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ORIGINAL ARTICLE

Genetic and physical mapping of the avirulence gene *Avr-Pik*^{m} in *Magnaporthe oryzae*

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Abstract The interaction between rice and the rice blast fungus *Magnaporthe oryzae* follows a gene-for-gene model. The pathotype of a strain is determined by its avirulence gene content. In this study, we crossed avirulent strain S1522 and virulent strain S159 to generate 108 progenies. We subsequently isolated the avirulence gene *Avr-Pik*^{m} through classical genetic analysis. The segregation ratio in F1 population was 1:1 and confirmed the presence of a single locus in the genome of S1522. Two SCAR and five SSR markers linked to the avirulence gene were identified from the segregated population, and *Avr-Pik*^{m} was mapped on the chromosome of *M. oryzae*. A genomic library of avirulence parental strain S1522 was then constructed; the library was screened using the markers $SCE12_{1406}$ and SSR47A18, which were linked to the *AVR* gene as probes. Using these procedures, a fine physical map was assembled to include five TAC clones. TAC clone 35C5 is 32 kb in length and contains the two above-mentioned $SCE12_{1406}$ and SSR47A18 probes, suggesting that the *Avr-Pik*^{m} gene spans across the two markers located on the clone. These results provide support towards *Avr-Pik*^{m} map-based cloning.

Keywords Avirulence gene · Genetic map · *Magnaporthe oryzae* · Physical map

Introduction

Rice blast is one of the most devastating diseases of rice worldwide and is caused by the fungus *Magnaporthe oryzae* (formerly *Magnaporthe grisea*) (Ou 1985; Rossman et al. 1990). The use of blast-resistant rice cultivars is an effective and economic approach for controlling blast disease, but the cultivars often lose their specific resistance after a few years in commercial production. The breakdown of blast resistance in rice cultivars may be attributable to the genetic instability of avirulence (*AVR*) genes (Ou 1980; Kiyosawa 1982; Bonman 1992; Chauhan et al. 2002). An individual pathogen strain may have multiple *AVR* genes, and the combination of these genes specifies the physiologic race of that strain (Leach and White 1996). Loss of *AVR* gene function usually results in the generation of virulent races in the pathogen population. The rice blast pathosystem has emerged as a seminal paradigm for gene-for-gene interactions in the past few decades (Flor 1971; Valent 1990). Interactions among blast resistance (*R*) and *M. oryzae* avirulence (*AVR*) genes trigger the host immune system and plant recognition processes. The discovery of genetic mechanisms that regulate the stability of *AVR* genes—as correlated with their resistance (*R*) gene interactions—would be indispensable towards developing rice cultivars with durable resistance.

Magnaporthe oryzae is an ascomycetous fungal phytopathogen which possesses a small genome and ample cross-fertility. *M. oryzae* can be used to isolate avirulence genes as a model of the fungi (Valent 1990). Several of the phytopathogen's avirulence genes have hitherto been mapped, including *AVR1-Ku86*, *AVR1-MedNoï*, *AVRPi15*, *AVR-Pit, AVR-Pia*, etc. (Valent and Chumley 1991; Notteghem et al. 1994; Hirayae et al. 1999; Dioh et al. 2000; Li et al. 2000; Lin et al. 2002; Wang et al. 2002; Luo et al. 2004; Yasuda et al. 2004; Chen et al. 2006; Ma et al. 2006; Chen et al. 2007). Four avirulence genes (*PWL2, AVR1-CO39*, *AVR-Pita* and *ACE1)* have been isolated from *M. oryzae* using map-based cloning (Sweigard et al. 1995; Farman and Leong 1998; Orbach et al. 2000; Bonert et al. 2004). *AVR-Pii* and *AVR-*

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Pia were also cloned by bioinformatics and comparative genome techniques (Yoshida et al. 2009). Therefore, physical mapping of *AVR* genes is the foundation of map-based cloning in *M. oryzae*.

In this study, we isolated the avirulence gene *Avr-Pik*^{m} from a cross between two rice blast field isolates (S1522 and S159). We constructed a genetic map containing two SCAR and five SSR markers and then screened the avirulent parental isolate's genomic library with two of the most tightly linked markers ($SCE12_{1406}$ and SSR47A18). The positive TAC clone 35C5 was found to contain the above-mentioned linked markers; the presence of these markers suggests the clone also contain the avirulence gene *Avr-Pik*^{m}. We have now obtained the entire sequence of the positive TAC clone35C5; genetic identification of the avirulence gene *Avr-Pik*^{m} is also under way.

