Genetic Mapping and Chromosomal Assignment of *Magnaporthe oryzae* Avirulence Genes *AvrPik*, *AvrPiz*, and *AvrPiz-t* Controlling Cultivar Specificity on Rice

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ABSTRACT

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A genetic map including three avirulence (*Avr*) genes, *AvrPik*, *AvrPiz*, and *AvrPiz-t*, was constructed in a genetic cross of two rice field isolates, 84R-62B and Y93-245c-2. The chromosomal locations of the *Avr* genes were determined by using selected markers to probe Southern blots of the parental chromosomes that had been separated by contour-clamped homogenous electric fields electrophoresis. Electrophoretic karyotyping showed that both parental isolates 84R-62B and Y93-245c-2 contained

seven chromosomes greater than 3.5 megabases (Mb) in size and 84R-62B possessed a small chromosome of ≈ 1.6 Mb. The linkage groups containing *AvrPiz* and *AvrPiz-t* were assigned to chromosomes 3 and 7, respectively. Some markers from the linkage group that contained *AvrPik* hybridized with chromosome 1 and the 1.6-Mb chromosome, yet all of the cloned RAPD markers that were closely linked to *AvrPik* hybridized exclusively to the 1.6-Mb chromosome in 84R-62B, the parent that possesses *AvrPik*. Thus, we conclude that *AvrPik* is located on the 1.6-Mb chromosome in 84R-62B.

Additional keywords: *Pyricularia grisea*, *P. oryzae*, rice blast fungus.

The ascomycetous fungus *Magnaporthe oryzae* B. Couch (formerly *Magnaporthe grisea* (Hebert) Barr.) (2) is the causal agent of rice blast, the most damaging disease of cultivated rice (*Oryza sativa* L.). The most effective way to control rice blast disease is by growing resistant rice cultivars; however, newly developed resistant cultivars are often defeated after only a few years of commercial production due to the emergence of new pathogenic variants (races) in *M. oryzae* populations. Creation of rice cultivars with durable resistance will require a firm understanding of the processes underlying pathogenic variation in *M. oryzae*.

Blast resistance in rice generally follows a gene-for-gene relationship (8,11,17) and, as such, is dependent on the presence in *M. oryzae* of a cognate avirulence (*Avr*) gene. Understanding the genetic mechanisms underlying *M. oryzae*'s ability to overcome host resistance requires molecular characterization of these *Avr* genes. To date, several *M. oryzae* *Avr* genes have been genetically mapped, and four genes (*PWL2*, *AVR1-CO39*, *AVR-Pita*, and *ACE1*) have been cloned by chromosome walking (1,7,16,20); and, although the gene has not yet been isolated, the locus containing *AVR1-MARA* has been characterized at the molecular level (13). Studies of the chromosomal organization of these genes have provided valuable insights into the genetic basis for pathogenic variability in *M. oryzae* (1,5,8,13,16,20). For example, *AVR-Pita* and *AVR-TSUY* both exhibit extremely high levels of mutability in vegetative culture, because the spontaneous virulent mutants of both genes were easily isolated after standard inoculation experiments (23). Significantly, both genes mapped near telomeres (21), and molecular characterization of *AVR-Pita* revealed

that it is located just 50 bp from the telomere repeat (16). By contrast, the *AVR1-CO39*, *ACE1*, and *AVR1-MARA* genes are much more stable and, although they have been lost or mutated in certain fungal lineages during the course of *M. oryzae* evolution, they do not exhibit instability in vegetative culture (1,5,13). Significantly, all of these genes reside at internal chromosomal locations (4,13,19). The association between chromosomal location and stability is not perfect, however, because spontaneous *PWL2* mutants arise in vegetative culture (9), yet this gene resides in the middle of chromosome 2 (21). At present, too few *Avr* genes have been mapped or cloned to know if *PWL2* is a rare exception, or if the correlation between stability and chromosome position is purely coincidental. It follows that a better understanding of factors affecting *Avr* gene mutation and loss in *M. oryzae* will require mapping, cloning, and characterization of many more genes. Here we describe the genetic mapping and chromosomal assignment of three *M. oryzae* *Avr* genes, *AvrPik*, *AvrPiz*, and *AvrPiz-t*.

MATERIALS AND METHODS

Fungal isolates. Parental isolates, 84R-62B (*MAT1-1*) and Y93-245c-2 (*MAT1-2*), and a subset of 60 progeny used in this study have been described previously (12).

