The *Magnaporthe oryzae* Avirulence Gene *AvrPiz-t* Encodes a Predicted Secreted Protein That Triggers the Immunity in Rice Mediated by the Blast Resistance Gene *Piz-t*

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The *Magnaporthe oryzae* avirulence gene *AvrPiz-t* activates immunity in a gene-for-gene fashion to rice mediated by the blast resistance gene *Piz-t*. To dissect the molecular mechanism underlying their recognition, we initiated the cloning of *AvrPiz-t* using a map-based cloning strategy. The *AvrPiz-t* gene was delimited to an approximately 21-kb genomic fragment, in which six genes were predicted. Complementation tests of each of these six candidate genes led to the final identification of *AvrPiz-t*, which encodes a 108-amino-acid predicted secreted protein with unknown function and no homologues in *M. oryzae* or in other sequenced fungi. We found that *AvrPiz-t* is present in the virulent isolate GUY11 but contains a Pot3 insertion at a position 462 bp upstream from the start codon. Complementation tests of *AvrPiz-t* genes driven by promoters of varying length revealed that a promoter larger than 462 bp is essential to maintain the *AvrPiz-t* function. These results suggest that a Pot3 insertion in GUY11 might interfere with the proper function of *AvrPiz-t*. Additionally, we found that *AvrPiz-t* can suppress the programmed cell death triggered by mouse BAX protein in *Nicotiana benthamiana*, identifying a mechanism by which *AvrPiz-t* may contribute virulence of *M. oryzae*.

In the long warfare between plants and phytopathogens, the plants have culminated in a highly effective immune system able to protect them from attack by a wide variety of pathogens. They have evolved two layers of immune responses referred to as pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al.

2006; Jones and Dangl 2006). The recognition of common features of microbial pathogens by diverse plant cell-surface receptors activates the PTI response to mount the primary defense. For example, both FLS2 and EFR can activate resistance to bacterial disease through perception of flagellin and EF-Tu, respectively (Zipfel et al. 2004, 2006). However, PTI can be effectively suppressed by so-called effector proteins encoded and delivered by phytopathogens into the plant cytosol. For example, several type III secretion system (T3SS) effectors from *Pseudomonas syringae* (e.g., HopM1, AvrPto, AvrRpm1, AvrRpt2, and HopAO1) were found to interfere with PTI in *Arabidopsis* (de Torres et al. 2006; He et al. 2006; Kim et al. 2005; Li et al. 2005; Nomura et al. 2006; Oh and Collmer 2005; Underwood et al. 2007). In order to monitor the presence of pathogen effectors, plants have developed the ETI, a more specialized immune response mediated by surveillance proteins, the so-called resistance (R) proteins. The resistance activated by this pairwise association of R and avirulence (Avr) genes was proposed as gene-for-gene resistance decades ago (Flor 1971). Only the coexistence of an R gene and its cognate Avr gene during interaction can activate the resistance, which is manifested as localized cell death (i.e., hypersensitive response [HR]). This response occurs at the site of infection to inhibit pathogen growth. The majority of R genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Dangl and Jones 2001). Comparative genomics has demonstrated that all plants have large collections of NBS-LRR genes (McHale et al. 2006). *Arabidopsis*, for instance, maintains approximately 150 NBS-LRR genes (Meyers et al. 2003).

It has been found that bacterial pathogens utilize a specialized T3SS to deliver the Avr proteins into the plant cytoplasm (Alfano and Collmer 2004; Grant et al. 2006). Most Avr genes in eukaryotic pathogens encode secreted proteins, including flax rust *AvrL567*, *AvrM*, *AvrP4*, and *AvrP123* (Catanzariti et al. 2006; Dodds et al. 2004), *AvrPi-ta* in *Magnaporthe oryzae* (Orbach et al. 2000), *Avr1b-1* in *Phytophthora sojae* (Shan et al. 2004), *Avr3a* in *P. infestans* (Armstrong et al. 2005), and *ATR13* (Allen et al. 2004) and *ATR1* (Rehmany et al. 2005) in *Hyaloperonospora parasitica*. Furthermore, expression of these Avr genes inside the host cytoplasm was found to be

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The genomic sequence of the *AvrPiz-t* gene was deposited at GenBank database (accession no. EU837058).

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables are published online.

essential for the activation of resistance mediated by their respective *R* genes encoding NBS-LRR protein (Allen et al. 2004; Armstrong et al. 2005; Catanzariti et al. 2006; Dodds et al. 2004; Jia et al. 2000; Rehmany et al. 2005).

