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Genome Organization and Evolution of the *AVR-Pita* Avirulence Gene Family in the *Magnaporthe grisea* Species Complex

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The avirulence (*AVR*) gene *AVR-Pita* in *Magnaporthe oryzae* prevents the fungus from infecting rice cultivars containing the resistance gene *Pi-ta*. A survey of isolates of the *M. grisea* species complex from diverse hosts showed that *AVR-Pita* is a member of a gene family, which led us to rename it to *AVR-Pita1*. Avirulence function, distribution, and genomic context of two other members, named *AVR-Pita2* and *AVR-Pita3*, were characterized. *AVR-Pita2*, but not *AVR-Pita3*, was functional as an *AVR* gene corresponding to *Pi-ta*. The *AVR-Pita1* and *AVR-Pita2* genes were present in isolates of both *M. oryzae* and *M. grisea*, whereas the *AVR-Pita3* gene was present only in isolates of *M. oryzae*. Orthologues of members of the *AVR-Pita* family could not be found in any fungal species sequenced to date, suggesting that the gene family may be unique to the *M. grisea* species complex. The genomic context of its members was analyzed in eight strains. The *AVR-Pita1* and *AVR-Pita2* genes in some isolates appeared to be located near telomeres and flanked by diverse repetitive DNA elements, suggesting that frequent deletion or amplification of these genes within the *M. grisea* species complex might have resulted from recombination mediated by repetitive DNA elements.

In certain plant species, different cultivars exhibit differential resistance to individual pathogen isolates through the gene-for-gene surveillance system (Flor 1971), in which a disease resistance (*R*) gene product detects the presence of the pathogen by recognizing a corresponding avirulence (*AVR*) gene-derived signal molecule. Presence of a matching *AVR-R* pair triggers rapid local defense responses, which limit the spread of the pathogen to cells immediately surrounding the initial infection site and also may evoke a systemic resistance (Dangl and Jones 2001). Although *R* gene-mediated resistance is highly effective once triggered, such resistance frequently loses its effectiveness in the field when new races capable of evading deployed *R* genes emerge (Kiyosawa 1982; Kolmer 1989; Leach et al. 2001; McDonald and Linde 2002; Mundt 1990, 1991). New pathogen races can emerge through multiple mechanisms, such as modifying the structure or expression of the *AVR* gene product or removing the *AVR* genes from their genome. Characterization of the distribution and genome organization of *AVR* genes within pathogen species will help understand the evolutionary mechanisms underpinning race variation. To this end, in the study reported here, we analyzed an *AVR* gene called *AVR-Pita* (Orbach et al. 2000) among isolates from diverse hosts (including both rice pathogens and those that are not pathogenic to rice) in the *Magnaporthe grisea* species complex.

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Members of the *M. grisea* species complex (Couch and Kohn 2002; Couch et al. 2005; Hirata et al. 2007), consisting of multiple phylogenetic species, cause diseases in a wide variety of weeds and cultivated cereal crops such as rice, wheat, barley, millet, and maize (Ou 1985). Rice blast, caused by certain *M. oryzae* isolates (Couch et al. 2005), is a classic gene-for-gene system (Silue et al. 1992; Valent and Chumley 1994) in which hundreds of races have been identified (Zeigler et al. 1994). Management of rice blast via breeding resistant cultivars often has had only short-term success due to the frequent appearance of new races that can overcome newly introduced *R* genes (Valent and Chumley 1994; Zeigler et al. 1994). Although the degree of race variation in this fungus remains a controversial subject (Kang et al. 2000), analysis of its *AVR* genes has begun to provide new insights into potential mechanisms underlying race variation (Bohnert et al. 2004; Couch et al. 2005; Farman et al. 2002; Fudal et al. 2005; Kang et al. 1995, 2001; Orbach et al. 2000; Sweigard et al. 1995; Tosa et al. 2005; Zhou et al. 2007).

The *AVR-Pita* gene, originally identified in rice isolate O-137, encodes a protein that exhibits similarity to fungal metalloproteases of the deuterolysin family (Orbach et al. 2000). AVR-Pita was predicted to be processed to a 176-amino-acid active form ($AVR\text{-}Pita_{176}$) based on the structure of other fungal metalloproteases, and only this processed form functioned to trigger *Pi-ta*-mediated resistance when expressed directly in rice cells (Jia et al. 2000). *AVR-Pita* in certain strains appears genetically unstable, frequently producing spontaneous mutants that gain virulence on *Pi-ta* rice cultivars under laboratory conditions (Valent and Chumley 1991). Molecular analysis of several such mutants identified a variety of mutation types in *AVR-Pita*, including deletion events ranging in size from 100 bp to over 12.5 kb (Orbach et al. 2000), point mutations (Orbach et al. 2000), and the insertion of a transposon (Kang et al. 2001). A survey of *AVR-Pita* among field isolates suggests that similar mechanisms generate virulent strains in the field (Zhou et al. 2007). We report here that *AVR-Pita* is a member of a gene family comprising functional and nonfunctional *AVR* genes. Based on this result, we renamed *AVR-Pita* to *AVR-Pita1*. Potential mechanisms underpinning the evolution of this family are discussed based on genomic contexts of *AVR-Pita1* and two other members in several isolates from diverse hosts.

RESULTS

AVR-Pita is a member of a gene family unique to the *M. grisea* species complex.

Twenty-nine isolates from diverse hosts (Table 1) were surveyed to determine whether *AVR-Pita* is present among strains that are not pathogenic to rice (Fig. 1). A phylogenetic tree inferred from sequences of the internal transcribed spacer (ITS) region of ribosomal RNA encoding genes suggests that these 29 isolates belong to at least three distinct lineages (Fig. 2): i) *M. oryzae* isolates from rice and other grass species, ii) *M. grisea* isolates from *Digitaria* spp., and iii) two isolates from *Cyperus* spp. that most likely belong to a new species distinct from the *M. grisea* species complex. This phylogeny is consistent with results from previous studies showing that *M. oryzae* isolates are genetically distinct from the other isolates in this complex (Couch and Kohn 2002; Hirata et al. 2007; Tosa et al. 2005). One isolate from genus *Leersia* (G-194) and three isolates from genus *Pennisetum* (G-78, G-123, and G-223) appear distinct from *M. oryzae* isolates. However, an in-depth phylogenetic analysis with more markers is required to test the hypothesis that these isolates form a lineage distinct from *M. oryzae* and *M. grisea*.

A Southern analysis of *Eco*RI (a restriction site absent in *AVR-Pita*)-digested genomic DNA of these isolates revealed the presence of zero to three DNA fragments hybridizing to *AVR-Pita* (Fig. 1A). In addition, there was substantial variation in the degree of hybridization intensities between and within some of the isolates, suggesting the presence of genes with varying degrees of sequence similarity to *AVR-Pita*. In O-315, an isolate from wild rice, the probe hybridized to three fragments, suggesting that there are multiple copies of *AVR-Pita*. In contrast, a few *M. oryzae* isolates from diverse hosts, including rice (O-135), wheat (T-5 and G-158), *Eleusine* spp. (G-22, G-77, and G-176), *Eragrostis* spp. (G-17), and *Panicum* spp. (G-218 and G-219), lacked a strong hybridization signal. G-194 from genus *Leersia*, one *Digitaria* isolate (G-11), and both isolates from genus *Cyperus* (G-229 and G-231) also appeared to lack *AVR-Pita*. In contrast, several isolates that are distantly related to rice pathogens, such as G-1, G-156, and G-189 from *Digitaria* spp. and G-78 and G-223 from *Pennisetum* spp., appeared to carry *AVR-Pita*. Many of the isolates apparently lacking *AVR-Pita* produced a weak hybridization signal, suggesting the presence of a gene that is distantly related to *AVR-Pita*. This survey result suggested that *AVR-Pita* might be a member of a gene family.

Low-stringency hybridization conditions were used to isolate *AVR-Pita* homologs from genomic DNA libraries of six field isolates that are nonpathogenic to rice, including two isolates from *Digitaria* spp. (G-1 and G-213), two from *Pennisetum* spp. (G-78 and G-223), one from *Eragrostis* spp. (G-17), and one from *Eleusine* spp. (G-22). DNA sequence analysis revealed that the *AVR-Pita* homologs from G-1, G-213, G-78, and G-223 had 92 to 98% DNA sequence identity to *AVR-Pita*, whereas those from G-17 and G-22 exhibited only 71 to 72% identity to *AVR-Pita* (Table 2). When the sequenced genome of strain 70-15 (Dean et al. 2005) was searched via BLASTN using the *AVR-Pita* gene sequence as a query, two different genes were identified. One (locus ID MGG_11081.4 in the gene set version 4; not included in version 5) had 98% DNA sequence identity to *AVR-Pita*, whereas the other one (locus ID unassigned to date) exhibited 71% DNA sequence identity to *AVR-Pita* but 97 to 98% identity to the *AVR-Pita* homologs from G-17 and G-22 (Table 2). The original *AVR-Pita* and its homologs from G-1, G-78, G-223, and 70-15 (98% identical to *AVR-Pita*) were renamed to *AVR-Pita1*. The homolog from G-213 (92% identical to *AVR-Pita1*) was designated tentatively as *AVR-Pita2*, and those from G-17, G-22, and 70-15 as *AVR-Pita3*. Together, these genes form a new gene family designated as the *AVR-Pita* family.

