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Genetic and physical mapping of *Pi5(t)*, a locus associated with broad-spectrum resistance to rice blast

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Abstract To gain an understanding of the molecular basis for resistance to rice blast (*Magnaporthe grisea*), we have initiated a project to clone *Pi5(t)*, a locus associated with broad-spectrum resistance to diverse blast isolates. AFLP-derived markers linked to *Pi5(t)*-mediated resistance were isolated using bulked segregant analysis of F_2 populations generated by crossing three recombinant inbred lines (RILs), RIL125, RIL249, and RIL260 with the susceptible line CO39. The most tightly linked AFLP marker, S04G03, was positioned on chromosome 9 of the fingerprint-based physical map of Nipponbare, a well-characterized rice genotype. Flanking BAC-based Nipponbare markers were generated for saturation mapping using four populations, the three initial RILs and an additional one derived from a cross between M202 and RIL260. A BIBAC (binary BAC) library was constructed from RIL260. Using these resources *Pi5(t)* was mapped to a 170-kb interval, and a contiguous set of BIBAC clones spanning this region was constructed. It had previously been suggested that *Pi3(t)* and *Pi5(t)* might be allelic, due to their identical resistance spectrum and tight linkage. We therefore compared genomic regions for lines containing *Pi3(t)* using the *Pi5(t)*-linked markers. DNA gel-blot analyses indicated that the region around *Pi3(t)* is identical to that of *Pi5(t)*, suggesting that *Pi3(t)* and *Pi5(t)* are the same resistance gene.

Keywords *Magnaporthe grisea* · Blast resistance · *Pi3(t)* · *Pi5(t)* · Genetic and physical mapping

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Introduction

Rice blast, which is caused by the fungus *Magnaporthe grisea*, is one of the most destructive diseases of rice, costing farmers $5 billion a year (Moffat 1994). The management of rice blast relies heavily upon the incorporation of single disease resistance genes. However, blast resistance in many cultivars is short-lived in environments that are conducive to the disease (Lee and Cho 1990), due to the high degree of pathogenic variability of the causative organism *M. grisea* (Ou 1979; Bonman et al. 1986). Therefore breeding for cultivars that display broad-spectrum resistance has become a priority for crop improvement. The genetic basis of broad-spectrum resistance is still not well understood. It may be controlled by single genes or multiple genes with cumulative effects (Johnson 1981).

Relatively broad spectrum or durable resistance has been observed in some rice cultivars. For example, the traditional African cultivar Moroberekan has been cultivated for many years in large areas of West Africa without high losses from blast (Bonman and Mackill 1988). ROK16, LAC23, IRAT13, OS6 and some Brazilian upland rice cultivars show durable resistance to blast in upland conditions (Bidaux 1978; Bonman and Mackill 1988; Lee et al. 1989; Ahn 1994; Fomba and Taylor 1994). Tetep, an *indica* rice cultivar, and

Pai-Kan-Tao (PKT), a temperate *japonica* cultivar, both exhibit broad-spectrum resistance to rice blast (Yu et al. 1987; Mackill and Bonman 1992; Ahn 1994,2000). Many of these rice lines have been used as resistance donors in breeding programs (Mackill and Bonman 1992; Inukai et al. 1994). Phenotypic evaluation of near-isogenic lines (NILs) suggested that as many as four major resistance genes could be identified in Tetep, three in PKT including *Pi3(t)*, two in Moroberekan and two in LAC23 (Mackill and Bonman 1992; Inukai et al. 1994; Wang et al. 1994). Isolation and characterization of the genes conferring resistance in these cultivars should provide insight into the genetic basis of broad-spectrum resistance and may be useful in developing new genotypes.

For this purpose, a recombinant inbred (RI) population consisting of 281 F_7 lines was produced by single-seed descent from a Moroberekan × CO39 cross (Wang et al. 1994). These lines were evaluated for resistance in the greenhouse and field, and analyzed with 127 restriction fragment length polymorphism (RFLP) markers. Two dominant loci, *Pi5(t)* and *Pi7(t)*, that segregated with complete resistance to five blast isolates were identified, while ten quantitative trait loci (QTLs) segregated with partial resistance.

Greenhouse inoculation tests showed that the RI lines (RILs) RIL125, RIL249 and RIL260, which carry *Pi5(t)*, were resistant to at least six races belonging to four lineages in the Philippines (Wang et al. 1994; Chen et al. 2000). These lines were then evaluated for field performance in the Philippines and Indonesia, in locations where the blast fungal populations have been shown to be diverse and broadly virulent (Wang et al. 1994; Chen et al. 1995; Zeigler et al. 1995). The field tests indicated that the *Pi5(t)*-containing RILs displayed resistance to diverse isolates. In a separate study, the recombinant inbred line RIL249 exhibited resistance to 26 of 29 Korean isolates tested (S.-S. Han, personal communication). Together, these studies suggest that the *Pi5(t)* locus itself confers broad-spectrum resistance to rice blast.

When the resistance profiles of the RILs carrying *Pi5(t)* were compared with those of the CO39 NIL lines carrying *Pi3(t)* (C104PKT), the reaction pattern was similar. Genetic and phenotypic analysis of an F_2 population derived from the cross between RIL249 and C104PKT indicated that the *Pi5(t)* resistance gene in RIL249 is allelic, or else closely linked, to *Pi3(t)* (Inukai et al. 1996).