Materials and methods

Fungal isolates and mapping population construction

Professor Shen Ying (National Rice Research Institute of China) provided the fungal isolates S1522 (MAT1-1) and S159 (MAT1-2) that were used to construct the mapping population. S1522 and S159 faced each other while being incubated at 26 °C in 6-cm Petri plates on oatmeal tomato medium (3 % oatmeal, 1.5 % agar, 15 % mature tomato juice). The isolates were incubated in these conditions until the hypha margin of the two colonies came in contact with one another, which were then transferred to 20 °C for further incubation under continuous fluorescent illumination. Following asci maturity, progeny ascospores were randomly selected to construct the mapping population for avirulence gene analysis. S1522 and S159 produced progenies (Z1-1, Z1-5, Z1-6 and Z41), which were in turn conserved by our laboratory.

Rice cultivar and avirulence analysis

The Tsuyuake rice cultivar was selected for the pathogenic tests in our study. *M. oryzae* rice seedling cultivation and inoculums were performed as previously described (Peng and Shishiyama 1988). Inoculation was conducted by spraying a conidial suspension of the pathogens (5×10^5 conidia/mL) at the fifth leaf stage of the seedling. To facilitate the production of lesions, the inoculated seedlings were kept in the dark for 36 h at 28 °C and 100 % humidity. The seedlings were then incubated under fluorescent illumination and 100 % humidity at 28 °C. After 5–7 days of incubation, seedling infection types were recorded by use of a 6-class scale. According to this scale, a progeny was characterized as avirulent when it caused lesions that were scored as 0–3 on the host cultivar, and progeny which created lesions scored as 4–5 were determined to be virulent.

Mycelia culture and DNA extraction

The mycelia that were incubated on oatmeal tomato medium were washed with sterile distilled water; the mycelia were then inoculated into 200 mL of CM (complete medium), which was contained in a 500-mL flask (Valent and Chumley 1991). The inoculations were incubated under constant agitation (at 200 rpm) for approximately 72 h at 28 °C. The mycelia were harvested after passing the inoculated media through filter paper and gauze mesh with a vacuum pump. The dried mycelia were then quickly frozen using liquid nitrogen and stored at −80 °C.

Approximately 1.0 g of dry frozen mycelia was ground into a fine powder in liquid nitrogen. DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Graham et al. 1994). We dissolved the isolated DNA in 1× TE buffer (10 mM Tris, pH 7.5, 0.5 mM EDTA) with RNaseI (100 μg/mL); the quality of the nucleic acid was then assessed by 1% agarose gel electrophoresis. DNA dilutions of 10–20 ng/μL were prepared for future use. The samples were then stored at −20 °C.

Linkage marker isolation and genetic map construction

We prepared two contrasting bulks based on the phenotype of avirulence and virulence from the above-mentioned populations. Each bulk contained the DNA from six avirulent or virulent progenies towards the rice cultivar Tsuyuake. Sample DNA concentrations were equaled prior to pooling. The two DNA pools—which had undergone bulked-segregant analysis (BSA) (Michelmore et al. 1991)—were used to screen for RAPD markers. A sample of the avirulent and virulent progenies was then used to test for these polymorphic markers. Candidate genes were confirmed as linkage markers for the avirulence gene *Avr-Pik*^{m} by testing all the isolates from the mapping populations. Finally, we determined the linkage relationships between *Avr-Pik*^{m} and these markers, and transferred RAPD to SCAR markers.

The DNA sequences and BlastN analysis of the SCAR markers were compared with the whole genome sequence of *M. oryzae* strain 70-15. This genome sequence had been previously released by the International Rice Blast Genome Project (Dean et al. 2005). SSR primers were designed to localize in the estimated *Avr-Pik*^{m} region with annealing temperatures between 55 and 60 °C; this design was expected to yield amplification products between 150 and 250 bp in size. Oligo nucleotide primers were synthesized by SBS Genetech (Beijing, China). PCR amplification was carried out in reaction volumes of 25 μL; each reaction contained 10 ng of genomic DNA, 1.5 mmol/L of Mg^{2+},

100 μmol/L of dNTP, 1 U of Taq polymerase, 0.4 μmol/L of primer, and 1× PCR buffer (20 mM Tris–HCl at pH8.4, 50 mM KCl, and 0.0001 % gelatin). The PTC-200 Peltier Thermal Cycler was used for running PCR reactions; the machine was programmed to run for 35 cycles at 94 °C for 30 s, and the annealing temperature was set at 72 °C.

