Development of DNA Markers Suitable for Marker Assisted Selection of Three \( Pi \) Genes Conferring Resistance to Multiple \textit{Pyricularia grisea} Pathotypes

Robert Fjellstrom,* Concetta A. Conaway-Bormans, Anna M. McClung, Marco A. Marchetti, A. Robert Shank, and William D. Park

ABSTRACT

Rice blast, caused by the fungus \textit{Pyricularia grisea} (Cooke) Sacc., is a serious rice (\textit{Oryza sativa} L.) disease causing considerable economic damage worldwide. DNA markers for rice blast resistance have been developed, but most are not suitable for routine use in a marker-assisted selection breeding program involving large numbers of progeny. After identifying candidate microsatellite markers from public database sources, we have mapped these markers near the blast resistance genes \( Pi-b, Pi-k, \) and \( Pi-ta^2 \) on rice chromosomes 2, 11, and 12, respectively, using segregation information from hundreds of progeny in several crosses. Two microsatellite markers, RM208 and RM224, were found to cosegregate with the \( Pi-b \) and \( Pi-k \) genes, respectively, while additional microsatellites were found to closely flank these two genes and the \( Pi-ta^2 \) gene. The new markers are polymorphic in the narrow crosses characteristic of applied breeding programs and appear to be ideally suited for marker assisted selection for blast resistance in rice because of their tight linkage with resistance genes and ease of use through analysis of amplification products. A dominant marker indicating the presence of the \( Pi-b \) gene, Pibdom, has also been developed on the basis of the sequence of the cloned \( Pi-b \) gene. These markers should facilitate the introgression and pyramiding of these three blast resistance genes into new rice cultivars and elite lines.

Rice blast is one of the most widespread and destructive diseases of rice (Ou, 1985). Losses from this disease can account for over 70% crop loss in the USA (Lee, 1994). The introgression of genes for blast resistance into improved varieties has been a cost effective and environmentally beneficial means of minimizing crop losses due to this disease. The blast pathogen, \textit{P. grisea} (sexual form being \textit{Magnaporthe grisea} Cav.), is highly variable and numerous; pathotypes (races) of blast are present in most field situations. Ten races of blast, designated as IB-1, IB-33, IB-45, IB-54, IC-17, IE-1, IE-1K (synonymous with IC-1k; Correll et al., 2000), JG-1, and IH-1, are commonly detected in the USA, although other races have been occasionally identified (Xia et al., 1993; Moldenhauer et al., 1998).

Several genes conferring resistance to rice blast have been identified in U.S. germplasm, nearly all of which display resistance to one or more, but not all, races of the blast fungus. Race-specific \( Pi \) resistance genes used in U.S. breeding programs for cultivar development include \( Pi-ta^t \) [allelic to \( Pi-ta \) and \( Pi-4(t) \); Kiyosawa, 1972; Inukai et al., 1994], used in the development of the cultivar Katy (Moldenhauer et al., 1992); two alleles of the \( Pi-k \) gene, \( Pi-k^s \), identified in ‘Dawn’ (Kiyosawa, 1974; Kiyosawa, 1978), and \( Pi-k^q \) (Kiyosawa, 1974), identified in ‘Caloro’ (Kiyosawa, 1969); and, more recently, what appears to be \( Pi-b \) (synonymous with \( Pi-s \) and \( Pi-tq^5 \); Kiyosawa, 1972; Tabien et al., 2000), identified in ‘Saber’ (McClung et al., 2004b) and ‘Bolivar’ (McClung et al., 2004a). These four genes confer resistance from one to eight of the 10 blast races found in the USA, with \( Pi-ta^t \) offering the widest spectrum of resistance and \( Pi-k^s \) providing resistance to only race IB-54 [Table 1; Marchetti et al., 1987; Tabien et al., 2000; McClung, 2002; McClung et al., 2003; summarized rice blast reactions from U.S. Uniform Regional Rice Nursery (URRN) data]. Since many \( Pi \) genes confer resistance to overlapping spectra of blast pathotypes, it is often difficult to monitor the presence of individual resistance genes and pyramid these in breeding lines using traditional phenotypic screening. DNA markers offer an efficient and rapid means to select for the presence of multiple blast resistance genes without performing extensive progeny testing or disease screening. Marker-based selection is particularly valuable in early generation selection where the amount of seed for disease phenotyping is limited and in locations where disease screening opportunities are lacking or prohibited (i.e., because of quarantine restrictions). DNA markers associated with several \( Pi \) blast resistance genes have been located on rice chromosome maps, including markers for \( Pi-b \) (Monna et al., 1997) and \( Pi-ta \) (Nakamura et al., 1997; Hittalmani et al., 2000; Jia et al., 2002). Unfortunately, the majority of DNA markers for blast resistance are restriction fragment length polymorphisms (RFLPs), which are labor intensive to analyze for use in a breeding program. Markers that can be analyzed by the polymerase chain reaction (PCR) are more amenable for breeding purposes, such as the ones developed for \( Pi-2 \) (Hittalmani et al., 2000) and \( Pi-ta \) (Jia et al., 2002). Although the \( Pi-b \) and \( Pi-ta \) genes have been cloned (Wang et al., 1999; Bryan et al., 2000), the published sequences of these genes do not readily provide PCR markers for
them. The presence of multigene families of individual resistance genes (Wang et al., 1999), sequence redundancy among different resistance genes throughout the rice genome, and unexpected PCR amplification products (Jia et al., 2002) results in the need of significant effort to develop usable markers from published sequence information.

