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Tagging genes for blast resistance in rice via linkage to RFLP markers

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Summary. Both *Pi-2(t)* and *Pi-4(t)* genes of rice confer complete resistance to the blast fungal pathogen *Pyricularia oryzae* Cav. As economically important plant genes, they have been recently characterized phenotypically, yet nothing is known about their classical linkage associations and gene products. We report here the isolation of DNA markers closely linked to these blast resistance genes in rice. The DNA markers were identified by testing 142 mapped rice genomic clones as hybridization probes against Southern blots, consisting of DNA from pairs of nearly isogenic lines (NILs) with or without the target genes. Chromosomal segments introgressed from donor genomes were distinguished by restriction fragment length polymorphisms (RFLPs) between the NILs. Linkage associations of the clones with *Pi-2(t)* and *Pi-4(t)* were verified using F_3 segregating populations of known blast reaction. Cosegregation of the resistant genotype and donor-derived allele indicated the presence of linkage between the DNA marker and a blast resistance gene. RFLP analysis showed that *Pi-2(t)* is closely linked to a single-copy DNA clone RG64 on chromosome 6, with a distance of 2.8 + 1.4(SE) cMorgans. Another blast resistance gene, *Pi-4(t)*, is 15.3 + 4.2(SE) cMorgans away from a DNA clone RG869 on chromosome 12. These chromosomal regions can now be examined with additional markers to define the precise locations of *Pi-2(t)* and *Pi-4(t)*. Tightly linked DNA markers may facilitate early selection for blast resistance genes in breeding programs. These markers may also be useful to map new genes for resistance to blast isolates. They may ultimately lead to the cloning of those genes via chromosome walking. The gene tagging approach demonstrated in this paper may apply to other genes of interest for both monogenic and polygenic traits.

Key words: Disease resistance – Molecular markers – *Oryza sativa* L. – *Pyricularia oryzae* Cav. – Restriction fragment length polymorphism

Introduction

Rice blast, caused by *Pyricularia oryzae* Cav., is one of the most destructive factors in rice production. It occurs in all rice-growing areas worldwide and causes severe damage under favorable conditions. Growing resistant cultivars has been the most economical and effective way of controlling this disease. Inheritance of resistance to rice blast has been studied extensively in both temperate and tropical countries (Kiyosawa 1981; Mackill et al. 1985). Complete resistance is generally conferred by major dominant genes. Very few recessive resistance genes (Woo 1965; Yu et al. 1987) and some modifying genes (Rosero 1967) have also been reported. However, no information on linkage associations is available for blast resistance genes, except for those identified in Japan (Kinoshita 1986). Although there have been many investigations, the gene products of blast resistance genes are not currently known. Consequently, fundamental studies on gene interaction between host resistance and pathogen virulence have been hindered.

The recent development of restriction fragment length polymorphism (RFLP) techniques offers a new tool to monitor gene transfer in breeding programs and potentially to clone the genes whose products are not currently known (Tanksley et al. 1989). Genes of interest can be located on an RFLP map through the use of nearly isogenic lines (NILs) (Young et al. 1988). The basis

of gene tagging using NILs lies in the way the gene of interest is introduced into a recurrent parent by introgression from its donor. After a number of generations of repeated backcrossing, the genome of the selected progeny becomes otherwise identical to that of the recurrent parent, except for the introgressed DNA segments from the donor variety in which the gene of interest resides. These introgressed segments, if polymorphic relative to the recurrent background, can be used as targets to determine whether a specific DNA clone is located on a flanking region of that gene. With a recently constructed rice RFLP map (McCouch et al. 1988) and a set of rice NILs developed at the International Rice Research Institute (IRRI) (Mackill et al. 1988), we have been conducting experiments to identify RFLP markers that are tightly linked to blast resistance genes. Results from that study are reported herein.

Materials and methods

Plant varieties

A set of rice NILs has been developed by backcrossing using the highly susceptible indica cultivar CO39 for more systematic studies of resistance genes in the host and avirulence genes in the pathogen (Mackill et al. 1988). All NILs are the product of six backcrosses followed by three selfings (B_6F_4). Morphologically, they are similar to the recurrent parent CO39. At least four nonallelic genes for complete resistance to blast have been identified in these NILs by allelism tests (D. J. Mackill and J. M. Bonman, in preparation). Among them, the *Pi-2(t)* gene was derived from resistant cultivar 5173, and the *Pi-4(t)* gene from Tetep (Table 1). Both donors are in indica background. In conformity with rules for gene designation in rice, all blast resistance genes newly identified at IRRI are indicated as being of a tentative nature (t).