PCR products were separated on 6 % SDS-PAGE and stained with Argentine dye. Polymorphic SSR primers were identified between two parental isolates and were used to analyze the mapping populations. Restriction enzymes were used to digest the SSR PCR products in attempt to differentiate between similar avirulent and virulent isolates.

Genomic library construction and physical mapping of the *Avr-Pik*^{m}

The TAC library contains 8,000 individual clones; approximately 68 % of this population harbors foreign DNA inserts that average 30 kb or five equivalents of the genome in size. The genomic library was constructed with S1522 (avirulent parental isolate toward Tsuyuake) according to methods previously described (Wei et al. 2003). The genomic TAC library of Y34 (another avirulent strain towards Tsuyuake) was also used in the study (Wei et al. 2003).

We identified a selection of markers linked to *Avr-Pik*^{m}, as well as the genetic distance between these markers and *Avr-Pik*^{m}. The linked markers were then used as probes to screen the TAC library. Positive TAC clones were digested with *Hind*III and fractionated on 0.8 % agarose; the clones were then used to assemble contig maps (Wei et al. 2003). The positive TAC clone was subcloned for sequencing, and ultimately reassembled to predict the candidate avirulence gene.

Results

Genetic analysis of *Avr-Pik*^{m}

A cross between the avirulent isolate S1522 and the virulent isolate S159 produced 108 random progenies; these progenies were used to determine avirulence/virulence activity towards the rice cultivar Tsuyuake. Disease testing of the progenies identified 56 and 52 avirulent and virulent isolates, respectively. The χ^2 is 0.0833 in the mapping population, and the 1:1 segregation ratio corresponds with avirulence/virulence. Mapping populations were constructed by crossing the avirulent isolate S1522 with the virulent progeny isolate Z41; an avirulent progeny isolate Z1-1 and two other virulent progeny isolates (Z1-6 and Z1-5) were also crossed (Table 1). The results suggest that S1522 has an avirulence gene, and is specific to the Tsuyuake cultivar which contains the R gene *Pik*^{m}. We designated the avirulent gene in S1522 as *Avr-Pik*^{m}.

Identification of SCAR markers and chromosome localisation of *Avr-Pik*^{m}

OPO12$_{946}$ and OPE12$_{1406}$ are two RAPD markers that have been linked to *Avr-Pik*^{m}. These markers were isolated and transferred into SCAR markers SCO12$_{946}$ (Fig. 1) and SCE12$_{1406}$; the genetic distance between *Avr-Pik*^{m} and SCO12$_{946}$ is 4.80 cM, while the distance from *Avr-Pik*^{m}-toSCE12$_{1406}$ is 1.89 cM. These SCAR markers are located on the same side of *Avr-Pik*^{m}.

Using a BlastN algorithm, SCO12$_{946}$ was located on Chr. IV and identified in Contig 2.4 of the *M. oryzae* 70-15 strain. SCE12$_{1406}$ was found in Contig 2.1544 and on Chr. I. Both the two SCAR markers are linked to *Avr-Pik*^{m}, despite the fact that the three genes are not located on the same chromosome, which implies that the genome sequence of S1522 is likely to have significant differences when compared to *M. oryzae* isolate 70-15

To confirm the chromosomal location of *Avr-Pik*^{m}, two SCAR markers that are linked to the avirulent gene were used as probes in genomic Southern blotting. The results indicate that the SCAR markers are single copy in the parental isolate S1522. The SCAR markers were also used as probes to screen the Y34 TAC library, in which SCO12$_{946}$ confirmed 16 positive clones; these clones were classified into four types (Fig. 2a–c). The end sequences of a particular clone that shared a positive fingerprint with S1522 were used to BlastN 70-15 draft sequences. These sequences were also located on Chr. I. SCE12$_{1406}$ identified 11 positive

Table 1 Genetic analysis of the avirulence gene *Avr-Pik*^{m} of *Magnaporthe oryzae* in different crosses

