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Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence

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Abstract

The avirulence gene *AVR-Pita* of *Magnaporthe oryzae* determines the efficacy of the resistance gene *Pi-ta* in rice. The structures of the *AVR-Pita* alleles in 39 US isolates of *M. oryzae* were analyzed using polymerase chain reaction. A series of allele-specific primers were developed from the *AVR-Pita* gene to examine the presence of *AVR-Pita*. Orthologous alleles of the *AVR-Pita* gene were amplified from avirulent isolates. Sequence analysis of five alleles revealed three introns at identical positions in the *AVR-Pita* gene. All five alleles were predicted to encode metalloprotease proteins highly similar to the AVR-Pita protein. In contrast, the same regions of the *AVR-Pita* alleles were not amplified in the most virulent isolates, and significant variations of DNA sequence at the *AVR-Pita* allele were verified by Southern blot analysis. A Pot3 transposon was identified in the DNA region encoding the putative protease motif of the AVR-Pita protein from a field isolate B2 collected from a *Pi-ta*-containing cultivar Banks. These findings show that transposons can contribute to instability of *AVR-Pita* and is one molecular mechanism for defeating resistance genes in rice cultivar Banks.
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1. Introduction

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) (Couch and Kohn, 2002; Kato et al., 2000), is one of the most economically devastating diseases worldwide. Resistance (*R*) genes have been identified and incorporated into rice cultivars for managing rice blast disease throughout the world. Resistance conditioned by a single major *R* gene is typically effective in preventing infection by races of *M. oryzae* containing the corresponding avirulence (*AVR*) gene (Silué et al., 1992). To date, forty *R* genes for blast resistance have been described (Chauhan et al., 2002), and some of them, such as *Pi-b*, *Pi-ta, Pi-d2* and *Pi9*, have been cloned (Bryan et al., 2000; Chen et al., 2006; Qu et al., 2006; Wang et al., 1999). The *Pi-b* and *Pi9* genes encode putative NBS-LRR proteins, respectively (Qu et al., 2006; Wang et al., 1999). *Pi-d2* encodes a receptor-like kinase protein with a predicted extracellular domain of a bulb-type mannose specific binding lectin (B-lectin) and an intracellular serine–threonine kinase domain (Chen et al., 2006). The *Pi-ta* gene encodes a protein with a NBS and leucine rich domain (LRD) that is located near the centromere of rice chromosome 12 a region that is typically stable in the rice genome (Bryan et al., 2000; Chen et al., 2002). In the southern US, *Pi-ta*-based resistant cultivars including Katy, Drew, Kaybonnet, Madison, Cybonnet, Ahrent and Banks have been widely utilized to control the disease since the release of the first *Pi-ta*-containing cultivar Katy in 1990 (Jia et al., 2004b; Moldenhauer et al., 1990).

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Control of blast disease via conventional breeding has achieved only short-term success due to the frequent breakdown of resistance under field conditions. Frequent appearance of new races (or pathotypes) of the fungus that are virulent to previously resistant cultivars has been proposed as the principal cause for the loss of resistance. *M. oryzae* is known to reproduce asexually in nature and instability of *AVR* genes was then proposed to be an adaptive advantage for the pathogen (Valent, 1997). To date, 25 *AVR* genes have been described (Dioh et al., 2000), and five of them [*PWL1* (Kang et al., 1995), *PWL2* (Sweigard et al., 1995), *AVR1-CO39* (Farman and Leong, 1998), *AVR-Pita* (*AVR2-YAMO*) (Orbach et al., 2000) and *ACE1* (Böhnert et al., 2004)] have been molecularly characterized. The *AVR-Pita* gene is located in the telomeric region of chromosome 3 in *M. oryzae,* and encodes a putative neutral zinc metalloprotease (Orbach et al., 2000). *Pi-ta* and *AVR-Pita* are the first matched pair of *R*/*AVR* genes cloned in the rice blast system (Bryan et al., 2000; Orbach et al., 2000), and their interactions, genetics and variations have been studied intensively (Jia et al., 2000, 2002, 2003, 2004a,b, 2005; Kang et al., 2001).