The *Eco*RI-digested genomic DNA blot hybridized with *AVR-Pita1* was stripped and rehybridized with *AVR-Pita2* and *AVR-Pita3* (Fig. 1B and C). Because of the high sequence

Table 1. Fungal isolates used in this study

Strain	Host	Country of origin	Comments[a]
O-135	*Oryza sativa*	China	Valent et al. 1991
O-137	*O. sativa*	China	Sweigard et al. 1995
O-284	*O. sativa*	Guiana	Guy11, Leung et al. 1988
G-160	*Leersia hexandra*	Philippines	Lh-A8401, J. M. Bonman
O-315	*O. longistaminata*	Cameroon	CM13, J. L. Notteghem
T-5	Wheat	Brazil	S. Igarashi (1988)
G-158	Feral *Triticale*	Brazil	K. Kmetz (1989)
G-200	*Eleusine indica*	Ivory Coast	CD156, J. L. Notteghem
G-77	*E. indica*	Philippines	J. M. Bonman (1982)
G-17	*Eragrostis curvula*	Japan	K76-79, H. Yaegashi (1976)
G-22	*Eleusine coracana*	Japan	WGG-FA40, H. Yaegashi (1977)
G-176	*E. coracana*	Nepal	NP10-17-4-1-3, H. Kato
G-219	*Panicum maximum*	India	IN5, J. L. Notteghem
G-165	*Brachiaria distachya*	Philippines	Bd-A8401, J. M. Bonman
G-218	*P. coloratum*	Japan	JP31, J. L. Notteghem
G-220	*P. repens*	India	IN3, J. L. Notteghem
G-221	*Pennisetum clandestinum*	Japan	JP35, J. L. Notteghem
G-156	*Digitaria horizontalis*	Brazil	A. S. Prabhu (1986)
G-11	*D. sanguinalis*	Japan	A26, H. Yaegashi (1975)
G-1	*D. sanguinalis*	United States	F. M. Latterell (1981)
G-213	*D. smutsii*	Japan	JP34, J. L. Notteghem
G-32	*Digitaria* sp.	Japan	H. Kato (1982)
G-189	*Digitaria* sp.	United States	J. A. Sweigard (1991)
G-123	*P. glaucum*	United States	84P-19, H. Wells
G-78	*P. polystachyon*	Philippines	R. Gopinath (1983)
G-223	*P. typhoideum*	Burkina Faso	BF17, J. L. Notteghem
G-194	*L. oryzoides*	Japan	JP36, J. L. Notteghem
G-229	*Cyperus brevifolus*	Philippines	PH54, J. L. Notteghem
G-231	*C. rotundus*	Philippines	PH52, J. L. Notteghem
CP987	Laboratory strain	…	Sweigard et al. 1995

[a] Reference, alternative name, collector, and year of collection.

similarity between *AVR-Pita1* and *AVR-Pita2*, the probes cross-hybridized. However, in some strains (G-220, G-213, G-32, and G-189), *AVR-Pita2* hybridized to fragments that had not been detected clearly by *AVR-Pita1*, suggesting the presence of an additional family member. Unlike *AVR-Pita1*, *AVR-Pita3* was present only in *M. oryzae* isolates (Fig. 1C, first 17 lanes). Although G-223 (*Pennisetum* isolate) also hybridized to the probe, given the weak signal it probably is not *AVR-Pita3* but a potential new member. The *AVR-Pita1* gene consists of four exons (Orbach et al. 2000). *AVR-Pita2* and *AVR-Pita3* contained the introns at the same positions with consensus splice sites that were identical to those in *AVR-Pita1*. The *AVR-Pita3* gene

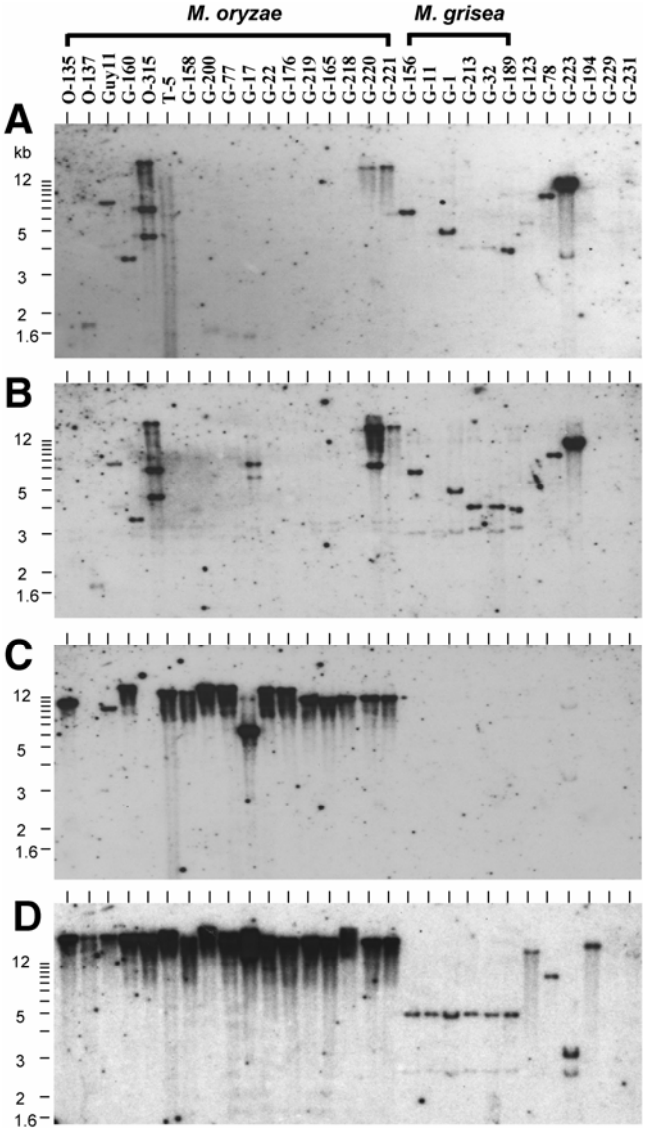

Fig. 1. Distribution of members of the *AVR-Pita* family. Genomic DNA from 29 isolates was digested with *Eco*RI, electrophoretically separated, blotted, and hybridized with **A,** O-137 *AVR-Pita1*, **B,** G-213 *AVR-Pita2*, **C,** G-22 *AVR-Pita3*, and **D,** MGG_10927.5.

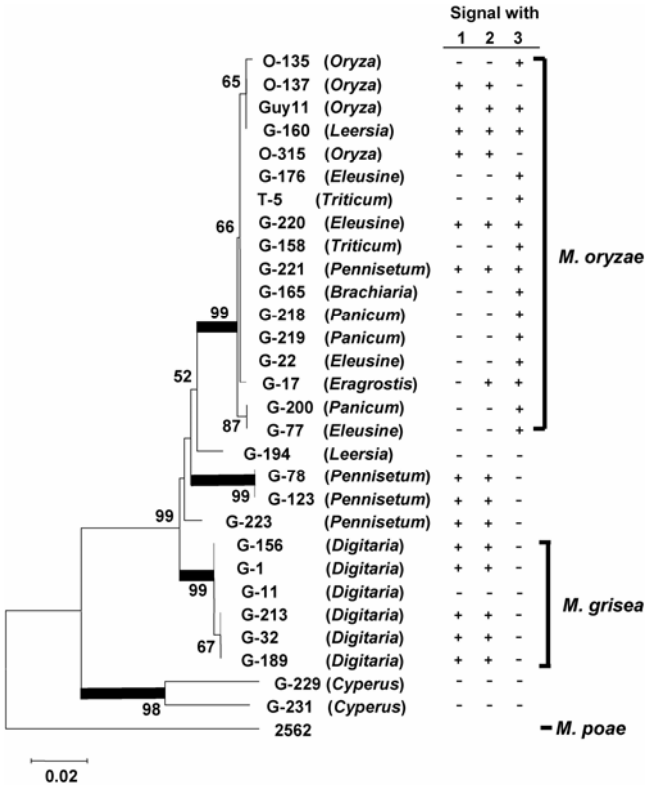

Fig. 2. Phylogenetic relationships among 29 isolates in the *Magnaporthe grisea* species complex and distribution of three members of the *AVR-Pita* gene family among these isolates. The phylogenetic tree was inferred from internal transcribed spacer sequences using the neighbor-joining method. Bootstrap values >50% are indicated at individual nodes of the tree. The tree is rooted to *M. poae* (GenBank accession number AJ010042). Presence (+) or absence (–) of signal with the probes *AVR-Pita1* (1), *AVR-Pita2* (2), and *AVR-Pita3* (3) is indicated. Because *AVR-Pita1* and *AVR-Pita2* cross-hybridize due to a high degree of sequence similarity between them, the positive signal does not necessarily mean the presence of both genes. Also note that the *AVR-Pita1* hybridization signals for G-77 and G-200 are faint, and that there is no corresponding hybridization with the *AVR-Pita2* probe for these strains.

Table 2. Pairwise sequence comparison between *AVR-Pita* genes and gene products[a]

	AVR-Pita1					*AVR-Pita2*	*AVR-Pita3*	
Isolates	**O-137**	**G-78**	**G-223**	**G-1**	**70-15**	**G-213**	**70-15**	**G-22**
AVR-Pita1	…	…	…	…	…	…	…	…
G-78	98/92	…	…	…	…	…	…	…
G-223	98/94	99/97	…	…	…	…	…	…
G-1	98/94	99/97	99/99	…	…	…	…	…
70-15	98/94	98/94	99/96	98/96		…	…	…
AVR-Pita2								
G-213	92/81	92/84	91/83	91/83	91/82	…	…	…
AVR-Pita3								
70-15	71/55	70/57	70/57	70/57	70/56	69/53	…	…
G-22	72/56	71/57	70/57	70/57	70/56	70/54	97/92	…
G-17[b]	71	70	70	70	70	70	98	97

[a] Percent identities of DNA sequences to the left and amino acid sequences to the right of the slash.
[b] This protein sequence was not used for comparison because of the presence of a premature stop codon in the gene.

in G-17 contained a frameshift mutation near the start codon, resulting in a truncated protein (18 amino acids), if translated. The sequence similarity among members of the gene family at the amino acid level (53 to 99%) is lower than that at the DNA level (69 to 99%) (Table 2).

