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Characterization of rice blast resistance genes in rice germplasm with monogenic lines and pathogenicity assays

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ABSTRACT

Resistance (*R*) genes have been effectively deployed in preventing rice crop losses due to the fungus *Magnaporthe oryzae*. In the present study, we studied the interaction between 24 monogenetic lines, carrying at least one major *R* gene, *Pia*, *Pib*, *Pii*, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, *Pik-s*, *Pish*, *Pit*, *Pita*, *Pita-2*, *Piz*, *Piz-t*, *Pi1*, *Piz-5*, *Pi3*, *Pi5*(t), *Pi7*(t), *Pi9*, *Pi11*(t), *Pi12*(t), *Pi19*, or *Pi20*, with 14 commonly found races (isolates) of *M. oryzae*. We demonstrated that the monogenic lines carrying *Pi9* and *Pita-2* provided broad spectrum resistance and their resistance frequencies were 92.9% and 78.6%, respectively. The races (isolates), IB33(FLN9), IE1(ZN13), IB1(ZN15), IC1(120), IE1K(TM2), IA1(ARB20112-114), IB49(unnamed), IH1(unnamed), ID1(ZN42), and IC17(ZN57) of *M. oryzae* were highly virulent to monogenic lines, and more than 50% of the isolates were pathogenic across all monogenic lines, respectively. Genetic diversity of 16 races (isolates), two as controls, of *M. oryzae* was verified by repetitive element palindrome polymerase chain reaction (Rep-PCR). Based on these data, a useful strategy of pyramiding blast *R* genes against pathogenic *M. oryzae* races was proposed. Eight rice germplasms were identified to carry more than two blast *R* genes with pathogenicity assays and *R* gene specific primers. These findings are important for breeding for improved blast resistance in the USA and worldwide.

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1. Introduction

The study of plant–pathogen interactions can benefit crop protection. In theory, plants have evolved sophisticated innate immune systems mediated by resistance *(R)* genes to fight against pathogens (Jia et al., 2000). For the past two decades, successful cloning of plant *R* genes has revealed insights on the molecular basis of disease resistance; however, only a few *R* genes have been tested for long term effectiveness against a diverse pathogen population. Searching for effective *R* genes has been one of the major efforts for plant scientists worldwide.

Rice blast caused by *Magnaporthe oryzae* has been one of the major constraints of rice production worldwide (Dean et al., 2012). Resistance to *M. oryzae* follows a gene for gene system where the plant *R* gene will coexist and function with the corresponding avirulence (*AVR*) gene (Flor, 1971). Genetic screening of rice germplasm has identified numerous major blast *R* genes, some of them have been cloned, and molecular markers have been identified for their deployments (RoyChowdhury et al., 2012b). Similarly, cloning of *AVR* genes from the pathogens not only sheds the molecular basis of pathogen recognition but also predicts which *R* gene may be effective in preventing blast disease (Jia et al., 2000; Orbach et al., 2000; Zhou et al., 2007; Dai et al., 2010; Xing et al., 2013). Knowing the genetic identity of *M. oryzae* pathotypes (races) may also help in selecting effective *R* genes (Ou, 1980; Correll et al., 2000; Xia et al., 2000; Xing et al., 2013). A method such as Rep-PCR is a fast PCR-based method that can also be used to detect genetic identity of *M. oryzae* isolates (George et al., 1998).

The monogenic and digenic lines have been important assets to identify blast *R* genes (Jia and Moldenhauer, 2010). The first international rice blast differential system (DS) was developed in the 1960s to determine physiological races of *M. oryzae* (Atkins et al., 1967). Subsequently, DSs have been substituted with local rice

Abbreviations: AVR, Avirulence; DB NRRC, Dale Bumpers National Rice Research Center; GSOR, Genetic stock *oryza*; LTH, Lijiangxintuanheigu; *M. oryzae*, *Magnaporthe oryzae*; MLs, Monogenic lines; NILs, Near-isogenic lines; MAS, Marker assisted selection; R gene, Resistance gene; Rep-PCR, Repetitive element palindrome polymerase chain reaction; DS, Differential system; USDA, United State Department of Agriculture.