M. oryzae, the causal agent of the destructive blast disease to rice, is an ideal model with which to investigate the molecular mechanisms underlying the interaction between fungi and plants (Martin et al. 2002; Valent 1990). The recently completed genome sequences of both rice and *M. oryzae* have advanced the dissection of the molecular basis of genetic identification of both *R* genes and *Avr* genes (Dean et al. 2005; International Rice Genome Sequencing Project 2005). Currently, over 50 rice blast *R* genes have been genetically analyzed, with 8 of them being characterized at the molecular level (Bryan et al. 2000; Chen et al. 2006; Lin et al. 2007; Liu et al. 2007; Qu et al. 2006; Wang et al. 1999; Zhou et al. 2006). Interestingly, only *Pi-d2* encodes a non-NBS-LRR protein, suggesting that the majority of the blast resistance genes may encode NBS-LRR R proteins (Chen et al. 2006; Dai et al. 2007). In the pathogen, more than 40 *M. oryzae* *Avr* genes have been genetically analyzed (Ma et al. 2006), with 5 being characterized at the molecular level. *PWL1* and *PWL2* both confer resistance specificity to weeping lovegrass and encode sequence-related proteins without ascribed function (Kang et al. 1995; Sweigard et al. 1995). *Avr1-CO39* was confined to a 1.05-kb region containing several potential open reading frames (ORF) (Farman et al. 2002). *Avr-Pita* encodes a 223-amino-acid (aa) polypeptide with features typical of metalloproteases and was found to trigger *Pi-ta*-specific immunity inside rice cells (Orbach et al. 2000). *ACE1* encodes a large cytoplasmic enzyme of 4,035 aa that is unlikely to be secreted and was found to be required for triggering the *Pi33*-specific resistance response (Böhnert et al. 2004). Of these blast *Avr* genes, *PWL1*, *PWL2*, and *Avr-Pita* encode secreted proteins, suggesting that the secretion is required for their *Avr* function (Jia et al. 2000; Kang et al. 1995; Orbach et al. 2000).

Despite significant progress on the genetic analyses of both *R* genes and *Avr* genes in the rice blast pathosystem, only one matched pair of *R* and *Avr* genes, *Pi-ta/Avr-Pita*, has been analyzed in the rice blast pathosystem (Bryan et al. 2000; Jia et al. 2000; Orbach et al. 2000). The *Pi-ta* gene encodes a predicted cytoplasmic receptor with a centrally localized NBS and C-terminal leucine-rich domain (LRD), while *Avr-Pita* encodes a putative metalloprotease (Bryan et al. 2000; Orbach et al. 2000). Both *Pita* and *Avr-Pita* were found to interact physically by both yeast-two hybrid experiments and an in vitro assay (Jia et al. 2000). To elucidate more deeply the molecular mechanism underlying the interaction between *R* genes and *Avr* genes, more *R/Avr* pairs will need to be studied.

Both *R* genes *Pi2* and *Piz-t* encode highly related NBS-LRR proteins and confer resistance to an overlapping but different set of diverse *M. oryzae* strains (Zhou et al. 2006). Only 8 aa differences exclusively confined within three consecutive LRR distinguish *Pi2* from *Piz-t*, indicating that the LRR domain is a primary specificity determinant (Zhou et al. 2006). *M. oryzae* *Avr* gene *AvrPiz-t* recognized by *Piz-t* was previously mapped between two markers, MAT1 and CH5-75H, on chromosome 7 (Luo et al. 2005). The final cloning of *AvrPiz-t* will be helpful in unraveling the molecular mechanism underlying the recognition between *Piz-t* and *AvrPiz-t*. In this study, we describe the isolation of *AvrPiz-t* using a map-based cloning strategy.

RESULTS

Map-based cloning of *AvrPiz-t*.

An F₁ population consisting of 137 single ascospores derived from a cross between avirulent isolate 81278ZB15

(MAT1-1) collected from a field in Fujian province, China and virulent isolate GUY11 (MAT1-2) was constructed for the genetic analysis of *AvrPiz-t*. Of the 52 single ascospores tested, 29 progeny were avirulent to transgenic rice plants carrying *Piz-t* whereas 23 were virulent. The segregation ratio of a virulence versus virulence was found to fit a 1:1 ratio in the χ^2 test of a goodness-of-fit ($\chi^2 = 0.69$, $P = 0.4$), indicating that *AvrPiz-t* is controlled by a single locus.

A polymerase chain reaction (PCR)-based marker system to distinguish between the presence or absence of a transposable element (PATE) was developed in this study (discussed below). Using this system, we identified a total of 11 PATE markers showing polymorphisms on chromosome 7 between two parental isolates (Table 1). *AvrPiz-t* was first mapped between two PATE markers, P21.1698.1 and P4.520.1, and was found to co-segregate with another two PATE markers, P21.1709.1 and P21.1709.2 (Fig. 1A; Table 1). Another two single nucleotide polymorphism (SNP) markers, SNP1702 and SNP514, derived from transposable elements P21.1702.1 and P4.514.1, respectively, were used to further narrow the location of *AvrPiz-t* (Fig. 1A; Supplementary Table 1). By aligning the sequences of SNP1702 and SNP514 on the sequence of chromosome 7 (GenBank: CM000230), we found that the genomic region between these two markers is approximately 145 kb. Therefore, the construction of a BAC contig that covers the *AvrPiz-t* locus in avirulent parental isolate 81278ZB15 was initiated (Fig. 1B). One BAC clone, BAC07bg07, was then completely sequenced. Fine mapping of *AvrPiz-t* finally confined it to an approximately 21-kb genomic interval flanked by two SNP markers, SNP12 and SNP78 (Fig. 1C). In all, six genes, designated 7bg7.12 to 7bg7.17, were predicted in this region (Fig. 1D). The colinear regions containing these genes were amplified from the virulent parental isolate GUY11 and their sequence was determined. It was found that the coding and promoter sequence of both 7bg7.12 and 7bg7.17 in GUY11 were identical to the ones in 81278ZB15, suggesting that neither of them could be the *Piz-t* candidate. In the remaining four genes, sequence polymorphisms were identified in either coding or promoter sequence between 81278ZB15 and GUY11 (Supplementary Table 2).