DNA extraction. Each fungal isolate was grown in 50 ml of potato sucrose broth (200 ml of broth from 200 g of potato, 20 g of sucrose per liter) for 4 to 5 days at 25°C on an orbital shaker (120 rpm). Mycelia were harvested from the liquid culture by filtration through Kimwipe paper (Kimberly-Clark Corporation, Tokyo) and then frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Three- to four-hundred milligrams of mycelial powder was transferred to 0.5 ml of extraction buffer (0.5% sodium dodecyl sulfate [SDS], 100 mM LiCl, 100 mM EDTA, and 10 mM Tris [pH 7.5]) in a micro-centrifuge tube. After mixing, the mixture was incubated at 55°C for 30 min,

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and then centrifuged at 12,000 rpm for 15 min. The supernatant was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with isopropanol. The pellet was washed in 70% ethanol and resuspended in 0.5 ml of TE buffer (1 mM EDTA and 10 mM Tris [pH 8.0]) containing 20 ng of RNase A (Nippon Gene, Toyama, Japan). After RNA digestion, the solution was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) and once with chloroform/isoamyl alcohol (24:1, vol/vol). Final supernatant was precipitated with 11/16 volumes of 20% polyethylene glycol containing 2.5 M NaCl. After washing in 70% ethanol, the DNA pellet was resuspended in TE buffer.

RAPD analysis. A total of 780 primers (Kit A to Z and Kit AA to AM, Operon Technologies, Alameda, CA) was used in random amplified polymorphism DNA (RAPD) analysis. Polymerase chain reaction (PCR) was carried out in one of two methods: with primer combinations (Kit A to Z and Kit AA to AM), or single primers (Kit A to F) in each reaction. The amplification reaction was performed in a 20- μ l reaction containing 0.5 units of Ex. *Taq* DNA polymerase (TaKaRa, Otsu, Japan), 1 $\times$ PCR buffer provided by the manufacturer, 125 μ M each dNTP, 30 ng of template DNA, and 0.5 μ M of each primer for dual primer reactions or 1.0 μ M primer for single primer reactions. Amplification was performed in a 9600 thermocycler (Perkin-Elmer, Norwalk, CT) programmed for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C followed by 5 min at 72°C.

Amplification products were separated in a 0.8% agarose gel in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer (44.5 mM Tris-borate and 1 mM EDTA [pH 8.0]), using a 50- to 2,500-bp DNA Marker (TaKaRa, Otsu, Japan) and Smart ladder (Nippon Gene, Toyama, Japan) as molecular size markers.

Cloning of RAPD markers and sequencing. Markers of interest were separated from other amplified fragments by agarose gel electrophoresis and extracted from the gel by QIAquick Gel Extraction Kit (Qiagen Sciences, Germantown, MD) and cloned into pGEM-T Easy vector (Promega, Madison, WI). Transformation of plasmids into DH5 α -competent cells (Nippon Gene, Toyama, Japan) was performed following the manufacturer's instructions. The cloned RAPD marker was sequenced using BigDye terminator version 3.1 cycle sequencing (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. Sequences were acquired using the Genetic Analyzer DNA Model 310 (Applied Biosystems).

Southern hybridization analysis. Total genomic DNA was digested with restriction enzyme and fractionated through a 0.8% agarose gel in 0.5 $\times$ TBE buffer. The fractionated DNA was transferred to an MSI nylon membrane (Osmonics, Westborough, MA) and fixed by UV irradiation following the manufacturer's instructions. Plasmid clones containing RAPD markers as described previously and plasmid clones representing restriction fragment length polymorphism (RFLP) markers in the map of Skinner et al. (18) were used as probes. The membrane was hybridized overnight with biotin-labeled probes in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 5 $\times$ Denhardt's solution, 0.5% SDS, and 100 μ g of heat denatured salmon sperm DNA per ml at 68°C. The hybridized membrane was washed twice in 2 $\times$ SSC, 0.1% SDS at room temperature for 5 min, and then washed twice in 0.1 $\times$ SSC, 0.1% SDS at 68°C for 15 min. Detection of target DNA sequences was performed using the NEBlot Phototope Kit (New England Biolabs, Beverly, MA).