We have initiated a positional cloning approach to clone the *Pi5(t)* locus. Major advances in rice genomics over the last few years have made positional cloning in rice much more efficient. A high-density genetic linkage map and a YAC- and BAC-based contig map have been constructed for the rice cultivar Nipponbare (Harushima et al. 1998; Chen et al. 2002; Wu et al. 2002). Over 110,000 sequence-tagged connectors (STCs) have been generated by sequencing both ends of every BAC clone (Mao et al. 2000). A fingerprint-based contig (FPC) of BAC clones has been anchored with RFLP markers onto the genetic map (Yuan et al. 2000). Using these resources and four mapping populations, we have set up an efficient procedure to construct a genetic and physical map for the *Pi5(t)* locus. We have assembled a 170-kb Binary BAC (BIBAC) contig containing *Pi5(t)*, and shown that the *Pi5(t)* locus is identical to the *Pi3(t)* locus, and that PKT and Moroberekan are not the donors of *Pi3(t)* and *Pi5(t)*, respectively.

Materials and methods

Plant materials and mapping populations

Three *Pi5(t)* RI lines, RIL125, RIL249 and RIL260, derived from a cross between Moroberekan and CO39 (Wang et al. 1994) were selected for this study. These lines were completely resistant to the blast isolate PO6-6. RIL260 was used as the resistance control in all analyses, and for the construction of the genomic DNA library. Crosses of each of these lines with CO39, the susceptible parent of the RIL population, were performed to develop segregating F_2 populations for DNA marker and resistance segregation analysis. Each of the three F_2 populations consisted of 50 to 70 individual plants. F_3 families were developed from the F_2 populations for further DNA marker and resistance analysis. The segregating F_3 families derived from the cross between RIL260 and CO39 were advanced to develop an F_4 segregating population consisting of 2000 individuals. An additional cross of RIL260 to M202, a susceptible parent, was performed to develop an F_3 family consisting of over 1000 individuals for high-resolution mapping. The cultivars C104PKT and PKT were provided by the National Small Grains Research Facility, USDA-ARS, Aberdeen, Idaho.

Inoculation and disease evaluation

The *M. grisea* isolate PO6-6 was used for all phenotypic analyses. PO6-6 is a pathogenically stable isolate from the Philippines, and was used to detect the *Pi5(t)*locus in the RIL population (Wang et al. 1994). All inoculations and disease evaluations were conducted in greenhouses at the International Rice Research Institute (Los Banos, Philippines) and at Ohio State University, as described in Chen et al. (1996).

BSA and AFLP analysis

Four pooled DNAs representing homozygous resistance (RR) and homozygous susceptible (rr) lines were made for bulk segregant analysis (BSA) (Michelmore et al. 1991) using amplified fragment length polymorphism (AFLP) screening. Each bulk DNA pool contained equivalent amounts of DNA from ten DNA samples extracted from the bulked leaves of the F_3 homozygous families. Total DNA was extracted from young leaves following the protocol described by McCouch et al. (1988). Polymorphic AFLP markers associated with resistance in the bulks were confirmed in the F_2 individuals from which the bulks were constructed. The segregation data for both AFLP loci and resistance phenotypes obtained from the F_2 population and F_3 families were used to estimate the genetic distance between the molecular marker loci and the resistance locus.

AFLP markers were used to screen DNA markers linked with the resistance according to the following procedure (Chen et al. 1999). Rice genomic DNAs digested with *Eco*RI and *Mse*I were ligated with double-stranded adapters. The *Eco*RI adapter was a 1:1 mixture of the primers 92A18 (5′-GACGATGAGTCCTGAG-3′) and 92A19 (3′-TACTCAGGACTCAT-5′). The *Mse*I adapter was an equal mixture of the primers 91M35 (5′-bio-

CTCGTAGACTGCGTACC-3′) and 91M36 (3′-CTGACG-CATGGTTAA-5′). Signal detection was carried out by end-labeling the primer specific for the *Eco*RI adapter with [γ-^{33}P]ATP. The linked AFLP markers were converted into RFLP probes by cloning the DNA fragment into pCRII (Invitrogen), a TA cloning vector, and then analyzed by DNA sequencing.

RFLP analysis

For RFLP analysis, samples of over 3 μg of rice genomic DNA were digested with restriction enzymes and fractionated by electrophoresis in a 0.8% agarose gel. DNA gel-blot analyses were carried out according to standard procedures under high-stringency hybridization conditions (Sambrook et al. 1989). The filters were then scanned with a Phosphoimager (Molecular Dynamics). *Dra*I-digested genomic DNA was used for RFLP analysis of the markers 17I18-12, 39B24-1, 4D13-2-2, G103, and C1454. *Eco*RI and *Hin*dIII were used for the markers 34E14-10 and 47J03f, respectively.

CAPS analysis

For CAPS (cleaved amplified polymorphic sequence) analysis, rice genomic DNA was isolated from young leaves using a simple miniprep method (Chen and Ronald 1999). CAPS analysis was performed in a volume of 30 μl (100 pmol of each primer, 200 μM each of dNTPs, 10 mM TRIS-HCl pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 0.5 U of Taq polymerase) using 50 ng of genomic DNA as template. The digested PCR products were subsequently size-fractionated on 2% agarose gels.

BIBAC library construction

High-molecular-weight (HMW) DNA was prepared from young leaves of RIL260 by the CTAB method (Murray and Thompson 1980). The isolated HMW DNA was partially digested with *Hin*dIII and then size-fractionated using a pulsed-field gel electrophoresis device (CHEF DR II system, Bio-Rad) as described previously (Wang et al. 1995). The purified DNA from the low-melting point agarose slice containing fragments of 30–50 kb was ligated to the *Hin*dIII-digested and dephosphorylated pBIGRZ vector provided by Dr S. Kawasaki (Tsunoda et al. 2000). The ligation mix was transformed by electroporation using a Cell-Porator (Gibco-BRL) into *Escherichia coli* DH10B. About 500 clones were resuspended in 4 ml of the freezing buffer (Peterson et al. 2000) to form a BIBAC minipool.