The *Pi-ta* resistance gene has been effective in managing rice blast in southeast production areas of the US since its release in 1990 (Moldenhauer et al., 1990). Although field and greenhouse isolates of *M. oryzae* have been recovered that have been virulent on *Pi-ta*-containing cultivars since the mid 1990's (Correll et al., 2000a), no blast epidemics had been observed on any *Pi-ta*-containing cultivars in commercial production fields. However, in 2004, epidemics were observed on Banks in several fields in several different counties in Arkansas, USA (Lee et al., 2005). Banks contained *Pi-ta* and was released to Arkansas seed growers in 2004 (Moldenhauer et al., 2004; Lee et al., 2005). The objectives of this study were to develop *AVR-Pita*-specific primers in order to monitor the presence of *AVR-Pita*, to analyze the structures of *AVR-Pita* alleles in virulent isolates of *M. oryzae*, and to explain how isolates of *M. oryzae* overcame the *Pi-ta*-based resistance used in cultivars grown throughout the southeastern rice production areas of the US.

2. Materials and methods

2.1. Fungal isolates, culture and pathogenicity assays

A total of 39 isolates of *M. oryzae* were used in the study (Table 1). The isolates include wild-type field isolates (WT); race-shift isolates (RS) which were virulent isolates on *Pi-ta*-containing cultivars that were originally recovered from the susceptible lesions on Katy after inoculated with a parental isolate which was avirulent on Katy (Correll et al., 2000a), and nitrate (nit) and sulfate (sul) non-utilizing mutants that were used as marked strains to confirm that the race-shift isolate originated from the avirulent parental isolate (Correll et al., 2000b). Isolate FL9 was recovered as a race-shift isolate from cultivar Katy grown in greenhouse. Isolates 1188R and 1188S as well as the isolates with a "B" prefix were collected from the cultivar Banks from several counties in Arkansas during severe rice blast epidemics in commercial fields in 2004. All isolates were stored at −20 °C on desiccated filter papers, and grown on plates containing oatmeal agar for production of conidial inocula at room temperature under blue and white fluorescence lighting, or grown in complete medium broth at 24 °C for producing mycelia to be used for DNA preparation.

The virulence of isolates of *M. oryzae* was evaluated on the *Pi-ta*-containing cultivars Katy and Drew as well as the non-*Pi-ta*-containing cultivars C101A51 and M202. Standard pathogenicity assays were performed as previously described (Xia et al., 2000; Valent et al., 1991a). Conidial concentration was measured with a hemacytometer, and adjusted to 2×10^5 conidia per milliliter. Rice seedlings at 4-leaf stage were inoculated with 15 ml of conidial suspension five plants per pot (6 cm diameter) using an airbrush sprayer. After inoculation, the seedlings were incubated in a dark dew-chamber at 21 °C for 24 h, and then returned to the greenhouse. Disease reactions were scored 7 days post-inoculation on either a 0–5 (Valent et al., 1991a) or a 0–9 (Xia et al., 2000) rating scale. A mean disease reaction of either 0–2 or 0–3 was considered a resistant reaction on the 0–5 or 0–9 scale, respectively, whereas a 3–5 or 4–9 disease reaction was considered susceptible.

2.2. DNA extraction, PCR, rep-PCR amplification, cloning and sequence analysis

DNA of *M. oryzae* was isolated from frozen mycelia using a Qiagen DNeasy® Plant Mini Kit according to a protocol recommended by the manufacturer (Qiagen Inc., Valencia, CA, USA). Three forward and five reverse primers from different regions of the *AVR-Pita* gene were designed and synthesized based on the genomic DNA sequence of *AVR-Pita* (GenBank Accession No. AF207841) (Orbach et al., 2000) (Fig. 1 and Table 2). With these forward and reverse primers, six pairs of specific primers were used to detect the existence of *AVR-Pita* (Table 3). To detect the genetic diversity in *M. oryzae* race-shift isolates, another primer pair (Pot2-1 and Pot2-2) was designed and synthesized based on an inverted repeat of transposon in *M. oryzae* for the repetitive element-based PCR (rep-PCR) experiment (Table 2, George et al., 1998; Kachroo et al., 1994). All PCRs were performed using *Taq* PCR Master Mix (Qiagen Inc., Valencia, CA, USA). Each PCR consisted of the following components: 10 μl of *Taq* PCR Master Mix (contains 5 U of *Taq* DNA polymerase, 2× Qiagen PCR buffer, 3 mM $MgCl_2$ and 400 μM of each dNTP), 1 μl of each 10 μM primer, 10 ng of fungal genomic DNA and distilled water (provided by Qiagen Kit) in a final reaction volume of 20 μl. Reactions were performed in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA, USA) with the following PCR program: 1 cycle at 95 °C for 3 min for initial denaturation, 29 cycles at 95 °C for 30 s, 52–60 °C