Identification and characterization of telomeric restriction fragments. Total DNA of each isolate was digested with *Eco*RI or *Hind*III and separated in 0.8 and 0.4% agarose gels in 0.5 $\times$ TBE buffer. The fractionated DNA was transferred to Hybond N+ membranes (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK) and then fixed by drying at 80°C for 2 h. A synthetic oligonucleotide (TTAGGG)₁₀ was labeled with alkaline phosphate using AlkPhos Direct Labeling Reagents (Amer-

sham Biosciences UK Limited, Buckinghamshire, UK) and used as probes for hybridization. Hybridization was performed at 55°C in Gene Images AlkPhos Direct hybridization buffer (Amersham Biosciences UK Limited) including 0.5 M NaCl and 4% blocking reagent (Amersham Biosciences UK Limited). After hybridization, the membrane was washed twice in primary wash buffer (2.0 M urea, 0.1% SDS, 50 mM NaH₂PO₄ [pH 7.0], 150 mM NaCl, 1 mM MgCl₂, and 0.2% blocking reagent) at 55°C for 10 min and then washed twice in secondary wash buffer (50 mM Tris [pH 10.0], 100 mM NaCl, and 2 mM MgCl₂) at room temperature for 5 min. Target DNA was detected using the CDP-star reagent (Amersham International plc, Buckinghamshire, UK) according to the manufacturer's instructions.

*Bal*31 nuclease (New England Biolabs) digestion of genomic DNA was performed as described by Farman and Leong (6). The *Bal*31-treated DNA sample was subsequently cleaved with *Hind*III. After electrophoresis, the fractionated DNA was transferred to Hybond N+ membrane and hybridized with the oligonucleotide probe (TTAGGG)₁₀.

Mapping of RFLP and RAPD markers. Telomeric restriction fragments were observed as the presence of a hybridizing band of a given size. If a fragment was present in isolate 84R-62B, occurrences of this fragment in the progeny were scored as "A"; occurrences of fragments from isolate Y93-245c-2 were scored as "B". The segregation data for each telomere consisted of a consensus derived by scoring fragments produced in the *Eco*RI and the *Hind*III digests. For progeny with inconsistent segregation of particular telomeres due to rearrangement, data were scored as "-".

Linkage analysis and construction of the genetic map were performed using Mapmaker version 2.0 for Macintosh (E. S. Lander, P. Green, J. Abrahamson, A. Barlaow, M. J. Daly, S. E. Lincoln, and L. Newburg, Whitehead Institute, Cambridge, MA). Parameters for map construction were a minimum logarithm of odds (LOD) score of 3.0 and a maximum recombination fraction of 0.4. The Kosambi function was used to compute recombination distances in centimorgans (cM).

Preparation of chromosome-size DNA and contour-clamped homogenous electric fields (CHEF) electrophoresis. Protoplasts were prepared from mycelial cultures as described previously by Tosa et al. (22). The resulting protoplasts were suspended in SE buffer (1.0 M sorbitol, 50 mM EDTA [pH 8.0]) at a density of approximately 1 $\times$ 10⁹ per milliliter and were then added to an equal volume of 1.5% low melt agarose (Bio-Rad Laboratories, Hercules, CA) in 0.125 M EDTA (pH 7.5). The suspension was mixed by pipetting and then poured into a mold to solidify into an agarose/protoplast plug. The solidified plugs were poured into 10 ml of LET buffer (0.01 M Tris [pH 7.5], 0.45 M EDTA [pH 8.0], and 7.5% β -mercaptoethanol) at 37°C for 16 to 24 h. After washing three times in 10 ml of 0.05 M EDTA (15 min per wash), 10 ml of NDS buffer (0.01 M Tris [pH 7.5], 0.45 M EDTA [pH 8.0], and 1.0% lauryl sarcosine) containing 10 mg of proteinase K (Invitrogen Life Technologies, Carlsbad, CA) was added and incubated at 50°C for 16 to 24 h. The plugs were then washed twice with 10 ml of 0.05 M EDTA for 15 min at room temperature before equilibrating in 10 ml of 0.05 M EDTA at room temperature for 16 to 24 h. Finally, the EDTA buffer was discarded and 5 ml of fresh 0.05 M EDTA buffer was added, and the plugs were stored at 4°C until use.

CHEF electrophoresis was performed in 0.8% Certified Megabase Agarose (Bio-Rad Laboratories) using a running buffer of 0.5 $\times$ TBE at 14°C. The CHEF electrophoresis was carried out in a Bio-Rad DR III system (Bio-Rad Laboratories) with switching intervals of 90 min for 5 days and then 60 min for 2 days at 35 V. *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* whole chromosomal DNAs (Bio-Rad Laboratories) were used as size markers. After electrophoresis, the resolved chromosomes were blotted to MSI nylon membranes following the protocol in the Bio-Rad DR III system applications manual. Hybridization

with biotin-labeled probes and detection of target DNA were performed as described previously.

