Identification of a New Locus, *Ptr(t)*, Required for Rice Blast Resistance Gene *Pi-ta*-Mediated Resistance

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Resistance to the blast pathogen *Magnaporthe oryzae* is proposed to be initiated by physical binding of a putative cytoplasmic receptor encoded by a nucleotide binding site-type resistance gene, *Pi-ta*, to the processed elicitor encoded by the corresponding avirulence gene *AVR-Pita*. Here, we report the identification of a new locus, *Ptr(t)*, that is required for *Pi-ta*-mediated signal recognition. A *Pi-ta*-expressing susceptible mutant was identified using a genetic screen. Putative mutations at *Ptr(t)* do not alter recognition specificity to another resistance gene, *Pi-k^s*, in the *Pi-ta* homozygote, indicating that *Ptr(t)* is more likely specific to *Pi-ta*-mediated signal recognition. Genetic crosses of *Pi-ta Ptr(t)* and *Pi-ta ptr(t)* homozygotes suggest that *Ptr(t)* segregates as a single dominant nuclear gene. A ratio of 1:1 (resistant/susceptible) of a population of BC1 of *Pi-ta Ptr(t)* with *pi-ta ptr(t)* homozygotes indicates that *Pi-ta* and *Ptr(t)* are linked and cosegregate. Genotyping of mutants of *pi-ta ptr(t)* and *Pi-ta Ptr(t)* homozygotes using ten simple sequence repeat markers at the *Pi-ta* region determined that *Pi-ta* and *Ptr(t)* are located within a 9-megabase region and are of *indica* origin. Identification of *Ptr(t)* is a significant advancement in studying *Pi-ta*-mediated signal recognition and transduction.

Additional keywords: disease resistance.

Plant resistance (*R*) genes play important roles in preventing diseases. The molecular mechanism of *R* gene-mediated resistance is believed to follow a gene-for-gene specificity in which an *R* gene in plants is effective in preventing infection by pathogen races containing the corresponding pathogen avirulence genes (Flor 1971). Most *R* genes are known to encode putative proteins with nucleotide binding sites (NBS), kinase activity, and leucine-rich repeats (LRR) that are consistent with their predicted roles as receptors in detecting the elicitors of the pathogen directly or indirectly through the products of the pathogen avirulence genes (Hulbert et al. 2001). Direct interactions of *R* proteins with avirulence proteins have been documented in four host-pathogen systems (Deslandes et al. 2003; Dodds et al. 2006; Jia et al. 2000; Scofield et al. 1996; Tang et al. 1996). In contrast, *R* proteins were found to interact with

other plant proteins modified in the presence of avirulence proteins (Axtell and Staskawicz 2003; Mackey et al. 2002, 2003).

The study of genes involved in signal transduction processes associated with disease resistance may yield new knowledge for understanding the cellular actions of *R* genes. To date, a number of genetic methods have led to the identification of genes involved in transducing a resistance response, including yeast two-hybrid screening using cloned genes to identify interacting proteins (Zhou et al. 1997) and screening for mutants affecting the resistance phenotype, specific defense responses, or both (Innes 1998). Such screening has identified a tomato gene, *Prf*, encoding a putative NBS-LRR protein (Salmeron et al. 1994, 1996). *Prf* is predicted to be involved in the *Pto/AvrPto* complex that transduces defense signals (Mucyn et al. 2006). Subsequently, the *Arabidopsis* *NDR1* and *EDS1* genes and the barley *Rarl* gene were determined to be involved in multiple *R* gene-mediated signaling pathways (Century et al. 1997; Innes 1998; Shirasu et al. 1999). Mutants with partial defects were also identified in the *Xa21*-mediated signaling pathways (Wang et al. 2004). Thus far, mutants completely abolishing *R* gene-mediated disease resistance, other than mutations in the *R* gene itself, have not been reported in rice.

In the rice blast system, 40 *R* genes have been described (McCouch et al. 1994), and several, such as *Pi-b*, *Pi-ta*, *Pi-d²*, *Pi-9*, and *Pi-36* have been molecularly characterized (Bryan et al. 2000; Chen et al. 2006; Liu et al. 2007; Qu et al. 2006; Wang et al. 1999). The *Pi-b*, *Pi-9*, and *Pi-36* genes encode predicted NBS-LRR proteins (Qu et al. 2006; Wang et al. 1999). *Pi-d²* is predicted to encode a receptor-like kinase (Chen et al. 2006). We have focused on the study of the *Pi-ta* gene encoding a NBS major *R*-gene type that confers resistance to *Magnaporthe oryzae* races containing the corresponding avirulence gene *AVR-Pita* (Bryan et al. 2000; Valent 1997). In the U.S., the tropical *japonica* cultivar Katy was released containing *Pi-ta* introgressed from the landrace *indica* cultivar Tetep from Vietnam (Moldenhauer et al. 1990). Historically, rice cultivars containing the resistance gene *Pi-ta²* exhibit a broader spectrum of resistance to pathogens than those containing *Pi-ta* (Rybka et al. 1997). The identification of the *Pi-ta* gene in rice cultivar containing *Pi-ta²* suggests that the *Pi-ta* gene may be needed for *Pi-ta²*-mediated pathogen recognition (Bryan et al. 2000; Jia et al. 2003). High resolution physical and genetic maps of *Pi-ta²* near the centromere of chromosome 12 were constructed; whether or not both *Pi-ta²* and *Pi-ta* are located at the same chromosomal location is undetermined (Nakamura et al. 1997; Rybka et al. 1997). However, among *R* genes molecularly characterized, the *Pi-ta* gene is extremely valuable and is still useful in preventing blast diseases worldwide (Jia et al. 2004a; Moldenhauer et al. 1990; Wang et al. 2007). Sequence analysis of the coding regions of the *Pi-ta* alleles in 16 rice cultivars