BIBAC library screening

Aliquots (50 μL) of *E. coli* cells from each of 186 BIBAC minipools were used for preparation of total BIBAC plasmids. Total BIBAC DNAs were isolated by standard alkaline lysis procedures (Sambrook et al. 1989). Equivalent amounts of DNAs purified from 11 or 12 minipools were mixed to make a super pool, producing 16 super pools. The first round of PCR was performed using 16 super pools and *Pi5(t)* marker-specific primers (see below). In the second round of PCR, the 11 or 12 minipools making up the super pool showing a positive hit were then screened individually. Over 2000 individual clones of the identified mini-pool were further screened by colony blot hybridization (Sambrook et al. 1989) using the products amplified in the second PCR as probes.

Sequence analysis of BIBAC ends

The isolated plasmids were sequenced using T3 and T7 primers to obtain both end sequences of the cloned insert. The BIBAC-end sequences of each clone were amplified by PCR using specific primers and were subsequently used for chromosome walking.

Sublibrary construction of BAC or BIBAC clones

Size-fractionated DNAs (~2 kb) were obtained after partial digestion with *Sau* 3AI, ligated to the *Bam*HI site of pBluescriptII SK+, and transformed into *E. coli* DH10B. The inserts were used for RFLP and CAPS analysis.

Linkage analysis

Linkage analysis was performed using Mapmaker software (Lander et al. 1987) on a Macintosh computer. The segregation dataset generated from the 204-member F_2 population of Black Gora/Labelle (Redoña and Mackill 1996) and the 186 Nipponbare/Kasalath plants (Harushima et al. 1998) were combined with the established AFLP data set for the analysis. Map distances presented in cM between markers were derived using the Kosambi function (Kosambi 1944).

Results

Genetic and phenotypic analysis of RI lines

Based on the molecular and phenotypic dataset from a previous study, three resistant parents, RIL125, RIL249 and RIL260, were selected for fine-scale mapping of *Pi5(t)*. All three lines showed complete resistance to the blast isolate PO6-6, giving scores of 0 to 1 on a scale of 0–5 with 5 being the most susceptible. CO39 and M202, the susceptible parents used in the study, were compatible with PO6-6, displaying a score of 4–5 in repeated inoculations.

The resistance and susceptibility phenotypes of the three F_2 populations developed from crosses of RIL125, RIL249, RIL260 with CO39 were determined by monitoring the effects of inoculating the F_2 plants and the corresponding F_3 families. The distribution of resistance in the F_2 population was compatible with a 3:1 segre-

Table 1 Chi square test for the segregation of resistance and susceptibility in F2 populations inoculated with the rice blast isolate PO6-6

Cross	Total number of F2 plants observed	Resistant	Susceptible	X^2	P[a]
RIL125/CO39	49	39	10	0.55	0.46
RIL249/CO39	68	53	15	0.31	0.58
RIL260/CO39	50	39	11	0.24	0.62
RIL260/M202	59	45	14	0.05	0.82

[a]The goodness of fit to a 3:1 ratio is indicated

gation ratio for all three populations (Table 1). The three genotypes in the F_3 families (RR, Rr and rr) fit a 1:2:1 segregation ratio (data not shown). In each of the heterozygous F_3 families, the resistance/susceptibility phenotypes followed a 3:1 segregation pattern. The resistance in the additional F_2 population derived from a cross between RIL260 and M202 also segregated in a 3:1 ratio (Table 1). These results indicate that in each of the selected lines a single dominant locus confers resistance to PO6-6.

Identification of AFLP markers linked with the resistance

An AFLP experiment was employed to identify markers linked to the resistance, using two pooled DNAs for each homozygous resistance and for homozygous susceptibility. Of the 579 AFLP primer combinations screened, one primer pair, S04G03, identified a polymorphic marker associated with resistance in RIL125. Out of 350 primer pairs surveyed, 11 AFLP markers were associated with resistance in the RIL249-derived F_2 population. Out of 750 primers surveyed, 12 markers were associated with resistance in the RIL260-derived F_2 population. These AFLP markers were converted into RFLP markers by cloning the bands into a TA cloning vector. For S04G03 it was difficult to obtain a hybridization signal in DNA gel-blot analyses with the 48-bp AFLP fragment. To obtain a larger fragment including this marker, we isolated a BIBAC clone, JJ5, by screening a Moroberekan library (J.-S. Jeon, D. Chen and P. C. Ronald, unpublished data) with SO4GO3 as a probe. Sequence analysis of JJ7, a 1.9-kb subclone of JJ5, revealed that the clone contains not only SO4G03 but also S10F07, which was isolated from the RIL260-derived F_2 population, another AFLP marker linked to *Pi5(t)*. Thereafter, the subclone JJ7 was used as a probe to represent the S04G03 and S10F07 markers in the present study.

To verify that the AFLP markers co-segregate with the resistance phenotype in *Pi5(t)* lines, we carried out DNA gel-blot analysis using F_2 homozygous susceptible and resistant lines. The result showed that S04G03, identified in all three populations, co-segregated with resistance in 15 individuals (Fig. 1A). DNA gel-blot analysis with the co-dominant 47J03f marker (see below) also co-segregated with S04G03 (Fig. 1B). Of all AFLP-derived markers from the three populations, S04G03 was the most tightly linked to *Pi5(t)* in 48 RIL249-derived F_2 lines. The other markers gave one to four recombination events between *Pi5(t)* and each marker (data not shown).