Determination of *AvrPiz-t* by gene complementation tests.

In order to identify the *AvrPiz-t* candidate from these four genes, gene complementation tests were conducted, where each gene was transformed into GUY11. Pathogenicity reactions of the derived transformants were analyzed using both susceptible rice cv. Nipponbare and *Piz-t*-transgenic Nipponbare. It was found that only transformants into which 7bg7.15 was introduced showed avirulence to *Piz-t*-transgenic Nipponbare (Fig. 2; Table 2). Nevertheless, the transformants derived from the other three genes showed virulence, suggesting that they did not receive *AvrPiz-t* (Fig. 2; Table 2). Based on the pathogenicity reaction test, all the transformants showed virulence to Nipponbare. Therefore, it has been clearly demonstrated that the *Piz-t*-mediated resistance requires the introduction of 7bg7.15.

AvrPiz-t encodes a predicted secreted protein.

AvrPiz-t is an intronless gene of 327 bp in length and encodes a predicted 108-aa polypeptide. It is a single copy gene in the 70-15 genome confirmed by homology search. The deduced *AvrPiz-t* product shows no substantial homology to any known proteins in *M. oryzae* as well as in any other organism. An 18-aa secretion signal was identified at the amino-terminus by using both SignalP 3.0 and ProtComp programs. In order to further demonstrate the importance of this N-terminal secretion signal, a construct, designated pAvrPiz-t₉₀,

was created by removal of the secretion signal (Table 2). The derived construct was then transformed into virulent isolate GUY11. Pathogenicity tests of the transformants showed that they were virulent to *Piz-t*-transgenic rice, indicating that AvrPiz-t₉₀ is no longer capable of triggering immunity medi-

ated by *Piz-t* (Table 2). Therefore, it was concluded that the N-terminal secretion signal is critical for the Avr function of AvrPiz-t. However, it is unknown whether removal of the secretion signal makes AvrPiz-t₉₀ unable to be mobilized outside the cell or not.

Table 1. Distribution of transposable elements (TE) and presence or absence of a TE (PATE) markers on chromosome 7 of *Magnaporthe oryzae*^a

Supercontig, contig	Contig length (bp)	TE location (bp)	TE length (bp)	Primer sequences ^b	PATE designation	Polymorphism
25						
1829	21,909	2,081–3,944	1,864	...	...	...
1842	14,880	8,898–10,760	1,863	...	...	...
1850	51,013	2,715–4,481	1,767	F gcagtgaacaccttgc	P25.1850.1	Yes
	...	...	...	R cgtcgtcgtaatcacac	...	...
	...	15,111–16,978	1,868	F ttgcacctaccagctgc	P25.1850.2	Yes
	...	...	...	R ttcaacccccaggcagac	...	...
1856	27,674	16,319–18,185	1,867	F aaaggcagaggcattgtg	P25.1856.1	Yes
	...	...	...	R cgggtgcagacatttactg	...	...
37						
2031	37,033	2,472–2,949	478	F attgtacacatcactcc	P37.2031.1	Yes
	...	...	...	R caagctcaactcgaagc	...	...
21						
1698	30,783	23,589–25,451	1,863	F ttgcttgagccattgac	P21.1698.1	Yes
	...	...	...	R cataacaatgtccactc	...	...
1702	9,614	3,914–5,773	1,860	F atccgacctaactcgtgac	P21.1702.1	No
	...	...	...	R acgggtgcaatcatgaag	...	...
1703	5,193	3,040–4,904	1,865	...	...	...
1705	5,505	2,394–4,257	1,864	...	...	...
1709	21,702	6,512–8,382	1,871	F gaaggtcattttctcacag	P21.1709.1	Yes
	...	...	...	R atccaattcatgaacacgac	...	...
	...	11,736–13,601	1,866	F taacctgtcaacgaagatag	P21.1709.2	Yes
	...	...	...	R aagtgacgggatttggttc	...	...
1713	2,908	715–1,169	455	...	...	...
1719	16,164	2,472–5,167	2,696	...	...	...
1720	19,931	8,502–8,963	462	...	...	...
1723	25,973	17,430–19,295	1,866	...	...	...
1725	20,877	6,663–8,497	1,835	...	...	...
1738	10,202	5,518–5,967	450	...	...	...
1739	23,489	7,832–9,698	1,867	F cggagaggggaatggatc	P21.1739.1	No
				R gaaaagctgcgtaaagg	...	...
4						
514	8,058	3,670–5,532	1,863	F ttgcactgggtaccgttacc	P4.514.1	No
	...	...	...	R atgcgtgaaaccaaactcgg	...	...
520	36,000	14,769–16,633	1,865	F ttcgatggcagtgctggag	P4.520.1	Yes
	...	...	...	R tatgcaaggaggatggcttg	...	...
522	32,999	603–2,467	1,865	...	...	...
523	72,540	35,372–37,258	1,887	F actctatggaatcgtgctc	P4.523.1	Yes
	...	...	...	R aagttcaggtcatctgtag	...	...
	...	42,640–44,504	1,865	F ctctctagctatctacatg	P4.523.2	Yes
	...	...	...	R aaggtcagactaatgtgcc	...	...
527	27,274	7,598–7,726	129	...	...	...
537	21,003	18,106–18,227	122	...	...	...
542	21,366	19,597–19,720	124	F caagcgggtgttttatgag	P4.542.1	No
	...	...	...	R atctgacgtgaccattgtag	...	...
557	35,303	19,492–19,647	156	F gaggatgtcatcgaagag	P4.557.1	No
	...	...	...	R catgtctcgacacattcc	...	...
561	92,635	2,042–3,904	1,863	F ataaccggtatgccaatcg	P4.561.1	Yes
	...	...	...	R actccaatccgttaagcc	...	...
	...	63,542–64,000	459	F cagcttgataagagagg	P4.561.2	No
	...	...	...	R aggtattgttgatcgc	...	...
565	32,557	5,460–5,587	128	...	...	...
581	10,725	7,535–7,994	460	...	...	...
595	60,696	53,111–55,802	2,692	...	...	...
600	80,506	9,714–12,404	2,691	...	...	...
605	56,017	39,488–39,617	130	...	...	...
608	8,622	7,295–8,148	854	...	...	...
611	13,828	4,751–7,442	2,692	...	...	...
32						
1963	16,649	8,252–10,115	1,864	...	...	...
33						
1983	57,283	10,163–10,621	459	...	...	...
	...	52,183–52,630	448	...	...	...
1990	33,944	12,320–14,136	1,817	...	...	...