RESULTS

Characterization of RAPD markers showing close-linkage to *Avr* genes. In a cross between 84R-62B and Y93-245c-2, we previously identified 17 RAPD markers that were linked to three *Avr* genes, *AvrPik*, *AvrPiz*, and *AvrPiz-t* (12). Four markers (*AE6AF6/900*, *AE12AF12/1700*, *AI6AJ6/1200*, and *AK11AL11/280*) linked to the *Avr* gene *AvrPik* (12) were cloned into the pGEM-T Easy vector, to give the plasmids pAE6AF6, pAE12AF12, pAI6AJ6, and pAK11AL11. One other marker *N6O5/200* was also converted to RFLP marker *N6O5-1*. Clones containing the *AvrPik*-linked markers were then used to probe genomic DNA from both parents. The inserts in plasmids pAE6AF6 and pAK11AL11 produced a single major hybridization signal in the parental DNA samples (Fig. 1A and D) and are, therefore, single-copy sequences. pAE6AF6 produced additional fragments with very weak signals in parental isolate 84R-62B, suggesting that partially related sequences exist in the 84R-62B genome. Clone pAE12AF12 also contained a single-copy sequence, although more than one band appeared in the *Hind*III and *Dra*I digests (Fig. 1B), due to the presence of these restriction sites in the region spanned by the probe (data not shown). pAI6AJ6 hybridized to more than one fragment in both parental genomes but an extra fragment was consistently produced with DNA from 84R-62B, the parent that gave the RAPD fragment (Fig. 1C). This, together with the facts that the common fragments exhibited weaker hybridization, indicated that the extra fragment contained the original *AI6AJ6/1200* RAPD fragment, whereas related partial sequences exist in both parental isolates. The clone derived from *AM13A13/1900*, which was linked to *AvrPiz-t*, contained repetitive sequences and so this marker was not used for mapping. Attempts to clone the RAPD fragments linked to *AvrPiz* were unsuccessful.

The inserts in the four clones (pAE6AF6, pAE12AF12, pAI6AJ6, and pAK11AL11) were sequenced, and data were submitted to DNA Data Bank of Japan (DDBJ) under accession nos. AB175829, AB175830, AB175831, and AB175832, respectively. DNA sequences of the inserts were compared with genome contigs in *M. grisea* database (available online from Broad Institute) using the BLASTN algorithm. As a result, almost the whole sequence (1,619 out of 1,656 bp) of pAE12AF12 could be aligned to the region (position 6948 to 8597) in contig 2.1572 (45,775 bp in length), which is on chromosome I. For pAE6AF6 (953 bp in length) and pAI6AJ6 (1,227 bp), no unique matches were identi-

fied, yet both inserts yielded BLASTN hits to multiple contigs. The lengths of the hits were less than 81 and 208 bp, respectively, indicating that these marker fragments contain small repetitive elements. However, the sequence of the insert in pAK11AL11 (264 bp) did not produce any significant BLASTN hits to the *M. grisea* genome. The sequences were also used to query the GenBank, EMBL, DDBJ, and PDB databases through a nucleotide sequence search site (available online from the National Center for Biotechnology Information [NCBI]). No significant matches were found in any of these databases.

Fig. 2. Identification of telomeric restriction fragments in parental isolates 84R-62B (lane 1) and Y93-245c-2 (lane 2). The enzymes used to generate the restriction profiles are shown above lanes. Digested genomic DNA was separated in 0.8% agarose gel, and the blot was hybridized with telomeric sequence (TTAGGG)₁₀. The sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λDNA) are indicated on the left. Abbreviations: Bam, *Bam*HI; Eco, *Eco*RI; Hin, *Hind*III; and Dra, *Dra*I.

Fig. 1. Genomic hybridization patterns of parental isolates revealed by cloned random amplified polymorphic DNA markers. Genomic DNAs from parental isolates 84R-62B (lane 1) and Y93-245c-2 (lane 2) were digested by restriction enzymes as described above lanes and separated by 0.8% agarose gel. The blot was hybridized with plasmid clones **A**, pAE6AF6, **B**, pAE12AF12, **C**, pAI6AJ6, and **D**, pAK11AL11, respectively, as probes. The sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λDNA) are indicated on the left. Abbreviations: Bam, *Bam*HI; Eco, *Eco*RI; Hin, *Hind*III; and Dra, *Dra*I.