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identified cultivars with one resistant *Pi-ta* haplotype and three susceptible *pi-ta* haplotypes (Jia et al. 2003). High conservation of DNA sequences in the *Pi-ta* gene led to the development of markers from *Pi-ta* for marker-assisted *Pi-ta* incorporation (Jia et al. 2002, 2003, 2004b).

The *AVR-Pita* gene determining the efficacy of *Pi-ta* was predicted to be a metalloprotease (Orbach et al. 2000). *AVR-Pita* is located near the telomere in some *M. oryzae* races, and was identified in archived and contemporary collections worldwide (Zhou et al. 2007). *AVR-Pita* encodes a putative metalloprotease, and it was shown that the product of *AVR-Pita* appears to function inside the plant cell in triggering *Pi-ta*-mediated defense responses (Bryan et al. 2000; Jia et al. 2000). However, virulent isolates were identified with significant sequence alterations at the *AVR-Pita* allele, and the presence of transposon Pot3 in the promoter region of *AVR-Pita* in a lab strain and in the coding region of *AVR-Pita* in a field strain suggests that *AVR-Pita* is highly unstable and is still under intensified selection (Kang et al. 2001; Zhou et al. 2007). Evidence on the product of *Pi-ta* binding to the product of *AVR-Pita* (Jia et al. 2000) led us to investigate how *Pi-ta* detects this highly unstable pathogen determinant (*AVR-Pita*) in triggering effective resistance.

In this study, we have taken a genetic approach to investigate other plant genes involved in the recognition of the *AVR-Pita* gene and transduction of defense signaling in the *Pi-ta*-mediated defense pathway. We have isolated one rice mutant from Katy altered in the ability to resist *M. oryzae* races expressing *AVR-Pita*. Genetic analyses indicate we have isolated mutations in a new locus, designated *Ptr(t)* for *Pi-ta* resistance. It was also found that *Pi-ta* and *Ptr(t)* cosegregated as a single locus; thus, this new information and materials provide a starting point for map-based cloning of *Ptr(t)*.

RESULTS

Isolation of a rice mutant susceptible to *M. oryzae*.

Among 30,000 M₂ plants derived from 5,781 M₁ seeds of Katy, a total of 19 susceptible Katy lines were initially identified, and their genotypes were analyzed using nine selected simple sequence repeat (SSR) markers to verify their origins

(Table 1). Five blast-susceptible mutants, M2494, M2500, M2354, M2912, and M5415, were confirmed to be from Katy. The remaining 14 susceptible lines were identified to be volunteer seeds in rice fields. These five susceptible Katy lines were further genotyped, using a panel consisting of 67 SSR markers from all 12 rice chromosomes (their chromosomal locations are listed in Table 2) to verify that they were derived from Katy. Subsequently, all five of these mutants were advanced to M₇. The susceptibilities of different mutant lines to *M. oryzae* infection were consistently observed over multiple generations (data not shown).

Analysis of the *Pi-ta* alleles in rice mutants.

The *Pi-ta* alleles, including 5' and 3' regions in five mutants, were amplified by polymerase chain reaction (PCR) (data not shown). Amplification products of the identical size as *Pi-ta* indicate that there were no detectable deletions in these mutants. PCR products of the expected sizes were obtained using various PCR primer pairs designed using the sequence of the *Pi-ta* gene (GenBank accession number AF207824) (Fig. 1). Sequence analysis of 870 bp of 5' untranslated region, the open reading frame, and the intron of the *Pi-ta* gene in Figure 1 using *Pi-ta*-specific primers listed in Table 3 revealed an identical *Pi-ta* sequence from mutant 2354 to the *Pi-ta* gene. This result indicates that the putative mutations have not occurred in the *Pi-ta* gene in mutant 2354. However, susceptible *pi-ta* alleles were identified in M5415, M2500, M2494, and M2912, suggesting that these lines contain susceptible *pi-ta* alleles from a susceptible parent of Katy (data not shown).