Seeds of the parents (5173, Tetep, and CO39), corresponding NILs, and B_6F_3 populations segregating for blast resistance were provided by IRRI and grown at Cornell University for fresh-leaf tissue harvest.

Fungus inoculum and disease evaluation

Five *P. oryzae* isolates collected from The Philippines were used for development of NILs. They were designated 101, 102, 103, 104, and 105, all belonging to different international races IA-125, IF-3, IB-47, ID-15, and IA-127, respectively. These isolates are genetically stable and routinely used for studies at IRRI. The same isolates were used to inoculate the subset of B_6F_3 segregating populations.

Inoculum was prepared as described previously (Bonman et al. 1986). At about the four-leaf stage of the rice seedling, the inoculum suspension of ca. 5×10^4 conidia/ml was sprayed onto rice leaves in a controlled dew chamber. Inoculated seedlings were transferred to an air-conditioned room after 1-day incubation. Blast disease was monitored about 1 week after inoculation, when typical lesions developed on the leaves of CO39.

Screening of RG clones

An RFLP genetic map of the rice genome was constructed (McCouch et al. 1988) and has been recently augmented with additional DNA markers (S.D. Tanksley, unpublished data). These DNA markers are mostly produced from a rice genomic DNA library by digesting the IR36 variety with the restriction enzyme *Pst*I. According to estimates of Hanson (1959), the average length of introgressed segments at B_6F_4 is at least 15 cMorgans long. One hundred and forty-two mapped rice genomic clones, well-destributed on the map at this interval, were thus chosen for the NIL survey.

Table 1. Nearly isogenic lines (NILs) for complete blast resistance

No.	NIL	Reaction pattern group[a]	Gene designation[b]
1.	C101A51-2	2	*Pi-2(t)*
2.	C102A51-4	2	*Pi-2(t)*
3.	C103A51-24	2	*Pi-2(t)*
4.	C104A51-4	2	*Pi-2(t)*
5.	C105A51-2	2	?
6.	C101TTP-3	3	?
7.	C102TTP-20	3	?
8.	C105TTp-1	3	*Pi-4(t)*

[a] Reaction pattern of each NIL to a set of *P. oryzae* isolates was phenotypically grouped (Mackill et al. 1988)
[b] More allelism studies for three NILs with a question (?) mark remain necessary (D.J. Mackill and J.M. Bonman, in preparation)

Table 2. Percent polymorphism detected between rice resistant donor lines and susceptible recurrent parent (CO39), and proportion genome covered by polymorphic clones

Donor	Race	Origin	Polymorphic clones	Genome coverage[a]
5173	Indica	Colombia	41/142 (29%)	32%
Tetep	Indica	Vietnam	46/142 (32%)	40%

[a] The genome coverage means total polymorphic regions over the entire genome in terms of cMorgans (based on Hanson 1959)

Chromosome numbering system

A newly unified chromosome numbering system has been recently established at the Second International Rice Genetics Symposium in The Philippines (T. Kinoshita, personal communication). The system is also based on the pachytene length of the chromosomes, but unifies all previously used chromosome numbering systems. We adopt this system here, which is different from chromosome numbering in the rice RFLP map published by McCouch et al. (1988).

Plant DNA extraction, restriction digests, electrophoresis, and Southern analysis

Plant DNA was prepared from fresh-frozen leaf tissues according to McCouch et al. (1988). Total genomic DNA was digested with five restriction enzymes found to be most efficient in detecting polymorphism: *Eco*RV, *Xba*I, *Eco*RI, *Hind*III, *Dra*I (Wang and Tanksley 1989). For the F_3 verification filters, only those enzymes giving positive results were used. Electrophoresis and Southern analysis were according to McCouch et al. (1988). Autoradiograms from the hybridization of Southern blots to the RFLP markers were analyzed on a locus-by-locus basis.