Cross type	Parents	No. of progeny	Progeny segregation (A:V)	Genetic ratio	χ^2 for 1:1
F1	S1522(A)×S159(V)	108	56:52	1:1	0.0833
Backcross	S1522(A)×Z41(V)	37	20:17	1:1	0.1081
Sib-cross	Z1-1(A)×Z1-6(V)	53	30:23	1:1	0.6792
Sib-cross	Z1-1(A)×Z1-5(V)	50	25:25	1:1	0.0000

Backcross progeny Z41 derived from the cross S1522×S159; sib-cross F2 progeny of mating between siblings from the cross S1522×S159

A Avirulent isolate towards rice cultivar Tsuyuake, *V* virulent isolate towards rice cultivar Tsuyuake

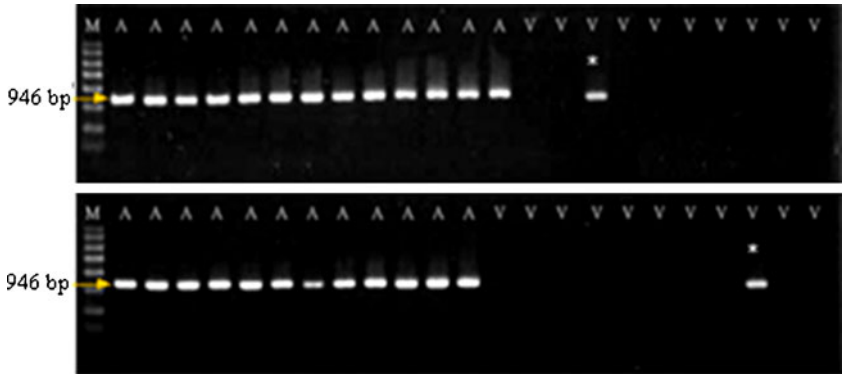

Fig. 1 PCR amplification in genomic DNA with $SCO12_{946}$ SCAR markers; amplification of progeny isolates from the mapping population. *A*, *V* Avirulence and virulence towards Tsuyuake, respectively. *Recombinant

clones and classified to two clone types. The ends of one clone were sequenced and found to be the same as the S1522 fingerprint; the ends are also located on Chr. I. These results reveal that both of the SCAR markers are located on Chr. I.

Identification of SSR markers linked with *Avr-Pik*^{m}

According to the whole genome sequence of the *M. oryzae* strain 70-15, as released by the International Rice Blast Genome Project (IRBGP, www.riceblast.org), *Avr-Pik*^{m} is linked with SSR markers. These markers (SSR56A28, SSR52TAGG18, SSR50CA24 and SSR47T34) are located on chromosomes I and IV. This linkage relationship is also corroborated by our findings from the positive clone end sequences. The genetic distances between SSR56A28, SSR52TAGG18, SSR50CA24, SSR47T34 and *Avr-Pik*^{m} are 21.94, 19.12, 7.01, and 4.90 cM, respectively.

Four additional SSR primers were designed to find a marker that is linked even more tightly with *Avr-Pik*^{m}. Among the four SSR primers, SSR47A18 was the only one found to hybridize between virulent and avirulent BSA. Cloning and sequencing results showed mere two-base pair difference between virulent and avirulent BSA. DNAMAN 5.0 sequence analysis also revealed a *Hae*III enzyme restriction site in this region; the PCR products of the SSR47A18 primer were subsequently digested with *Hae*III. Only two recombinants were identified among the 108 mapping progenies via primer analysis. SSR47A18 localized to the same region as the SSR markers listed in Table 2; the genetic distance between SSR47A18 and *Avr-Pik*^{m} is 1.89 cM, which demonstrates a tighter linkage