Since the identical *Pi-ta* gene was identified in M2354, the possibility of mutations at the promoter regions was also investigated. To rule out the possibility of the promoter mutations, reverse transcription (RT)-PCR was used to amplify the region between the intron, using healthy rice leaves (Fig. 1). Similar expression of *Pi-ta* in both 2354 and Katy was detected by RT-PCR (Fig. 1). Real-time RT-PCR analysis was used to determine the expression dynamics of the *Pi-ta* alleles in M2354 and Katy after pathogen inoculation using *Pi-ta*-specific primers. As shown in Figure 2, expression of the *Pi-ta* allele in M2354 is similar to that in Katy at different time-points up to 76 h postinoculation. These results suggest that

Table 1. Analyses of nine selected simple sequence repeats (SSR) of putative rice mutants derived from Katy

Mutant ^a	Marker								
	RM149	RM190	RM22	RM225	RM481	RM231	RM303	RM484	RM489
1989	241	113	185	143	217	192	NAD	299	270
2354	NAD ^b	NAD	192	143	217	181	144	293	270
2494	241	113	192	143	NAD	181	144	293	270
2500	241	113	192	143	217	181	144	293	270
2966	241	113	185	143	217	192	150	299	270
2912	241	113	192	143	217	181	144	293	270
3136	NAD	NAD	185	143	217	192	150	299	270
4395	241	113	192	143	217	181	144	293	270
4927	241	113	195	143	217	NAD	NAD	293	270
5171	241	113	185	143	217	192	150	299	270
5240	241	113	185	143	NAD	192	NAD	299	270
5322	241	113	192	143	217	181	NAD	299	270
5323	241	...	185	143	NAD	192	150	299	270
5326	241	113	185	143	217	192	150	299	270
5415	241	113	192	143	217	181	144	293	270
6868	241	113	185	143	217	192	150	299	270
6878	241	113	185	143	217	192	150	299	270
7025	241	113	185	143	217	192	150	299	270
7033	241	113	185	143	217	192	NAD	299	270
7040	241	113	185	143	217	192	150	299	270
Katy	241	113	192	143	217	NAD	144	293	270

^a Bold indicates that all SSR profiles of the line are identical to the parent Katy.

^b NAD indicates that the datum was not of sufficient quality for analysis.

there was no mutation in the promoter region that impacts the expression of the *Pi-ta* allele in 2354. Taken together, the present data are consistent with the notion that the susceptibility of M2354 is due to a mutation at a locus other than *Pi-ta*.

To determine the inheritance pattern of this mutation, M2354 was genetically fixed through selfing to the M₇ generation and was then crossed with the parent Katy and with Tetep, the donor for *Pi-ta* in Katy (Table 4). In all crosses, F₁ progeny were resistant. A segregation ratio of 3:1 (resistant/susceptible) was observed in F₂ progeny, indicating that mutations in M2354 is controlled by a single, recessive nuclear gene, henceforth tentatively denoted as *ptr(t)*.

***Pi-ta* and *Ptr(t)* confer resistance to the same spectrum of *M. oryzae*.**

Katy containing *Pi-ta*, *Ptr(t)*, and *Pi-k^s* is historically known to be resistant to the common races of *M. oryzae* IA1, IB1, IB45, IB54, IB49, IC17, ID1, IG1, IE1, and IH1 and is susceptible to IE1k. We found that M2354 was susceptible to all the above-mentioned isolates except IB54 (Table 5). IB54 was previously shown to trigger resistance mediated by another blast resistance gene, *Pi-k^s*. This result indicates that the defect in M2354 is related to *Pi-ta* resistance but not to *Pi-k^s* and not to the common component downstream of defense responses. *Pi-ta* is effective in preventing infection by *M. oryzae* races that contain the *AVR-Pita* gene. We then confirmed the existence of the *AVR-Pita* allele, using a pair of *AVR-Pita*-specific PCR primers for avirulent races IB1, IB45, IB1, IB49, IB54, IC17, IG1, IE1, and IH1 and also confirmed that the *AVR-Pita* allele

was not detected in the virulent race IE1k (data not shown). These results suggest that both *Pi-ta* and *Ptr(t)* in Katy confer resistance to a similar spectrum of resistance to the common races of *M. oryzae* in the U.S..

The *Ptr(t)* gene cosegregates with the *Pi-ta* gene.

The resistance of F₁ progeny of the cross of Katy with the susceptible cultivar M202 and the 1:1 resistant/susceptible segregation ratio (with *pi-ta*) among 100 individuals of backcrossing

Table 3. Oligonucleotides used for polymerase chain reaction amplification and sequencing of *Pi-ta* alleles

Name	Position ^a	Sequence
YL62	1510 to 1531	5'-GGGAGACAGCACCATCGGTGA-3'
YL93	2391 to 2371	5'-AAGATCAGATCAGAGCTAGT-3'
KG2	2,341 to 2,360	5'-ATCAGCAACTAACGAGGCAT-3'
F8-5	2,571 to 2,589	5'-TCCTCAGAGGCGATCTCCG-3'
YL68	3,091 to 3,110	5'-GGCCGACGGCGAGCCGAAGC-3'
YL69	3,310 to 3,329	5'-GGATGTTTGGGAGGTTGATC-3'
YL90	3,751 to 3,770	5'-GTATGAAACATGTACTTTTCA-3'
YL71	3,940 to 3,959	5'-AGCAGTAAGTTGTAATTTAT-3'
YL155	4,409 to 4,428	5'-AGCAGGTTATAAGCTAGGCC-3'
YL88	4,931 to 4,950	5'-TACAGGTTCAATTTCTGTTG-3'
YL87	5,431 to 5,450	5'-CTACCAACAAGTTCATCAAA-3'
YL86	5,699 to 5,718	5'-CATACACTTGACTTGTCCTGA-3'
YL80	6,091 to 6,110	5'-CAGGATGACCTTGACACTCT-3'
YL82	6,511 to 6,530	5'-AGAGACTTGGATGAAGATTT-3'
KG4	6,851 to 6,870	5'-GACTAGGAACCACCTTCT-3'

^a Position based on GenBank accession number (AF207842).