Results

Identification of RFLP markers putatively associated with blast resistance genes

The polymorphism rate within rice subspecies (i.e., japonica and indica types) is generally lower than between subspecies (Wang and Tanksley 1989). Because CO39 and both donors 5173 and Tetep are indica rice varieties, only about 30% of total tested rice genomic clones showed different restriction fragment patterns on the NIL survey filters (Table 2). Once a clone is tested to be polymorphic, it represents 15 cMorgans each side of the polymorphic chromosomal segment (Hanson 1959). Therefore, all tested polymorphic clones cover about 40% of the rice genome being searched (maps not shown). The majority of the polymorphic clones produced identical restriction fragment patterns with DNA from pairs of NILs [i.e., same RFLPs between CO39 and CO39-*Pi-2*(t), or between CO39 and CO39-*Pi-4*(t) digests]. At such loci, the resistant isogenic line inherits its alleles from the recurrent parent rather than from the donor. Thus, resistance genes are not likely to be near these markers. However, a few clones exhibited different RFLP patterns between NILs. The size of hybridizing restriction fragments was identical in both the resistant isogenic line and the donor, but different from the susceptible isogenic line CO39. Figure 1 shows four NILs containing the same allele as their donor parent 5173, when probed with RG64. Two heterozygous NILs may be due to lack of fixation in the selfed generations or recombination between the linked RFLP and resistance gene. Because the *Pi-2(t)* gene in NILs was introduced from the donor 5173, the common RFLP band is a good indication that RG64 is located in the polymorphic segment of DNA flanking *Pi-2(t)*. Such clones were considered potential positive markers associated with the blast resistance genes.

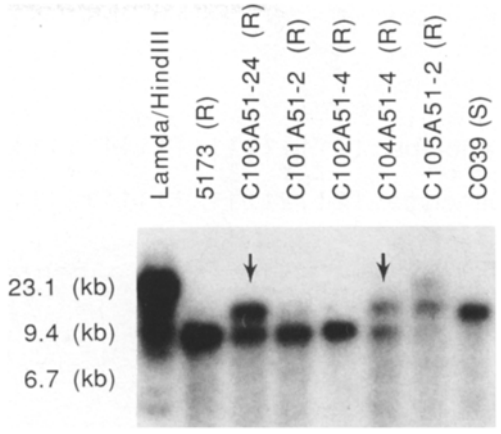

Fig. 1. A survey of the DNA clone RG64 with *EcoR*I digests of the resistant donor 5173 (*left*), susceptible recurrent parent CO39 (*right*), and five resulting NILs: C103A51-24, C101A51-2, C102A51-4, C104A51-4, C105A51-2 (from 5173 to CO39). Note that C103A51-24 and C104A51-4 had received alleles from both 5173 (*lower*) and CO39 (*upper*). This was probably due to heterozygosity at RG64 locus in these NILs. A total of 142 rice genomic clones was tested for the presence of RFLPs between each of five pairs of NILs by hybridization to *Pi-2(t)* survey filters

Verification of linkage and estimation of map distance between positive RFLP markers and blast resistance genes

Wherever putatively positive clones were identified, they were verified on populations segregating for blast resistance. Confirmation of linkage between an RFLP marker and the resistant genotype was obtained through use of segregating B_6F_3 families, derived from single B_6F_2 populations for this purpose. Individual lines of a B_6F_3 family were scored at IRRI for blast reactions [Resistant (R), Segregating (Seg), Susceptible (S)]. Remnant seed from the same F_3s were growing at Cornell University for fresh-leaf tissue harvest. Normally, only homozygous resistant and susceptible members of an F_3 family were included in the verification filters. A cosegregation of the scored genotypes for blast reaction and the restriction fragment pattern was monitored after hybridization with putatively positive clones. If the donor-derived fragment cosegregated with the resistance and the CO39-derived fragment with the susceptibility, the linkage was then established. The map distance between the RFLP markers and resistance genes was estimated from the number of crossovers in the F_2 generation.