AVR-Pita1 exhibits sequence similarities to neutral metalloproteases in *Aspergillus* spp. and *Penicillium citrinum* that belong to families M35 and M36 in the superfamily of zinc-dependent metalloproteases (Orbach et al., 2000). According to InterProScan analysis (Quevillon et al. 2005), all three *AVR-Pita* gene products contain InterPro profile IPR006025 (zinc-binding region signature; corresponding to residues of 173 to 182 in O-137 AVR-Pita1) (Fig. 3) and belong to InterPro family IPR001384 (deuterolysin metalloprotease [M35] family). To investigate evolutionary relationships between AVR-Pita proteins and fungal members of the M35 family, we searched known fungal proteomes for proteins that are closely related to AVR-Pita proteins (BLASTP E value <1.0 e^{-3}) and belong to the M35 family (discussed below). There was substantial variation in the number of putative M35 proteins in different fungi (e.g., one in *Fusarium graminearum* versus seven in *Coccidioides immitis*). In the 70-15 genome, three putative M35 proteins were identified. Similar to *AVR-Pita* gene products, two of them (MGG_03029.5 and MGG_10927.5) contain a predicted signal peptide at their N-terminus and InterPro Profile IPR006025.

A phylogenetic analysis of putative M35 proteins revealed three clusters (bootstrap support >70%) that contain orthologs from at least three species (Fig. 4). MGG_10927.5 was used as a probe to investigate its distribution in the *M. grisea* species complex (Fig. 1D). Unlike members of the *AVR-Pita* family, this gene seems to be present in all isolates, with the exception only of G-229. Putative orthologs of MGG_10927.5 were found in *Neurospora crassa* (NCU05071.2) and *F. graminearum* (FG08289.1). However, no fungi sequenced to date seem to carry orthologs of members of the *AVR-Pita* family, suggesting that the gene family may be unique to the *M. grisea* species complex.

Functional analysis of *AVR-Pita* family members.

Clones carrying individual family members were introduced via transformation into CP987, a laboratory strain lacking *AVR-Pita1*, to determine whether they confer avirulence. The *AVR-Pita1* gene from O-137, G-1, G-78, and G-223 and the *AVR-Pita2* gene from G-213 converted CP987 to avirulence toward rice cultivars carrying *Pi-ta*, but the *AVR-Pita3* gene from G-17 and G-22 failed to do so (data not shown). Possible reasons for the failure of the G-22 *AVR-Pita3* gene to confer avirulence would be improper expression of the gene or production of a protein that may not interact with Pi-ta. To investigate the underlying cause of the G-22 *AVR-Pita3*'s failure to confer avirulence, we produced a series of chimeric genes. First, to test whether the G-22 *AVR-Pita3* coding sequence (designated as $C_{AVR\text{-}Pita3}$) can produce a functional avirulence protein, we fused it to the O-137 *AVR-Pita1* and G-213 *AVR-Pita2* gene promoters (designated as $P_{AVR\text{-}Pita1}$ and $P_{AVR\text{-}Pita2}$, respectively), producing $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita3}$

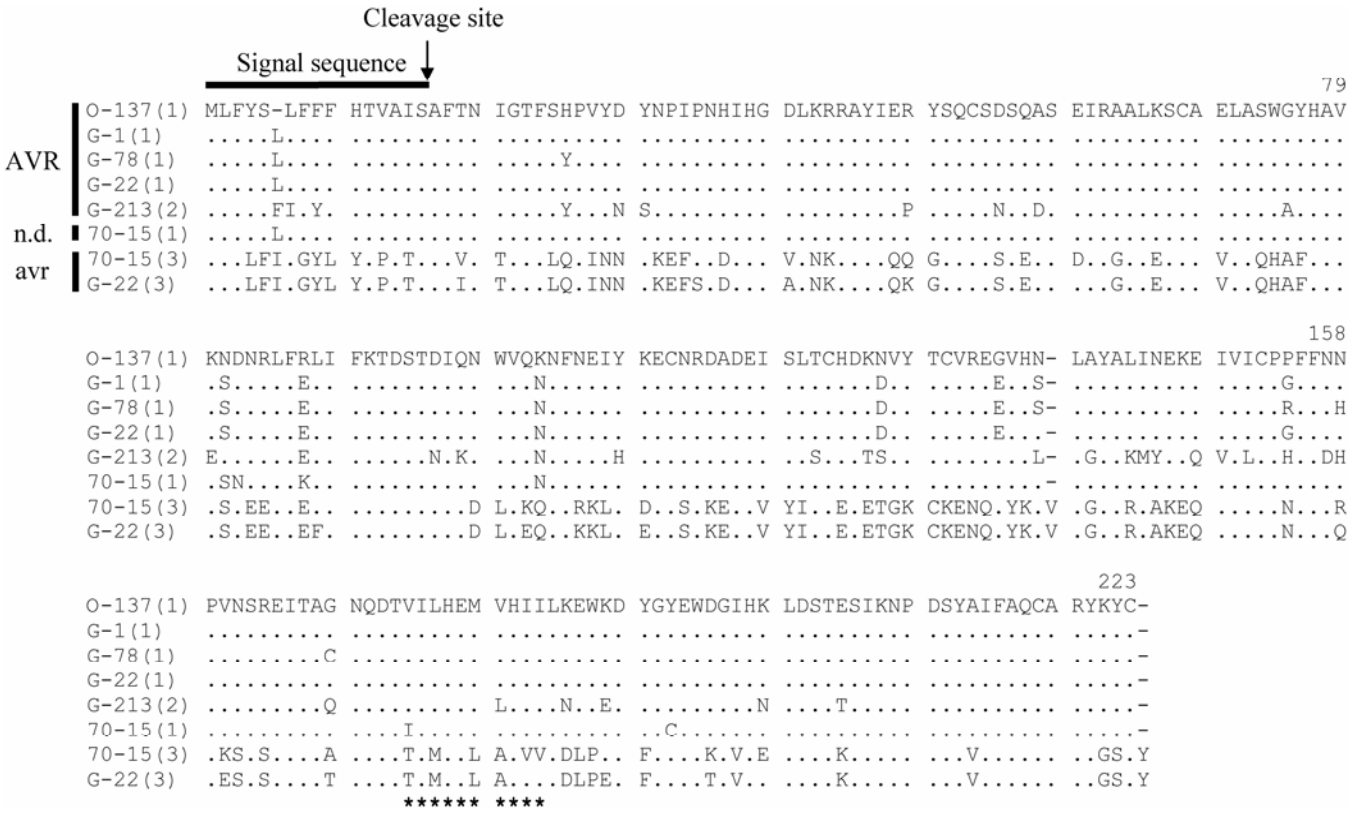

Fig. 3. Sequence comparison among members of the *AVR-Pita* family. The number (1, 2, or 3) next to the strain name indicates AVR-Pita1, AVR-Pita2, or AVR-Pita3, respectively. *AVR-Pita1* from O-137, G-1, G-78, and G-223 and *AVR-Pita2* from G-213 confer avirulence (AVR), whereas *AVR-Pita3* from 70-15, labeled as 70-15(3), and G-22(3), lack avirulence function (avr). Although the avirulence function of *AVR-Pita1* from 70-15, labeled as 70-15(1), was not determined (n.d.) in this study, considering that 70-15 is virulent on *Pi-ta*-carrying cultivars, the gene probably is nonfunctional as an *AVR* gene. Sequence positions were numbered according to O-137 AVR-Pita1. Asterisks indicate the protease motif that is shared by the majority of zinc-dependent metalloprotease (InterPro profile IPR006025; corresponding to residues of 173 to 182 in O-137 AVR-Pita1), [GSTALIVN]-x(2)-**H**-**E**-[LIVMFYW]-{DEHRKP}-**H**-x-[LIVMFYWGSPQ], in which [] indicates that any one of the listed amino acid residues is allowed, x indicates that any residue is allowed, and {} indicates that any one of the listed residues is not allowed. Boldfaced resides above (two histidines and one glutamic acid) correspond to putative zinc ligands and the active site residue, respectively. The putative signal peptide sequence is overlined with an arrow marking its predicted cleavage site.

and $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita3}$. Functionality of the G-22 *AVR-Pita3* gene promoter also was tested by fusing it to the O-137 *AVR-Pita1* and G-213 *AVR-Pita2* coding sequences (yielding $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita1}$ and $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita2}$, respectively). Five additional constructs, including $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita1}$, $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita2}$, $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita3}$, $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita2}$, and $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita1}$, were produced as controls to determine whether the fusion process affected gene function.

Individual chimeric constructs were introduced to CP987, and randomly chosen transformants were analyzed by polymerase chain reaction (PCR) to confirm that the integrated genes were intact. Five to six confirmed transformants with each construct were inoculated on *Pi-ta* rice, and host responses were scored. The $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita1}$ and $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita2}$ constructs successfully conferred avirulence, as did $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita2}$ and $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita1}$. As expected, the $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita3}$ construct failed to confer avirulence. These results showed that individual modules retained their original functionality after the construction process. All transformants carrying either $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita3}$ or $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita3}$ caused typical blast symptoms on *Pi-ta* rice, suggesting that G-22 *AVR-Pita3* encodes a protein that does not trigger *Pi-ta*-mediated resistance. Interestingly, two of six transformants carrying the $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita1}$ construct and four of six transformants carrying $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita2}$ construct caused intermediate resistance responses, suggesting the expression of *AVR-Pita1* and *AVR-Pita2*. However, the remaining transformants caused blast disease on *Pi-ta* rice. Considering that all of the transformants had an intact transgene, differences in gene expression among transformants with $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita1}$ or $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita2}$ probably were caused by position effects (i.e., genome sequences flanking individual insertion sites differentially affecting expression of the transgene).