Microsatellites are PCR markers that provide an attractive high-throughput and labor-saving means to tag blast resistance genes, particularly for breeding programs that screen thousands of lines annually. Comprehensive microsatellite maps developed in rice have been combined with maps of disease resistance genes (Gramene internet site, http://www.gramene.org; verified 23 April 2004). Preliminary studies indicated that several microsatellite markers that had been developed with information from publicly available databases were closely linked with the \( Pi-b, Pi-k, \) or \( Pi-ta2 \) resistance genes (Conaway et al., 1998; Fjellstrom et al., 2002). In this study, we have tested these and several newly developed markers in a number of crosses and report the identification of DNA markers associated with three \( Pi \) genes that can be used to develop new cultivars having resistance to all of the predominant races of blast found in the USA.

**MATERIALS AND METHODS**

**Development of Markers**

The DNA markers reported here were developed by four different methods. Five of them (RM101, RM138, RM144, RM155, and RM166) are based on an initial set of microsatellites identified at Texas A&M University by screening the NCBI public DNA sequence database for repeated sequences as reported in Ternykh et al. (2000). These markers were noted to map near the reported location of \( Pi-b, Pi-k, \) and \( Pi-ta' \) and their actual linkage was confirmed by mapping in some of the populations described below (Conaway et al., 1998). By mapping these initial markers relative to microsatellite markers developed by traditional methods at Cornell University (Ternykh et al., 2000), three additional tightly linked markers were subsequently identified—RM208, RM224, and RM266. Public release of the Monsanto rice microsatellite database then allowed the development of two additional linked microsatellite markers, which were subsequently released as RM1233 and RM7102 (McCouch et al., 2002).

Because the primers for markers RM101 and RM155 gave weak amplification of DNA under our standard conditions, we developed markers with new primers that gave stronger amplification of DNA from these same sequence regions, named OSM89 and OSM91, respectively. We identified OSM89 as a separate locus from RM101 because the amplification products for OSM89 represent a different polymorphism than RM101, although it was derived from the same cDNA sequence (GenBank accession D17586). Following the nomenclature system established by McCouch et al. (2002), because the amplification products for OSM91 represent the same polymorphism identified by RM155, RM155 was used to identify the locus studied and the OSM91 primer pair is considered a different marker reagent for this locus. The forward (F) and reverse (R) primer sequences for OSM89 and OSM91, listed in 5' to 3' direction are OSM89F, TTGTGTC AAGGT TAGGC ATGGGAGGG; OSM89R, TTTGAACCGGGTGCCCA

<table>
<thead>
<tr>
<th>Blast race</th>
<th>( Pi-b )</th>
<th>( Pi-k^b )</th>
<th>( Pi-k^a )</th>
<th>( Pi-ta^a )</th>
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<tbody>
<tr>
<td>IB-1</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
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<td>IB-33</td>
<td>S</td>
<td>S</td>
<td>S</td>
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</tr>
<tr>
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<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>IB-49</td>
<td>M</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>IB-54</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<tr>
<td>IC-17</td>
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<td>S</td>
<td>S</td>
<td>R</td>
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<td>IE-1</td>
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<td>S</td>
<td>R</td>
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<tr>
<td>IE-1K</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td>IG-1</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>HI-1</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

† Genes conferring a blast reaction of 0 to 2 are listed as ‘R’, a score of 3 to 4 are listed as ‘M’, and a score of 5 or more is listed as ‘S’.

CATG; OSM91F, AACGTCGCTCCCTCCAGGGC; and OSM91R, CTCCACGGTGAGCGGTGGC. A marker for \( Pi-b, Pibdom, \) was also developed on the basis of the sequence of the cloned \( Pi-b \) blast resistance gene (GenBank accession AB013448; Wang et al., 1999). The primers for the Pibdom marker were constructed to specifically amplify a 365-bp fragment from the 3' terminus of the \( Pi-b \) gene (corresponding to bases 8699 to 9063 in GenBank accession AB013448) and not to amplify a similar product from a homologous sequence in rice (GenBank accession AB013450; Wang et al., 1999) because of multiple mismatches between the two homologs in this region. The Pibdom primer sequences are: PibdomF, GAACAATGCCAAAACACTTGAGA, and PibdomR, GG GTCCACATGTCAGTGAGC.