Because the NILs used in this study were at a relatively early stage of backcrossing, many of the potential candidate clones turned out to be false positives upon testing of small populations segregating for the genes (data not shown). In the case of false positives, the RFLP patterns and disease reactions segregated independently. These apparently reflect residual donor chromosomal segments still present in the isolines and unrelated to the resistance genes. It was confirmed that two DNA clones were linked to different blast resistance genes. RFLP analysis has confirmed that the *Pi-2(t)* gene is closely linked to a single-copy DNA clone RG64 on chromosome 6 (as chromosome 3 in McCouch et al. 1988). It was possible to determine all three genotypes in the lines of a B_6F_3 family, since heterozygotes could be identified by segregation for resistance within a line. The use of only homozygous genotypes resulted in an easy observation of cosegregation. Three crossovers were found in the F_2 generation among 36 F_3 lines (26R:2Seg:8S) from a cross between CO39 and a NIL C101A51-2 (Fig. 2a), and only one crossover was found among 36 F_3 lines (22R:1Seg:13S) from a cross between CO39 and a NIL C104A51-4 (Fig. 2b). Since earlier genetic studies discovered that C101A51-2 and C104A51-4 may have the same *Pi-2(t)* gene from their donor variety 5173, the combined

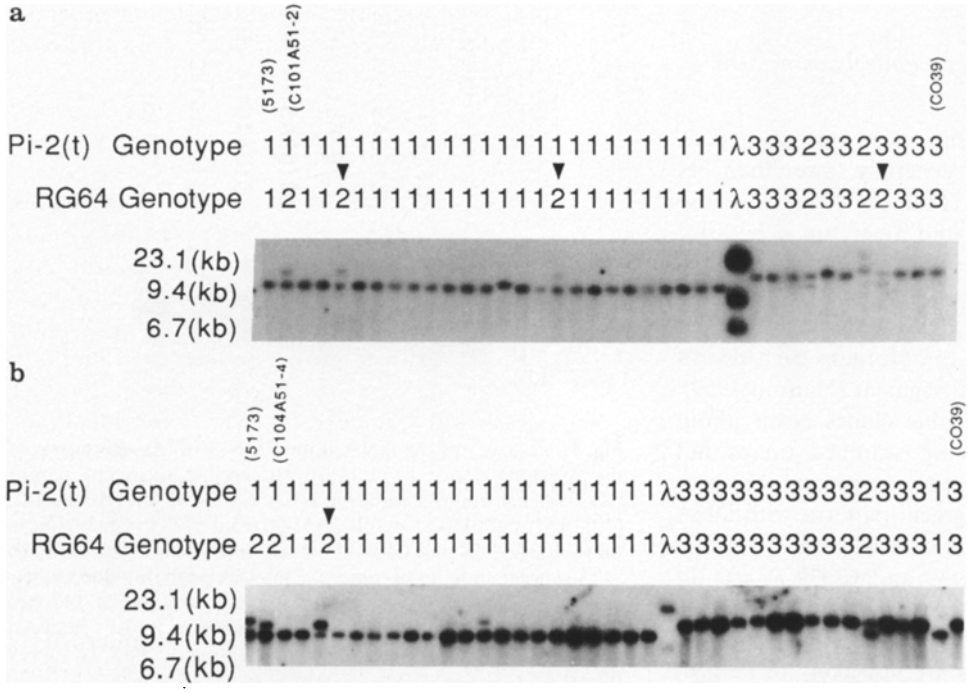

Fig. 2a and b. Cosegregation of the DNA clone RG64 and *Pi-2(t)* gene in the B_6F_3 family of C101A51-2 (**a**) and C104A51-4 (**b**). These filters consisted of *Eco*RI digested DNA mainly from homozygous-resistant (*left* to lamda) and homozygous-susceptible F_3 lines (*right*). The *arrows* mark the occurrence of crossover events between the RG64 locus and the *Pi-2(t)* gene. In **a**, two lines were phenotypically segregating and accidentally included in the filter. In **b**, there were one segregating line and one resistant line in the S group. Genotypes: 1 = 5173/5173; 2 = 5173/CO39; 3 = CO39/CO39