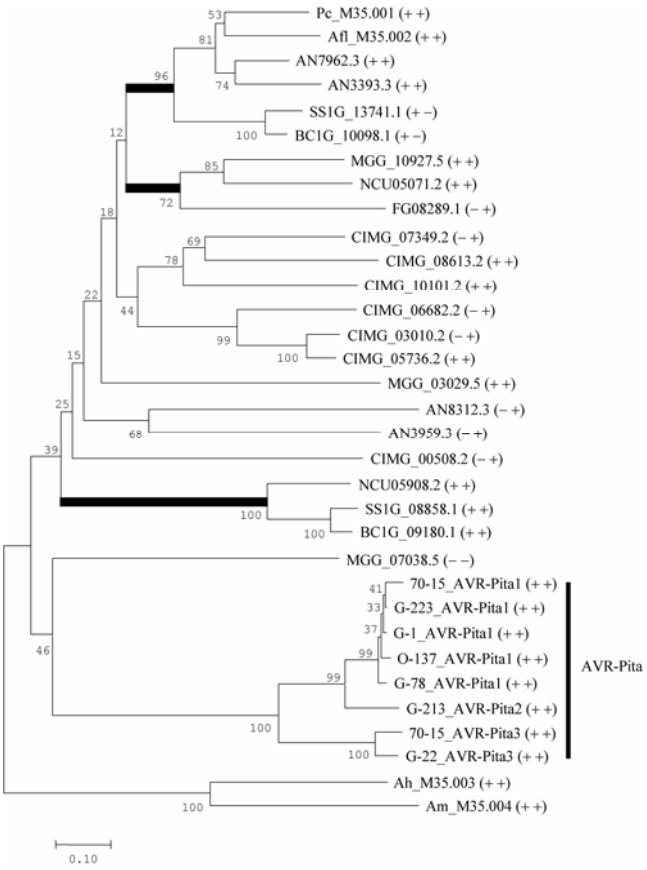

Fig. 4. Neighbor-joining tree of AVR-Pita proteins and several members of the fungal M35 (IPR001384) metalloprotease family. The complete deletion and Poisson correction options were used. The following proteins were included in the analysis: i) four M35 type peptidases from *Penicillium citrinum* (Pc_M35.001), *Aspergillus fumigatus* (Afl M35.002), *Aeromonas hydrophila* (Ah_M35.003), and *Armillaria mellea* (Am_M35.004); ii) eight AVR-Pita proteins; and iii) 21 annotated proteins from *Aspergillus nidulans* (AN), *Botrytis cinerea* (BC1G), *Coccidioides immitis* (CIMG), *Fusarium graminearum* (FG), *Magnaporthe grisea* (MGG), *Neurospora crassa* (NCU), and *Sclerotinia sclerotiorum* (SS1G). Percent bootstrap values are shown at the nodes. Highly supported clusters (>70% bootstrap support) that contain orthologues from at least three species are indicated with a solid line. Presence (+) or absence (–) of the InterPro profile IPR006026 and the signal peptide is indicated sequentially.

Some members of the *AVR-Pita* family are telomeric.

Similar to the O-137 *AVR-Pita1* gene, which is located near a telomere (Orbach et al. 2000), the *AVR-Pita1* gene in *Digitaria* isolate G-1 also appears to be telomeric based on chromosome walking via inverse PCR. However, its orientation relative to the telomeric repeat was reversed (Fig. 5A): in O-137 *AVR-Pita1*, the telomeric repeat is located 48 bp downstream from its stop codon, whereas the telomeric repeat ($(TTAGGG)_{29}$) is located at the 5′ region of G-1 *AVR-Pita1* (1,689 bp upstream from the start codon). Considering the telomeric location of the *AVR-Pita1* gene in these two distantly related isolates (Fig. 2), we hypothesized that members of the gene family in other isolates also might be telomeric. The close linkage between the telomere and G-223 *AVR-Pita1* and G-213 *AVR-Pita2* was confirmed by cloning of fosmid genomic clones containing these genes. Restriction analysis of these clones indicated that, in both G-213 and G-223, the distance between the gene and the telomeric repeat appeared to be much greater than that of the *AVR-Pita1* gene in O-137 and G-1, but less than 30 kb (data not shown). No clones containing *AVR-Pita3* hybridized to the telomeric repeat.

We used genetic and physical maps available in the genome database of 70-15 to find the chromosomal positions of *AVR-Pita1* and *AVR-Pita3* in this strain. The *AVR-Pita1* gene was found in the contig 2.2232 (3,251 bp) that belongs to the supercontig 124 (5,281 bp). Based on version 4 of the genome assembly, supercontig 124 has not yet been mapped on any linkage group. The *AVR-Pita3* gene was found in the supercontig 179 (version 5), mapped on chromosome 7. Recently, a new chromosome 7 sequence assembly was released (approximately 4 mb; GenBank accession number CM000230). The *AVR-Pita3* gene is separated from the telomeric repeat by more than 300 kb, indicating that it is not telomeric, and is located within one of the transposable element clusters (Thon et al. 2006).

Multiple transposable elements are closely associated with the *AVR-Pita* family.

Members of the *AVR-Pita* family are closely associated with diverse transposons (Fig. 5). They include i) two different inverted terminal repeat (ITR) transposons that exhibit high similarity to Pot2 (Kachroo et al. 1994) and Pot3 (Farman et al. 1996), respectively; ii) a highly degenerated ITR transposon (tentatively termed ψ transposon); iii) a solo long-terminal repeat (LTR) derived from retrotransposon Pyret (Nakayashiki et al. 2001); iv) two novel retrotransposons; and v) three undefined, short repetitive DNA elements, including MGR619 (79 bp), MGR608 (126 bp), and REP1 (188 bp). The presence of MGR619, MGR608, and REP1 was intriguing because they are closely associated with other *AVR* genes, including *PWL* (Kang et al. 1995) and *AVR1-CO39* (Farman et al. 2002). The MGR619 and MGR608 elements are highly polymorphic and dispersed sequences (Hamer et al. 1989) and frequently appear together as a contiguous sequence (Kang et al. 1995; Orbach et al. 2000).

Some of the MGR619/MGR608 and REP1 elements flanking members of the *AVR-Pita* gene family were attached to sequences exhibiting significant homology to reverse transcriptase of LTR-type retrotransposons. In addition, the lengths of MGR619/MGR608 (205 bp) and REP1 (188 bp) were similar to those of fungal retrotransposon LTRs (ranging from 198 bp to 596 bp in size) (Galun 2003). To determine whether the MGR619/MGR608 and REP1 elements were solo LTRs (i.e., LTRs missing the internal domains of retrotransposon) derived from novel retrotransposons, we searched the genome database of 70-15 using MGR619/MGR608 and REP1 as queries. In many positive contigs, the internal domains of LTR retrotransposon (e.g., the *gag* and *pol* genes) were sandwiched between copies of MGR619/MGR608 or REP1. However, none of the positive contigs contained an intact open reading frame of the *pol* gene, suggesting that they correspond to degenerate retrotransposons.

The borders of the LTRs corresponding to MGR619/MGR608 and REP1 were defined based on sequence alignment among multiple copies of retrotransposons from different contigs (data not shown). We used one REP1-associated retrotransposon and a retrotransposon copy associated with MGR619/MGR608 to determine whether they correspond to any LTR-retrotransposons previously characterized (Dean et al. 2005; Thon et al. 2006). The REP1-associated copy exhibited 91.8% sequence identity with RETRO6-1 and the MGR619/MGR608 associated copy had 91.7% sequence identity with RETRO7-1. The REP1 element (158 bp) was identical in sequence to that of the RETRO6-1 LTRs (193 and 194 bp, respectively, with one additional nucleotide in 3′ LTR), suggesting that REP1 is a truncated derivative from RETRO6-1. Similarly, the MGR619/608 element (205 bp) is a deletion derivative of the RETRO7-1 LTRs (215 bp).

Comparison of the regions flanking members of the *AVR-Pita* family.