**Development of Genetic Populations**

As part of the URRN evaluation process, USDA-ARS researchers at Beaumont, TX, (among other researchers) have routinely determined the resistance of rice cultivars on the basis of their reactions to races of blast that occur in the USA. The corresponding reaction of established cultivar differentials helps to determine which major blast resistance genes are present in U.S. cultivars. These disease reactions have served to identify cultivars for use in crosses to generate populations segregating for specific blast resistance genes (Table 1). The cultivar Maybelle possesses no major resistance genes (Bollich et al., 1991) and was used by Marchetti and Lai (2000) as a susceptible background to isolate individual \( Pi \) genes found in U.S. cultivars. The isolation of individual \( Pi \) genes was initiated through backcrossing resistant cultivars to Maybelle, followed by phenotypic screening for resistance to individual blast races. Their work demonstrated that ‘Le- mont’ possesses the \( Pi-d \) and \( Pi-k^b \) genes, ‘Kaybonnet’ possesses both \( Pi-k^b \) and \( Pi-ta^2 \) genes, ‘Bengal’ has \( Pi-z \) and \( Pi-k^a \), and ‘M201’ has only \( Pi-k^b \). They also used the Chinese cultivar, Te-Qing (also referred to as Teqing), which has resistance to all known races of blast that occur in the USA, although which \( Pi \) gene(s) it possessed were unknown, and crossed it with Maybelle in the same manner. In the current study, selfed progeny from each of the Maybelle backcross populations were screened for resistance with races indicative of specific \( Pi \) genes and were used to associate with DNA markers.

Three additional segregating populations were also developed for the current study. These comprised the 'M201', which possesses no major resistance genes, and Kaybonnet was used to generate and \( F_2F_3 \) progeny for marker evaluation of the \( Pi-ta^2 \) gene. A population of \( F_{13} \) families derived from Gulfmont*2/Te-Qing was also used for determination of the \( Pi-b \) gene from Te-Qing. Gulfmont is a conventional long-grain
cultivar, widely grown in the southern USA during the 1990s, and carries the Pi-kt resistance gene, but is susceptible to several blast races to which Te-Qing confers resistance. A breeding line that was developed from the cross Vista/Lebonnet (PI592507)/Rosemont was found to have the same blast resistance spectrum as its sibling ‘Jefferson’ (McClung et al., 1997), indicating that it possesses the Pi-kt and Pi-z genes. This breeding line was crossed with Katy, which has the Pi-ta gene. Since some of these genes have overlapping resistance spectra, progeny with isolated Pi genes were determined by testing with races that are indicative of certain Pi genes. For example, in the population derived from (Vista/Lebonnet/Rosemont)/Katy, progeny having resistance to IB-49 (i.e., Pi-ta) or resistance to IE-1K (i.e., Pi-z; McClung, 2002) were disregarded and only the remaining progeny showing resistance or susceptibility to IB-45 were used in the analysis for Pi-kt. Seed of parent materials used in these genetic studies are available as accessions through the USDA-ARS National Plant Germplasm System (http://www.ars-grin.gov/npgs; verified 23 April 2004).

Disease Resistance Screening

 Cultures of pathotypes maintained by the USDA-ARS at Beaumont were used to inoculate parents, progeny, and controls of resistant and susceptible cultivars to test for their disease reaction. These included isolate 93M25 of race IE-1K for testing for the presence of the Pi-b resistance gene; isolates 75L5 of race IB-45, 429 of race IB-54, and 74T3 of race IG-1, for the Pi-kt gene; isolate 429 of race IB-54, for the Pi-kt gene; and isolate 793 of race IB-49, for the Pi-ta gene (Marchetti et al., 1987). Standard seedling inoculation methods were used to evaluate plants for their disease resistance reaction with a score from 0 to 9 (Marchetti et al., 1987). Plants scored with a reaction of less than 3 were rated as resistant, whereas plants with a reaction greater than 3 were rated as susceptible. Plants with a reaction of 3 were considered inconclusive and were left out of subsequent genetic analyses. Leaves were harvested from scored seedlings, transferred into a −80°C freezer, lyophilized, and then stored at −20°C.

Marker Analysis

DNA was isolated through an adaptation of the method of Williams and Ronald (1994) with 20 to 40 mg lyophilized leaf tissue cut up into small (about 4 mm3) pieces and put into 2-mL microcentrifuge tubes. A 900-μL aliquot of PEX/CTAB extraction buffer containing 6.25 mM potassium ethyl xanthogenate, 0.5% cetyltrimethyl ammonium bromide, 700 mM NaCl, 10 mM EDTA, and 100 mM Tris (pH 7.5) was added to each and lightly vortexed. Tubes were placed in a 65°C water bath for 1 to 1.5 h, cooled briefly, and extracted with 700 μL 100% chloroform. Tubes were centrifuged at top speed and 800 μL of isopropanol was added to precipitate nucleic acids. Nucleic acid pellets were washed with 400 μL ethanol, dried, and resuspended in 100 μL TE buffer (10 mM Tris, pH 7.5, 0.5 mM EDTA). Microsatellite markers were PCR-amplified from 1 to 2 μL DNA extract by the protocol of Ayres et al. (1997) with primers listed in Temnykh et al. (2000), McCouch et al. (2002), or those listed above for OSM89 and OSM91/RM155. Briefly, PCR was performed in 20-μL volumes with 0.2 mM dNTPs, 2.5 mM MgCl2, 0.1 μM of forward and reverse primers, approximately 5 ng DNA, and 0.5 U Taq polymerase (Life Technologies, Rockville, MD) in 1× PCR buffer supplied by manufacturer. PCR used 30 or 26 cycles of thermocycling for products analyzed with gel or capillary electrophoresis, respectively. The Pibdom gene marker was amplified under similar conditions, but with a 65°C annealing temperature. Markers prepared for capillary electrophoresis were amplified with fluorescently labeled (FAM or HEX) forward primers.