^a Release 2, not 5, of the *M. oryzae* genome assembly (*Magnaporthe grisea* database) was used for sequence analysis because there is some misassembly found in release 5.

^b F = forward and R = reverse.

The Pot3 insertion disturbs the proper function of the promoter of *AvrPiz-t*.

Sequence comparison of the *AvrPiz-t* gene between isolates 81278ZB15 and GUY11 revealed no sequence variation in their coding regions. However, a Pot3 transposon is located at a position 462 bp upstream from the start codon of *AvrPiz-t* in GUY11 (Fig. 3A). It is 1,861 bp in length and contains two 48-bp inverted terminal repeats (ITR) and one ORF exhibiting significant homology to transposases encoded by TC1/Mariner transposons (Farman et al. 1996). Moreover, it shares 99% identity in nucleotide sequence to the one reported in the *Avr-Pita* locus (GenBank: AF333034) (Kang et al. 2001). Transposition of Pot3 in *AvrPiz-t* in GUY11 resulted in the duplication of the target sequence (5' TA 3'). After removal of Pot3 and one copy of the target sequence, the promoter sequence of *AvrPiz-t* in GUY11 was found to be identical to the one in 81278ZB15.

To determine the minimum size of promoter which is required for the proper function of *AvrPiz-t*, we made four constructs, including one mimicking the version in GUY11, and transformed them into GUY11. It was found that both pP537 and pP736 constructs, but neither pP454 nor pP462, were able to complement the *AvrPiz-t* function (Fig. 3B; Table 2). These results indicated that the minimum size of the promoter to properly maintain the function of *AvrPiz-t* should be larger than 462 bp, the position at which Pot3 inserted in GUY11. Therefore, sequences essential for *AvrPiz-t* function are located

upstream from the insertion site. The transcription of *AvrPiz-t* in both 81278ZB15 and GUY11 at mycelium, conidium, appressorium, and in planta stages was investigated to further validate the hypothesis. However, due to undetectable levels of expression of *AvrPiz-t* by reverse-transcription PCR in the conditions tested, we were not able to demonstrate whether the insertion of Pot3 interferes with the expression of *AvrPiz-t* in GUY11.

Virulent isolates exhibit either a Pot3 insertion or amino acid substitution in *AvrPiz-t*.

To investigate the sequence variation and resulting phenotypes of *AvrPiz-t* in diverse *M. oryzae* isolates, we analyzed the *AvrPiz-t* sequence in nine virulent and avirulent isolates (Table 3). It was found that no sequence changes existed in *AvrPiz-t* in the avirulent isolates GDS2 and KJ201 (Table 3). However, two types of sequence variations were revealed in the virulent isolates. *AvrPiz-t* in the isolate 70-15 had the same Pot3 insertion in its promoter region as the one identified in GUY11 (Table 3). In the remaining six isolates, as shown in Table 3, *AvrPiz-t* had the same single nucleotide substitution (C to T), resulting in an amino acid change of valine for alanine at position 41 (A41V). It is worthwhile to note that all the six virulent isolates might have originated from the same isolate because they all came from the same province in China. In order to further confirm that substitution A41V caused the loss of function of *AvrPiz-t*, the p*AvrPiz-t*^{A41V} allele from the