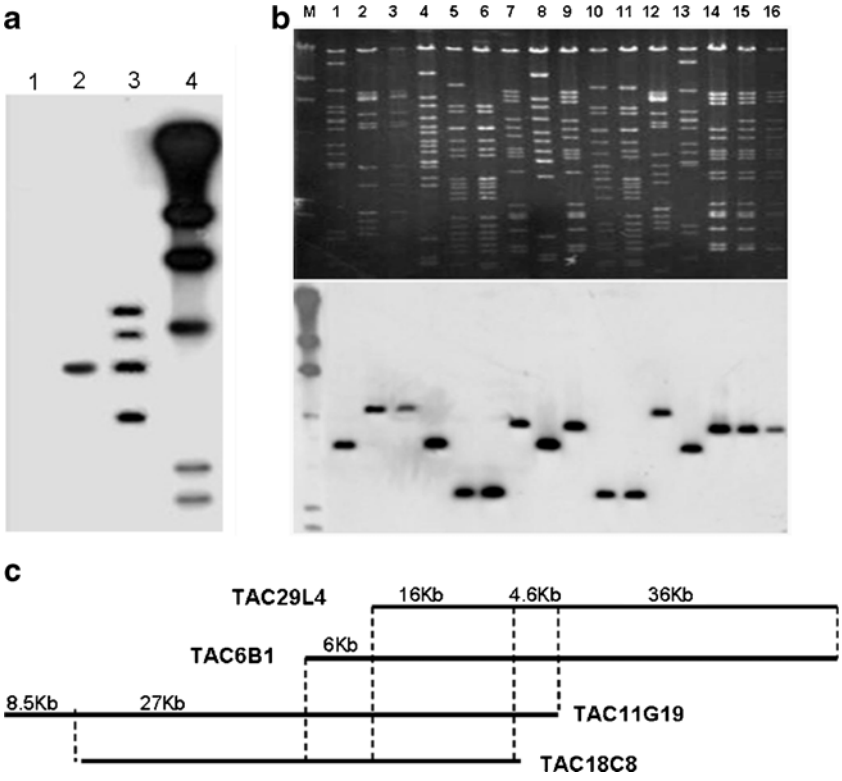

Fig. 2 Genomic Southern blot and the Y34 TAC genomic library screened by the $SCO12_{946}$ probe. **a**. Genomic Southern with the $SCO12_{946}$ probe. *Line 1* S159, *line 2* S1522, *line 3* Y34, *line 4* λDNA digested by *Hind*III S159, S1522 and Y34 are all digested by *Hind*III. **b**. The fingerprint of the positive clones, as screened with the probe $SCO12_{946}$ (*upper*) and its Southern blot (*lower*). *M* λDNA digested by *Hind*III; *1–16* different clones screened with the probe $SCO12_{946}$. **c**. The contig map assembled with key positive clones, as screened by the $SCO12_{946}$ probe

Table 2 Primers of polymorphic markers mentioned in the study

Primer name	Primer sequence
$SCE12_{946}$	Forward: 5-CTGTGGACTAAGTAGCATGCTTCT-3
	Reverse: 5-TAGGCAATCAAGAGAAAGCCAGTA-3
$SCE12_{1406}$	Forward: 5-TGCAGCCTTGTCTCTCTGGG-3
	Reverse: 5-TTGACACACGGTTGACGACC-3
SSR56A28	Forward: 5-CCGAGACAAGAACTGTGCG-3
	Reverse: 5-GGGATTGCGCGGTAATTAG-3
SSR52TAGG18	Forward: 5-AGTTAGCAGGTACCGGGC-3
	Reverse: 5-CAAGGACTTTGCTCAGAC-3
SSR50CA24	Forward: 5-TGCGGCAAGCTGAACTTT-3
	Reverse: 5-CATTCGCATATGGCCGA-3
SSR47T34	Forward: 5-GCAGAGCAATTTGGCCAG-3
	Reverse: 5-CATGGCAACACGTGGTTCT-3
SSR47A18	Forward: 5-CCGGTCCAACAGAACGGTT-3
	Reverse: 5-GAGGCACGACGTCAAATGT-3

relationship than that between SSR47T34 and *Avr-Pik*^{m} (separated by 4.90 cM). Alternative co-segregating SSR markers were not identified in this region.

We constructed a genetic map of *Avr-Pik*^{m} (Fig. 3a), which contains two SCAR and five SSR markers (marker primers listed in Table 2). The SSR47A18 and $SCE12_{1406}$ markers are the most closely linked to *Avr-Pik*^{m} and are located on opposing sides of the gene. The recombinant progenies of the cross S1522×S159 (with different markers) are listed in Table 3.