Pi5(t) is located on chromosome 9

Primer pairs generating the linked markers were used to analyze the parents of the Black Gora/Labell mapping population (Redoña and Mackill 1996). Nine primer pairs (S04G03, S10F07, S08G24, S10M04a, S10M04b, B11G19, R01G23a, R01G23b, P03G07) detected polymorphism between Black Gora and Labell at the loci associated with the resistance to PO6-6. Linkage analysis with Mapmaker showed that all the markers from the RIL125-, RIL 249- and RIL260-derived populations were located on chromosome 9 (Fig. 2). These results suggested that the loci that conferred resistance to PO6-6 in the three RILs were probably the same as, or tightly linked to, *Pi5(t)* on chromosome 9.

The *Pi5(t)* locus was previously reported to be linked to the DNA marker RG788 on chromosome 4 (Wang et al. 1994). However, DNA gel-blot analysis using RG788 in the F_2 population derived from the RIL249 cross indicated that *Pi5(t)* was not linked to RG788 (data not shown). Our data thus confirmed the results of another study that also showed that the resistance gene in RIL249 was unlinked to RG788 (Inukai et al. 1996).

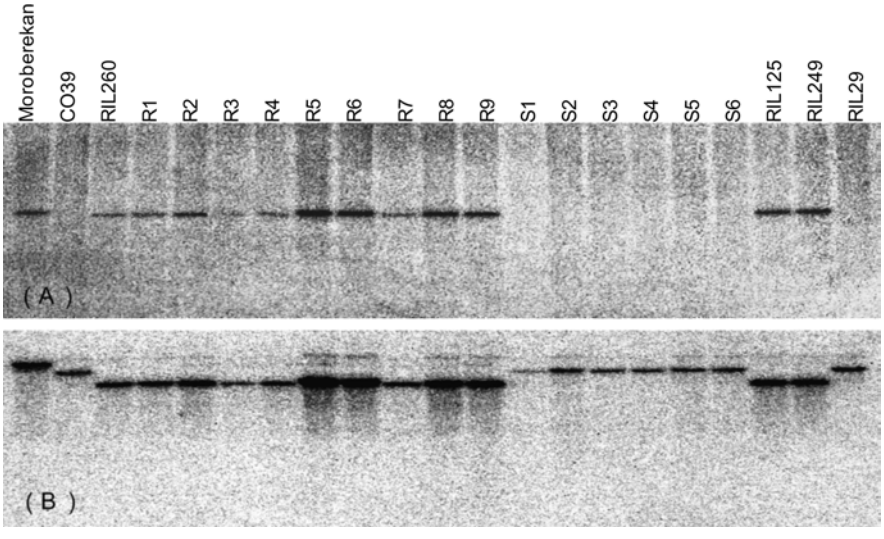

Fig. 1A, B Co-segregation of two markers, S04G03 (**A**) and 47J03f (**B**), with *Pi5(t)*resistance. Genomic DNA was digested with *Hin*dIII and fractionated in a 0.8% agarose gel. R1 to R9, and S1 to S6 are homozygous resistant and homozygous susceptible lines from the RIL260/CO39 F_2 population, respectively. DNA from Moroberekan, CO39, RIL125, RIL249, RIL260 and RIL29 [lacking *Pi5(t)*] were included as controls

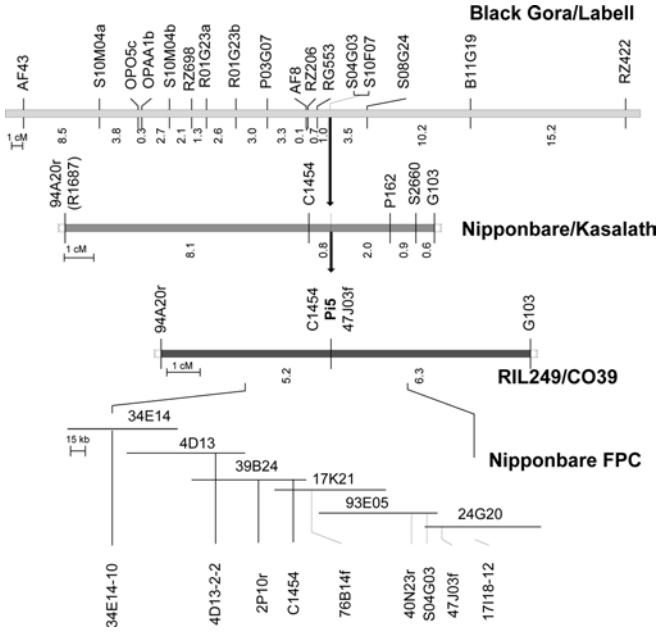

Fig. 2 Genetic and physical maps of the *Pi5(t)*locus on rice chromosome 9. The nine AFLP markers identified (S10M04a, S10M04b, R01G23a, R01G23b, P03G07, S04G03, S10F07, S08G24, B11G19) were localized on the genetic linkage map of Black Gora/Labell (*top*). The *numbers below* this map are relative genetic distances in cM. S04G03, which co-segregates with *Pi5(t)* resistance, was also mapped on the high-density genetic map of Nipponbare/Kasalath (*upper middle*). These data were used to identify BAC subclones from the CUGI database that spanned the region (*bottom*). Markers were developed from these BAC subclones and used to localize *Pi5(t)*on the RIL249/CO39 map (*lower middle*). The key markers used in this study were mapped on the Nipponbare BAC contig as shown in the *bottom panel*

High-resolution mapping of *Pi5(t)*: use of Nipponbare BAC clones

Saturation mapping of a small genomic region is an essential tool for positional cloning (Monna et al. 1997). To pinpoint the *Pi5(t)*genomic region, we mapped the co-segregating marker S04G03 onto the linkage map of Nipponbare/Kasalath to identify RFLP markers linked to *Pi5(t)* (Harushima et al. 1998) (Fig. 2). These results indicated that the marker C1454 is tightly linked to S04G03, only 0.8 cM away. We then used C1454 as a probe in colony hybridization experiments with two BAC libraries of Nipponbare provided by the Clemson University Genome Institute (CUGI). These experiments enabled us to identify a large physical region of more than 1000 kb consisting of Nipponbare BAC clones carrying the C1454/SO4G03 genomic region (Chen et al. 2002) (Fig. 2).