Sequence similarity at the *AVR-Pita1* locus among different strains, and between *AVR-Pita1* and *AVR-Pita2*, extended beyond the coding sequence at both the 5′ and 3′ sides but was interrupted frequently by retrotransposons or solo LTRs (Figs. 5A and 6). For instance, between G-213 *AVR-Pita2* and O-137 *AVR-Pita1*, the synteny in the promoter region was interrupted by insertion of a 192-bp solo LTR of RETRO6-1 in the *AVR-Pita2* promoter at the position corresponding to –332 of the O-137 *AVR-Pita1* promoter. The synteny continued after this solo LTR but was interrupted again, at position –447 in the O-137

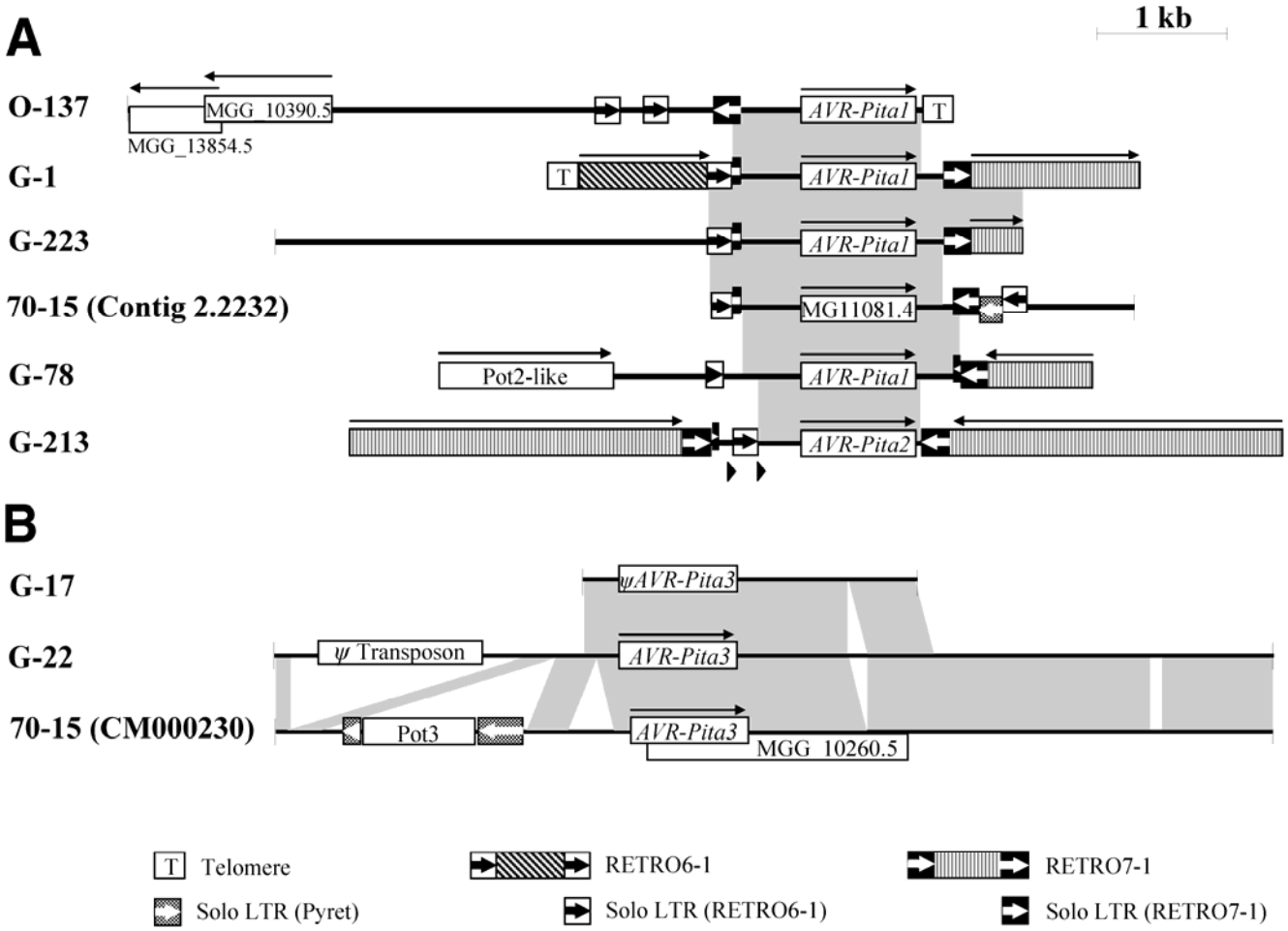

Fig. 5. Comparison of the genomic context among members of the *AVR-Pita* family in seven field isolates and one laboratory strain. GenBank accession numbers for the loci used here are as follows: O-137 (AF2207841), G-1 (DQ855953), G-223 (DG855954), G-78 (DG855955), G-213 (DG855956), G-17 (DG855957), and G-22 (DG855958). For 70-15 sequences, contig 2.2232 and GenBank accession number CM000230 (sequences between 3668019 and 3676012) were used. A putative coding sequence is indicated by an open box with a gene name. A highly degenerate transposon is indicated by *ψ*. The box with T indicates the telomeric repeat (not drawn in scale). Long-terminal repeat (LTR) retrotransposons RETRO6-1 and RETRO7-1 and solo LTRs derived from them are marked with boxes filled with different patterns. A solo LTR (or its derivative) adjacent to another LTR is indicated in a staggered manner. A 5-bp target site duplication (G-213) is marked with a pair of filled triangles. The orientation of transcription for individual genes is indicated by an arrow. Gray areas between genes indicate the syntenic regions with >96% sequence identity. **A,** The *AVR-Pita1* and *AVR-Pita2* loci among six isolates were compared. **B,** The *AVR-Pita3* locus was compared among three different isolates.

AVR-Pita1 promoter, by the presence of a solo LTR of RETRO7-1 (Fig. 6). At the corresponding site of *AVR-Pita2*, a partial solo LTR of RETRO7-1 was found (oriented opposite to that in O-137), which was followed immediately by a copy of RETRO7-1. At the 3′ end, O-137 *AVR-Pita1* contained the telomeric repeat 48 bp downstream from the stop codon, whereas the G-213 *AVR-Pita2* was flanked by a copy of RETRO7-1 that exists in a head-to-head orientation with the RETRO7-1 element at the 5′ side of the gene (98% sequence identity between them).

The *AVR-Pita1* gene in other strains also was surrounded by retrotransposons and solo LTRs. A truncated copy of RETRO6-1 was located between the telomeric repeat and the 5′ end of the G-1 *AVR-Pita1* gene (Fig. 5A); when compared with an intact copy of RETRO6-1 (5,871 bp), it contained only the 3′ 1,181 bp of RETRO6-1, immediately followed by the telomeric repeat at the 5′ end. Although *AVR-Pita1* in other strains and *AVR-Pita2* in G-213 do not have RETRO6-1 at their 5′ region, the presence of a solo RETRO6-1 LTR suggests that RETRO6-1 once existed at this location but has been deleted. Numerous stop codons exist in the internal domains of the *AVR-Pita*-associated RETRO6-1 and RETRO7-1 elements, indicating that they have degenerated.

Between *AVR-Pita1/AVR-Pita2* and *AVR-Pita3*, noticeable sequence identity (65%) existed only up to approximately 180 bp upstream from the translation start codon but no significant similarity was found downstream of the stop codon. However, at the *AVR-Pita3* locus in G-17, G-22, and 70-15, there was strong sequence conservation (more than 93% identity) at both sides of the gene (Fig. 5B). The synteny between G-22 and 70-15 at the *AVR-Pita3* locus was disrupted by transposons (G-22 contained a highly degenerated transposon whereas 70-15 carried Pot3 and a solo-LTR of Pyret) as well as deletions (or insertions) of two DNA segments; the *AVR-Pita3* locus in G-17 and 70-15 lacks a 197-bp segment that is present at the 3′ region of the *AVR-Pita3* locus in G-22. A 329-bp segment at the 5′ region of the gene in 70-15 appears to be missing in both G-17 and G-22. BLAST searches with these segments as queries did not reveal any significant matches in GenBank, nor did these segments exhibit any distinct structural features such as direct repeats at their ends or flanking regions.

Signs suggesting multiple transpositions and subsequent rearrangements near members of the gene family were abundant. A partial solo LTR of RETRO7-1 immediately flanked the 3′ end of the truncated RETRO6-1 in G-1 and a solo LTR of RETRO6-1 in G-223 and 70-15 (Fig. 5A). In contrast, at the same location in O-137, only an intact solo LTR of RETRO7-1 was present (Fig. 5A). These data suggest that an LTR of RETRO7-1 existed at this location in the strain ancestral to G-1, G-223, and O-137, and transposition of RETRO6-1 into this LTR occurred later in the lineage leading to G-1 and G-223 but not in the lineage leading to O-137. This RETRO6-1 element in G-1 and G-223 subsequently has undergone additional changes (e.g., chromosomal breakage and addition of the telomeric repeat in G-1 and deletion of the internal domain of RETRO6-1 probably mediated by recombination between its LTRs in G-223). The orientation of RETRO7-1 in the 3′ region of the *AVR-Pita1* gene in G-1 and G-223 was opposite to that in G-78 and G-213, suggesting independent transposition events at this location.

DISCUSSION

Similar to the *PWL AVR* gene family in the *M. grisea* species complex (Kang et al. 1995), members of the *AVR-Pita* family are widely distributed among isolates from diverse hosts (Figs. 1 and 2), including those that are not pathogenic to rice. Based on the genomic context and distribution of members of the *AVR-Pita* family, we discuss their function, evolution, and variation.

Do members of the *AVR-Pita* gene family have more than AVR function?