Amplification products were analyzed by vertical gel electrophoresis or capillary electrophoresis. Nonfluorescently labeled amplification products were loaded on nondenaturing 7% (w/v) polyacrylamide (29:1, acryl: bis) 1× Tris-borate gels (TBE), run overnight at 220 V, and stained directly on separated, water-rinsed plates for 10 min with 50 mL solution of GelStar (Bio Whittaker Medical Applications, Rockland, MA) nucleic acid stain diluted 10 000 fold in 1× TBE. Stained plates were rinsed with water and placed on a blue-light-emitting transilluminator (DarkReader, Clare Chemical Research, Denver, CO). Images of fluoresced DNA bands were captured with a digital camera and recorded directly on computer. Fragment size data were interpreted from molecular weight ladders and controls included on each gel image. For capillary electrophoresis, 1 μL of fluorescently labeled amplification product was diluted into 99 μL water. One microliter of this mixture was diluted into 9 μL of a formamide-400HD ROX mixture (ABI, Foster City, CA), placed in a 96-well microplate, denatured for 2 min at 94°C, immediately cooled to 4°C, and placed in the a capillary electrophoresis instrument (3100 Genetic Analyzer, ABI). Samples were run with POP-4 matrix using the GeneScan36 POP4 Default Module, with run conditions of 60°C, 15 kV, and 25 min. Fragment size data were automatically stored on computer and analyzed by Genotyper Version 3.7 software (ABI) to determine progeny genotypes.

Genetic linkages among microsatellite marker genotypes, Pibdom marker phenotypes, and disease resistance phenotypes or genotypes were determined using Map Manager QTX (Manly et al., 2001). Disease resistance was scored as a dominantly controlled trait (where plants homozygous for disease resistance are not differentiated from heterozygous plants), except for the Kaybonnet cross populations scored for IB-49 resistance, which were completely classified for disease resistance genotype (where homozygous and heterozygous plants are differentiated apart) by F1 family analysis. Recombination estimates for differing levels of progeny (i.e., F2, F3, and F8) were taken into account using the advanced intercross option from the linkage evaluation menu of Map Manager QTX. Recombination distance data (Morgan map units) between resistance genes and markers from separate crosses were combined by averaging distances weighted according to the number of progeny.

RESULTS

Markers for Pi-b

Two crosses were analyzed to determine linkages between four microsatellite markers and the Pi-b blast resistance gene on chromosome 2 of rice. Some 200 F1; families from the cross Gulfmont*/2/Te-Qing were scored as being uniformly susceptible, uniformly resistant, or segregating in their reaction to inoculation with the IE-1K pathotype. Plants within each family were combined according to their disease reaction, separating segregating lines into resistant and susceptible pools before bulking, and then tissue samples were scored for marker genotypes. Markers RM138, RM166, and RM266 on chromosome 2 were all closely linked to the IE-1K resistance gene, while no recombination was seen.
between IE-1K resistance and marker RM208 (Table 2). In greenhouse tests using the same families from the above cross, but inoculated with blast race IC-17, resistance to race IC-17 appeared to cosegregate with IE-1K resistance and RM208 (data not shown). However, since symptom severity appeared to be minimal in several inoculated flats of seedlings (including susceptible controls), the potential high number of infection escapes precluded confident linkage analyses. It was concluded that a few $F_{12}$ progeny lines appeared to have partial resistance to IC-17 when they were susceptible to IE-1K because of escape. Moreover, no progeny were observed to be susceptible to IC-17 and resistant to IE-1K.

To investigate if the gene conferring resistance to race IE-1K on chromosome 2 was the $Pi-b$ gene, we developed the dominant Pibdom marker on the basis of published sequence information of the cloned $Pi-b$ gene (Wang et al., 1999). We found the 365-bp marker product present in Te-Qing, but not in Gulfmont (Fig. 1), the parental sources of IE-1K resistance and susceptibility, respectively, used in this study. This marker was also present in the cultivar BL-1, documented to possess the $Pi-b$ gene (Kiyosawa, 1972), and in other cultivars suspected to carry $Pi-b$ (Fig. 1) and absent in over 120 U.S. germplasm and international accessions believed to lack $Pi-b$ on the basis of their phenotypic reactions (data not shown). Segregation analysis using the presence or absence of the Pibdom marker in the Gulfmont*2/Te-Qing population implies that this marker identifies a single copy gene. The marker cosegregated with IE-1K resistance suggesting that the marker is amplifying the $Pi-b$ gene that, in turn, confers IE-1K resistance. Sequence analysis indicates that a single 365-bp sequence identical to the published $Pi-b$ gene (Wang et al., 1999) was produced from BL-1 and Te-Qing DNA templates amplified with the Pibdom marker primers (data not shown).