Fig. 1. Map-based cloning of *AvrPiz-t*. **A**, Genetic analysis of *AvrPiz-t*. Molecular markers are listed above the string and the corresponding recombinants from *AvrPiz-t* are listed underneath. The number of the recombinants at P21.1698.1, P4.520.1, and P4.523.1 derived from two rounds of mapping analyses as described in Materials and Methods is indicated in parenthesis. **B**, Physical mapping of *AvrPiz-t*. The BAC contig spanning the *AvrPiz-t* locus consisted of two BAC clones indicated in gray rectangles with their names above. **C**, Fine mapping of *AvrPiz-t*. Molecular markers are listed above the string and corresponding recombinants from *AvrPiz-t* are listed underneath. **D**, Candidate genes for *AvrPiz-t*. Genes are represented by filled arrows indicating direction of transcription. The gene designation is listed above the arrows. The figure is not drawn to scale.

virulent strain, GDS3, was transformed into GUY11 (Table 2). Transformants were found to be virulent to *Piz-t*, further indicating that *AvrPiz-t*^{41V} was not able to produce the *AvrPiz-t* phenotype (Tables 2 and 3). Taken together, both insertion of *Pot3* and the substitution A41V can cause the loss of function of *AvrPiz-t*.

***AvrPiz-t* can suppress the cell death induced by BAX in tobacco.**

Many bacterial and filamentous pathogen effectors were found to be able to suppress programmed cell death (PCD) induced by the proapoptotic protein BAX, identifying a mechanism by which these effectors contribute positively to the virulence of pathogens (Dou et al. 2008; Jamir et al. 2004). We tested whether *AvrPiz-t* could also suppress BAX-triggered PCD in *Nicotiana benthamiana*. Obvious cell death symptoms were observed when *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* cells carrying pGR106:BAX (Fig. 4A). Filtration of *N. benthamiana* leaves with *A. tumefaciens* cells containing a green fluorescent protein (GFP) gene (Fig. 4B) or MgCl₂ buffer (data not shown) did not affect the BAX-triggered cell death. Infiltration of *N. benthamiana* leaves with *A. tumefaciens* cells harboring *AvrPiz-t* alone did not cause obvious cell death symptoms (Fig. 4C).

However, we found that *N. benthamiana* leaves infiltrated with *A. tumefaciens* cells harboring the *AvrPiz-t* gene 24 h prior to infiltration with the pGR106:BAX-harboring cells did not produce symptoms (Fig. 4D). The expression of BAX was detected at 24 h and later, after infiltration, which ruled out the possibility of expression failure of BAX in tests (data not shown). These results indicated that *AvrPiz-t* could suppress the BAX-mediated PCD in *N. benthamiana*.

DISCUSSION

In this study, we described our effort to clone the *M. oryzae* *Avr* gene *AvrPiz-t*, complementary to the previously cloned rice blast resistance gene *Piz-t* (Zhou et al. 2006), using a map-based cloning strategy. The *Piz-t/AvrPiz-t* pair is the second *R/Avr* pair in the rice and rice blast system to be characterized at the molecular level. The cloning of *AvrPiz-t* as well as *Piz-t* will be very helpful in gaining insight into mechanisms of rice resistance to *M. oryzae*.

In the last decade, over 20 *Avr* genes have been isolated from filamentous microorganisms, including fungi and oomycetes (Kamoun 2007). Most of them encode small secreted proteins without known functions (Kamoun 2006). In *M. oryzae*, four *Avr* genes—*Avr-Pita* (Orbach et al. 2000) *ACE1*

Fig. 2. Complementaion tests of *AvrPiz-t*. Pathogenicity tests of strains transformed with two candidate genes, 7bg7.15 or 7bg7.14, were used for illustration. Results for three independent transformed isolates for each candidate gene are illustrated from left to right. The photos were taken 7 days after inoculation. NPB, Nipponbare; *Piz-t*, Nipponbare containing *Piz-t* transgene. **A**, GUY11 transformants with 7bg7.15 are virulent to Nipponbare but avirulent to Nipponbare containing *Piz-t* transgene. **B**, GUY11 transformants with 7bg7.14 are virulent to both Nipponbare and Nipponbare containing *Piz-t* transgene.