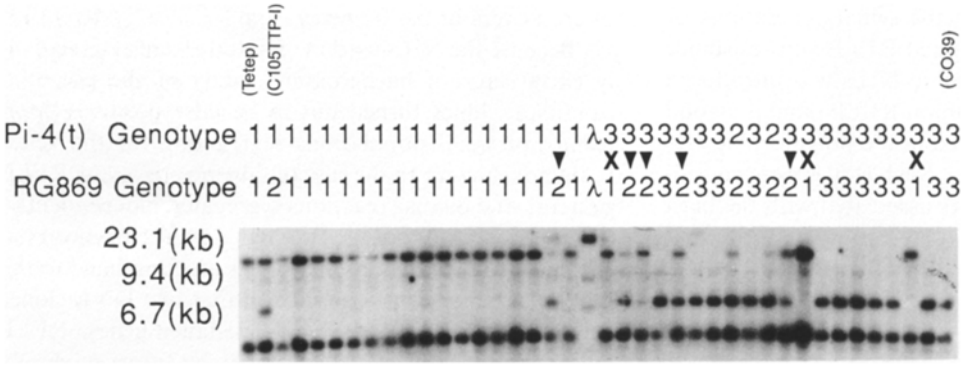

Fig. 3. Cosegregation of the DNA clone RG869 and *Pi-4(t)* gene in the B_6F_3 family of C105TTP-1. *Eco*RV-digested DNA from homozygous-resistant and homozygous-susceptible lines was blotted onto the filter. As in Fig. 2, two segregating lines were included in the S group accidentally. There were two copies of RG869 DNA sequence in these indica rices (Tetep and CO39); the fragment with a higher molecular weight was identified as residing on chromosome 12. In this case, more recombinant lines (see *arrows*) were observed. "X" marks the occurrence of the double-crossover in the line. This would be possible if the distance between an RFLP marker and the resistance gene is large. Genotypes: 1 = Tetep/Tetep; 2 = Tetep/CO39; 3 = CO39/CO39

data from the two populations were used to estimate the map distance. Figure 4a shows an RFLP map of rice chromosome 6, where the *Pi-2(t)* gene and RG64 marker are linked at a distance of 2.8 + 1.4 (S.E.) cMorgans.

In addition, it was discovered that the *Pi-4(t)* gene was significantly linked to a DNA clone RG869 on chromosome 12 (as chromosome 6 in McCouch et al. 1988). Five single and three double crossovers were observed in the F_2 generation among 36 F_3 lines (17R:2Seg:17S) from a cross between CO39 and a NIL C105TTP-1 (Fig. 3). The estimated distance between *Pi-4(t)* and RG869 is about 15.3 + 4.2 (S.E.) cMorgans. As seen in Fig. 4b, the introgressed segment containing the *Pi-4(t)* gene on chromosome 12 might be as large as 40 cMor-