We used the Pibdom dominant marker to map the $Pi-b$ gene location relative to the microsatellite markers RM138, RM166, RM208, and RM266 using over 120 plants from an $F_{2}$ population of Maybelle*2/Te-Qing that was not inoculated with blast. Again, the Pibdom marker cosegregated with RM208 and was closely linked to markers RM138 and RM166 (Table 2). Recombination distances between microsatellite markers and the $Pi-b$ resistance gene were combined from the two segregating populations to develop a fine map of the $Pi-b$ gene region. Markers RM138 and RM266 both map on the telomeric side of the $Pi-b$ gene, RM208 cosegregates with this gene, while RM166 flanks the.

### Table 2. Segregation of DNA markers with resistance to blast race IE-1K ($F_{0}$ progeny) and microsatellite markers with the Pibdom marker ($F_{2}$ progeny) arising from the $Pi-b$ gene of Te-Qing.

<table>
<thead>
<tr>
<th>Cross, Generation</th>
<th>Microsatellite Recombinants</th>
<th>Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulfmont*2/Te-Qing $F_{12}$</td>
<td>RM 138 13/209</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>RM 166 9/199</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>RM 208 0/201</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>RM 266 9/203</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Pibdom 0/199</td>
<td>0.0</td>
</tr>
<tr>
<td>Maybelle*2/Te-Qing $F_{2}$</td>
<td>RM 138 2/132</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>RM 166 2/130</td>
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</tr>
<tr>
<td></td>
<td>RM 208 0/126</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>RM 266 2/130</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Fig. 1. Image of amplification products resulting from the Pibdom primers in selected rice cultivars commonly grown in U.S. producer fields electrophoresed on a non-denaturing polyacrylamide gel. Lane identities are as follows: Lanes 1 and 20, molecular weight marker; Lanes 2 to 19, rice cultivars Bengal, BL-1, Bolivar, Cocodrie, Cypress, Francis, Gulfmont, Hildalgo, Jasmine 85, Katy, L-204, Maybelle, M-202, M-205, Saber, Te-Qing, and Wells. Note that BL-1, Bolivar, Saber, and Te-Qing are the only cultivars displaying the 365-bp amplification product found in the $Pi-b$ gene sequence, with its position shown by an arrow to the left of the image.
Table 3. Segregation of microsatellite markers with resistance to blast races IB-54, IG-1, or IB-45 arising from the Pi-\(k^h\) allele of Kaybonnet, Lemont, or Vista, respectively, and to blast race IB-54 arising from the Pi-\(k^t\) allele of Bengal and M-201.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cross, generation</th>
<th>Microsatellite</th>
<th>Recombinants</th>
<th>Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi-(k^h)</td>
<td>Maybelle*2/Kaybonnet F(_2)</td>
<td>RM144</td>
<td>5/98</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RM224</td>
<td>0/98</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RM1233</td>
<td>4/94</td>
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</tr>
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<td></td>
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<td>RM144</td>
<td>3/173</td>
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<tr>
<td></td>
<td></td>
<td>RM224</td>
<td>0/180</td>
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</tr>
<tr>
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<td></td>
<td>RM1233</td>
<td>3/179</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>(Vista/Lebonnet//Rosemont)/Katy F(_5)</td>
<td>RM144</td>
<td>3/88</td>
<td>0.9</td>
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<tr>
<td></td>
<td></td>
<td>RM224</td>
<td>2/88</td>
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</tr>
<tr>
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<td>2/65</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>RM1233</td>
<td>0/65</td>
<td>0.0</td>
</tr>
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<td>Maybelle*2/M-201 F(_2)</td>
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<td>5.4</td>
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<td></td>
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<td>0/90</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RM1233</td>
<td>4/88</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Pi-\(b\) gene on its centromeric side (Fig. 2A). It can be noted that RM48 also has been mapped to this region ([http://www.gramene.org](http://www.gramene.org)); however, our analyses indicate that this marker amplifies sequences identical to or near the Pi-\(b\) blast resistance gene on rice chromosome 12. Segregation in the F\(_2\) generation discriminates the difference between resistant F\(_2\) plants that are either homozygous or heterozygous for the dominant resistance allele. Initial linkage analysis experiments placed the Pi-\(t_{a2}\) resistance gene in Katy that confers resistance to blast race IB-49, near markers RM101 and RM155 ([Consaway et al., 1998]) which are located near the centrome of the short arm of chromosome 12. Several microsatellite markers mapping near this region ([http://www.gramene.org](http://www.gramene.org)) were then analyzed for their linkage to the Pi-\(k^h\) and Pi-\(k^t\) blast resistance alleles at the Pi-\(k\) locus. Using F\(_2\) plants from four Maybelle backcross populations, we found two microsatellite markers, RM1233 and RM144, to be closely linked with resistance to race IG-1, conferred by Pi-\(k^h\), and race IB-54, conferred by Pi-\(k^t\), while RM224 cosegregated with resistance to both races in all 433 progeny analyzed (Table 3). IB-45 resistance also was found to map very close to RM224 in the (Vista/Lebonnet//Rosemont)/Katy F\(_2\) cross ([Consaway et al., 1998]).