Table 2. All the key constructs used in the study

Constructs ^a	Description	Phenotype
p7bg7.13	Entire region of the predicted gene 7bg7.13	Virulent
p7bg7.14	Entire region of the predicted gene 7bg7.14	Virulent
p7bg7.15	Entire region of the predicted gene 7bg7.15	Avirulent
p7bg7.16	Entire region of the predicted gene 7bg7.16	Virulent
pAvrPiz-t ₉₀	AvrPiz-t without N-terminal 18-amino-acid secreted signal	Virulent
pP454	AvrPiz-t driven by a 454-bp promoter	Virulent
pP462	AvrPiz-t in GUY11 containing a portion of <i>Pot3</i> at the position of 462-bp promoter site	Virulent
pP537	AvrPiz-t driven by a 537-bp promoter	Avirulent
pP736	AvrPiz-t driven by a 736-bp promoter	Avirulent
pAvrPiz-t ^{41V}	AvrPiz-t carrying a single amino acid change (A to V) at position 41	Virulent

(Böhnert et al. 2004), *AvrI-CO39* (Farman et al. 2002; Peyyala and Farman 2006), and *AvrPiz-t* in this study—complementary to different rice blast resistance genes have been cloned. Both *Avr-Pita* and *AvrI-CO39* encode predicted secreted proteins (Orbach et al. 2000; Peyyala and Farman 2006). *AvrPiz-t* described in this study also encodes a small protein predicted to be secreted. Taken together, we speculated that the major repertoire of *M. oryzae* *Avr* genes might encode secreted proteins.

It has been found that more than 9.7% of the *M. oryzae* genome is made up of repetitive DNA based on the analysis of the whole-genome sequence (WGS) of 70-15 (Dean et al. 2005). Even a higher proportion (nearly 14%) of the repetitive DNA composition was identified when using the combined BAC-WGS sequence of chromosome 7 of 70-15 (Thon et al. 2006). Moreover, virtually all of the repetitive DNA sequences found on chromosome 7 consisted of transposable elements (TE), which were distributed as clusters (Thon et al. 2006). The abundance of the TE has been implicated as a major contributor to genome rearrangements and as a source of genetic variations (Nishimura et al. 2000; Kang et al. 2001). Here, we took advantage of polymorphisms of TE with respect to their presence and absence in parental isolates and designed PATE markers for genetic analysis of *AvrPiz-t*. A significant portion (11 of 17) of the tested PATE showed polymorphisms between parental strains (Table 1), suggesting that polymorphisms with respect to presence or absence of TE are significant among diverse *M. oryzae* strains. In addition to other DNA-based markers associated with TE, (e.g., rep-PCR and retrotransposon-microsatellite amplified polymorphism) (Chadha and Gopalakrishna 2005; Wang et al. 2002), the PATE marker system described herein provided another tool for genetic analysis of *M. oryzae*.

It has been reported that *M. oryzae* has relatively more genetic instability in *Avr* determinants corresponding to certain rice blast *R* genes, making the utility of *R* genes in controlling rice blast disease limited (Bonman et al. 1987; Kiyosawa 1976). However, mechanisms underlying the instability of *Avr* determinants in *M. oryzae* are largely unknown. It is compelling that TE were always found to be associated with the loss of function or rearrangement of *M. oryzae* *Avr* genes (i.e., *AvrI-CO39*, *Avr-Pita*, and *ACE1*) (Böhnert et al. 2004; Farman et al. 2002; Kang et al. 2001). An unknown retrotransposon and a repetitive element were identified at right- and left-hand

borders, respectively, of deletion breakpoints of the *AvrI-CO39* locus in GUY11 as well as in other virulent isolates (Farman et al. 2002). An insertion of the MGL line-like retroposon was identified in the last exon of *ACE1*, which caused a loss of function in the virulent isolate 2/0/3 (Böhnert et al. 2004). A Pot3 insertion was identified in the promoter of *Avr-Pita*, which caused the gain of virulence in the mutant strain CP1632 (Kang et al. 2001). Zhou and associates (2007) identified a Pot3 transposon in the DNA region encoding the putative protease motif of the *Avr-Pita* protein from field-virulent isolate B2. Analyses of the genomic organization and evolution of the *Avr-Pita* gene family further showed family members to be closely associated with diverse TE, including ITR transposons, retroposons, solo long-terminal repeats (LTR), and undefined short repetitive DNA elements (Khang et al. 2008). Here, we found that a Pot3 insertion in the promoter region caused the loss of function of *AvrPiz-t*, providing another example for the association of TE with *Avr* genes. It is reasonable to hypothesize that TE function as an on/off switch controlling the expression of *Avr* genes for defeating rice resistance genes in nature. Given that the *M. oryzae* genome encodes a large number of effectors contributing cumulatively to virulence, loss of function of a particular *Avr* gene does not compromise the entire virulence phenotype of *M. oryzae*. However, loss of function of a particular *Avr* gene by insertion of a TE will make a strain unrecognized by rice cultivars harboring the complementary *R* gene. Therefore, insertion of TE in *Avr* genes may possibly be one of the strategies that *M. oryzae* has evolved to maintain high instability in *Avr* determinants in the field.

Table 3. Sequence variation of the *AvrPiz-t* gene in different isolates

Isolates	Sequence variation	Phenotype
KJ201	No	Avirulent
70-15	Insertion of Pot3	Virulent
GDS2	No	Avirulent
GDS3	A single SNP	Virulent
GDS4	A single SNP	Virulent
GDS6	A single SNP	Virulent
GDS8	A single SNP	Virulent
GDS10	A single SNP	Virulent
GDS11	A single SNP	Virulent

^a SNP = single nucleotide polymorphism

Fig. 3. Schematic determination of the promoter size required for proper function of *AvrPiz-t*. *AvrPiz-t* is indicated by filled arrows and Pot3 is indicated by a filled rectangle. The position at which the Pot3 inserted is marked with vertical arrows and base pair positions. **A**, Pot3 located at 462 bp upstream from start codon (ATG) of *AvrPiz-t* in the virulent isolate GUY11. **B**, Pathogenicity tests of each strain transformed with four *AvrPiz-t* variants with different size of promoter. The name of each construct is listed at the left and the phenotype to *Piz-t* transgenic rice plants of the derived transformants is at the right. A: avirulent; V: virulent.