Addition of telomere, RAPD, and RFLP markers to expand the current genetic map. For telomere analysis, *Bal31*-digested genomic DNA of isolate 84R-62B was subsequently cleaved with *HindIII*, electrophoresed in a 0.8% agarose gel, and blotted to a membrane. The membrane was probed with the telomere oligonucleotide (TTAGG)₁₀. Fourteen hybridizing fragments were observed all of which were sensitive to *Bal31* digestion (data not shown). This confirmed that all of the restriction fragments detected by the oligonucleotide probe are truly telomeric. A preliminary survey using the enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Dra*I revealed that the telomeres of the parental isolates 84R-62B and

Y93-245c-2 were highly polymorphic (Fig. 2). Since the *Eco*RI and *Hind*III digests resulted in the largest number of discrete fragments, these digests were used to generate the telomeric markers for mapping. Electrophoresis through 0.4 and 0.8% agarose gels allowed resolution of 33 telomeric restriction fragments, whose segregation patterns could be combined into 14 consensus data sets, which corresponded to the 14 chromosome ends (data not shown).

Further expansion of the genetic map was achieved by adding 39 new RAPD markers and 14 RFLP markers, which included six markers generated by hybridizing with the five cloned RAPD markers and eight from the map of Skinner et al. (18). Analysis of

Fig. 3. Genetic map was constructed based on the segregation data of 60 progeny isolates from the cross 84R-62B and Y93-245c-2. Kosambi centimorgan units are shown on the left, and random amplified polymorphism DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers, *Avr* loci, and mating-type locus are listed on the right. For the RAPD markers, the numbers of other markers mapped at the same locations are shown in parenthesis. The seven RAPD markers at the same location with *AE6AF6/900* included *AI6AJ6/1200* and *AK11AL11/280* (12). Probes of boxed RFLP markers were used for hybridization to electrophoretically separated chromosomes. RFLP markers generated by cloned RAPD markers are indicated by a star. RAPD markers are designated as a primer or a primer combination used for amplification/fragment size.

the segregation data using Mapmaker version 2.0 yielded eight linkage groups (LGs) (Fig. 3). Twelve of the fourteen telomere markers mapped to the ends of LGs and two remained unlinked. LGs A and G, which contained *AvrPik* and *AvrPiz-t*, respectively, possessed telomere markers on each end; and LG D containing *AvrPiz* had a telomere marker at one end. These results strongly suggest that the three *Avr* genes reside on different chromosomes. Segregation analysis of the converted RAPD markers was performed using the *Bam*HI RFLPs (Fig. 1). As shown in Figure 4, pAE12AF12 hybridized to DNA from both parental isolates, producing hybridizing bands of ≈ 2.8 - and ≈ 3.2 -kb in 84R-62B and Y93-245c-2, respectively. However, these fragments did not segregate as expected for a single-copy locus because 10 of the 60 F_1 progeny inherited both fragments. Closer inspection of the data revealed that the 2.8-kb fragment segregated 1:1 (present/absent) among the progeny but the 3.2-kb band segregated 2:1. The overabundance of progeny inheriting the 3.2-kb band suggested that the *AE12AF12* locus might be duplicated in the Y93-245c-2 genome, which would explain why some progeny possessed both alleles. However, when the segregation data for the 3.2-kb band were used to map the fragment as though it were a single-copy locus, the fragment was placed in the interval between markers *CH4-116H* and *N8O7/2200* (Fig. 3). This achievement did not require inference of an unacceptable number of double crossovers between the flanking markers, thereby providing a strong indication that the map location is valid and, hence, that the 3.2-kb band represents a single-copy locus in Y93-245c-2. Accordingly,

Fig. 4. A, Segregation of random amplified polymorphic DNA (RAPD) marker *AE12AF12/1700*, and B, restriction fragment length polymorphism (RFLP) markers *AE12AF12-1* and *AE12AF12-2*. Segregation patterns of these markers in 10 F_1 isolates are shown as examples. A, RAPD amplifications were performed with genomic DNA from parental isolates 84R-62B (lane 1) and Y93-245c-2 (lane 2), and F_1 progeny isolates (lanes 3 to 12) using primer combination OPE-AE12/OPE-AF12. Amplified products were separated in a 0.8% agarose gel. "A" and "V" indicate avirulence and virulence toward rice cv. Kanto 51 (*Pik*) (12). Arrow shows the RAPD marker *AE12AF12/1700*. B, Genomic DNA from the same isolates was digested with *Bam*HI and electrophoresed in 0.8% agarose gel. The blot was hybridized with the plasmid clone pAE12AF12 as a probe. Arrows show RFLP markers *AE12AF12-1* (2.8 kb) and *AE12AF12-2* (3.2 kb).

the segregation data are best explained by the assumption that the 2.8- and 3.2-kb fragments represent distinct, single-copy loci that occupy nonallelic positions in the parental chromosomes, and that progeny lacking both alleles were not recovered because the translocated chromosome segment contains an essential gene. The markers corresponding to the 2.8- and 3.2-kb bands were designated as dominant RFLP markers *AE12AF12-1* and *AE12AF12-2*, respectively. The remaining RAPD fragments all yielded dominant RFLP markers because major hybridization signals were detected only in 84R-62B (Fig. 1).