Table 4. Segregation of microsatellite markers with resistance to blast race IB-49 arising from the Pi-\(t_{a2}\) gene of Katy or Kaybonnet.

<table>
<thead>
<tr>
<th>Cross, generation</th>
<th>Microsatellite</th>
<th>Recombinants</th>
<th>Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Vista/Lebonnet//Rosemont)/Katy F(_2)</td>
<td>OSM89</td>
<td>22/250</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>RM155</td>
<td>17/251</td>
<td>3.5</td>
</tr>
<tr>
<td>Maybelle*2/Kaybonnet F(_2)</td>
<td>OSM89</td>
<td>16/339</td>
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<tr>
<td></td>
<td>RM155</td>
<td>5/335</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>RM7102</td>
<td>7/334</td>
<td>1.1</td>
</tr>
<tr>
<td>Kaybonnet/M-204 F(_2)</td>
<td>OSM89</td>
<td>13/443</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>RM7102</td>
<td>7/440</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Lafitte R# R S 212 421 163
/H11002
Saber R R M 199 315 177
L-205 S S S 212 419 163
/H11002
Gulfmont S R S 212 419 163
/Drew S R R 212 419 163
Pi-b
# Resistance not conferred by
Pi-ta
marker. Our results suggest that the presence of the
Pi-RM208
/ haplotype of RM138
and Saber is demonstrated by the unique marker allele 5) and appear to be ideally suited for marker assisted
resistance gene. The presence of the
races of the blast fungus are also polymorphic for the associated resistance genes using mapping information
germplasm differing in susceptibility to
assayed marker genotypes in a wide range of U.S. rice loci have been identified for marker-assisted selection
the
Pi-ta²
associates with the RM224
/ haplotype, associated with the RM224
/ haplotype, Pi-ks
and
Pi-ks
are tightly linked to the centromere of chromosome 12
(Fig. 2C).

Marker Polymorphism in U.S. Rice Germplasm
To examine the utility of these markers in developing new U.S. varieties with enhanced resistance to blast, we
assayed marker genotypes in a wide range of U.S. rice germplasm differing in susceptibility to
P. grisea (Table 5). Varieties which differ in susceptibility to different races of the blast fungus are also polymorphic for the DNA markers closely linked to the corresponding blast resistance gene. The presence of the
Pi-b
gene in Bolivar and Saber is demonstrated by the unique marker allele genotype of RM138 = 199 nt, RM166 = 315 nt, and RM208 = 177 nt, as well as the presence of the Pibdom marker. Our results suggest that the presence of the
Pi-k
and
Pi-k
alleles can be differentiated by their allelic signatures at RM224 and RM144. The
Pi-k
gene is associated with the RM224 = 139 nucleotide (nt) and RM144 = 255 nt alleles, whereas the
Pi-k
allele is associated with the RM224 = 120 nt and RM144 = 255 nt alleles. The table also indicates that ‘Madison’
(McClung et al., 1998) is heterogeneous for the
Pi-k
and
Pi-k
alleles derived from its parents, Lemont and
Katy, respectively. It can be seen that discrimination of the
Pi-ta²
gene requires the use of more than one of the microsatellite markers near its locus on rice chromosome
12. Cultivars M-104, M-202, M-204, and M-205 possess the resistance allele (= 269 nt) only at RM155 and do not possess
Pi-ta²
, whereas cultivars Ahrent, Drew, and Madison possess the resistance alleles at
OSM89, RM155, and RM7102 and demonstrate the race resistance spectra that is associated with
Pi-ta².

DISCUSSION
Codominant, rapidly analyzed, polymorphic markers closely flanking the
Pi-b
, 
Pi-k
, and
Pi-ta²
blast resistance loci have been identified for marker-assisted selection of blast resistance genes. In most crosses, the reported markers have been found to be 0.0 to 2.9 cM from their associated resistance genes using mapping information from hundreds of segregating progeny. These markers are polymorphic over a wide range of germplasm (Table
5
) and appear to be ideally suited for marker assisted selection for blast resistance in rice because of their tight
linkage with resistance genes and ease of use through analysis of amplification products. The existence of multiple markers for each gene allows more latitude in the selection of closely related parents for crosses. Having multiple markers, each of which is highly polymorphic, is especially important in breeding programs that use a narrow germplasm base, where polymorphism between parents can be minimal.