MATERIALS AND METHODS

Fungal isolates and rice cultivars used in this study.

The *M. oryzae* isolate 81278ZB15 that is avirulent to *Piz-t*-harboring rice cultivars was collected from a field in Fujian province, China. It was used as one of the parental isolates for F₁ population construction. The isolate GUY11 that is virulent to *Piz-t*-harboring rice cultivars was used as another parental isolate. It was also used as the recipient isolate for *M. oryzae* transformation experiments in this study. To create the population, 137 single ascospores were randomly picked and rescued for genetic analysis. Rice cv. Nipponbare and a *Piz-t* transgenic rice line in Nipponbare background were used for the pathogenicity tests. The *Piz-t* donor line IRBL11 and susceptible rice cv. CO39 were also used as control rice cultivars for the pathogenicity tests.

Pathogenicity tests and disease assessment.

Infection assays were conducted on 21-day-old rice seedlings and the resulting resistance or susceptible phenotype was evaluated 7 days after inoculation as described previously (Zhou et al. 2006). The pathogenicity tests for parental, progeny, and transformant isolates were repeated two times.

The development of PATE markers for genetic analysis.

A set of molecular markers representing PATE in two parental strains were developed for genetic analysis. TE with typical features (e.g., ITR, LTR, and target duplicated sequences) pre-

sent in the sequence of chromosome 7 of *M. oryzae* were identified by BLAST search. In order to make the PATE suitable for molecular analysis, we selected TE which were smaller than 3 kb in size. Primers were then designed from the flanking sequences of TE. PCR-amplified products were analyzed for polymorphisms regarding the presence or absence of the TE between two parental isolates. Verified polymorphic PATE were used for the genetic analysis.

Genetic analysis of *AvrPiz-t*.

We adopted two rounds of genetic analyses to delimit the location of the *AvrPiz-t* gene. At the first round, we mapped *AvrPiz-t* between two markers, P21.1698.1 and P4.523.1, at which one and two recombinants, respectively, were identified from 52 progeny isolates. At the second round, we identified 13 recombinants between these two markers at the DNA level from the remaining 85 progeny isolates. After phenotype evaluation, seven and six more recombinants at P21.1698.1 and P4.523.1, respectively, were counted. Therefore, we used these 16 recombinants to further narrow down the location of *AvrPiz-t*. Two SNP markers, SNP514 and SNP1702, developed based on the reference sequence of 70-15 helped to delimit *AvrPiz-t* to an approximately 145-kb genomic interval. Both BAC contig construction and sequence determination of the *AvrPiz-t* locus facilitated the development of more SNP markers. *AvrPiz-t* was finally delimited into an approximately 21-kb genomic interval flanked by two SNP markers, SNP12 and SNP78.

Fig. 4. Suppression of BAX-mediated programmed cell death (PCD) by *AvrPiz-t* in *Nicotiana benthamiana* leaves using *Agrobacterium* infiltration. The photographs of a representative leaf were shown. **A**, BAX triggered the PCD. **B**, Green fluorescent protein (GFP) did not interfere with the BAX-mediated PCD. **C**, *AvrPiz-t* alone did not induce the cell death. **D**, *AvrPiz-t* significantly suppressed the BAX-mediated PCD.

Screen of the BAC clone by pool method.

The whole BAC library of 81278ZB15 saved in 24 384-well plates was divided into 24 pools (i.e., one pool per 384-well plate) and DNA from each pool was isolated for the first round of screening. The markers proximal to or co-segregating with *AvrPiz-t* were used to screen the positive pools by PCR method. Each of the 384-well plates corresponding to a positive pool was further divided into four ordered 96-well plates. The DNA was isolated from each 96-well plate and was saved as subpools. The subpools were used for the second screen with the same markers by PCR method. At the final step, the 96-well plate corresponding to the positive subpool was divided into 20 DNAs based on the 12-by-8 format for the isolation of a single positive BAC clone.

BAC clone sequencing, sequence assembly, and gene prediction.

A BAC clone, BAC07bg07, identified by the pool method as described above was selected for sequencing using a shot-gun approach as described previously (Zhou et al. 2006). More than 1,200 subclone ends were sequenced and processed for sequence assembly using the software packages Phred/Phrap/Gap4 (Ewing and Green 1998; Ewing et al. 1998). Gaps present in the assembled sequence were further filled as described previously (Zhou et al. 2006). Genes of the derived BAC sequence were predicted using the Fgenesh program.

Vector modification for fungal transformation.