Six of these *Pi5(t)*-linked Nipponbare BAC clones were used to develop additional markers that flank *Pi5(t)*. The BAC clones were selected using information based on the primary genetic map of *Pi5(t)*, the location of RFLP markers, and the FPC map consisting of Nipponbare BAC contigs (Fig. 2). They were digested partially with *Sau* 3AI for subcloning. Out of 150 subclones tested, 25 showed clear polymorphisms between the three RILs and CO39 as determined by DNA gel-blot analysis. The remaining clones included those that were monomorphic between the parents, those that gave comparatively weak hybridization signals or those with multiple bands, which were considered inappropriate for further analysis (data not shown). Eight polymorphic markers, 34E14-10, 4D13-2-2, 2P10r, C1454, 76B14f, 40N23r, 47J03f, and 17I18-12, were used because they were evenly distributed or homologous to known resistance genes (Fig. 2). Thus, the amino acid sequences deduced from the DNA sequences of 76B14f and 40N23r showed similarity to a 'nucleotide binding site plus leucine-rich repeat' (NBS-LRR) motif. To use the markers most efficiently in the analysis of a large population, they were converted to CAPS markers using marker-specific primers (Table 2). Because C1454 and S04G03 could not be converted to CAPS markers, we instead amplified the corresponding regions of both markers from RIL260 and M202, and directly compared the sequences of the PCR products. Both markers showed a few nucleotide differences between RIL260 and M202 (Table 2; data not shown).

Table 2 PCR-based markers linked to the Pi5 locus

Marker[a]	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Enzyme	Mapping population
94A20r	AATTCCATTCGCCACCGAGTGCTC	TCTCAGTATAGAACACTAACTCTA	*Ava*II	RIL/CO39
34E14-10	CCTACCACCACAGGACATAACA	GTTTCTTCTCTTATCCCCTCTC	*Ase*I	RIL/CO39
17I18-12	TACACGAACAACCAAATCGACC	AGGCGTTTGGTTTTGGTGGAGA	*Hin*dIII[b], *Hin*PII[c]	RIL/CO39, RIL260/M202
2P10r	ATTGTCAAGCTCTTCTGCTGTC	TGAACTGATCATCAAATCAATC	*Dpn*II	RIL/CO39, RIL260/M202
76B14f	GTCTTGGACTTAAAGCACTACC	TGAGAAACTGGTTCAAATTGGC	*Dra*I	RIL260/M202
40N23r	TGTGAGGCAACAATGCCTATTGCG	CTATGAGTTCACTATGTGGAGGCT	EcoRI	RIL260/M202
C1454	CACCTGAAGGCTGAAAATCTGAAT	CCGTTGATAGCGCTTAATGTTCTT	–	RIL260/M202
S04G03	CTTAACAATCAATGTTTAATGAAA	GTTATATTATACTAATTGTTTATC	–	RIL260/M202

[a]With the exception of C1454 and S04G03, all markers are CAPS markers. For these two markers, sequence analysis of PCR products was used to survey polymorphisms
[b]RIL/CO39
[c]RIL260/M202

We used the flanking markers 94A20r and G103 to narrow down the region carrying *Pi5(t)* on the genetic map of RIL249/CO39. This was easily accomplished because the parents of the RIL249/CO39 F_2 population were polymorphic for these markers. The marker 94A20r was developed from a Nipponbare BAC clone that contains the previously mapped marker R1687. R1687 was monomorphic between the parents. The result revealed that *Pi5(t)* mapped to an 11.5-cM interval (11 recombinations in 96 meiotic events) between the markers 94A20r [5.2 cM proximal to *Pi5(t)*] and G103 [6.3 cM distal to *Pi5(t)*] in the RIL249/CO39 F_2 population (Fig. 2).

To identify more recombination events at the *Pi5(t)* locus, two markers flanking *Pi5(t)* (34E14-10 and 17I18-12) within the 11.5-cM region defined by the above analysis, were screened in 731 susceptible individuals and 515 resistant and segregating individuals of BC_2F_4 RIL260/CO39 using CAPS analysis. Since the susceptibility score is more reliable than the resistance estimate (due to escapes from the inoculum), we first analyzed all susceptible plants to make an accurate map, and the remaining plants were analyzed later. In the primary screen, 22 and 8 recombination events were identified between 34E14-10 and *Pi5(t)* and between 17I18-12 and *Pi5(t)*, respectively (Fig. 3). The recombinant lines were further analyzed using internal PCR markers. The analysis identified a recombination event between 4D13-2-2 and 2P10r. The other six markers, 2P10r, C1454, 76B14f, 40N23r, S04G03 and 47J03, co-segregated with *Pi5(t)*. To identify rare recombination events at the *Pi5(t)* locus, F_2 segregating populations of RIL125/CO39 and RIL249/CO39 were analyzed further (Fig. 3). This identified a recombination event between 4D13-2-2 and 2P10r and two between 47J03f and 17I18-12 from the RIL125/CO39 population, and one between 34E14-10 and 4D13-2-2 and two between 47J03f and 17I18-12 from the RIL249/CO39 population (Fig. 3). Phenotypic analyses of all recombinant lines identified demonstrated that the resistance locus is most likely to be same in all three RILs, RIL125, RIL249 and RIL260 (Fig. 3). On the basis of the Nipponbare contig (Chen et al. 2002) it is estimated that the genomic region between 17I18-12 and 4D13-2-2 is approximately 300 kb long.

