *Pi-1(t)* was located within an introgressed region on chromosome 11, defined by marker loci *RZ424, RZ536* and *RG303* (Fig. 2). *RZ536* was the closest marker, located at a distance of 14.0±4.5 cM. Among the 36 BC_6F_3 lines used to confirm linkage between *Pi-1(t)* and *RZ536,* (23R:2Seg:11S), 4 lines showed single crossovers (one gamete only) and 3 lines showed crossovers occurring in both gametes (Fig. 1). *RZ424* was located at a distance of 19.6±5.3 cM from *Pi-1(t)*, and 5.5±2.8 cM from *RZ536.* The LAC 23 allele was also detected at *RG303* in the NIL. but a monomorphic pattern was observed in the 36 progeny tested, making it impossible to confirm linkage. RG303 appears to have recombined away from the resistance gene in the specific NIL selected from the BC_6F_3 population.

RFLP mapping of blast resistance in the DH population

Linkage analysis for the 75 DH lines was performed using RFLP, isozyme, and phenotypic resistance scores. A major blast resistance locus was detected on chromosome 12, located at 15.8 cM from *RG457* and 23.6 cM from *RG869* using MAPMAKER software (Lander et al. 1987) (Fig. 3b). When Allard's two-point linkage formula (1956) was used, the same order of markers was detected along the chromosome, but the distance was estimated to be only 13.5±4.3 cM between the resistance gene and *RG457*, and 17.7±4.5 cM from *RG869* (Table 1). Using two-point analysis with isozyme markers and different individuals from the same DH population, Abadassi et al. (1991) reported that blast resistance was linked to *Sdh1* at a distance of 15 cM.

Clustering of blast resistance genes on chromosomes 11 and 12

When all of the available linkage information from the previous study about *Pi-4(t)*(Yu et al. 1991) *Pi*-ta (Inukai et al. 1994) and from the current study of blast resistance in cv 'Apura', was combined, it became clear that these genes are either closely linked or allelic (Fig. 3). Clusters of dis-

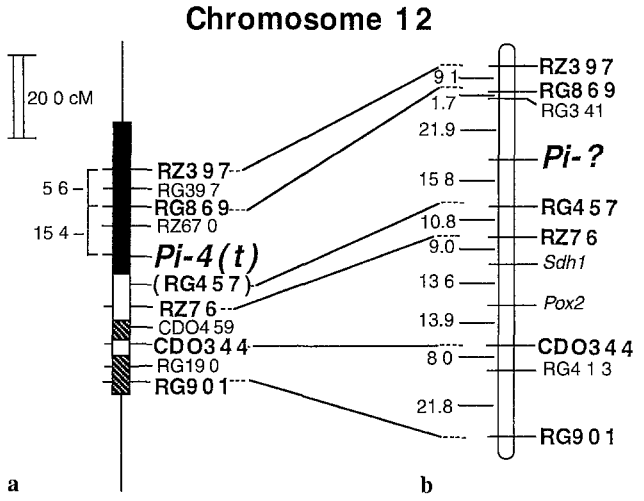

Fig. 3 a RFLP map of rice chromosome 12 [from Causse et al. (1994) but in reverse orientation with short arm up (Singh et al. 1996)] defining the region around *Pi-4(t)* (from Yu et al. 1991 with additional marker data from this study). Yu et al. (1991) reported that *Pi-4(t)* was linked to *RG869* at a distance of 15.4±4.7 cM. Linkage to *RZ397* at a distance of 18.1±5.1 cM was confirmed in this study. *RG457* is placed in *parentheses* just below *RZ76*, signifying that its placement in this interval is with LOD<2.5 (Causse et al. 1994). Markers appearing in *bold* were mapped on both the segregating population described in Causse et al. (1994) and on the 'Apura'/'IRAT177' DH population (described in this paper). The *black area* overlaid on the Causse et al. (1994) map represents the putative region containing *Pi-4(t)* introgressed from the resistant donor ('Tetep') in the NIL, C105TTP-1. Map distances (to the left of the chromosome) were estimated from the segregation of BC_6F_3 individuals. The *dark, diagonally hatched* areas represent regions in the NIL containing only recurrent parent ('CO39') DNA. The *white* areas contain markers on the Causse et al. (1994) map that were not tested on the C105TTP-1 NIL. **b** Molecular map of rice chromosome 12 based on the segregation of isozymes. RFLP markers and blast resistance in the 'Apura'/'IRAT177' DH population. The locus controlling blast resistance in this population was located between *RG457* and *RG869*, in the same position relative to all co-mapped markers as that previously reported for *Pi-4(t)* by Yu et al. (1991). These results suggest that the *Pi-4(t)* and *Pi-?*, the 'Apura'-derived gene for blast resistance, are either closely linked or allelic to one another