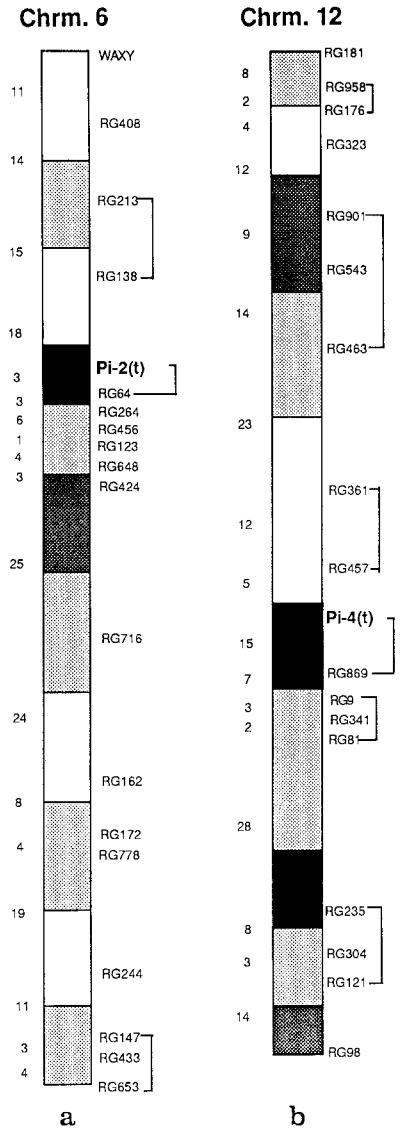

Fig. 4. **a** RFLP map of chromosome 6 (as chromosome 3 in McCouch et al. 1988) showing the region near *Pi-2(t)*. Note that *Pi-2(t)* is linked ot RG64 with a distance of about 3 cMorgans. **b** RFLP map of a main linkage group of chromosome 12 (as chromosome 6 in McCouch et al. 1988) showing the region near *Pi-4(t)*. The distance between *Pi-4(t)* and RG869 is about 15 cMorgans. On both maps, the orientation of *Pi-2(t)* and *Pi-4(t)* to RFLP markers remains to be further clarified. Marker designations are on the *right*, and the genetic distances are given in cMorgans on the *left*. The black areas represent the introgressed segment(s) from the donor. It was concluded that RG235 on chromosome 12 was a false-positive clone, upon verification of the segregation population. Thus, there were also possibly two independent introgressed segments on chromosome 12 from Tetep. The dark cross-hatched areas represent the negative regions, polymorphic between the donor and CO39. The light cross-hatched areas represent monomorphic chromosomal segments. The white areas were not tested due to repetitive DNA sequences

gans. This is not unexpected, since the breeding program introducing the *Pi-4(t)* gene into CO39 consisted of only six backcross generations.

Discussion

We are now focusing on these two chromosomal regions to define the precise locations of the *Pi-2(t)* and *Pi-4(t)* genes. Their orientations to the DNA markers and tighter linkages will be achieved as the rice RFLP map becomes saturated with additional markers. Tightly linked DNA markers may facilitate early selection for genes of interest in breeding programs. Since nearly all DNA markers are codominant in nature, individuals homozygous for the desired alleles can be identified in the F_2 generation of a cross, without F_3 progeny testing. This so-called marker-aided selection may be very useful for the genes controlling such traits, whose expression is influenced by the environment and/or are phenotypically difficult to be scored.

The linked DNA markers may also be employed as powerful tools to map new resistance genes to *P. oryzae* isolates in both tropical and temperate countries. It has been difficult to compare blast resistance genes identified in different countries. One major obstacle is that the blast fungus is not easily obtained from abroad, and thus a universally susceptible cultivar that is accepted internationally has not yet been developed. However, gene comparisons become possible with the help of tightly linked DNA markers. For example, the *Pi-z* gene, resistant to Japanese *P. oryzae* isolates, is also on the middle region of chromosome 6, based on a classical genetic study in Japan (Kinoshita 1986). The relationship of the *Pi-2(t)* and *Pi-z* genes should be clarified soon.

The mechanism of host-pathogen interaction is of great interest to plant scientists, but as yet remains unsolved. Information about which genes and gene products confer resistance in the plant and avirulence in the pathogen is essential for such studies. In the case of blast disease, cloning avirulence genes from the blast fungus is in progress (Valent et al. 1991). Currently, the gene products for blast resistance are unknown. With the development of RFLP technology, a map-based cloning procedure may be used to isolate economically important genes (Tanksley et al. 1989). High resolution mapping and gene tagging are the first steps towards the eventual cloning of important genes in plants. Although most of the DNA markers near the *Pi-2(t)* and *Pi-4(t)* genes were monomorphic between donors and CO39 (Fig. 4a, b), the orientation of the genes to rice RFLP markers and closer linkage may be achieved with the additional restriction enzymes and additional polymorphic DNA clones. Tightly linked RFLP markers can be used to begin chromosome walking or to isolate very large DNA fragments containing the genes.

After six generations of backcrossing, some pieces of introgressed segments still existed that were not related to the blast resistance genes. For this reason, we found several false-positive clones. These clones gave positive results on the NIL survey filters, showing different restriction fragment patterns between a pair of NILs. However, they gave negative results on the verification filters, showing independent segregation between the donor-derived allele and resistance genotype. Identification of such clones can be used to "purify" the NILs by removing those introgressed DNA segments upon further backcrossing.

Finally, partial resistance to disease has received increasing attention in plant breeding. Partial resistance to blast seems more durable. The mechanism of such resistance is not well understood. Expression of partial resistance is influenced by several factors and is generally inherited quantitatively. Based on the measurement of leaf blast by lesion number and lesion size, Wang et al. (1989) studied the inheritance of partial resistance to blast in two indica cultivars. They found that the heritability estimates were so low that breeding programs could not easily introduce them to other rice cultivars. RFLP markers may help locate various Mendelian loci and determine their relative contribution to partial resistance.

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