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Diversification and evolution of the avirulence gene *AVR-Pita1* in field isolates of *Magnaporthe oryzae* ☆

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ABSTRACT

Rice blast disease is the single most destructive plant disease that threatens stable rice production worldwide. Race-specific resistance to the rice blast pathogen has not been durable and the mechanism by which the resistance is overcome remains largely unknown. Here we report the molecular mechanisms of diversification and the instability of the avirulence gene *AVR-Pita1* in field strains of *Magnaporthe oryzae* interacting with the host resistance gene *Pi-ta* and triggering race-specific resistance. Two-base-pair insertions resulting in frame-shift mutations and partial and complete deletions of *AVR-Pita1* were identified in virulent isolates. Moreover, a total of 38 *AVR-Pita1* haplotypes encoding 27 AVR-Pita1 variants were identified among 151 avirulent isolates. Most DNA sequence variation was found to occur in the exon region resulting in amino acid substitution. These findings demonstrate that *AVR-Pita1* is under positive selection and mutations of *AVR-Pita1* are responsible for defeating race-specific resistance in nature.

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1. Introduction

Race-specific plant resistance (*R*) genes have evolved to detect the products of the corresponding avirulence (*AVR*) genes and trigger effective resistance to plant pathogens. In nature, the effector molecules encoded by *AVR* genes were thought to facilitate disease development and were hypothesized to be under selection. It is theorized that new virulent races of the pathogen emerged by genetic modification of *AVR* genes using diverse mechanisms. Point mutation (Joosten et al., 1994), deletion (Schurch et al., 2004; Dodds et al., 2006), and frame-shift (Ridout et al., 2006) of *AVR* genes have been documented in strains of several pathogens. Further analysis of the *AVR* genes in naturally-occurring field isolates of a plant pathogen may yield valuable information for the deployment of *R* genes in field crops (Stukenbrock and McDonald, 2009).

Rice blast disease, caused by the ascomycete fungal pathogen *Magnaporthe oryzae* [formerly *Magnaporthe grisea* (Hebert) Barr], is one of the most devastating plant diseases worldwide. The disease has been managed using both major and minor resistance genes integrated with effective cultural practices. The major *R* genes, or *Pi*-genes, for *M. oryzae* in rice are able to prevent the infection of races of *M. oryzae* that contain a corresponding avirulence (*AVR*) gene (Silue et al., 1992). To date, more than 80 *Pi* genes have been reported from rice and some of them have been used to control blast disease (Ballini et al., 2008). Among these *Pi* genes, eleven have been cloned including *Pi-ta* (Bryan et al., 2000), *Pi-b* (Miyamoto et al., 1996; Wang et al., 1999), *Pi-2*/*Pi-zt* (Zhou et al., 2006), *Pi-d2* (Chen et al., 2006), *Pi-9* (Qu et al., 2006), *Pi-36* (Liu et al., 2007), *Pi-37* (Lin et al., 2007), *Pikm* (Ashikawa et al., 2008), *Pi-d3* (Shang et al., 2009), *Pi-5* (Lee et al., 2009b), and *Pi-t* (Hayashi and Yoshida, 2009). These cloned genes encode putative cytoplasmic NBS–LRR proteins, with the exception of the product of *Pi-d2*, which is a putative transmembrane B-Lectin-TM-Kinase (Chen et al., 2006).

The fungus *M. oryzae* is highly variable and often can overcome the deployed resistant cultivars in a short period of time when resistance is dependent on one major *R* gene. The ability to defeat the *R* gene has been hypothesized to be due to the instability of *AVR* in *M. oryzae* (Khang et al., 2008). Thus far, 25 *AVR* genes in *M. oryzae* have been genetically mapped (Dioh et al., 2000), nine of which were recently cloned: *AVR-Pita* (Orbach et al., 2000), *AVR1-CO39* (Farman and Leong, 1998), *PWL1* (Kang et al., 1995) *PWL2* (Sweigard, 1995), *ACE1* (Fudal et al., 2005), *AVR-Pizt* (Li et al., 2009), *AVR-Pia*, *AVR-Pii*, and *AVR-Pik/km/kp* (Yoshida et al., 2009). The *AVR* genes in *M. oryzae* are highly diversified and are predicted to be capable of rapid changes in nature (Jia et al., 2000). Farman et al. (2002) reported that three types of diversification occurred at the *AVR1-CO39* locus in *M. oryzae*. The G types, including G1