In the (Vista/Lebonnet/Rosemont)/Katy cross, the apparent genetic distances between the IB-49 resistance factor and the markers analyzed were larger than the distances observed in the Maybelle/Kaybonnet and Kaybonnet/M-204 crosses (e.g., 3.5 vs. 0.8 cM, respectively, from RM155 for the two crosses; Table
4
). While substantial differences in map distances between crosses are common, we suspect that much of the apparent recombination is actually due to difficulties in accurately
distinguishing resistant and susceptible reactions in this cross. This cross is known to have the \( \text{Pi-}k^h \), \( \text{Pi-}k^k \), \( \text{Pi-ta}^2 \), and \( \text{Pi-}z \) resistance genes segregating in it and is thus difficult to distinguish resistant and susceptible phenotypes with complete accuracy due to overlapping resistance spectra. This is made more difficult by the presence of field resistance, in which the progress and severity of infection are reduced in the absence of race-specific resistance genes (Marchetti, 1983; Wang et al., 1994; Tabien et al., 2000; Tabien et al., 2002). One variety exhibiting such resistance is Lebonnet (Bollich et al., 1975), a parent of both Katy and the Vista/Lebonnet/Rosemont parent in this cross. As discussed in Bormans (2002), the fact that phenotypic data is traditionally scored on a continuous 0-to-9 scale further complicates the issue.

Although the above stated difficulties were present for this cross when mapping resistance factors at the \( \text{Pi-}k^h \) gene, it could also be easily argued that the use of different blast races can account for these map position changes. The \( \text{Pi-}k^h \) locus may have several resistance genes found at or near it and the IB-45 resistance factor mapped in the (Vista/Lebonnet/Rosemont)/Katy cross may be encoded by a separate gene near the IG-1 or IB-54 resistance factor(s) mapped in the other crosses. It is likely that the large number of alleles found at this locus (\( \text{Pi-k}^k \), \( \text{Pi-k}^h \), \( \text{Pi-}k^w \), \( \text{Pi-}k^z \), and \( \text{Pi-}k^b \); Kiyosawa, 1972; McCouch et al., 1994) are actually different disease haplotypes (Collins et al., 1999) differentiated by the presence or absence of several resistance gene factors found at this chromosomal location.

Although not directly presented in this research, variability within pathogen races for aggressiveness can also confuse resistance gene mapping efforts. We observed that cultivars carrying the \( \text{Pi-b} \) resistance gene display different levels of resistance to race IB-49 on the basis of the blast isolate used for inoculation (McClung et al., 2004b; compiled URRN blast reaction data, 1995–2003). The IB-49 isolate (USDA-793) we used in our mapping populations was fairly aggressive on the \( \text{Pi-b} \) gene, making it difficult to clearly discern segregating resistant and susceptible families and, thus, prevented definitive resistance gene mapping (results not shown). The moderate levels of IB-49 resistance conferred by the \( \text{Pi-b} \) gene can be contrasted with the \( \text{Pi-ta}^2 \) gene, which displays uniformly high levels of resistance to various IB-49 isolates and allows for straightforward mapping of markers.

The blast resistance markers identified in this research are currently being used at the TAES/USDA-ARS rice breeding program at Beaumont, TX, for the development of improved rice germplasm with combinations of blast resistance genes that have given broad spectrum resistance to U.S. blast races (e.g., cultivars Saber and Bolivar; McClung et al., 2004a, 2004b). These markers were also used to detect the presence of the \( \text{Pi-}k^h \) gene in the recently released cultivar Madison (McClung et al., 1998; McClung, 2002) where the contribution of \( \text{Pi-}k^h \) had been obscured by the presence of the \( \text{Pi-ta}^2 \) gene. The presence of both of these genes suggests that Madison may have more durable resistance to blast disease than previously expected.

One can question the value of microsatellite markers near a disease resistance gene when the same gene has already been cloned and sequenced. Such a case exists for the IE-1K resistance gene on chromosome 2 where IE-1K resistance, RM208 and a dominant marker developed from the cloned \( \text{Pi-b} \) gene sequence all appear to co-segregate. This situation allows one to choose between selection based on a cloned resistance gene sequence or linked microsatellite markers, each of which has advantages and disadvantages.

The main disadvantage of using a single microsatellite marker such as RM208 is that recombination between the marker and the resistance gene would result in erroneous selection of a susceptible plant. There are, however, several advantages of using closely linked microsatellite markers. Since these markers are codominant, a single amplification reaction can detect the absence of resistance-associated allele bands because of failed reactions, the presence of heterozygotes, and the relative proportion of resistance-associated alleles in a bulked population. With flanking codominant markers, it is also possible to detect recombination within the resistance locus, which would indicate potential losses of genetic factors necessary for disease resistance.