An intriguing question about *AVR* genes in general is why, during evolution, plant pathogens have maintained genes that limit their ability to infect potential hosts. Accumulating evidence indicates that, in both bacterial and fungal pathogens, the maintenance of certain *AVR* genes is due to their function as a virulence factor in hosts lacking corresponding *R* genes. A number of *AVR* genes in bacterial pathogens have been shown to contribute to virulence (Abramovitch et al. 2003; Axtell et al. 2003; Bai et al. 2000; Chang et al. 2000; Chen et al. 2004; Greenberg and Vinatzer 2003; Leach et al. 2001; Lorang et al. 1994; Marois et al. 2002; Ritter and Dangl 1995; Yang et al. 1994, 1996). Fungal *AVR* genes demonstrating a dual role include *ecp2* (Laugé et al. 1997) and *Avr4* (van den Burg et al. 2006; van Esse et al. 2007) in *Cladosporium fulvum*, *nip1* in *Rhynchosporium secalis* (Knogge 1996; Wevelsiep et al. 1991),

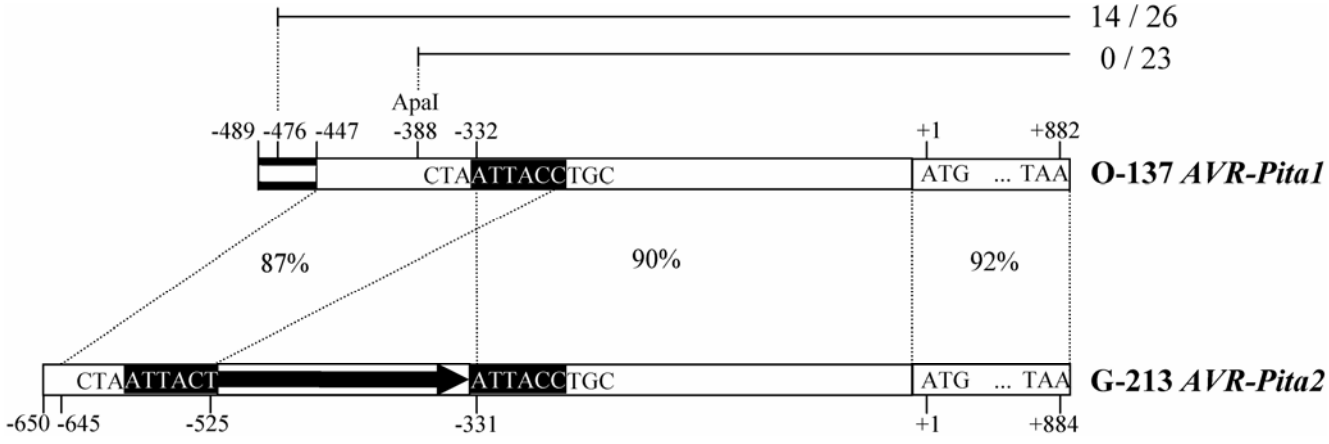

Fig. 6. Comparison of the promoter and coding regions of O-137 *AVR-Pita1* (-489 to +82) and G-213 *AVR-Pita2* (-650 to +884). Numbers above the boxes indicate the positions defined relative to the translation start site (A of ATG as +1). The ratios indicate the number of avirulent transformants among the total number of transformants using the corresponding subclones in the complementation analyses (derived from Orbach et al. 2000). Percent identities between the corresponding regions are indicated. A partial copy of RETRO7-1 long-terminal repeat (LTR) (43 bp) and a complete copy of RETRO6-1 LTR (192 bp), present at the 5′ ends of the O-137 *AVR-Pita1* promoter and the G-213 *AVR-Pita2* promoter, respectively, are indicated. A putative 6-bp target site duplication (ATTACT and ATTACC with a change at the sixth position) generated during transposition is highlighted.

AVRa10 and *AVRk1*in *Blumeria graminis* f. sp. *hordei* (Ridout et al. 2006), and *SIX1* in *F. oxysporum* (Rep et al. 2004, 2005). Several *AVR* gene products of *Melampsora lini* are expressed in haustoria, suggesting their roles in virulence (Catanzariti et al. 2006). The *Avr2* gene product of *C. fulvum* may contribute to virulence by inhibiting apoplastic cysteine proteases in tomato (Rooney et al. 2005). Certain *AVR* genes in oomycete species also appear to contribute to virulence (Kamoun 2006). Although there is no direct evidence supporting members of the *AVR-Pita* gene family as virulence factors, circumstantial evidence suggests such roles.

Rice pathogens that entirely lack members of this family were rarely found among approximately 200 isolates from several countries (Fig. 1; *unpublished data*), suggesting the presence of positive selection pressure for their maintenance. Expression from *AVR-Pita1* was not detected under various axenic culture conditions but was induced in rice during an early stage of infection (B. Valent and S. Kang, *unpublished data*). Although new races that specifically overcame resistance conferred by *Pi-ta* in rice cv. Katy have been isolated from rice fields in the United States, this cultivar has been stably resistant to rice blast since its introduction in the late 1980s (Jia et al. 2004). Preservation of functional members of the gene family in *M. grisea* isolates that are not pathogenic to rice, such as G-1, G-78, G-213, and G-223, also suggests that this family may carry out functions unrelated to avirulence factors in such strains. Presence of rice-specific *AVR* genes among isolates nonpathogenic to rice is not unique to the *AVR-Pita* family (Tosa et al. 2005; Valent et al. 1991; Yaegashi and Asaga 1981). For instance, *AVR* genes *AVR1-CO39, AVR1-M201*, and *AVR1-YAMO* were identified in a weeping lovegrass pathogen (Valent et al. 1991). Gene-for-gene interactions of rice cultivars with *M. oryzae* likely have evolved through the specific recognition by rice of preexisting fungal gene products that originally had roles other than triggering rice defense as avirulence factors.

Possible mechanisms underlying the evolution of the *AVR-Pita* family.

The presence of both *AVR-Pita1* and *AVR-Pita3* in several isolates (Fig. 2) suggests that they were derived from an ancestral gene duplication event. Although we designated *AVR-Pita1* and *AVR-Pita2* as distinct members of the gene family, the validity of this designation has not yet been firmly proven. We have not yet found strains that carry both genes, suggesting the possibility that they are highly divergent alleles of the same gene. However, the degree of sequence variation within *AVR-Pita1* from diverse isolates was limited to 2% at most (even when genes from strains distantly related to rice pathogens were compared), and a similar pattern existed within *AVR-Pita2* genes in several strains (*unpublished data*). In contrast, the degree of sequence difference between *AVR-Pita1* and *AVR-Pita2* was approximately 8 to 9%. We also have not yet found variants that fall between *AVR-Pita1* and *AVR-Pita2*. All sequenced *AVR-Pita* genes have the introns at same positions, ruling out retrotransposition (i.e., insertion of a reverse transcribed cDNA of mRNA from a member of the gene family) as a mechanism for gene duplication. Gene conversion initiated by recombination between repetitive elements flanking a family member and homologous repeats at different chromosomal locations is a possible mechanism for the generation of a new gene family member. This mechanism appears to have involved in the amplification of the *PWL2 AVR* gene (S. Kang, *unpublished data*). Interestingly, the *AVR-Pita3* gene appears to be present only among *M. oryzae* isolates, suggesting that it was generated after the separation of *M. oryzae* from *M. grisea*. Alternatively, this gene may have been present in the ancestral population of the *M. grisea* species complex but has been completely deleted in *M. grisea*. The latter possibility (requiring multiple independent deletions of the gene in different host-specific lineages) seems unlikely. The *AVR1-CO39* gene also appears to be absent in *M. grisea* (Tosa et al. 2005). Interestingly, *AVR1-CO39* is absent among rice isolates (Farman et al. 2002).

The *AVR-Pita1* and *AVR-Pita2* genes in certain strains seem to be located in subtelomeric regions, at distances ranging from 48 bp up to approximately 30 kb from the telomeric repeat. Variation in the distance and orientation between the telomeric repeat and members of the gene family suggests the dynamic nature of the subtelomeric regions associated with *AVR-Pita1* and *AVR-Pita2*. Other subtelomeric regions in *M. oryzae* also appear highly dynamic, as illustrated by the telomere-linked helicase (*TLH*) gene family (Gao et al. 2002; Rehmeyer et al. 2006). Most *TLH* family members are located within 10 kb of the telomeric repeat, and undergo frequent deletion and amplification events, which likely have been mediated by interchromosomal recombination between the repetitive elements flanking them. It remains to be determined whether similar recombination mechanisms contribute to the proliferation of the *AVR-Pita* family. Telomeres of the *M. oryzae* isolates from turfgrass also exhibited hypervariability (Farman and Kim 2005). Frequent deletions and rearrangements at subtelomeric regions have been observed in diverse eukaryotes (Carlson et al. 1985; Charron and Michels 1988; Heather and Trask 2002). In general, those genes whose frequent variation can confer an advantage for adapting in certain niches often are present in telomeric regions. In *Saccharomyces cerevisiae*, members of the *SUC* and *MAL* gene families that are involved in metabolizing specific carbon sources (sucrose and maltose, respectively) were found at subtelomeric regions (Carlson et al. 1985; Charron and Michels 1988). The presence and locations of these subtelomeric genes vary widely from strain to strain, with individual strains carrying only a subset of gene family members at different chromosomes, suggesting frequent recombination between subtelomeric regions. Similarly, subtelomeric regions of the human malarial parasite *Plasmodium falciparum* undergo frequent ectopic recombination, promoting the variation of *var* genes in these regions and thus helping the parasite evade the host immune system (Freitas-Junior et al. 2000).

In addition to the *AVR-Pita* family, *M. oryzae* has several *AVR* genes, such as *AVR1-Ku86, AVR1-MedNoi,* and *PWL1,* that are mapped to the telomere (Dioh et al. 2000; Kang et al. 1995). Given the dynamic nature at the subtelomeric regions, the presence of *AVR* genes in these regions may provide an advantage to the fungus by helping it evade newly deployed *R* genes via frequent loss or modification of *AVR*. Considering the possibility that certain *AVR* genes also might function as a virulence factor, we speculate that two opposing selection pressures (increased virulence on host cultivars lacking the corresponding *R* gene versus incompatibility with those carrying the *R* gene) have driven the direction of change (amplification versus deletion or modification) in fungal populations. We should note that certain *AVR* genes in *M. oryzae*, such as *AVR1-Mara* (Mandel et al. 1997), *AVR1-CO39* (Farman and Leong 1998), *PWL2* (Sweigard et al. 1995), *AVR-Pik* (Yasuda et al. 2005), and *ACE1* (Bohnert et al. 2004), are not telomeric.