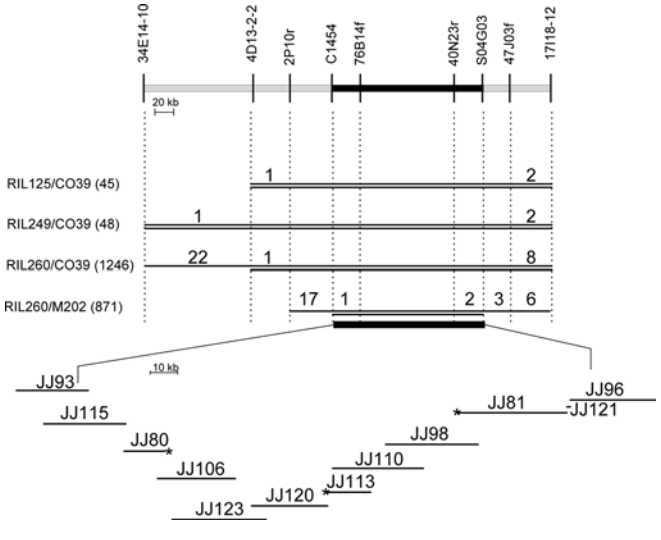

Fig. 3 Genetic and physical mapping of the *Pi5(t)* locus. High-resolution genetic map of the *Pi5(t)* locus (*top*). The four populations used for this analysis are indicated on the *left*. The *numbers in parentheses* indicate the numbers of plants analyzed for recombination events at the *Pi5(t)* locus. The numbers of recombinants obtained are indicated *between* the relevant markers. The physical region containing the *Pi5(t)* resistance locus is shown by the *double lines* for each population. The *black bar* indicates the minimal genomic region carrying *Pi5(t)* delimited by this analysis of all four mapping populations. This region was physically covered by a contiguous set of RIL260 BIBAC clones (*bottom*). The *asterisks* indicate the positions of the BIBAC-end markers JJ80-T3, JJ81-T3 and JJ113-T3, which were used in the DNA gel-blot hybridization experiment shown in Fig. 5

We were not able to localize the gene to a smaller physical region even when the mapping population was enlarged, because skewed recombination events were observed at the *Pi5(t)* locus. Thus, we developed another F_2 segregating population derived from a cross between RIL260, a resistant cultivar, and M202, a susceptible cultivar, and analyzed 871 F_3 individuals to further delimit *Pi5(t)* to a small physical region. Since we have limited quarantine facilities for blast inoculations, we employed a prescreening strategy to identify plants with rare recombination events around the *Pi5(t)* region using the flanking CAPS markers 2P10r and 17I18-12. These experiments identified 23 recombinants between 2P10r and 17I18-12: 17 recombination events between 2P10r and *Pi5(t)*, and six recombination events between 17I18-12 and *Pi5(t)* (Fig. 3). Phenotypes of all the identified lines displaying these rare recombination events were confirmed in the progeny from each line. Through further analyses of these recombinants with the internal markers, one recombinant between C1454 and *Pi5(t)* and two recombinants between S04G03 and *Pi5(t)* were identified. This high-resolution mapping experiment thus revealed that *Pi5(t)* is located in a ~170-kb interval between the markers S04G03 and C1454. The markers 76B14f and 40N23r co-segregated with *Pi5(t)* (Fig. 3).

Construction of a BIBAC contig spanning the *Pi5(t)* locus

Nipponbare does not carry *Pi5(t)*. Therefore we constructed a BIBAC library from RIL260 using the pBIGRZ vector (Tsunoda et al. 2000) to clone the region carrying *Pi5(t)*. The library comprised approximately 93,000 clones with an average DNA insert size of 25 kb, corresponding to five genome equivalents (data not shown). To identify positive clones from the library, we adapted a pooling system for a PCR-based procedure (see the Materials and methods for the details). This strategy is efficient for screening a large library with

small insert sizes. The alternative strategy of picking over 90,000 individual clones would have been much more time-consuming and laborious.

To span the physical region containing *Pi5(t)*, four markers, S04G03, C1454, 76B14f, and 40N23r, were initially used for the library screening, yielding the BIBACs JJ96, JJ93, JJ80, and JJ81, respectively (Fig. 3). We sequenced each end of the four isolated BIBAC clones and used this information to generate PCR products to screen the library again in order to extend the region. Using this approach we identified three more clones (JJ98, JJ106, and JJ115) in the region. We repeated this step and identified an additional three clones (JJ110, JJ113, and JJ120) from the region (Fig. 3). Some BIBAC-end sequences were homologous to transposable elements and therefore could not be used as probes for BIBAC library screening due to their repetitive nature. This was the case for the BIBAC-end sequences obtained from JJ106 and JJ120. To close the gap between JJ106 and JJ120, a subclone, JS624-T7, isolated from the *Sau3*AI shotgun library of JJ106, was utilized to find the linking clone JJ123. In another case, a small region (183 bp) between JJ81 and JJ96 was amplified, cloned (JJ121), and its position confirmed by sequence analysis (data not shown). PCR analysis confirmed that both JJ113 and JJ120 are consecutively linked at the *Hin*dIII site used for cloning. All 12 clones could be arranged into a single contig spanning the 170-kb region between C1454 and S04G03 (Fig. 3).