The four RFLP markers *AE6AF6-1*, *AE12AF12-1*, *AI6AJ6-1*, and *AK11AL1-1*, created from RAPD markers closely linked to *AvrPik*, were mapped at the same loci as their corresponding RAPD markers (Fig. 3). *AE12AF12-2*, which represents the second allele identified by RFLP marker *AE12AF12/1700*, was not linked to *AE12AF12-1*, *AvrPik*, or the *AvrPik*-linked RAPD markers (Fig. 3).

Chromosomal assignments of LGs revealed by hybridization of markers to CHEF-separated chromosomes. CHEF electrophoresis was used to separate intact chromosome-size molecules in both parental isolates. It was not possible to resolve all of the chromosomes from one another, because both isolates had at least one pair of chromosomes that co-migrated (Fig. 5A). The separated chromosomes were blotted and hybridized with 12 selected RFLP markers. Results for nine markers are shown in Figure 5B. Despite the co-migration, hybridization made it possible to distinguish seven chromosomes greater than 3.5 megabases (Mb) in length in both of the parental isolates. For example, RFLP markers *CH4-116H* and *4-10* hybridized to the largest band in both parents. However, *CH4-116H* is on LG A, which is fully delimited by telomeric markers, whereas *4-10* is on LG B (Fig. 3). This indicates that the largest band in both parents actually consists of two separate chromosomes that co-migrated. We designate these chromosomes 1 and 2, respectively. Chromosomes 3 and 4 had different mobilities in parent 84R-62B, and hybridized to markers *4-194* (LG C) and *CH3-125H*, respectively. In Y93-245c-2, however, the two markers both hybridized to the second largest band, indicating that this band actually comprises chromosomes 3 and 4. Chromosomes 5, 6, and 7 were clearly resolved following CHEF electrophoresis (Fig. 5A). In addition to the seven chromosomes greater than 3.5 Mb, isolate 84R-62B contains a small chromosome of approximately 1.6 Mb. Figure 5C shows an interpretive diagram of the chromosomes in isolates 84R-62B and Y93-245c-2, as inferred from CHEF electrophoresis (Fig. 5A) and hybridization analysis (Fig. 5B).

LG C was clearly assigned to chromosome 3 based on the hybridization result for RFLP marker *4-194*. Marker *N6O5-1* also hybridized to chromosome 3 in 84R-62B, indicating that LG D also belongs to this chromosome, a conclusion that is supported by the fact that LGs C and D both have telomeric markers at just one end. *N6O5-1* is a dominant marker and failed to detect any signals in Y93-245c-2 (Fig. 5B). RFLP markers *CH3-12H* (LG E), *4-69* (LG F), and *CH5-75H* (LG G) hybridized with chromosomes 5, 6, and 7, respectively. LGs A, E, F, and G are all delimited by telomeric markers on each end and, therefore, chromosomes 1, 5, 6, and 7 can be considered fully "assigned." So, although a lack of appropriate probes prevented LG H from being physically mapped by CHEF hybridization, it can only reside on chromosome 2, 4, or 8. Chromosomal assignments for the RFLP markers and corresponding LGs are summarized in Table 1.

Based on the chromosomal assignments of markers linked to *AvrPiz* and *AvrPiz-t*, we conclude that these *Avr* genes reside on chromosomes 3 and 7, respectively (Table 1). All of the *AvrPik*-linked markers mapped to LG A, which was assigned to the chromosome 1, based on the hybridization results for *CH4-116H* (Fig. 5B). Surprisingly, however, not all LG A markers were present on the largest chromosomal band in parent 84R-62B (Table 1; Fig. 6). For example, markers *AE6AF6-1*, *AI6AJ6-1*, and

AK11AL11-1, which were closely linked to *AvrPik* (1.7 cM, Fig. 3), all hybridized to the 1.6-Mb chromosome in 84R-62B. These markers failed to detect signals on any of the chromosomal bands in Y93-245c-2. By contrast, marker *AE12AF12-1*, which mapped at 8.4 cM from *AvrPik*, hybridized to the 1.6-Mb chromosome in 84R-62B, whereas the *AE12AF12-2*, which mapped in the internal location between the markers *CH4-116H* and *N8O7/2200*, hybridized to the chromosome 1 in Y93-245c-2 (Fig. 6), indicating that the markers *AE12AF12-1* and *AE12AF12-2* are located on the 1.6-Mb chromosome in 84R-62B and chromosome 1 in Y93-245c-2, respectively.