Repetitive DNA elements have been implicated as a major source for genetic variation, which can modify the host range of affected strains. A transposition event caused a loss-of-function mutation in two *AVR* genes of *M. oryzae*, leading to a change in virulence spectrum (Fudal et al. 2005; Kang et al. 2001; Zhou et al. 2007). Repetitive elements also can cause genome rearrangements through recombination between dispersed repeats located

on the same or different chromosomes, resulting in deletion, duplication, inversion, and translocation, depending on the relative orientation and position of recombining repeats. Some of these changes potentially can affect host range by mutating an *AVR* gene at or near the recombination site. Members of the *PWL AVR* gene family are flanked by diverse transposons, and such associations appear to be responsible for deletions or amplifications in the gene family (Kang et al. 1995, 2000; Sweigard et al. 1995). The *AVR1-CO39* gene also is closely associated with transposons that likely were responsible for the deletion of the gene among rice-pathogenic isolates of *M. oryzae* (Farman et al. 2002; Tosa et al. 2005). Two *AVR* genes in *Leptosphaeria maculans*, *AvrLm1* and *AvrLm6*, are embedded in long chromosomal regions mainly consisting of several types of LTR retrotransposons and their remnants (Fudal et al. 2007; Gout et al. 2006).

Transposons also can modify the expression pattern of *AVR* genes near their insertion sites. An insertion of a Pot3 ITR transposon into the promoter region of *AVR-Pita1* (–302 bp) abolished its avirulence activity (Kang et al. 2001). Since the discovery that LTRs carry promoter and enhancer motifs (Sverdlov 1998), many studies have shown that LTRs can influence the expression of adjacent genes both spatially and temporally (Brosius 1999). The *AVR-Pita1* and *AVR-Pita2* genes have one or more LTRs at their 5′ region; the O-137 *AVR-Pita1* gene has three solo LTRs in its 5′ region (Fig. 5). A deletion analysis of the putative promoter region of the O-137 *AVR-Pita1* gene (Orbach et al. 2000) showed that the two tandem copies of RETRO6-1 solo LTR were not essential for avirulence activity of the gene. A small part of a RETRO7-1 solo LTR (5′ 30 bp) is included in a region that is required for avirulence activity (Fig. 6, between –476 and –388), but its absence in the *AVR-Pita2* and G-78 *AVR-Pita1* promoter regions (Fig. 5) suggests that this sequence might not be essential. A solo LTR of RETRO6-1 (192 bp) was inserted into an *AVR-Pita2* promoter region (at –332 in the O-137 *AVR-Pita1* promoter) that is essential for the activity of O-137 *AVR-Pita1*. Because measuring avirulence activity based on plant infection is not a sensitive assay, determining if and how this solo LTR affects the expression of *AVR-Pita2* requires gene expression analysis.

MATERIALS AND METHODS

Strains and growth conditions.

Host and geographical origins of the 29 field isolates used in this study are described in Table 1. The laboratory strain 70-15 was derived from a cross between rice-pathogenic isolate Guy 11 and a weeping lovegrass (*Eragrostis curvula*)-pathogenic isolate, followed by backcrosses to Guy 11 (Chao and Ellingboe 1991; Leung et al. 1988). Another laboratory strain, CP987, is a spontaneous mutant that lacks functional *AVR-Pita1* and, thus, is virulent on *Pi-ta* rice (Orbach et al. 2000). *Escherichia coli* XL1-blue MRF′ was used for maintaining plasmids. *Agrobacterium tumefaciens* EHA105 (Klee 2000) was used for fungal transformation. Fungal cultures were grown at 25°C on oatmeal agar plates to produce conidia for infection assays. Mycelial cultures for genomic DNA extraction were grown in liquid complete medium (6 g of yeast extract, 6 g of casein acid hydrolysate, and 10 g of sucrose per liter).

Phylogenetic analyses.

The ITS region was amplified from each strain listed in Table 1 with the primers ITS1 and ITS4 (Table 3) using the FailSafe PCR system (Epicentre Technologies, Madison, WI, U.S.A.). The PCR cycling program included an initial denaturation for 2 min at 94°C; 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C; followed by a final extension for 10 min at 72°C. PCR products were purified using QIAquick spin columns (Qiagen, Valencia, CA, U.S.A.) and sequenced on both strands with the same primers used in the PCR amplification. ITS sequences were aligned using the Clustal W program (Thompson et al. 1994) with default parameters. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in the MEGA3 program (version 3.1) (Kumar et al. 2004) with the following parameters: complete deletion of gaps, Jukes-Cantor model, both transitions and transversions substitutions included, and 1,000 bootstrap replicates.

Proteins related to AVR-Pita in other fungi were identified through the following steps. First, multifungi BLASTP (E value <1 e^{-03}) against a database of "all fungal proteins" at the

Table 3. Polymerase chain reaction (PCR) primers used in this study

Target	Primer	Sequence (5′→3′)[a]
Internal transcribed spacer (ITS)	ITS1	TCCGTAGGTGAACCTGCGG
	ITS4	TCCTCCGCTTATTGATATGC
Insert of pSK1059	LF9	GCGATTTCGGCCTTCACC
	Pita1	CCCTCTATTGTTAGATTGA
Insert of pSK1183	Fun1	ATAAAATCGA(C/G)CGTTTCCG
	Fun2-2	GCCGTGACAATTCCCTTTAT
Inverse PCR	iP-1	TCATGCTCCCTGACTTTTGA
	iP-2	GCCGCGCTAAAAAGGTAAAT
Insert of pSK2544	MG10927-F	ATCGGAATTCGTCATGAGCAAGCGTG
	MG10927-R	ACCGTCTAGAGCAACCGACCTTGATAG
$P_{AVR\text{-}Pita1}$	Fs-P1	CGGAATTCGCCGAGTCGTTCTGA
	Fs-P2	CGGGATCCTGTTAATTGTGCAGAAGTTTTT
$C_{AVR\text{-}Pita1}$	Fs-C1	CGGGATCCTTGCAATTATGCTTTTTTATTC
	Fs-C2	CATGCATGCCCCTCTATTGTTAGATTGAC
$P_{AVR\text{-}Pita2}$	Fw-P1	CGGAATTCTACGCATATTTTTGTAAATTTC
	Fw-P2	CGGGATCCTATGAATTGTGCAAAAGTTTTT
$C_{AVR\text{-}Pita2}$	Fs-C1	Same as the Fs-C1
	Fw-C2	CATGCATGCCTGGCCGTGACAATTC
$P_{AVR\text{-}Pita3}$	nF-P1	CGGAATTCAAGTAAGCAGAAAATACAAG
	nF-P2	CGGGATCCTACAAATTGTGCAGATTTTTC
$C_{AVR\text{-}Pita3}$	nF-C1	CGGGATCCCCGCAATTATGCTTTTTTTATTT
	nF-C2	CATGCATGCCCCTCCAAAACAAAAGG

[a] Underlined sequences correspond to restriction enzyme sites introduced for cloning purpose: *Eco*RI (GAATTC), *Xba*I (TCTAGA), *Bam*HI (GGATCC), and *Sph*I (GCATGC).

Broad Institute using O-137 AVR-Pita1, G-213 AVR-Pita2, and G-22 AVR-Pita3 as queries. This led to the identification of 23 proteins, including four from *Aspergillus nidulans*, two from *Botrytis cinerea*, seven from *Coccidioides immitis*, one from *F. graminearum*, five from *M. grisea* (not including AVR-Pita1 and AVR-Pita3), two from *N. crassa*, and two from *Sclerotinia sclerotiorum*. These protein sequences were analyzed using InterProScan to detect the presence of signatures present in AVR-Pita proteins (IPR001384, IPR006025, and signal peptide). Two *M. grisea* proteins (MGG_03808.5 and MGG_12056.5) lacked IPR001384 and IPR006025 profiles and were excluded from further analyses. From the MEROPS database, a manually curated information resource for proteolytic enzymes (Rawlings et al. 2006), four representative M35 members (or called type peptidase) were retrieved; these include penicillolysin (M35.001; *Penicillium citrinum*), deuterolysin (M35.002; *A. fumigatus*), extracellular peptidase (M35.003; *Aeromonas hydrophila*), and peptidyl-Lys metallopeptidase (M35.004; *Armillaria mellea*). After aligning protein sequences, they were adjusted manually based on conserved sites of the type peptidases. Because of significant sequence divergence at the N- and C-terminal regions of these proteins, only parts that overlap with the peptidase units in the type peptidases were included. A phylogenetic tree was constructed using the neighbor-joining method in the MEGA3 program (version 3.1) with the following parameters: complete deletion of gaps, Poisson correction model, and 1,000 bootstrap replicates.

Southern analysis.

Fungal genomic DNA was prepared and purified by CsCl gradient centrifugation as previously described (Kang 2001). *Eco*RI-digested genomic DNA was separated by 0.8% agarose gel electrophoresis and blotted to Hybond N+ membrane (Amersham, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions. The blot was hybridized successively with ^{32}P-labeled probes of *AVR-Pita1* (0.5-kb *Ssp*I fragment of pSK1059), *AVR-Pita2* (1.0-kb *Eco*RI fragment of pSK1183), *AVR-Pita3* (0.6-kb *Kpn*I-*Bgl*II fragment of pSK924), and MGG_10927.5 (1.3-kb *Eco*RI-*Xba*I fragment of pSK2544) prepared by random priming; each probe DNA fragment was purified from gels using QIAquick spin columns. After hybridization at 65°C overnight, the blots were washed twice in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO4, and 1 nm EDTA [pH 7.7]) and 0.1% sodium dodecyl sulfate (SDS) for 15 min at 65°C, followed by two additional washes in 0.1× SSPE and 0.1% SDS for 15 min at 65°C. Following exposure to X-ray film, the blot was stripped by a combination of hot SDS (after boiling in 0.1% SDS, it was allowed to cool for 30 min) and alkali (0.2 M NaOH for 10 min at 42°C; repeated three times).

Cloning and DNA sequence analysis.