A fragment containing the *ccdB* gene and chlorophenol resistance gene from the gateway destination vector pPANDA developed by Miki and Shimamoto (2004) was amplified by PCR with the following primers: forward, 5'-ggcgccgcccatttttaatggcgccgcattagacc-3' and reverse, 5'-tttaattaccatttttaatggtcgacctgcagactgctg-3', with the restriction sites for *AscI* and *PacI* underlined, and cloned into pGEMT-easy vector (Promega Corp., Madison, WI, U.S.A.). The derived plasmid was designated pGEM-T-ncgr. A fragment released from pGEM-T-attR by digestion with *EcoRI* was further cloned into pBluescript KSII predigested with the same enzyme and the derived vector was named PKS-ncgr. A fragment released from PKS-ncgr by digestion with *HindIII* and *SacI* was finally cloned into the vector pCSN43 developed by Staben and associates (1989). The derived vector for *M. oryzae* transformation was designated pCSN43-ncgr.

Fungal transformation and complementation test.

All the constructs listed in Table 1 were subcloned into pCSN43-ncgr because the modified vector is highly efficient for cloning, with two 8-nucleotide restriction enzymes, *AscI* and *PacI*, introduced into the multiple cloning sites. The DNA corresponding to each candidate gene was amplified by PCR using pfu polymerase (Promega Corp.) with the primers listed in Supplementary Table 3. The amplified fragment either was cloned into pGEM-T-attR to introduce *AscI* and *PacI* for subsequent cloning in pCSN43-ncgr, or directly cloned into pCSN43-ncgr by digestion with the appropriate restriction enzymes to obtain the constructs for the complementation tests (Table 2). All the constructs were confirmed by sequencing. Fungal transformation was performed by a polyethylene glycol (PEG)-mediated approach as described previously (Sweigard et al. 1995). The virulent isolate GUY11 was used as the receipt isolate for transformations in this study. Five independent hygromycin-resistant transformants were selected for pathogenicity tests.

Sequence analysis of virulent and avirulent isolates.

The sequences of *AvrPiz-t*, including 5' regulatory and coding sequences, were amplified by PCR with the following primers:

forward, 5'-tttaattaagctccaacgtagacgtatgc-3' and reverse, 5'-aaatttgagctccgttgaagcaacaggatc-3'. The amplified DNA was analyzed by electrophoresis on a 1% agarose gel to determine any visible size difference compared with the one amplified from the wild-type strain. The *AvrPiz-t* coding sequence from each tested isolate was completely determined for sequence comparison.

A. tumefaciens infiltration assays.

We adopted the *Agrobacterium*-mediated transient expression system as described previously (Bos et al. 2006) to analyze whether *AvrPiz-t* was able to suppress the BAX-mediated PCD. The 35S:*AvrPiz-t* construct was generated by inserting a fragment encompassing the complete *AvrPiz-t* ORF amplified by PCR from isolate 70-15 of *M. oryzae* using the primers *AvrPiz-tF* (5'-ccatcgatATGAGCTTCGTACAATGCAATC-3', *ClaI* site underlined) and *AvrPiz-tR* (5'-cggtcgacCTATTGGCGCTGAGCCTGAGGG-3', *SalI* site underlined) between the *ClaI* and *SalI* sites in the *Potato virus X* binary vector pGR106 (pGR106:*AvrPiz-t*). Similarly, BAX was cloned into pGR106 (pGR106:BAX) after being amplified using primers BAXF (5'-ccatcgatATGGACGGGTCCGGGGAG-3', *ClaI* site underlined) and BAXR (5'-cggtcgacTCAGCCCATCTTCTTCCAG-3', *SalI* site underlined). After verification by sequencing, the constructs were introduced into the *A. tumefaciens* GV2260. Overnight cultures in Luria-Bertani media supplemented with kanamycin at 50 mg/ml were harvested by centrifugation. The harvested cells were washed three times in 10 mM MgCl₂ and resuspended in 10 mM MgCl₂ to an optical density at 600 nm of 0.3. After incubation at room temperature for 2 h, the cells were used to infiltrate into 4- to 6-week-old *N. benthamiana* leaves using a 1-ml needleless syringe as described previously (Romeis et al. 2001). Plants were grown and maintained throughout the experiments in a greenhouse with an ambient temperature of 25°C and 12 h of day light with high light intensity. *A. tumefaciens* cells carrying the BAX gene (pGR106:BAX) were infiltrated into the same site 24 h later. As controls, *A. tumefaciens* strains carrying BAX or *AvrPiz-t* were replaced with *A. tumefaciens* strains carrying the GFP gene (pGR106:GFP). Symptom development was monitored from 3 to 8 days after infiltration, and pictures were taken after 8 days. The experiments were repeated at least three times. Although it is likely that PVX replication occurred in the transformed plant cells, resulting in amplified expression of the genes in the PVX vector, no attempt was made to quantify PVX replication in this experiment.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

SignalP 3.0server: www.cbs.dtu.dk/services/SignalP
 Softberry ProtComp and Fgenesh programs: www.softberry.com
Magnaporthe grisea database:
www.broad.mit.edu/annotation/fungi/magnaporthe