Table 2. The 68 simple sequence repeat (SSR) markers assembled to verify the origin of selected Katy mutants^a

Name	Location in Chromosome	Genetic distance	Name	Location in chromosome	Genetic distance
RM1	1	29.7	RM345	6	145.2
RM312	1	71.6	RM481	7	3.2
RM5	1	94.9	RM418	7	42.1
RM315	1	166.3	RM455	7	65.7
RM431	1	178.3	RM234	7	88.2
RM555	2	34.7	RM420	7	115.3
RM452	2	58.2	RM408	8	0
RM475	2	92.5	RM25	8	52.2
RM106	2	123.2	RM310	8	57
RM112	2	166	RM72	8	60.9
RM250	2	170.1	RM210	8	90.3
RM22	3	13	RM149	8	103.7
RM231	3	15.7	RM296	9	0
RM489	3	29.2	RM316	9	1.8
RM7	3	64	RM219	9	11.7
RM232	3	76.7	RM409	9	45.6
RM338	3	108.4	RM215	9	99.9
RM55	3	168.2	RM474	10	0
RM468	3	202.3	RM171	10	73
RM85	3	231	RM228	10	96.3
RM307	4	0	RM484	10	97.3
RM335	4	21.5	RM147	10	99.8
RM471	4	53.8	RM333	10	110.4
RM303	4	116.9	RM120	11	31.7
RM317	4	118.3	RM181	11	54
RM348	4	134.5	RM287	11	68.6
RM153	5	0	RM224	11	120.1
RM413	5	26.7	RM144	11	123.2
RM13	5	31.4	RM19	12	22.9
RM289	5	56.7	RM512	12	43.2
RM164	5	78.7	Pita ^b	12	60
RM421	5	111.2	RM17	12	109.1
RM190	6	7.4			
RM225	6	26.2			
RM541	6	75.5			
RM340	6	133.5			

^a Five mutants M2354, M2494, M2500, M2912, M5415, and parent Katy were genotyped using these additional SSR markers, all markers were selected from the Gramene database.

^b A perfect marker for *Pi-ta* was used (Jia et al. 2004b).

with M202 suggests that *Pi-ta* cosegregate with *Ptr(t)* in the backcross progeny of Katy and M202 (data not shown). To verify the *Ptr(t)* is absent in M202, the plants of F₁ progeny of the cross of M 2354 and M202 were evaluated with a race of *M. oryzae* containing *AVR-Pita*. The susceptibility of F₁ progeny of the cross of M2354 and M202 indicates that the resistant phenotype cannot be restored by the *Ptr* allele in M202. Thus, we suggest that *Ptr(t)* is from Katy and it is defective in M2354.

To determine whether or not *ptr(t)* in M2354 can be complemented by *Ptr(t)* from other susceptible *indica* cultivars, PI 373122 (from Sri Lanka), which is known to express *Pi-ta*, was used for allelism tests. The likelihood was to find no fully resistant progeny, and this expectation was borne out as all 200 F₂ progeny examined were susceptible (data not shown). This result suggests that the *Ptr(t)* allele in PI373122 is also non-functional. To determine the allelism of mutations in all blast-susceptible mutants, a series of crosses was performed among all mutants, the parent Katy, and M202 (Table 6). The susceptibility of the F₁ progeny of the crosses of M2354 with M5415, M2500, M2494, and M2912 and the crosses among all five mutants suggest that they contain allelic *ptr(t)* in the remaining mutants that confer susceptibility.

Genotyping using 10 SSR markers spanning a 10-megabase (MB) region of the *Pi-ta* gene and PCR-based sequence analysis using *Pi-ta* specific primers determined the physical location of *Ptr(t)* in these four putative mutants. As shown in Fig-

Fig. 1. Graphic presentation of the structure and expression of the *Pi-ta* alleles in mutant 2354 and the parent Katy, using reverse transcription-polymerase chain reaction (RT-PCR). **A**, Graphic presentation of the *Pi-ta* gene showing the start codon of the translation (ATG) and the stop codon (TGA). Arrows indicate the locations of indicated primers used for RT-PCR and PCR. **B**, Gel showing the *Pi-ta* alleles in Katy (2) and in 2354 (4); RT-PCR product of the *Pi-ta* allele in Katy (3) and in 2354 (5). The Bench Top 1-kb DNA ladder (Madison, WI, U.S.A.) (1) was used to estimate the size of products of PCR and RT-PCR.

ure 3, a region that is identical to a *japonica* parent, spanning 9 MB of the *Pi-ta*, *Ptr(t)*, and centromere, was found in all mutants except M2354. These results suggest that this 9-MB region in M2354 was from Tetep and was from one of the susceptible parents in other mutants. The identical susceptible *pi-ta* allele to the susceptible parent Lebonnet was identified. These data suggest that *Ptr(t)* cosegregates with *Pi-ta* and that both genes are located within a 9-MB region from Tetep. We conclude that a 9-MB region including susceptible *pi-ta* alleles in M5415, M2500, M2494, and M2912 was inherited from a susceptible parent during the breeding process.