Clones containing an *AVR-Pita1* homolog were isolated from genomic DNA libraries of G-1, G-17, G-22, G-78, G-213, and G-223, which were constructed in the λGEM12 vector (Promega Corp., Madison, WI, U.S.A.) as previously described (Kang et al. 1995). Fosmid genomic libraries of selected strains were constructed using the CopyControl Fosmid Library kit (Epicentre, Madison, WI, U.S.A.) according to the manufacturer's instruction. These libraries were screened with ^{32}P-labeled *AVR-Pita1* under low-stringency conditions (hybridization at 55°C, followed by two 30-min washings at 55°C in 2× SSPE and 0.1% SDS), and positive clones were isolated. DNA was extracted from the positive lambda clones using the plate-lysate method (Sambrook et al. 1989). After restriction enzyme mapping, appropriate restriction fragments were cloned into pGEM3Zf (Promega Corp.) for sequencing (Table 4).

Inverse PCR (Ochman et al. 1988) was performed to obtain DNA sequences flanking the 5′ region of *AVR-Pita1* in isolate G-1. Genomic DNA of G-1 (1 µg) was digested with *Xmn*I at 37°C for 12 h in a total volume of 100 µl. Completely digested DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase was recovered by centrifugation for 5 min at 4°C. DNA was precipitated by adding 10 µl of 3 M sodium acetate, pH 5.5, and 200 µl of 100% ethanol and incubating for 30 min at –20°C; pelleted by centrifugation; washed with 70% ethanol; air dried; and resuspended in 50 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The digested DNA was self-ligated for 16 h at 16°C in a 500-µl reaction, ethanol precipitated, and subsequently resuspended in 30 µl of TE buffer. PCR was performed in a 20-µl reaction mixture containing 10 µl of template DNA and 2 µl (10 pmol/µl) of primers iP-1 and iP-2 (Table 3), using the FailSafe PCR system. The PCR cycling program consisted of an initial denaturation for 5 min at 95°C; 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 2 min of extension at 72°C; followed by a final extension for 10 min at 72°C. PCR products were cloned into pGEM-T Easy vector (Promega Corp.).

Plasmid clones were sequenced using primers based on the cloning vector or the EZ::TN transposon insertion system

Table 4. Key plasmids used in this study

Name	Description, reference
pSK646	pCB780; 6.5-kb *Bgl*II blunt telomere fragment from 4224-7-8 in *Bam*HI-*Eco*RV sites of pBluescript SK+ (Orbach et al. 2000)
pSK647	3.0-kb *Eco*RI fragment carrying *AVR-Pita1* from G-1 in *Eco*RI site of pGEM3Zf
pSK648	5.0-kb *Eco*RI fragment carrying *AVR-Pita1* from G-78 in *Eco*RI site of pGEM3Zf
pSK649	3.6-kb *Eco*RI fragment carrying *AVR-Pita2* from G-213 in *Eco*RI site of pGEM3Zf
pSK650	5.2-kb *Bam*HI fragment carrying *AVR-Pita1* from G-223 in *Bam*HI site of pGEM3Zf
pSK923	2.5-kb *Eco*RI fragment carrying *AVR-Pita3* from G-17 in *Eco*RI site of pGEM3Zf
pSK924	8.1-kb *Bam*HI-*Eco*RI fragment carrying *AVR-Pita3* from G-22 in *Bam*HI-*Eco*RI sites of pGEM3Zf
pSK1059	0.9-kb Polymerase chain reaction (PCR) product from O-137 (Primers LF9 and Pita1) in pGEM-T Easy
pSK1183	1.0-kb PCR product from G-213 (Primers Fun1 and Fun2-2) in pGEM-T Easy
pSK1813	1.7-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita1}$::T in *Eco*RI- *Hin*dIII sites of pBHt2
pSK1814	1.7-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita2}$::T in *Eco*RI- *Hin*dIII sites of pBHt2
pSK1815	1.7-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita1}$::$C_{AVR\text{-}Pita3}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1816	1.9-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita1}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1817	1.9-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita2}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1818	1.9-kb *Eco*RI-partial *Hin*dIII fragment carrying $P_{AVR\text{-}Pita2}$::$C_{AVR\text{-}Pita3}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1819	1.8-kb *Eco*RI-*Hin*dIII fragment carrying $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita1}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1820	1.8-kb *Eco*RI-*Hin*dIII fragment carrying $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita2}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK1821	1.8-kb *Eco*RI-*Hin*dIII fragment carrying $P_{AVR\text{-}Pita3}$::$C_{AVR\text{-}Pita3}$::T in *Eco*RI-*Hin*dIII sites of pBHt2
pSK2544	1.3-kb PCR product from 70-15 (Primers MG10927-F and MG10927-R) in pGEM-T Easy

(Epicentre); sequence gaps were closed by primer walking. Sequencing reactions were performed using the ABI Prism BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and analyzed with an ABI377 sequencer. Sequences were edited manually to remove vector and ambiguous sequences. Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). Contigs and supercontigs of 70-15 containing *AVR-Pita* genes and transposons were retrieved from the *M. grisea* genome sequence database at the Broad Institute. The sequence of chromosome 7 of strain 70-15 was obtained from GenBank (accession number CM000230).

Sequence features were identified using the following programs: i) SignalP v.3.0 to predict the signal peptide, ii) MegAlign software (Lasergene version 5.01; DNASTAR Inc., Madison, WI, U.S.A.) to calculate percentage sequence similarities (Clustal W method) and to detect regions of similarity within a clone or between clones (DotPlot method), and iii) BLAST (Altschul et al. 1997) to search GenBank databases and the fungal genome databases at the Broad Institute.

Construction of chimeric *AVR-Pita* genes and functional analysis.

The primers used to amplify the promoters and coding sequences of the *AVR-Pita1*, *AVR-Pita2*, and *AVR-Pita3* genes are shown in Table 3. Each primer contains a restriction enzyme site at the 5′ end to facilitate subsequent construction of chimeric genes in a modular structure: *Eco*RI and *Bam*HI sites at the 5′ and 3′ ends, respectively, of the promoter, and *Bam*HI and *Sph*I sites at the 5′ and 3′ ends, respectively, of the coding sequences. The location of putative promoter sequences was defined relative to the translation start site (A of ATG as +1). The promoter fragments for the *AVR-Pita1*, *AVR-Pita2*, and *AVR-Pita3* genes included the sequences from –9 to –489 (481 bp), –9 to –650 (642 bp), and –9 to –573 (565 bp), respectively. The last six nucleotides at the 3′ ends of the promoters were altered from 5′-TTATTT-3′ to 5′-GGATCC (*Bam*HI site)-3′. PCR amplifications were carried out using the FailSafe PCR system. The PCR cycling program included an initial denaturation for 5 min at 95°C; two cycles of 30 s of denaturation at 95°C, 30 s of annealing at 44°C, and 1 min of extension at 72°C; and 25 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 1 min of extension at 72°C; followed by a final extension for 10 min at 72°C. PCR products were isolated from gels using QIAquick spin columns and were cloned in pGEM-T Easy (Promega Corp.). All clones were verified by sequencing. Nine combinatorial chimeric constructs were generated by cloning a promoter (*Eco*RI-*Bam*HI fragment) and a coding sequence (*Bam*HI-*Sph*I fragment) between the *Eco*RI-*Sph*I sites of pSK1213, which contains the *N. crassa* β-tubulin terminator between the *Sph*I and *Hin*dIII sites of pGEM-3Zf (Promega Corp.). Subsequently, individual chimeric genes were isolated as *Eco*RI-*Hin*dIII fragments; clones containing the *AVR-Pita1* or *AVR-Pita2* promoters, which contain a *Hin*dIII site in the promoter, were partially digested with *Hin*dIII following a complete *Eco*RI digestion. The *Eco*RI-*Hin*dIII fragments were cloned between the *Eco*RI and *Hin*dIII sites of binary vector pBHt2 (Mullins et al. 2001). Transformation of CP987 with these clones was conducted using *Agrobacterium tumefaciens* as previously described (Rho et al. 2001).

Rice cvs. YT14 (*Pi-ta/Pi-ta*) and YT16 (*pi-ta/pi-ta*) were used in infection assays as previously described (Bryan et al. 2000). Rice seed were treated with 1% NaClO for 20 min for surface sterilization, followed by three washes with sterile water. The sterilized seed were germinated in sterile water for 2 days at room temperature. Five seedlings were transplanted to each plastic pot (5 in. in diameter) filled with Metro-mix 360 (The Scotts Company, Marysville, OH, U.S.A.). Plants were grown to the four-leaf stage (approximately 2 weeks) in a greenhouse at 25 to 30°C with a daily cycle of 16 h of light and 8 h of darkness. Conidia were collected from 10-day-old fungal cultures by scraping with 0.01% Tween 20, followed by filtration through two layers of cheesecloth to remove mycelia and other debris. The concentration of conidia was adjusted to 5×10^4 conidia/ml. Spore suspension (5 ml) was applied per pot using an artist's airbrush. Plants were kept inside a sealed plastic bag for 24 h to maintain high humidity and then removed to a greenhouse bench holding 2 to 3 in. of water. For the control experiment, plants were sprayed with 0.01% Tween 20, O-137 (avirulent), or CP987 (virulent).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute: www.broad.mit.edu
Broad Institute's Fungal Genome Initiative: www.broad.mit.edu/annotation/fungi/fgi
Broad Institute's *Magnaporthe grisea* genetic maps website: www.broad.mit.edu/annotation/fungi/magnaporthe/maps.html
EMBL-EBI InterProScan website: www.ebi.ac.uk/InterProScan
Cetner for Biological Sequence Analysis SignalP 3.0 Server: www.cbs.dtu.dk/services/SignalP
National Center for Biotechnology Information's Blast server: www.ncbi.nlm.nih.gov/BLAST