The *Pi3(t)* and *Pi5(t)* genomic regions are identical

It has been proposed that *Pi3(t)* is allelic to *Pi5(t)*, as both confer similar resistance spectra to a variety of blast lineages (Inukai et al. 1996). To determine if the genomic regions of *Pi3(t)* and *Pi5(t)* are indeed similar, DNA gel-blot analyses were carried out using the *Pi5(t)*-flanking RFLP markers 34E14-10 and 17I18-12. The hybridization patterns indicated that the region in C104PKT, the *Pi3(t)*-containing line, is identical to that in RIL260 and RIL249 harboring *Pi5(t)* (Fig. 4), but markedly different from those in Moroberekan the putative donor of *Pi5(t)* and CO39, the susceptible parent. Additional DNA gel-blot analyses with 2P10r, 76B14f, C1454, 40N23r, S04G03, and 47J03f indicated that C104PKT, RIL260 and RIL249 are all monomorphic for these markers (data not shown). This suggests that the lines containing the *Pi3(t)* and *Pi5(t)* loci share a common origin. In agreement with this hypothesis, the nucleotide sequences of the *Pi5(t)*-linked marker 40N23r from C104PKT and RIL260 were completely identical (data not shown). The sequence of the 40N23r region differed significantly from that in the presumptive donors of resistance, PKT and Moroberekan. This further confirmed that the *Pi3(t)* and *Pi5(t)* regions are identical, and are not derived from PKT or Moroberekan.

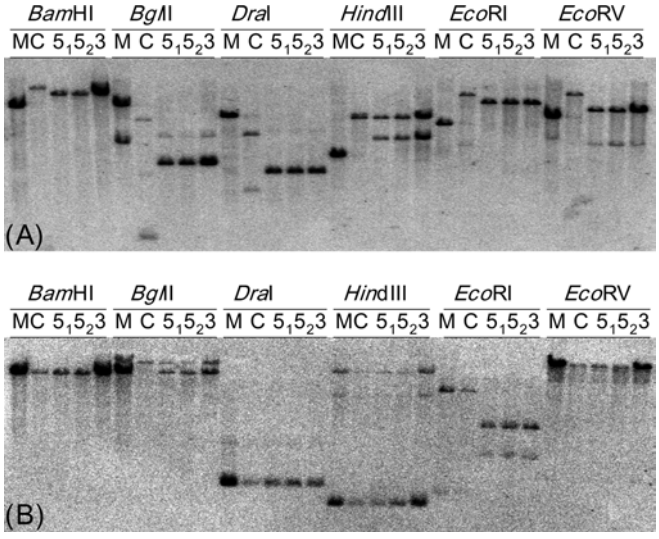

Fig. 4A, B DNA gel-blot analysis of the Moroberekan (M), CO39 (C), RIL260 (5_1), RIL249 (5_2), and C104PKT (3) genomes. Genomic DNAs were digested with six restriction enzymes, *Bam*HI, *Bgl*II, *Dra*I, *Hin*dIII, *Eco*RI and *Eco*RV. The *Pi5(t)*-flanking markers, 17I18-12 (**A**) and 34E14-10 (**B**) were used to probe for polymorphisms

The *Pi5(t)* resistance locus in the RI lines does not correspond to the Moroberekan allele

As noted above, all loci in the *Pi5(t)* genomic region differed from their counterparts in Moroberekan, which had been assumed to be the donor of *Pi5(t)*. It has been reported that PKT is susceptible to the rice blast strain PO6-6 (Mackill and Bonman 1992). These data further support our hypothesis that PKT is not the source of *Pi3(t)*. It is not likely that the *Pi5(t)* resistance gene was created by a recombination event in the Moroberekan/CO39 cross because the identical non-parental alleles were observed in RIL125, RIL249, RIL260, and C104PKT, and many dominant sequences were missing in Moroberekan and PKT.

To estimate the frequency of non-parental alleles in the whole population of RILs derived from the Moroberekan/CO39 cross, we analyzed 30 RILs from the 281 F_7 recombinant inbred lines. CAPS analysis using the flanking markers 17I18-12 and 34E14-10 revealed that three (RIL8, RIL14 and RIL27) of 30 lines tested contain the *Pi5(t)*-specific non-parental allele (data not shown). DNA gel-blot analysis with the co-segregating marker 40N23r showed that the non-parental alleles in these three lines are identical to that of *Pi5(t)* (Fig. 5). Furthermore, in an inoculation experiment, RIL13 and RIL30, two of three lines containing Moroberekan alleles at the *Pi5(t)* locus showed a segregating phenotype upon inoculation with PO6-6 (Fig. 5), suggesting that the resistance to PO6-6 in the RI lines containing the *Pi5(t)*-specific non-parental allele is not conferred by the Moroberekan allele.

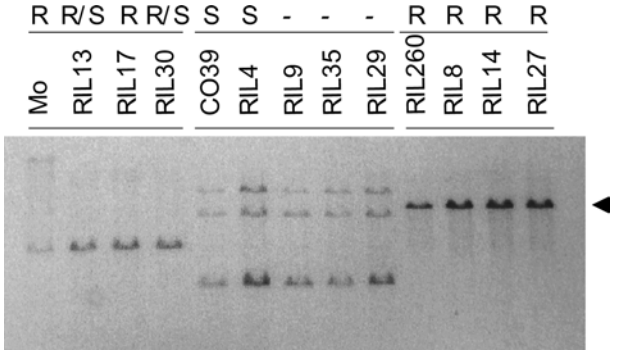

Fig. 5 Genomic DNA gel-blot analysis of RI lines using the *Pi5(t)* marker 40N23r. Moroberekan (Mo), CO39, RIL260, and ten preselected RILs (see text for details) were surveyed for polymorphism at the *Pi5(t)* locus. The *arrowhead* indicates a hybridizing band not present in CO39 or Moroberekan. R and S signify resistance and susceptibility to *M. grisea* PO6-6, and R/S denotes a segregating phenotype. RIL9, RIL35 and RIL29 were not used for phenotype analysis

