Avirulence (AVR) Gene-Based Diagnosis Complements Existing Pathogen Surveillance Tools for Effective Deployment of Resistance (R) Genes Against Rice Blast Disease

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ABSTRACT

Avirulence (AVR) genes in *Magnaporthe oryzae*, the fungal pathogen that causes the devastating rice blast disease, have been documented to be major targets subject to mutations to avoid recognition by resistance (R) genes. In this study, an AVR-gene-based diagnosis tool for determining the virulence spectrum of a rice blast pathogen population was developed and validated. A set of 77 single-spore field isolates was subjected to pathotype analysis using differential lines, each containing a single R gene, and classified into 20 virulent pathotypes, except for 4 isolates that lost pathogenicity. In all, 10 differential lines showed low frequency (<24%) of resistance whereas 8 lines showed a high frequency (>95%), inferring the effectiveness of R genes present in the respective differential lines. In addition, the haplotypes of seven AVR genes were determined by polymerase chain reaction amplification and sequencing, if applicable. The calculated frequency of different AVR genes displayed significant variations in the population. AVR-Piz-t and AVR-Pii were

detected in 100 and 84.9% of the isolates, respectively. Five AVR genes such as AVR-Pik-D (20.5%) and AVR-Pik-E (1.4%), AVR-Piz-t (2.7%), AVR-Pita (0%), AVR-Pia (0%), and AVR1-CO39 (0%) displayed low or even zero frequency. The frequency of AVR genes correlated almost perfectly with the resistance frequency of the cognate R genes in differential lines, except for International Rice Research Institute-bred blast-resistant lines IRBLzt-T, IRBLta-K1, and IRBLkp-K60. Both genetic analysis and molecular marker validation revealed an additional R gene, most likely Pi19 or its allele, in these three differential lines. This can explain the spuriously higher resistance frequency of each target R gene based on conventional pathotyping. This study demonstrates that AVR-gene-based diagnosis provides a precise, R-gene-specific, and differential line-free assessment method that can be used for determining the virulence spectrum of a rice blast pathogen population and for predicting the effectiveness of target R genes in rice varieties.

Rice (*Oryza sativa* L.) is the main staple food crop for more than 50% of the world's population. Rice blast disease, which is caused by an ascomycete pathogen, *Magnaporthe oryzae* B. C. Couch (anamorph: *Pyricularia oryzae* Cavara), is a major factor influencing stable rice production in many rice-growing countries. The harvests destroyed by rice blast are variously attributed to varietal resistance, environmental conditions, and disease management, including fungicide application, and could often reach 50% or more associated with outbreaks (Khush and Jena 2009). In addition to its economic importance, *M. oryzae* was ranked first in the top 10 scientifically important fungal phytopathogens in the field of molecular plant pathology (Dean et al. 2012).

The use of fungicides (Okamoto 1972; Yoshii 1949) and several cultural practices such as crop rotation (TeBeest et al. 2007), polyvarietal planting (Falvo 2000), nitrogen fertilizer application regulation (Hori 1898), silica application (Datnoff and Rodrigues 2005; Miyake and Ikeda 1932), and paddy field flooding (Kahn and Libby 1958) help in the management of the disease. However, the use of chemicals can lead to the development of resistance by the pathogen and the

production of toxic residues in treated plants that might cause environmental pollution (Pooja and Katoch 2014).

The most efficient tool for rice blast disease control is through the deployment of resistant rice varieties because it requires no additional cost to farmers and is environmentally safe (Leung et al. 2003). The resistance (R) genes in rice correspond to the avirulence (AVR) genes in *M. oryzae* in a gene-for-gene manner (Flor 1956), which entails that the interaction between a specific R protein in rice and the corresponding AVR effector in the pathogen would render a particular rice variety resistant to the disease. Despite the deployment of resistant varieties, blast epidemics can still occur, characterized by a lapse in host resistance and the emergence of new virulent pathotypes of the fungus (Lee and Cho 1990). It has been documented that *M. oryzae* mainly targets the AVR gene for various mutations to avoid recognition by the respective R gene, resulting in the erosion of resistance in rice varieties (Khang et al. 2008; Wu et al. 2015). Several genetic events that include point mutations, insertion of transposable elements, and deletion of partial or entire genes leading to the loss of function of AVR genes in *M. oryzae* have been documented (Chuma et al. 2011; Dean et al. 2005; Thon et al. 2006; Wu et al. 2015; Zhou et al. 2007).