Validation of the physical map of *Avr-Pik*^{m}

To physically map the *Avr-Pik*^{m} gene locus, we used $SCE12_{1406}$ and SSR47A18 as probes in screening the S1522 TAC library. TAC clones 35C5 and 35I9 were reconfirmed with probe SSR47A18 by dot blotting, while the former were also reconfirmed with probe $SCE12_{1406}$. Linked markers $SCE12_{1406}$ and SSR47A18 were found to be located on opposite sides of *Avr-Pik*^{m} in TAC clone 35C5. These results suggest that the *AVR* gene *Avr-Pik*^{m} is indeed located in the TAC clone 35C5. The clone was digested with *Hind*III, and the subclones were assembled into a full sequence 32 kb in size. TAC clone 35C5 was found to contain the sequences of S1522, $SCE12_{1406}$ and SSR47A18 (Fig. 3b).

Discussion

The integrated genetic map of filamentous fungus *M. oryzae* that has been constructed thus far (Nitta et al. 1997) contains 23 microsatellite markers (Kaye et al. 2003). The genetic map of the fungus will become increasingly intact following the integration of more markers. The five SSR and two SCAR markers that we found to be linked with *Avr-Pik*^{m} were identified based on the whole genome draft sequence of *M. oryzae* strain 70-15; this sequence was released by the

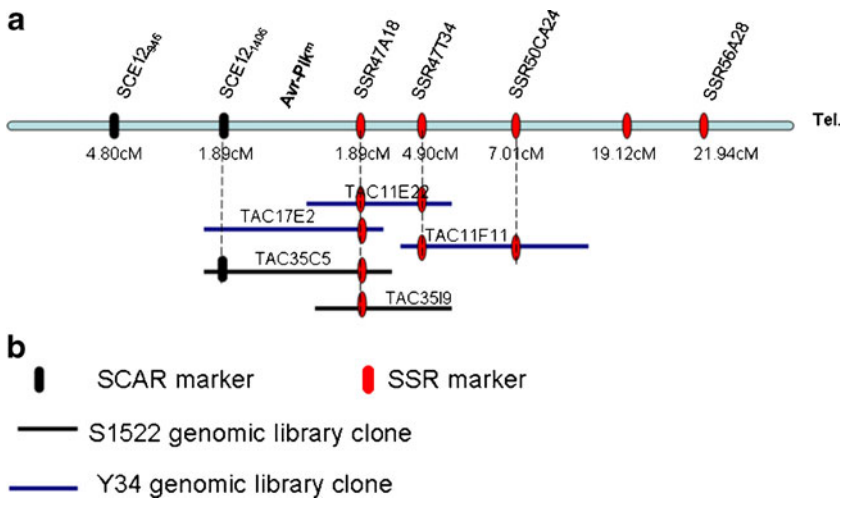

Fig. 3 Genetic and physical map of *Avr-Pik*m. **a** Genetic map of the avirulence gene *Avr-Pik*^{m}. **b** Physical map of the avirulence gene *Avr-Pik*^{m}

Table 3 Recombinant progenies of the cross S1522×S159 with different marker

Progeny	Markers						
	$SCO12_{946}$	$SCE12_{1406}$	SSR47A18	SSR47T34	SSR50CA24	SSR52TAGG18	SSR56A28
Z70	+	+	−	−	−	−	−
Z87	+	+	−	−	−	−	−
Z95	+	−	−	−	−	−	−
Z101	+	−	−	−	−	−	−
Z103	+	−	−	−	−	−	−
Z75	−	−	+	+	+	+	+
Z85	−	−	+	+	+	+	+
Z8	−	−	−	+	+	+	+
Z20	−	−	−	+	+	+	+
Z21	−	−	−	+	+	+	+
Z78	−	−	−	−	+	+	+
Z57	−	−	−	−	+	+	+
Z69	−	−	−	−	−	+	+
Z90	−	−	−	−	−	+	+
Z92	−	−	−	−	−	+	+
Z1-2	−	−	−	−	−	+	+
Z1-7	−	−	−	−	−	+	+
Z32	−	−	−	−	−	+	+
Z46	−	−	−	−	−	+	+
Z47	−	−	−	−	−	+	+
Z1-1	−	−	−	−	−	+	+
Z1-0	−	−	−	−	−	+	+
Z41	−	−	−	−	−	−	+
Z3	−	−	−	−	−	−	+

+Recombinant; −Non-recombinant

International Rice Blast Genome Project (IRBGP, www.riceblast.org). Newly developed markers can be used for population analysis or positional cloning of functional genes and ultimately integrated into the genetic map of *M. oryzae*.