DISCUSSION

We have identified a rice mutant harboring a defect in a critical gene controlling *Pi-ta*-mediated disease resistance. DNA sequences of the coding region of *Pi-ta* and its expression in a blast-susceptible mutant were determined to be identical to that of the resistant parent cultivar. Our data from sequencing and real-time PCR ruled out the possibility that mutations occurred within the *Pi-ta* gene. In the literature, however, most of the mutations identified in resistance pathways in plant species only partially suppress *R* gene-mediated resistance (Innes 1998; Wang et al. 2004). For example, the barley *Rar1* and *Rar2* genes have partial resistance phenotypes in cultivars carrying several powdery mildew *R* genes (Shirasu et al. 1999). The partial loss of resistance suggests that genes required for disease resistance are redundant or were part of branched pathways; thus, loss of any individual gene produces only small changes in resistance. Alternatively, mutations in some signal transduction components may be lethal, since these genes are essential for plant growth and development (Innes 1998). From the present work, it is evident that resistance mediated by the resistance gene *Pi-k^s* is not affected by defects at the *Ptr(t)* locus. *Pi-ta* gene-mediated resistance was completely abolished in a mutant harboring a defect at this new locus, *Ptr(t)*, expressing the *Pi-ta* gene. An additional critical nuclear gene was thus identified controlling *Pi-ta*-mediated resistance.

Fig. 2. Real-time polymerase chain reaction analysis of expression dynamics of the *Pi-ta* alleles in Katy and mutant 2354 at different timepoints after inoculation. x axis = hours after inoculation of Katy and mutant with *M. oryzae* containing *AVR-Pita*; y axis = relative expression (fold changes). Similar results were obtained in three separate trials.

Table 4. Phenotypes of segregating F₂ progeny from crosses between M2354 and resistant parents^a

Cross	F ₂		χ^2 ^b	P
	Resistant	Susceptible		
2354/Katy	189	64	0.021	0.891
2354/Tetep	324	106	0.0494	0.760

^a All F₂ plants of each cross was inoculated with IB49 (ZN61) at conidial concentration of 1.5×10^6 conidia per milliliter in 150 ml.

^b A ratio of 3:1 was tested. χ^2 = chi square and P = probability.

Although we were unable to determine the level of the *Pi-ta* protein in vivo, data from RT-PCR and real time RT-PCR indicated that *Pi-ta* was expressed at similar levels in comparison to the original resistant parent after pathogen inoculation. Our data further support a previous observation that expression of *Pi-ta* is constitutive (Bryan et al. 2000). Constitutive expression and no induction after pathogen inoculation have been observed as a common feature of blast *R* genes molecularly characterized thus far (Bryan et al. 2000; Chen et al. 2006; Liu et al. 2007; Qu et al. 2006).

Physical binding of the *Pi-ta* protein to AVR-Pita during resistance responses suggests that *Pi-ta* is a functional receptor for AVR-Pita (Jia et al. 2000). Differential responses to *M. oryzae* races could be due to another factor that distinguishes *Pi-ta* from *Pi-ta*². In the most common races of *M. oryzae* in the U.S., highly similar AVR-Pita proteins were identified with only minor differences found among all avirulent races. However, significant structural alterations within AVR-Pita alleles have been identified in virulent and race-shift isolates, suggesting that the AVR-Pita alleles in U.S. pathotypes trigger resistance mediated by *Pi-ta*/*Pi-ta*². Using a race of *M. oryzae* specifically recognizes the *Pi-ta*² gene; it was previously determined that Katy also contains *Pi-ta*² (Bryan et al. 2000; Jia

et al. 2003). It is known historically that *Pi-ta*²-containing rice cultivars exhibit a broader spectrum of resistance to different races of *M. oryzae* than those containing *Pi-ta*. Sequence analysis of selected *Pi-ta*²-containing accessions identified an

Table 6. Allelism tests of Katy mutants, their F₁ phenotypes, and the confirmation of crosses using a codominant marker for *Pi-ta*

Cross	No. of F ₁ plants	Disease reaction ^a	Confirmation ^b
2354/2494	5	S	+
2354/2500	5	S	+
2354/5415	1	S	+
5415/2500	1	S	–
2912/2354	5	S	+
2912/2494	4	S	–
2912/2500	4	S	–
2912/5415	5	S	–

^a Disease reaction was determined using isolate ZN59 (race IC-17). R indicates resistance and S indicates susceptible.

^b A codominant marker for *Pi-ta* (Jia et al., 2004b) was used to confirm the true hybrids if both parents were susceptible. + indicates the true hybrids were confirmed, and – indicates the marker for *Pi-ta* was not useful for confirming the true hybrids due to lacking polymorphism of *pi-ta*.