The determination and monitoring of effective R genes by analyzing the virulence (VIR) spectrum of a pathogen population is a prerequisite for breeding rice varieties resistant to rice blast. Several tools, including field assessment and pathotype analysis using differential lines, were introduced for this purpose (Correa-Victoria and Zeigler 1993; Fukuta et al. 2014). Given that the effectiveness of R genes is tightly dependent on the respective AVR gene, the latter

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could be used as a diagnostic reagent for providing more specific and DNA-based information on the virulence spectrum of the pathogen population. This study aims to introduce an AVR-gene-based diagnostic tool and use it for determining the virulence spectrum of isolates collected from a multienvironment trial (MET) field in the Philippines. The virulence spectrum determined through AVR-gene-based diagnosis will be compared with the one determined by other methods to verify the reliability and consistency of using different methods for pathogen surveillance.

MATERIALS AND METHODS

Collection and preservation of rice blast isolates from the MET field in Bohol, Philippines. Several plots located in one region of a 1.25-ha MET field, operated by the International Rice Research Institute (IRRI) in Bohol, Philippines, were selected for the collection of leaf samples infected by rice blast in March 2014. In total, 77 isolates were generated from the collection and named according to the code of its host variety, except for BZ64-1 (Supplementary Table S1). All purified isolates were preserved on filter paper and stored at -20°C in a freezer at IRRI. In 2015, one of the plots in the same region was developed as a hot spot for evaluating the blast resistance of different varieties, including the IRRI-bred blast-resistant lines (IRBL). The incidence of rice blast disease at this site was constant and severe in the past several years. Severity of the disease was evaluated using the standard evaluation system for rice leaf blast (IRRI 2014).

Pathogenicity testing of the isolates. Lijiangxintuanheigu (LTH)-derived IRBL and LTH were used for the pathogenicity test, as described previously (Telebanco-Yanoria et al. 2010). Spores of each isolate were harvested using 0.02% (vol/vol) Tween 20 in distilled water. The resulting spore concentration was adjusted to 1×10^5 conidia/ml. Rice seedlings at the three- to four-leaf stage were used for blast inoculation, as described previously (Telebanco-Yanoria et al. 2008). Disease assessment was performed 7 days after inoculation by following the standard evaluation system for rice (IRRI 2014). Generally, infection types 0 to 2 are considered to be a resistant reaction and infection types 3 to 5 are a susceptible reaction. However, in the case of IRBLh-S, the scores 0 to 3 are categorized as resistant and 4 to 5 are susceptible.

Diversity analysis of the rice blast population. The isolates were grouped using their reaction pattern to the 25 rice varieties by cluster analysis following Ward's hierarchical method (R software; stats package; hclust function). The 24 LTH IRBL were divided into five groups (U, i, k, z, and ta), as described previously (Hayashi and Fukuta 2009). Simpson's index of diversity was computed based on the reaction of the isolates to the five groups of IRBL (Fukuta et al. 2014; Peet 1974; Simpson 1949).

Molecular characterization of haplotypes of seven AVR genes. Fungal isolates were grown in one-half complete medium (Talbot et al. 1993) for 4 days, after which the mycelia were collected through filtration and then dried. Genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) buffer, as described previously (Scott et al. 1993). The DNA concentration of each isolate was determined and normalized to 10 ng/ μl . Primer pairs specific to seven AVR genes (*AVR1-CO39*, *AVR-Pita*, *AVRPiz-t*, *AVR-Pi9*, *AVR-Pik*, *AVR-Pia*, and *AVR-Pii*) were designed and used for polymerase chain reaction (PCR) amplification. The rTaq enzyme (TaKaRa) was used for PCR amplification following the manufacturer's instructions.

Genetic analysis of resistance of IRBLkp-K60 to rice blast. The F_2 progenies derived from the cross between LTH and IRBLkp-K60 were inoculated with three blast isolates: 4029-1, 5029-1, and 9475-1. The leaves of susceptible plants were collected for DNA extraction using the CTAB method (Scott et al. 1993). To further confirm the genes controlling resistance in IRBLkp-K60, a flanked simple-sequence repeat (SSR) marker, RM224, as used for the linkage analysis of a *Pik* allele in LTH (Singh et al. 2015), was

used to analyze the linkage between the genotype and the phenotype of the F_2 -derived progenies.