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and G2, were characterized by an approximate deletion of 20 kb, while the J type was characterized by a deletion of the 5′ half of *AVR1-CO39* in addition to point mutations. Meanwhile, the insertion of a 1.9 kb MINE retrotransposon in the last exon of *ACE1* in a virulent isolate was hypothesized to be responsible for conversion from avirulence to virulence (Fudal et al., 2005). Virulent isolates were found to exhibit either a Pot3 insertion in the promoter region or single nucleotide substitution resulting in an amino acid change in *AVR-Pizt* (Li et al., 2009). Transposon and telomeric sequences in the genomic regions neighboring *AVR-Pia* and *AVR-Pii* lead to the enhanced likelihood of gene loss and horizontal transfer (Silva et al., 2004; Rehmeyer et al., 2006).

The processed *AVR-Pita* protein from the rice blast fungus was demonstrated to interact directly with the translated product of the host *R* gene *Pi-ta* in rice triggering resistance (Bryan et al., 2000; Jia et al., 2000). The *AVR-Pita* alleles in isolates of the *M. grisea* species complex, including *M. oryzae*, were reported to be a member of a gene family which is comprised of one to three members, and as a result, *AVR-Pita* from *M. oryzae* was renamed *AVR-Pita1* (Khang et al., 2008). Many *Pi-ta*-containing cultivars have been effectively deployed to control rice blast disease worldwide. However, epidemics of rice blast disease have occurred on *Pi-ta*-containing cultivars, suggesting that the *Pi-ta* gene has been defeated (Lee et al., 2005). Evidence of the structural variation of *AVR-Pita1* in laboratory strains that alters *Pi-ta* recognition specificity has been documented. Point mutations, insertions, and deletions of *AVR-Pita1* detected in some lab strains rendered the fungus able to avoid triggering resistance responses mediated by *Pi-ta* (Orbach et al., 2000). Specific alteration of the active site from glutamic acid to aspartic acid (E177D) and substitution of methionine to tryptophan (M178 W) in the putative protease motif of the strain 4360-R-62 resulted in the loss of avirulence (Jia et al., 2000). Sequence analysis of the virulent strain CP1632 identified a Pot3 transposon in the promoter region of *AVR-Pita1*, suggesting that transposition is another mechanism for altering the avirulence of the *AVR-Pita1* gene (Kang et al., 2001). Deletions and a 31 bp duplication of the functional copy of *AVR-Pita1* were identified in the derived mutants of a single isolate (Takahashi et al., 2010). To date, the documented *AVR-Pita1* mutation identified from field isolates of *M. oryzae* from the US has been the Pot3 transposon insertion (Zhou et al., 2007). Whereas in field isolates from Japan, a deletion of functional copies and base substitutions have occurred (Takahashi et al., 2010).

In order to obtain a comprehensive understanding of the structural and functional variation of the *AVR-Pita1* gene in *M. oryzae*, we investigated a collection of field isolates of the pathogen from major rice-producing areas around the globe (Fig. 1a). Our findings support that a functional *AVR-Pita1* possesses diversified sequence structures and is under positive selection pressure in nature. We also demonstrate that frame-shift and deletions of *AVR-Pita1* are responsible for the loss of the gene's avirulence, which eventually renders the corresponding field isolates capable of avoiding the deployed *Pi-ta* gene in rice cultivars, resulting in disease. These results are consistent with previous findings in laboratory strains, and shed light on the molecular mechanisms of *AVR-Pita1* diversification in nature.