ease resistance genes in rice have been reported in other studies (McCouch et al. 1994). Shen et al. (1986) identified 4 closely linked (or allelic) dominant genes for blast resistance derived from 'Tetep' and three other resistant cultivars, and this gene cluster has recently been located to chromosome 12, linked to *RG869* (K. Zheng, CNRRI, Hangzhou, China, personal communication). In another study, Wang et al. (1994) identified a quantitative trait locus (QTL) for partial resistance to blast, also linked to *RG869*. These independent studies suggest that several blast resistance genes may be clustered in this region of chromosome 12, and it is possible that there are multiple alleles at each locus. Similar results have been reported for other blast resistance loci: among 13 blast resistance genes identified in Japan (Kiyosawa 1981), 5 genes are located at the *Pi-k* locus, which is closely linked or allelic to the *Pi-1(t)* locus (Inukai et al. 1994) at the top of chromosome 11 (reported here). Inukai et al. (1994) also observed a possible allelic relationship, or gene cluster, for blast resistance genes *Pi-z* and *Pi-2(t)*, previously mapped independently by Kinoshita (1986) and Yu et al. (1991) on chromosome 6.

Crosses between 'Tetep', or the derived NIL containing *Pi-4(t)* (=*Pi*-ta) (C105TTP-1), and 'Apura' are being made to further test for allelism for the cluster of genes on chromosome 12, and between 'LAC23', or the derived NIL containing *Pi-1* (C101LAC-4), and the *Pi-k*-containing differential developed by Kiyosawa (1981) to test for allelism of genes on chromosome 11. The lines will be evaluated for their blast reaction patterns in response to inoculation with various *P. grisea* races (including those used in this study) in order to help confirm the relationship between these pairs of genes.

Comparisons of the two mapping approaches employed in the study

Two different approaches to molecular mapping of blast resistance genes were employed in this study. *Pi-1(t)* was mapped by analyzing introgressed segments in the NILs and then confirming linkage between the resistance gene and RFLP markers in a small segregating population. The resistance gene from 'Apura' was mapped using a DH population segregating for blast resistance. NILs take considerable time and effort to develop, but they provide a way of dissecting complex genotypes and evaluating the effect of individual genes in a common genetic background. If available, they are excellent material for molecular analysis because the target region containing a gene of interest is generally well-defined and readily identified, provided there is sufficient genetic diversity to detect useful levels of DNA polymorphism. In some cases, NILs may retain segments of donor DNA unlinked to the target genes. This is often difficult to avoid if the donor and the recurrent parent cannot be readily distinguished during the backcrossing process. DH populations are convenient because phenotypic assays can be replicated on identical genotypes by different workers in different locations. This strategy proved very useful in this study, where different blast isolates were available in France and in the Philippines. In addition, the use of populations of inbred lines offers the possibility of tagging several different characters simultaneously based on a single RFLP data set (McCouch and Doerge 1995).

Implications of gene mapping for resistance breeding and future research

Pi-1(t) and the apparent cluster of resistance genes at or near to *Pi-4(t)* are important in rice breeding programs. Though neither gene alone is effective for long in blast-prone environments, there is compelling evidence to suggest that a combination of *Pi-1(t)*, *Pi-4(t)* (=*Pi*-ta), and *Pi-2(t)* [probably allelic to *Pi-z* (Inukai et al. 1994)] would broaden the spectrum of resistance to include all six of the lineages identified in two locations in the Philippines (Zei-

Table 1 Recombination values (cM)[a] of molecular markers and blast resistance segregating in the 'Apura'/'IRAT177' doubled-haploid (DH) population

Marker	*CDO344*	*RZ76*	*RG457*	Blast resistance	*RG869*
RZ76	28.5 ± 6.1				
RG457	18.7 ± 4.5	7.8 ± 3.2			
Blast resistance	24.9 ± 5.5	20.0 ± 5.1	13.5 ± 4.3		
RG869	27.7 ± 5.3	13.9 ± 4.1	10.2 ± 3.2	17.7 ± 4.5	
RZ394	38.0 ± 7.1	20.0 ± 4.8	27.4 ± 6.1	28.2 ± 6.3	8.6 ± 3.1

[a] Recombination values were calculated using R. W. Allard's formula (1956)

gler et al. 1995) and is likely to confer more durable resistance than any of these genes alone. The most efficient way to accomplish this objective would be to use molecular markers as a selection tool. Although the reported distances between these genes and their linked RFLP markers are relatively large at present (Figs. 2 and 3), tighter linkages may be attained by targetting these regions with additional molecular markers. The characterization of genes based on their map location has facilitated the international coordination and systematic identification of blast resistance genes. It has also provided evidence of gene clustering and raised questions about the biological significance of this type of genome organization. Simultaneously, linked molecular markers provide a starting point for fine mapping and eventually for chromosome walking aimed at isolating genes of interest (Song et al. 1995).

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