RESULTS

Pathotypes of rice blast isolates collected in Bohol, Philippines. Pathogenicity testing revealed that 73 of 77 isolates showed pathogenicity to LTH, a susceptible check control. The other four isolates (5012-3, 9020-1, 9160-1, and 9248-3) were not pathogenic to any rice lines without known reasons and, thus, were not included in this study. The frequency of avirulent and virulent isolates to each of the 24 IRBL is varied (Table 1). The isolates displayed a relatively high virulence frequency ($>95\%$) to each of eight IRBL; namely, IRBLi-F5, IRBLkp-K60, IRBLz-Fu, IRBLh-S, IRBL3-CP4, IRBL5-M, IRBL9-W, and IRBL20-IR24. In contrast, they showed a relatively low virulence frequency ($<24\%$) to each of 10 other IRBL; namely, IRBLa-A, IRBLks-F5, IRBLk-Ka, IRBLkh-K3, IRBLta2-Re, IRBLb-B, IRBL11-Zh, IRBL1-CL, IRBL7-M, and IRBLkm-Ts. It is noted that all isolates were avirulent to IRBL9-W whereas they were virulent to IRBLks-F5, IRBLb-B, and IRBLt-K59. These data indicate that the isolates collected in Bohol displayed contrasting virulence and avirulence spectrums to different IRBL.

The 73 pathogenic isolates were further classified into 20 pathotypes based on the reaction pattern to different IRBL (Table 1; Fig. 1). Three pathotypes—numbers 4, 6, and 7—were found dominant in the population and were represented by 18, 11, and 22 isolates, respectively. It was evident that some isolates showed identical pathotypes but were collected from different rice lines. For example, 18 isolates in pathotype group 4 were collected from 16 different rice varieties (Table 1). Cluster dendrogram analysis further identified two distinct clades: clades I and II were composed of 57 and 16 isolates, respectively (Fig. 1). Interestingly, all three dominant pathotypes belong to clade I (Fig. 1). It was also found that the isolates purified from the same lesion belonged to distinct pathotypes; for example, 9113-1 and 9113-2 belonged in pathotype groups 6 and 7, respectively (Fig. 1). Based on the pathogenic reactions to five groups of IRBL, following the method developed by Hayashi and Fukuta (2009), it was found that the diversity of the population is relatively narrow, with the index ranging from 0.0 to 0.5 for the different groups. The cases with extremely low diversity were found in the isolates in subgroups U, i, and k in clade I, each displaying an almost zero diversity index (Supplementary Fig. S1).

Haplotypes of seven AVR genes in the isolates. To determine the haplotypes of seven AVR genes, PCR amplification and amplicon sequencing were conducted. PCR amplification using primer pairs (Table 2) targeting the coding or promoter regions of the AVR genes revealed two patterns: amplification with expected size and no amplification (Fig. 2). The *AVRPiz-t* fragment was present in all isolates. In contrast, neither *AVR-Pia* nor *AVR1-CO39* was amplified in any isolates. A varying number of isolates were resolved with PCR amplicon at the expected size for *AVR-Pik* ($n = 20$), *AVR-Pii* ($n = 62$), *AVRPiz-t* ($n = 2$), and *AVR-Pita* ($n = 10$). Amplicon sequencing revealed that the sequences of *AVRPiz-t*, *AVRPiz-t*, and *AVR-Pii* were identical to those of the respective avirulent strains, as described previously (GenBank accession numbers KM004023, EU837058, and AB498874, respectively). Three haplotypes of *AVR-Pik* (i.e., *AVR-Pik-D*, GenBank accession number AB498875; *AVR-Pik-E*, GenBank accession number AB498879; and *avrPik-F*, GenBank accession number KU593517) were identified in 15, 1, and 4 isolates, respectively. It was noted that *avrPik-F* was recently identified in Philippine isolates and was found incapable of activating resistance to any of the *Pik* alleles in IRBL (Table 3). The sequences of *AVR-Pita* in 10 isolates were identical to each other (Table 3). However, compared with *AVR-Pita* in an avirulent strain (GenBank accession number AF207841), it contained three nucleotide changes in the coding region (716C, 724C, and 840C), resulting in two amino acid substitutions of the predicted protein product: 191C (cysteine) and 194H (histidine). It is worth noting that the mutations of 191C and 194H were characterized to

TABLE 1. Pathotypes of different isolates against International Rice Research Institute-bred blast-resistant lines (IRBL)^a