Discussion

We have developed an efficient method for positional cloning in rice using genetic resources that have been developed recently. In the present study, we utilized the genetic resources of Nipponbare, a line lacking *Pi5(t)*, to develop markers required for saturation mapping of the *Pi5(t)* locus. First, an initial marker linked to *Pi5(t)* was mapped on the high-density genetic map of Nipponbare/Kasalath (Harushima et al. 1998). Secondly, Nipponbare BAC clones physically spanning the region were identified using the CUGI database. Third, additional genetic markers for saturation mapping were produced using subclones of the identified BACs. Fourth, a small interval of Nipponbare corresponding to *Pi5(t)* was delimited by determining recombination breakpoints. Finally, a physical map of the *Pi5(t)* genomic region was constructed using flanking markers and a BIBAC library generated from a *Pi5(t)*-containing line.

Our data indicate that the *Pi5(t)* genomic region in RIL260 is identical to the *Pi3(t)* genomic region in C104PKT. This region is markedly different from that in the putative parents, PKT and Moroberekan. Furthermore our data indicate that PKT and Moroberekan do not have a resistance allele at the *Pi3(t)*/*Pi5(t)* locus. It is not likely that *Pi5(t)* was generated through a genetic event such as a deletion, inversion, duplication, etc., because the same non-parental alleles exist in many of the RIL populations, with a frequency of about 10%, as well as in C104PKT carrying *Pi3(t)*.

Our data suggest that a rice cultivar carrying *Pi3(t)* was outcrossed to CO39 and then backcrossed to CO39 five more times to produce C104PKT. It is possible that the same outcross was performed to generate the RI lines carrying *Pi5(t)*, because the genomic region of interest in these three *Pi5(t)*-containing RI lines was identical to that in C104PKT for all markers tested. It is also possible that C104PKT contaminated the cross between Moroberekan and CO39, and that C104PKT carrying *Pi3(t)* is the donor for the three *Pi5(t)* RILs. The present study demonstrates the usefulness of genetic markers for discovering the source of a particular resistance gene.

We were not able to identify recombination events in the 170-kb interval encompassing *Pi5(t)* among over 2000 individuals from four different mapping populations. In the RIL260/M202 population, the marker C1454 mapped 0.06 cM away from *Pi5(t)* (1 recombination/1742 meiotic events) and the other flanking marker, S04G03, mapped 0.11 cM away from *Pi5(t)* (2/1742), giving a ratio of over 1000 kb/cM. This is much higher than the average physical/genetic ratio of 260–280 kb/cM estimated for the rice genome as a whole (Wu and Tanksley 1993). This result could be due to lack of pairing and subsequent strand exchange between homologous regions in the RIL260 and M202 parents used for the cross. This is supported by the fact that many of the RIL260 BIBAC-end sequences were not present in M202. Suppression of recombination has been observed in other introgressed regions associated with disease resistance, such as the *Mi* (van Daelen et al. 1993), *Mla* (Wei et al. 1999), and *Pita* [2] (Nakamura et al. 1997) loci. Thus, the *Pi5(t)* locus is highly diverged in disease-resistant and susceptible genotypes.

Over the past decade, a number of dominant *R* genes have been characterized from diverse plant species and their encoded proteins can be grouped into six classes based on structure (Wang et al. 1998; Dangl and Jones 2001). The largest class of *R* genes encodes an NBS-LRR class of proteins that can be further divided into two groups based on their N terminal domains. Sixty percent of the Arabidopsis NBS-LRR proteins carry a domain with homology to the intracellular signaling domains of the Drosophila Toll and mammalian interleukin (IL)-1 receptors (TIR-NBS-LRR), whereas 40% contain putative coiled-coil domains (CC-NBS-LRR) (Dangl and Jones 2001). Over 100 *R* gene sequences of the TIR-NBS-LRR class exist in the Arabidopsis genome, but this subclass has not yet been found in cereals and is not present in available rice sequences (Meyers et al. 1999). To date, five rice *R* genes have been cloned, including the blast resistance genes *Pib* and *Pita* encoding CC-NBS-LRR proteins and the bacterial blight *R* genes *Xa1*, *Xa21* and *Xa21D* which encode a CC-NBS-LRR, a receptor kinase and receptor-like protein, respectively (Song et al. 1995; Wang et al. 1999; Yoshimura et al. 1998; Bryan et al. 2000).

We analyzed ~150 BAC end sequences of Nipponbare corresponding to approximately 70 kb in the *Pi5(t)* locus (Chen et al. 2002). Through similarity searches against sequence data in public databases, we found that the amino acid sequences deduced from two genes in the region show similarity to the conserved NB- and/or LRR motifs. BIBAC end sequence analysis of RIL260 identified a third NBS-LRR sequence. These

data indicate that the *Pi3(t)/Pi5(t)* locus contains a cluster of NBS-LRR sequences. These three genes are good candidates for *Pi3(t)/Pi5(t)* and may constitute part of a "natural pyramid" of resistance genes that confer the broad-spectrum resistance. To test this hypothesis, all the isolated BIBAC clones of RIL260 are currently being used in transgenic complementation studies to identify the *Pi3(t)/Pi5(t)* coding region(s). These plants will be inoculated with diverse isolates to determine whether a single gene or multiple genes are required for the *Pi3(t)/Pi5(t)* broad-spectrum resistance.

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