DISCUSSION

Sequence characterization of RAPD fragments amplified from fungi often reveals the presence of repetitive sequences (4,10).

For example, Dioh and coworkers (4) cloned 10 RAPD markers linked to *M. oryzae* *Avr* genes and found that 7 spanned the junction between a transposon and flanking single-copy DNA. In the present study, only one of the five *Avr* gene-linked RAPD markers (*AM13A13/1900*) contained repetitive sequences and, therefore, we were able to convert most of the RAPDs to RFLPs. Interestingly, four of the five RAPD markers yielded dominant RFLPs that hybridized only to DNA from the avirulent parent 84R-62B; and three of these markers were tightly linked to *AvrPik*. This suggests that the virulent isolate lacks the *Avr* gene altogether, along with a significant region of flanking DNA. In this regard, *AvrPik* resembles the *M. oryzae* *Avr* genes *AVR1-CO39*, *AVR-Pita*, *PWL2*, and *AVR1-MARA*, which are often deleted in virulent isolates/races (5,13,16,20).

In the present study, we constructed a genetic map to determine the chromosomal locations of the three *Avr* genes *AvrPiz*, *AvrPiz-t*,

Fig. 5. Chromosomes in parental isolates revealed by contour-clamped homogenous electric fields (CHEF) electrophoresis and hybridization of selected markers. **A**, Chromosome-sized DNAs of parental isolates 84R-62B (lane 1) and Y93-245c-2 (lane 2) were separated with CHEF electrophoresis. Sizes (in megabases) of *Schizosaccharomyces pombe* chromosomes and the *Saccharomyces cerevisiae* chromosomes are indicated on the left and right, respectively. **B**, The CHEF-separated of both parental isolates were hybridized with the selected restriction fragment length polymorphism (RFLP) markers shown above lanes. **C**, Illustrated diagram of chromosomes in the parental isolates (1, 84R-62B; 2, Y93-245c-2) was inferred from **A** and **B**. The chromosomes are numbered from largest to smallest, and homologous chromosomes between parental isolates are indicated as the same number.

TABLE 1. Results of hybridization of selected markers to contour-clamped homogenous electric fields (CHEF)-separated chromosomes

Marker	Feature	Codominant/dominant	Linkage group ^a	Hybridized to chromosome
<i>4-10</i>	RFLP	Codominant	B	2
<i>4-194</i>	RFLP	Codominant	C	3
<i>4-69</i>	RFLP	Codominant	F*	6
<i>CH3-12H</i>	RFLP	Codominant	E*	5
<i>CH3-125H</i>	RFLP	Codominant	—	4
<i>CH4-116H</i>	RFLP	Codominant	A*	1
<i>CH5-75H</i>	RFLP	Codominant	G*	7
<i>N6O5-1</i>	Converted RAPD	Dominant	D	Null ^b (Y93-245c-2) 3 (84R-62B)
<i>AE12AF12-1</i>	Converted RAPD, 84R-62B allele linked to <i>AvrPik</i>	Dominant	A	Null (Y93-245c-2) 8 (84R-62B)
<i>AE12AF12-2</i>	Converted RAPD, Y93-245c-2 allele not linked to <i>AvrPik</i>	Dominant	A	1 (Y93-245c-2) Null (84R-62B)
<i>AE6AF6-1</i>	Converted RAPD, linked to <i>AvrPik</i>	Dominant	A	Null (Y93-245c-2) 8 (84R-62B)
<i>AI6AJ6-1</i>	Converted RAPD, linked to <i>AvrPik</i>	Dominant	A	Null (Y93-245c-2) 8 (84R-62B)
<i>AK11AL11-1</i>	Converted RAPD, linked to <i>AvrPik</i>	Dominant	A	Null (Y93-245c-2) 8 (84R-62B)

^a Asterisks indicate linkage groups that are delimited by telomeric markers on each end; a dash indicates that the marker was not mapped.