During the process of mapping *Avr-pik*^{m}, we used the sequences of the linked SCAR markers to BlastN the 70-15 draft sequence and linkage group. As the two SCAR markers were found to be located on different chromosomes, we had to assemble TAC contigs and sequence the positive TAC clones' ends. These sequences revealed a 70-15 chromosomal regional difference in the parental isolate. We constructed a physical map of *Avr-pik*^{m} using TAC clones of the Y34 and S1522 TAC libraries. During this process, we discovered a difference between Y34 and S1522 despite their shared avirulence towards the rice cultivar Tsuyuake. It maybe imprudent to use different strains' genomic libraries to positionally clone *AVR* genes in *M. oryzae*, although the method for map base cloning without parental isolate genomic library is common in other plants and microbes. While constructing physical map without parental isolate genomic library often meet many problems in *M. oryzae*; one of the reasons may be more repeat sequences in *M. oryzae.*

In this study, *Avr-Pik*^{m} and five new markers were mapped on chromosome I of *M. oryzae*. The five markers (SSR56A28, SSR52TAGG18, SSR50CA24, SSR47T34 and SSR47A18) have been found to be linked with *Avr-Pik*^{m}. Using these results, *Avr-Pik*^{m} was successfully mapped between SSR47A18 and $SCE12_{1406}$. SSR47A18 and $SCE12_{1406}$ were also used as probes to screen the TAC library of *M. oryzae*, from which we obtained a positive TAC clone that potentially contained the *Avr-Pik*^{m} locus. Restriction enzyme digestion was used for SSR analysis when there were a couple base pair differences between the PCR products of the two parent strains.

During the course of our research, we discovered an interesting pattern in the ratio between physical and genetic distances. The physical/genetic ratio between SSR56A28 and SSR52TAGG18 was 18 kb/cM, while the ratio between SSR52TAGG18 and SSR50CA24 was 2.8 kb/cM. SSR50CA24 and SSR47T34 had a physical/genetic ratio

of 17 kb/cM, and the physical/genetic ratio between SSR47T34 and SSR47A18 was 6.3 kb/cM. Finally, the physical/genetic ratio between SSR47A18 and $SCE12_{1406}$ was 8.0 kb/cM. These ratios are much lower in comparison with the average estimated *M. oryzae* genome physical/genetic ratio of 33.5 kb/cM (Hamer et al. 1989). One of the reasons for this discrepancy may be that our region of interest resides at the end of the chromosome I. There is a high frequency of recombination at the gene-rich ends of chromosomes (Bishop et al. 2000). As a result, these regions also possess elevated levels of genetic variability. *AVR-Pita*, *AVR-TSUY*, *AVR-Ku86*, *AVR-MedNoï*, and *PWL* are *M. oryzae* genes that have been mapped to the ends of chromosomes (Sweigard et al. 1993; Dioh et al. 2000; Gao et al. 2002; Kang et al. 1995; Valent and Chumley 1991). The location of these *AVR* genes may also explain the frequent loss of resistance-conferring R genes in newly produced rice races. Molecular identification and cloning of more *AVR* genes is necessary to gain further understanding into their gene variability.

During the construction of the physical map containing the *AVR* locus, we found that all probes, which included repeat DNA sequences, were unsuccessful in screening TAC libraries. In addition, these TAC libraries could only be screened if they belonged to an avirulent parental strain. We were unsuccessful in screening the TAC library of another avirulent strain towards rice cultivar Tsuyuake (Y34; data not shown). One of the reasons may be the sequences between Y34 and S1522 were different.

In conclusion, we have fine-mapped the avirulence gene *Avr-Pik*^{m} using both genetic and physical maps. This provides a firm foundation for the positional cloning of the *AVR* gene *Avr-Pik*^{m}. We are currently constructing complementary and knockout vectors to confirm the function of the candidate gene. In 2009, Yoshida reported that the presence/absence of *AVR-Pik*^{m} was associated with that of *AVR-Pik*/*AVR-Pik*, and another study also shows that this trio of AVR genes was not always linked (Luo et al. 2004) . An investigation into alternative alleles that share the same function as *AVR-Pik*^{m}, such as Pex31-D, is therefore warranted (Yoshida et al. 2009). If *AVR* genes were cloned and characterized more systematically in *M. oryzae*, we could gain further insight into their genetic variability mechanisms, which would in turn allow for the cultivation of rice races with greater resistance to rice blast.

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