Table 5. Disease reactions of Katy, Katy mutants, and M202 to the common *Magnaporthe oryzae* races

Name	<i>M. oryzae</i> race ^a								
	IB1	IB49	IB54 ^b	IB45	IH1	IG1	IC17	IE1	IE1K
Katy	R	R	R	R	R	R	R	R	S
FN2354	S	S	R	S	S	S	S	S	S
FN2494	S	S	R	S	S	S	S	S	S
FN2500	S	S	R	S	S	S	S	S	S
FN2912	S	S	R	S	S	S	S	S	S
FN5415	S	S	R	S	S	S	S	S	S
M202	S	S	S	S	S	S	S	S	S

^a *M. oryzae* races (isolates) are IB1 (ZN15), IB49 (ZN 52), IB45 (unknown), IH1(unknown), IG1 (ZN39), IC17 (ZN57), IE1 (ZN4), and IE1K (ZN19).

^b Race IB54 detects the presence of the *Pi-k*⁵ gene.

Fig. 3. Disease reactions and fine mapping of the *Pi-ta*/*Ptr(t)* region in rice mutants and parents. R = resistant, S = susceptible. Note: Simple sequence repeat (SSR) marker RM519 was not drawn in proportion to other markers. The SSR markers were obtained from the Gramene website.

identical resistant *Pi-ta* allele, suggesting that *Pi-ta* is required for the resistance function of *Pi-ta*² (Jia et al. 2003). In the present study, resistance was abolished even when *Pi-ta* was expressed. The *Pi-ta* protein in Katy is identical to that of Yashiro-mochi, in which *Pi-ta* was originally cloned. If Katy contains both *Pi-ta* and *Pi-ta*², a question arises as to whether *Ptr(t)* is *Pi-ta*. If so, then the *Pi-ta* gene may also be *Pi-ta*². A more straightforward explanation for this is that *Ptr(t)* is required for both the *Pi-ta* and *Pi-ta*² genes and *Ptr(t)* is a defect in mutant 2354. Further analysis of DNA sequences of the 9-MB region spanning the *Pi-ta* gene in both Katy and 2354 will yield more insight into this possibility.

In our studies, we were unable to separate the *Pi-ta* gene from *Ptr(t)* in a series of crosses, including the use of 2,000 F₂ progeny from a segregating population derived from a cross of *Pi-ta/Ptr(t)* with *pi-ta/ptr(t)* homozygotes (Y. Jia, unpublished data). Since the *Pi-ta* gene is 1.39 MB away from the centromere of chromosome 12, it is highly possible that recombination suppression occurs at this region. Suppression of recombination around centromeres has been well documented in a wide range of eukaryotes (Anderson et al. 2003; Beadle 1932; Lambie and Roeder 1986; Mather 1993). Analysis of DNA markers located between the *Pi-ta* locus and the centromere will help to determine the DNA sequence of *Ptr(t)*. Using SSR markers, a block within the 9-MB genomic fragment surrounding to the *Pi-ta* region was determined to be inherited in Katy. Genotypic analysis of a core collection (140 accessions) containing *Pi-ta* revealed a 2-MB block surrounding *Pi-ta* and *Ptr(t)* that are of *indica* origin, thus delimiting the physical location of the *Ptr(t)* gene within a 2-MB region between *Pi-ta* and the centromere. Searches of publicly available databases identified a NBS-LRR candidate gene for *Ptr(t)* positioned approximately 12 kb from *Pi-ta* (Y. Jia, unpublished data). Nevertheless, because of suppressed recombination within this region, specific markers for the *Pi-ta* gene should be effective for selecting both *Pi-ta*- and *Ptr(t)*-associated resistance (Jia et al. 2002, 2004; Wang et al. 2007).

Four mutants identified in this study contain a genomic region from a susceptible parent, suggesting the cultivar is homozygous at all loci, with minor differences resulting from recombination occurring during the breeding process. This is not surprising, given that, by the time a line is established as a cultivar, it is typically in the F₈ generation or later, by which time only a small amount of residual heterozygosity exists. However, it appears from the DNA sequence and genotyping data that small genetic differences resulting from crossover events have been maintained at an extremely low frequency (four out of 5,781 individuals). Although the possibility that mutations in other regions may have occurred in these four putative mutants cannot be ruled out, a more compelling possibility is that the 9-MB genomic region was inherited from a susceptible parent.

The identification of *Ptr(t)* represents a significant advance in the understanding of *Pi-ta*-mediated disease resistance. *Pi-ta* confers resistance to *M. oryzae* races expressing *AVR-Pita*, and *Ptr(t)* also confers resistance to these races, suggesting that either these races contain another avirulence gene or *Pi-ta* and *Ptr(t)* both confer resistance to the same races of *M. oryzae* expressing *AVR-Pita*. The presence of a *Pi-ta* allele of the same size and the similar expression levels of *Pi-ta* in mutant 2354 in comparison with the parent Katy indicate that the loss of resistance is independent of the expression of the *Pi-ta* gene. Further molecular and genetic analysis of *Pi-ta* and *Ptr(t)* should lead to a better understanding of pathogen recognition mediated by the *Pi-ta/Ptr(t)* pathway and should assist in the genetic engineering of disease resistance in rice and other crop plants.

MATERIALS AND METHODS

Plant materials.