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genotypes based on local pathogen populations. For example, in Japan, two sets of monogenic DSs were developed, and have been used for race identification of Japanese blast isolates (Yamada et al., 1976; Kiyosawa, 1984). The International Rice Research Institute (IRRI) in the Philippines developed a DS with *indica*-type genetic background resulting in four near-isogenic lines (NILs) with four *R* genes, *Pita*, *Piz-5*, *Pi1*, and *Pi3* (Mackill and Bonman, 1992). In China, a set of NILs was developed with five blast *R* genes, *Pib*, *Pik*, *Pik-m*, *Pik-p*, and *Pita-2*, using a susceptible *japonica*-type genetic background, Lijiangxintuanheigu (LTH) (Ling et al., 2001). A set of NILs derived from LTH with 24 *R* genes, including *Pia*, *Pii*, *Pik*, *Pik-s*, *Pik-m*, *Pik-h*, *Pik-p*, *Piz*, *Piz-5*, *Piz-t*, *Pita*, *Pita-2*, *Pib*, *Pit*, *Pish*, *Pi1*, *Pi3*, *Pi5*(t), *Pi7*, *Pi9*, *Pi11*, *Pi12*(t), *Pi19*, and *Pi20*(t), was developed (Tsunematsu et al., 2000). This differential set has been used to identify blast *R* genes worldwide (Koide et al., 2011; Takehisa et al., 2009; Wang et al., 2013).

DNA markers are either closely linked to or derived from portions of a blast *R* gene that has been used to breed for improved disease resistance using marker assisted selection (MAS) (Narayanan et al., 2002; Jia, 2003). The allele-specific, insertion-deletion (InDel) and linked DNA marker sets are available for several blast *R* genes including *Pita* (Jia et al., 2002); *Piz*, *Pib* (Hayashi et al., 2006), and *Pi9* (Suh et al., 2009) for gene identification and MAS.

The objectives of this study were to (i) determine the effectiveness of blast *R* genes using commonly found blast races (isolates) in the southern USA, (ii) detect the genetic identity of the blast races (isolates) using Rep-PCR technology, and (iii) determine blast *R* genes in germplasm with DNA markers and pathogenicity assays.

2. Materials and methods

2.1. M. oryzae race (isolate), media and culture

Fourteen isolates of *M. oryzae* were used in this study, the isolates belonged to the following races: IA1 (ARB20112-114), IA45 (75L14), IB1 (ZN15), IB33 (FLN9), IB45 (isolate unnamed), IB49 (unnamed isolate), IB54 (unnamed isolate), IC1 (120), IC17 (ZN57), ID1 (ZN42), IE1 (ZN13), IG1 (ZN39), IH1 (unnamed isolate), and IE1K (TM2) (Correll et al., 2000). Two new isolates ARB2012-63 (race undetermined) and ARB2012-64 (race undetermined) were only used as controls for fingerprinting. Race and isolate will be used interchangeably throughout this paper. The isolates in desiccated filter papers (3–5 cm in diameter) carrying mycelia and conidia were stored at −20 °C in sterile glass vials at the USDA Agricultural Research Service Dale Bumpers National Rice Research Center (DB NRRC). The oatmeal agar (OMA; BD Difco, Franklin Lakes, NJ) plates were inoculated with 1–3 desiccated filter papers (3–5 cm in diameter) carrying mycelia and conidia. The fungi on the inoculated plates were allowed to sporulate for approximately 7–10 days under white and black light at 20 °C–23 °C. Conidia were collected with 0.25% gelatin using a sterilized scalpel, and filtered through four layers of cheesecloth. After the filtration, the concentration of the conidial suspension was determined using the method described by Sambrook and Russell (Sambrook and Russell, 2001). Conidial inoculum for pathogenicity assays was prepared by diluting the suspension to 2×10^5 conidia/mL with 0.25% gelatin. Liquid YCS culture media (yeast extract, 3 g/L; casamino acids, 3 g/L; sucrose, 3 g/L) were inoculated with conidia from the filter papers carrying mycelia and conidia. Media were incubated at 20 °C–23 °C on a shaker at 14 rpm to produce fungal mycelia. After seven days, the media were filtered through four layers of cheesecloth and the retained mycelia were desiccated for genomic DNA extraction.