There are advantages in using either type of marker, and one may choose according to their own application. If one desired to run only one marker, it may be best to choose markers such as RM208 and RM224 that are both codominant and tightly linked to their associated blast resistance genes (\( \text{Pi-b} \) and \( \text{Pi-}k^b \), respectively). If one wants to detect recombination around a resistance gene, markers flanking a \( \text{Pi} \) locus can be tested, such as RM155 and RM7102 for the \( \text{Pi-ta}^2 \) gene. For crosses involving medium grain cultivars developed in California (e.g., M-104, M-202, M-204, or M-205) or their derivatives, one should use markers OSM89 and RM7102, since RM155 is not polymorphic between \( \text{Pi-ta}^2 \) carrying lines and these medium grain lines. If one wanted to use agarose gels in marker analysis, dominant markers such as Pibdom, or a recently developed \( \text{Pi-ta} \) marker (Jia et al., 2002), may be best as long as the susceptible parent of the progeny being selected does not produce a similar sized amplification product. The \( \text{Pi-ta} \) resistance factor conferring resistance to blast races CP753 and CP3337 (Bryan et al., 2000) maps to a similar location as the \( \text{Pi-ta}^2 \) factor that confers resistance to race IB-49. Results to date indicate that the cloned \( \text{Pi-ta} \) gene co-segregates with IB-49 resistance arising from Katy (Jia et al., 2004). Codominant gene markers are also advantageous in cases of loci like \( \text{Pi-ta}^2 \), where more than one resistance gene appears to be present in this region (i.e., \( \text{Pi-ta} \) and \( \text{Pi-ta}^2 \); Rybka et al., 1997) and one would want to select for a lack of recombination in markers flanking this gene region. It can also be noted that the Pibdom marker should not be considered as the \( \text{Pi-b} \) gene per se, and that recombination could occur between the Pibdom marker and the \( \text{Pi-b} \) gene. Since the \( \text{Pi-b} \) gene appears to belong to a small gene family in rice (Wang et al., 1999), we cannot definitively say that the amplification product from \( \text{Pi-b} \) containing lines, like BL-1, can only arise from the \( \text{Pi-b} \) gene itself and not a homol-
ogous sequence. On the basis of our experimental evidence showing lack of recombination between this marker and IE-1K resistance on rice chromosome 2, we have concluded that the Pbdom marker amplified from Te-Qing is tightly linked to the Pi-b gene and is probably part of the Pi-b gene found in Te-Qing.

As considerable progress has been made in sequencing the rice genome, the close linkage of RM208 to the Pi-b gene can be directly related to the physical distance between these two sequences. Both these sequences can be found on the same BAC clone (GenBank accession AP004048) and are separated by approximately 23 kb (data not shown). With the average physical distance of a single centimorgan in the rice genome being 244 kb (Chen et al., 2002), the lack of recombination seen between RM208 and the Pi-b gene was to be expected. Physical distances between other Pi-b markers on rice chromosome 2 can be estimated from publicly available physical maps, such as those presented by the International Rice Genome Sequencing Project (http://irgp.dna.afirc.go.jp/IRGPSP; verified 13 May 2004), Gramene (http://www.gramene.org), or the Arizona Genomics Institute (http://www.genome.arizona.edu/fpc/rice/; verified 23 April 2004). Since the relationship between the cloned Pi-ta resistance factor and the Pi-ta2 resistance gene is unclear, the Pi-k resistance gene or factors are yet to be identified, and because the markers we developed for these two resistance genes are not yet physically mapped onto ungapped contigs, it is presently not possible to determine physical distances among the markers and the Pi-k and Pi-ta2 resistance genes.

Clearly, the DNA markers presented here can greatly enhance selection efforts to incorporate multiple blast resistance genes into improved germplasm. Such markers could also be combined with markers developed for field resistance, which would provide several levels of protection against this destructive disease. Since race-specific and nonrace specific disease resistance genes can greatly confound resistance phenotyping efforts, DNA markers provide the most efficient means to combine such genetic factors.

ACKNOWLEDGMENTS

The authors thank the diligent contributions of Mickey Frank at the Texas Agricultural Experiment Station at Beaumont in assaying markers, Carl Johnson at the California Cooperative Rice Research Foundation for providing the Kaybonnet/M-204 progeny population analyzed for Pi-ta2 markers, Sam Cartinhour for the bioinformatics approach used to identify the initial set of microsatellite markers, and Grace Walker and Nicola Ayres for their early work in assaying and mapping these markers, Jodie Cammack, Eric Christensen, and Gerald Gipson from the United States Department of Agriculture-Agricultural Research Service-Rice Research Unit and the work of Xing-Hua Lai, Rick Boyd, Pat Carre, Jay Cockrell, and Cullen Minter from the Texas Agricultural Experiment Station at Beaumont. Financial support was partially provided by the Texas Rice Research Foundation, Texas Advanced Technology Program, Texas Grain and Grass Initiative, Texas Agricultural Experiment Station, The Rice Foundation, and the United States Department of Agriculture-Agricultural Research Service.

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