Katy containing *Pi-ta*, *Pi-ta*², and *Pi-k*^s was found to be resistant to the common *M. oryzae* U.S. races IB1 (ZN15), IB49 (ZN 52), IB 45 (unknown), IH1 (unknown), IG1 (ZN39), IE1 (ZN13), IC17 (ZN57), IE1 (ZN4), and IE1K (ZN19) (Moldenhauer et al. 1990; Y. Jia, unpublished data). Genetic analysis suggests *Pi-ta* in Katy confers resistance to the major races IB49 and IC17 in the U.S. (Jia et al. 2004). M202 is susceptible to all of the above races and was used as a parent for genetic analysis (Wang et al. 2007). A susceptible *indica* cultivar, PI 373122, from Sri Lanka expressing *Pi-ta* (X. Wang and Y. Jia, unpublished data) was used for allelism tests to verify that *Pi-ta* cosegregates with *Ptr(t)*.

Fast neutron mutagenesis and mutant screening.

Our approach to studying the interaction between rice and *M. oryzae* was to isolate rice mutants that had lost resistance to *M. oryzae*. Seeds of the cultivar Katy (*Pi-ta/Pi-ta/Pi-ta*²/*Pi-ta*², *Pi-k*^s/*Pi-k*^s) were treated with 26.3 Gy fast neutron irradiation at the Oak Ridge Laboratory (Oak Ridge, TN, U.S.A.). The M₁ plants were field-grown in Stuttgart (AR, U.S.A.). A total of 5,781 M₁ panicles were recovered, and M₂ seed families from individual M₁ plants were kept separate to facilitate screening and to ensure that mutants isolated from different M₂ seed lots were independent. M₂ plants were screened for disease susceptibility in the greenhouse. For blast screening, seven to ten seeds were grown in the greenhouse to three- to four-leaf stage before inoculation with a *M. oryzae* conidial suspension as described below. A total of 42 susceptible mutants were recovered from 30,000 M₂ seedlings, and the susceptibility was maintained in only 19 of these mutants in M₃.

Crossing and determination of hybrids.

Standard methods of crossing were used for all genetic crosses. The susceptible parents were used as females for allelism tests. If F₁ progeny were resistant, the crosses were confirmed to be true crosses, because *M. oryzae* races containing *AVR-Pita* were used for inoculation. If F₁ progeny were susceptible, verification of the hybrids was performed using a codominant marker for *Pi-ta* (Jia et al. 2004b).

Fungal isolates, culture, inoculation suspension, pathogenicity assays, and identification of the *AVR-Pita* allele.

M. oryzae races (isolates), IB1 (ZN15), IB49 (ZN 52), IB 45, IH1, IG1 (ZN39), IE1 (ZN 13), IC17 (ZN57), IE1 (ZN4), and IE1K (ZN19), were used to inoculate rice plants in this study (Correll et al. 2000). By scraping the fungal mycelia on culture plates, *M. oryzae* conidia were harvested in a sterile 0.25% gelatin solution; this conidial solution was then filtered through eight sheets of cheesecloth to remove mycelia and pieces of agar. The number of conidia per milliliter was determined by using a hemacytometer chamber. Plants were grown to the three- to four-leaf stage for inoculation and for DNA and RNA preparation. Inoculation was performed using an artist airbrush as described by Valent and associates (1991). Leaves were inoculated with layers of conidial suspensions using 10⁶ and 5 × 10⁶ conidia per milliliter. After inoculation, the plants were placed in plastic bags and were incubated at room temperature in low light for 24 h. Leaf-segment samples were harvested at 2, 6, 16, and 24 h. Each leaf segment, approximately 5 cm in length, was detached from the marked leaf and was rapidly freeze-dried. After removing the samples, the remaining plants were removed from the plastic bags, were returned to the greenhouse, and were maintained at a temperature of 24

to 32°C with a light and dark cycle of 16 and 8 h, respectively. Disease development was monitored daily until disease symptoms were observed (5 to 7 days).

The identification of the *AVR-Pita* allele in races of *M. oryzae* was performed by PCR using a pair of *AVR-Pita*-specific primers (YL149 and YL169), using a procedure described by Zhou and associates (2007). The resulting PCR product at 1 kb indicates that the *AVR-Pita* allele is present in this race, and the absence of PCR product indicates that the *AVR-Pita* allele is absent in this race.

SSR analysis.