2. Materials and methods

2.1. Rice cultivars, fungal isolates, culture, and pathogenicity assays

Two rice cultivars (Katy and Drew) containing the *R* gene *Pi-ta*, and one (M202) lacking *Pi-ta* were used for the pathogenicity assays for the US isolates (Fig. 1b). The cultivars Tetep and K1, which contain *Pi-ta*, were used for pathogenicity assays for some of the non US isolates. An international set of differential rice cultivars was used for race determination and included Raminad Str #3, Zenith, NP-125, Usen, Dular, Kanto 51, Sha-tiao-tsao and Caloro. A collection of 187 isolates was examined (Supplemental Table S1). All isolates were stored at −20 °C on desiccated filter paper and were grown at room temperature under blue and white fluorescence lighting on plates containing oatmeal agar for producing conidial inoculum. Isolates from the US were evaluated on Katy, Drew, and M202; isolates from China were evaluated on Tetep; isolates from Colombia were evaluated on K1. Standard pathogenicity assays were performed as previously described (Valent et al., 1991). The race identification and classification method followed the methods of Ling and Ou (1969). Disease reactions were recorded 7 days post-inoculation using a 5-scale rating system (0–2: resistance; 3–5: susceptible). Each experiment was repeated three times.

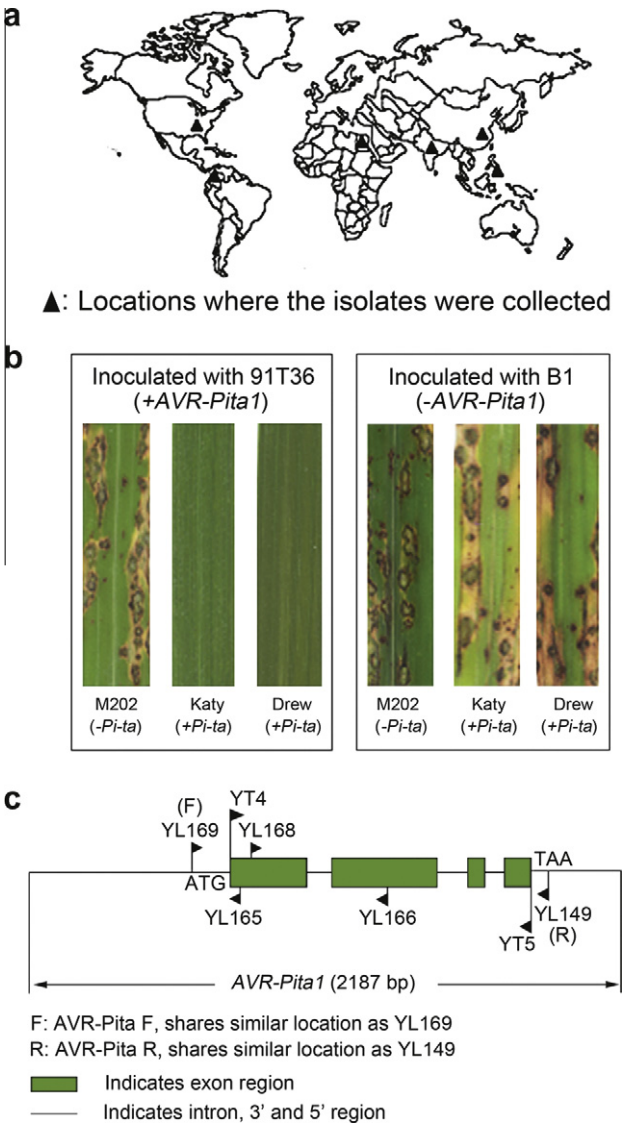

Fig. 1. Isolate collection and identification. (a) A world map showing the collection sites of *M. oryzae* isolates used in this study. The 187 isolates were collected from rice-producing countries. The US, China, Colombia, India, Egypt and the Philippines. (b) Disease reaction of rice cultivars to the US isolates of *M. oryzae*. (c) A graphic presentation of the *AVR-Pita1* allele showing the location of primers used in this study.

2.2. DNA preparation, PCR amplification, DNA sequencing and Southern blot analysis

Each isolate was grown in complete medium broth at 24 °C for approximately 7 days to produce mycelium. DNA of *M. oryzae* was