2.2. Rice material, plant growth, and pathogenicity assay

One hundred and eighty three accessions identified with a *Pi-ta* gene specific marker from a USDA core collection were used in the study (Wang et al., 2010), and were obtained from the Genetic stock *oryza* (GSOR) collection at DB NRRC (www.ars.usda.gov/spa/dbnrrc/gsor). Twenty four rice monogenic lines (MLs) and rice variety LTH were grown in a greenhouse at 20 °C–30 °C day/night until seedlings were at the three to four leaf stages, approximately two weeks after sowing, for DNA extraction and pathogenicity assays. The leaves (20 g per genotype) were collected in a 1.5 mL tube and stored in a −80 °C freezer for DNA extraction. Pathogenicity assays were conducted as previously described by Jia et al. (Jia et al., 2004). Plants were placed in plastic bags and sprayed with 35 mL of a conidial suspension (2×10^5 conidia/mL). The inoculated bags were sealed to maintain more than 90% humidity and maintained in the dark at 25 °C–28 °C. After 24 h, the inoculated seedlings were moved to a greenhouse and maintained at 20 °C–30 °C with 12 h light/dark for five to six days for disease development. Pathogenicity assays were performed twice and the average of disease reaction of 6–14 seedlings per genotype was used for disease rating. Disease reactions were rated with a scale of 0–5, with 0–2 indicating different resistant reactions (R), and 3 to 5 indicating different susceptible reactions (S), respectively.

2.3. DNA extraction

Plant and fungal DNA were prepared using the Qiagen DNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen Inc., Valencia, CA). All PCR reactions were performed in a Peltier Thermal Cycler (PTC-200; Bio-Rad Laboratories Inc., Carlsbad, CA). As controls, two new isolates, ARB2012-63 and ARB2012-64, collected from a rice field at Stuttgart, Arkansas in 2012, were analyzed by Rep-PCR.

2.4. Rep-PCR amplification

Rep-PCR was performed with *Pot2-1* and *Pot2-2* primers as previously described by George et al. (1998) (Table 1).

Briefly, each Rep-PCR was performed in a 30 μL volume containing 15 μL Taq PCR Master Mix (Qiagen Inc.), 2 μL of each primer (10 μM), about 30 ng of fungal genomic DNA, and distilled H_2O up to 30 μL. Rep-PCR was carried out as follows: 1 cycle of 95 °C for 2.5 min; 3 cycles of 94 °C for 1 min, 62 °C for 1 min, 65 °C for 10 min; then 25 cycles of 94 °C for 30 s, 62 °C for 1 min, 65 °C for 10 min; and a final cycle of 65 °C for 15 min. To visualize the DNA fingerprints, 10 μL of each PCR product were loaded in a 0.5% agarose gel. Gels were run at 120 V for 7 h with SYBR Safe DNA gel stain (Life Technologies Inc., Carlsbad, CA, USA) in 1 × TAE (tris-acetate-EDTA) solution, and then photographed with ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc.). Each Rep-PCR was conducted twice with the same result.

2.5. DNA marker for blast R gene and PCR amplification

DNA markers for *Pita-2*, *Pib*, *Piz*, *Pi-ta*, and *Pi9* and protocols for PCR amplifications were previously described (Table 1; Jia et al., 2002; Hayashi et al., 2006; Suh et al., 2009). In the present study, a modified protocol for *Pi-ta* was used as follows: 10 μL *Taq* PCR Master Mix (Qiagen Inc.), 2 μL of each primer (10uM), about 30 ng of genomic DNA, and distilled water up to 20 μL. PCR reactions were performed twice with the same result.