SSR analysis was used to determine the origin of susceptible mutants since M₀ was grown in rice fields where volunteer seeds are common. Information about the primer sequence and map position of these SSR markers was obtained from the Gramene database. Based on high peak values, nine SSR were developed for initial screening of the genotypes of mutants, and a panel of additional markers consisting of 67 SSR and one codominant marker for *Pi-ta* were assembled for final confirmation of the genotypes of mutants. Ten more SSR spanning a 10-MB region of the *Pi-ta* gene were developed for fine mapping. DNA was prepared from fresh leaves or leaves stored at -80°C, using a Qiagen kit (Qiagen Sciences, Germantown, MD, U.S.A.) following the manufacturer's protocol. PCR amplification of the markers were performed in 25-μl reaction volumes consisting of 20 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 300 nM of each primer, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.). For each marker, forward primers labeled with fluorescent dyes (6FAM, NED, and Hex) were purchased from Applied Biosystems (Foster City, CA, U.S.A.) or Integrated DNA Technologies (Coralville, IA, U.S.A.). DNA amplifications were performed with MJ Research Tetrad thermocyclers (Waltham, MA, U.S.A.) under the following PCR conditions: i) initial denaturation at 94°C for 5 min; ii) 35 cycles of 94°C for 1 min, 55°C to 67°C (marker dependent) for 1 min, 72°C for 2 min; and iii) 5 min final extension at 72°C. PCR products were pooled based on color and size range of amplified fragments (typically three markers per run along with a ROX-labeled size standard), and the DNA was denatured by heating at 94°C for 5 min. The samples were run on an ABI Prism 3700 DNA analyzer according to the manufacturer's instructions (Applied Biosystems). SSR fragment sizing was performed using the software GenSan Version 3.7NT (Applied Biosystems) and Genotyper Version 3.7NT (Applied Biosystems). Alleles were binned manually.

DNA sequence analysis.

DNA sequence analysis was used to determine DNA sequences of the *Pi-ta* alleles in mutants. Extractions of plant genomic DNA from Katy, 2354, 2912, 2494, 5415, 2500, and M202 were prepared from leaf tissues of greenhouse-grown plants using a DNeasy plant mini kit (Qiagen Sciences, Germantown, MD, U.S.A.). Primers KG2 and KG4 (sequences and positions in the *Pi-ta* gene are listed in Table 3) were designed based on the *Pi-ta* sequence from GenBank (accession number AF207842) and were used to amplify corresponding *Pi-ta* alleles. Amplification of 4,530-bp fragments was achieved using an Advantage genomic polymerase kit (Clontech, Mountain View, CA, U.S.A.). The reaction mixture for PCR consisted of 1× Advantage genomic polymerase buffer, 1.1 mM MgCl₂, 0.2 mM dNTP, 0.2 μM primers (KG2 and KG4), 0.5 ng of DNA template per microliter, 1× Advantage genomic polymerase mix, and PCR grade water to a final volume of 50 μl. Amplification was performed at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, 53°C for 30 s, 70°C for

5 min, and 1 cycle at 70°C for 5 min. Amplified fragments were cloned into the pGEM-T Easy vector (Promega) for sequencing. DNA sequencing was performed using an ABI 3700 automated DNA sequence in the genomic center at the University of Arkansas Medical Center (Little Rock, AR, U.S.A.). Primers and locations in the *Pi-ta* genes for amplifying the *Pi-ta* fragments and sequencing the *Pi-ta* alleles from mutants are listed in Table 3. DNA sequences of the *Pi-ta* alleles were verified by DNA sequences of PCR products of each overlapping fragment of the *Pi-ta* alleles in each mutant. Sequence analysis was performed using Vector NTI Advantage 10 software (Invitrogen, Carlsbad, CA, U.S.A.).

RT-PCR and real time RT-PCR.

RT-PCR was initially used to analyze the expression of the *Pi-ta* allele in mutants and the parent. Total RNA was isolated from leaf tissues using an RNeasy plant mini kit (Qiagen Sciences). RT-PCR was performed to examine the expression of *Pi-ta* in Katy and 2354. To avoid genomic DNA interference, the primers YL 69 and YL 88 amplify DNA sequences from each side of the intron in the *Pi-ta* gene. The SuperScript one-step RT-PCR with platinum *Taq* kit (Invitrogen) was used with primers YL69 and YL88 (Table 3) to generate a 178-bp product from the coding region. Reactions (total volume of 50 μl) included: 1× reaction buffer, 0.2 mM dNTP, 0.2 μM primers, 2.5 ng of total RNA per microliter, 1 μl RT/platinum *Taq* mix, and 21 μl of PCR-grade water. RT-PCR was performed with a thermal cycler using 1 cycle at 45°C for 30 min, 1 cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 52°C for 30 s, 68°C for 1 min with a final extension at 72°C for 5 min. The resulting products were subjected to electrophoresis using a 1% agarose gel and were photographed using a Chemi-Doc imaging system (BioRad, Hercules, CA, U.S.A.). All results were repeated in three independent trials.

Real time RT-PCR analysis was used to determine the expression dynamics of *Pi-ta* in Katy and mutant 2354 at different timepoints after infection. New primers (5'-ATCGACTCC TTGAACTATCC-3' and 5'-AGTATTCTACTGCAACTATTA TTATC-3') and hydrolysis probes were designed from opposite exons of *Pi-ta* (GenBank accession number AF 207842). Total RNA was prepared from leaf tissues of infected rice plants at the four-leaf stage at 6, 10, 16, 24, and 48 h post-inoculation, using an RNeasy plant mini kit (Qiagen Sciences). Real-time PCR was performed, using an iScript one-step RT-PCR kit for probes (Bio-Rad) according to the manufacturer's instructions. Reaction conditions were 50°C for 10 min, 95°C for 5 min, 95°C for 10 s, 51°C for 30 s. Relative quantification of gene expression was performed using the comparative cycle threshold method. 18S ribosomal RNA was used as an internal control. All results were repeated in three independent trials.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Gramene database: www.gramene.org