^b The parent had a null allele for the marker in question.

and *AvrPik*. Despite the relatively small number of genetic markers that was used, the map that was generated contained just eight LGs, seven of which were assigned to six chromosomes. In addition to chromosome 4, which did not appear on the genetic map as an LG, altogether seven chromosomes more than 3.5 Mb in size were identified in both parental isolates. This number corresponds to the euploid chromosome number in *M. oryzae*, as determined in previous mapping studies concerning the genetic cross of Guy11/2539 (6,14,18). In addition, the outer boundaries of four LGs were fully delimited by the presence of telomeric markers at each end. The ability to resolve LGs into discreet entities was particularly useful when interpreting the electrophoretic karyotypes because it enabled co-migrating chromosomes to be distinguished based on hybridization to markers that were known to be on separate LGs. Nine RFLP markers from the genetic maps of Guy11/2539 (6,14,18) were used for construction of the genetic map and identification of chromosomes in this study. Based on the chromosomal assignment of the markers, we inferred the correspondence of the seven chromosomes in both genetic crosses 84R-62B/Y93-245c-2 and Guy11/2539. Chromosomes 1, 2, 5, 6, and 7 in this study just correspond to chromosomes 1, 2, 5, 6, and 7 in Guy11/2539, respectively. Chromosomes 3 and 4 are inverted and correspond to chromosomes 4 and 3 in Guy11/2539, respectively. The other 23 RFLP markers from the maps of Guy11/2539, which did not provide the polymorphism between the parental isolates 84R-62B and Y93-245c-2, were selected and hybridized to the CHEF-separated chromosomes of both parental isolates (data not shown). These results confirmed the chromosomal correspondence between both genetic crosses.

Surprisingly, the hybridization of selected markers to electrophoretically separated chromosomes revealed that parent 84R-62B actually possesses an eighth chromosome that is approximately 1.6 Mb in length. Even more surprising was the finding that four markers that were linked to *AvrPik* and mapped to LG A did not actually hybridize with chromosome 1 but hybridized to the "extra" chromosome instead. At first glance the 1.6-Mb chromosome in 84R-62B would appear to be reminiscent of the supernumerary chromosomes that have been described in *M. grisea* and a number of other fungi (3). Supernumerary chromosomes

are characterized as small chromosomes (<2 Mb), containing sequences not present on the larger chromosomes. They are inherited in non-Mendelian fashion and are dispensable (3). However, our present data indicate that the 84R-62B 1.6-Mb chromosome only meets the first criterion because the insert in plasmid clone pAE12AF12 hybridized to the 1.6-Mb chromosome in parent 84R-62B but was present on chromosome 1 in parent Y93-245c-2. Furthermore, the 1.6-Mb chromosome was inherited in a Mendelian fashion. Orbach et al. (15) also characterized a similar small chromosome less than 2 Mb in the *M. oryzae* isolate, 4224-7-8, which was inherited in normal Mendelian manner and showed partial homology to the larger chromosomes in other isolates. Therefore, they concluded that the small chromosome in 4224-7-8 was not a dispensable but an essential chromosome (15). Not only the Mendelian segregation but the markers on the 1.6-Mb chromosome exhibited genetic linkage to markers on chromosome 1. The most plausible explanation for this situation is that the 1.6-Mb chromosome is actually a fragment of chromosome 1 that arose when the larger chromosome was broken. The fact that markers on the 1.6-Mb chromosome show linkage to markers on the remainder of chromosome 1 suggests that one or more essential genes reside on the 1.6-Mb chromosome and that progeny inheriting chromosome 1 from 84R-62B must also receive the 1.6-Mb chromosome if they are to survive. In the genetic cross of 84R-62B/Y93-245c-2, no more than two viable ascospores were observed in individual ascus. The low fertility in this genetic cross might be due to the cosegregation between the 1.6-Mb chromosome and chromosome 1 in 84R-62B. To further examine the relatedness between the 1.6-Mb chromosome and chromosome 1 in 84R-62B, single-copy genes located in the contig 2.1572 of *M. grisea* database might be used as probes to hybridize the CHEF-separated chromosomes, because the sequence in pAE12AF12 was found in this contig. Furthermore, such genes will potentially provide more closely linked markers to the *AvrPik*. Now we are cloning some genes located in contig 2.1572 as well as in neighboring contigs in *M. grisea* database. Likewise, to obtain more closely linked markers to *AvrPiz* and *AvrPiz-t*, it is necessary to find available contigs from the database. The markers linked to the *Avr* genes in this study promise to be good starting points for searching the available contigs.

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Fig. 6. Southern hybridization of contour-clamped homogenous electric fields (CHEF)-separated chromosomes of parental isolates 84R-62B (lane 1) and Y93-245c-2 (lane 2) with probes of restriction fragment length polymorphism (RFLP) markers linked to *Avr* gene *AvrPik*. The RFLP markers were created from cloned random amplified polymorphism DNA markers and indicated above lanes as probes. Arrows indicate positions of chromosome 1 and chromosome 8 (1.6-Mb chromosome) shown in Figure 5A.

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