		Reactions of IRBL to the isolates																											
PG	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	LTH			
1	9048-1	R	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	R	S	S			
2	9265-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	R	S	S			
3	5093-3	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
4	5054-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	5029-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	6021-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	8036-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	8081-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9056-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9124-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9186-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9190-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9193-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9252-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9316-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9323-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9334-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	BZ64-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
	9121-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
9310-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S				
5	5047-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	R	S	S	S			
6	7068-2	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	5033-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
7	4029-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	5156-3	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6033-3	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6054-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6057-3	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6119-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9113-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9149-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9262-3	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9202-1	S	R	S	S	R	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6030-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	5140-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6065-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6085-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	6116-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9113-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9157-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9221-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9233-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
	9245-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
9250-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9257-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9277-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9293-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9304-2	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9324-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9403-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9440-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9459-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
9533-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
7024-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S				
8	9212-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	R	S	S	S			
9	9264-1	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	R	R	S	S	S	S	S			
10	9333-3	S	R	S	S	R	S	R	R	R	R	S	S	R	S	R	R	S	R	S	R	S	S	S	S	S			
11	5127-1	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	S	R	R	R	S	S			
	5092-3	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	S	R	R	R	S	S			
12	5131-2	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	S	R	R	R	S	S			
	6050-3	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	S	S				
	6016-2	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	S	S				
9406-3	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	S	R	R	R	S	S				
9126-1	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	S	R	R	R	S	S				
12	6006-1	S	R	S	R	R	R	S	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	S				
13	6161-1	S	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	S				
14	9497-3	R	R	S	R	R	R	R	R	S	S	S	S	S	R	R	R	R	R	S	R	R	R	R	S				
15	6003-3	R	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	S				
16	5008-3	R	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	S				
17	9244-3	R	R	S	R	R	R	S	R	S	S	S	S	R	R	R	R	R	R	S	R	R							

correspond to the loss of function of avirulence of *AVR-Pita* (Chuma et al. 2011), indicating that the isolates containing *avrPita191C/194H* were virulent to rice varieties with *Pita*. We conclude that none of the 73 isolates characterized herein contained a functional *AVR-Pita*. Given that the promoter region was included in PCR validation, the probable presence of transposable elements in the promoter region of seven *AVR* genes could be disproved.

Correlation between the presence of *AVR* genes in rice blast isolates and the pathotypes to respective *R* genes. The correlation between the presence of *AVR* genes and the pathotypes to respective *R* genes is fundamental to apply the *AVR*-gene-based diagnosis for the determination of the virulence spectrum of rice blast isolates. The pathotypes of the isolates were hereby compared with those deduced from the haplotypes of *AVR* genes. As described above, all the isolates characterized in this study contained *AVRPiz-t* and also showed virulence to IRBL9-W in the pathotype test (Table 3),

reiterating a perfect correlation between the existence of *AVRPiz-t* in rice blast isolates and virulence to *Pi9*, as described previously (Wu et al. 2015). Moreover, the isolates containing any of the other six *AVR* genes were avirulent, without exception, to the IRBL containing the respective *R* genes (Table 3). However, a number of isolates without respective *AVR* genes were avirulent to IRBLi-F5 (5008-3 and seven other isolates), IRBL1-CL (7068-2), and IRBLa-A (5008-3 and four other isolates), suggesting that these lines most likely contained uncharacterized *R* genes (Table 3). It was also noticed that a set of 57 isolates (4029-1 and 56 other isolates) was avirulent coincidentally to three IRBL: IRBLzt-T, IRBLta-K1, and IRBLkp-K60. Intriguingly, these isolates did not contain *AVR* genes corresponding to respective *R* genes in these three IRBL (Table 3).

It is reasonable to speculate that the discrepancy of the virulence reaction of these 57 isolates to three IRBL could be attributable to the existence of additional genes in those rice lines. To validate our

Fig. 1. Cluster analysis of 73 pathogenic rice blast isolates. The dendrogram is built using cluster analysis based on the reaction of the isolates to 24 Lijiangxintuanheigu (LTH)-derived International Rice Research Institute-bred blast-resistant lines and LTH. The majority of the isolates belong to clade I (78%) whereas only 16 isolates (22%) belong to clade II. Isolates were classified into 20 pathotype groups and the effective resistance genes for each group were shown.

hypothesis, we selected IRBLkp-K60 as a representative line for the analysis. An F₂ population derived from the cross between IRBLkp-K60 and LTH was generated and used for the genetic analysis. An expected ratio of 3:1 (R to S) was observed in F₂ progenies against 3 selected isolates, including 2 from the aforementioned 57 isolates (Table 4), suggesting that only a single *R* gene was involved in the resistance in each interaction. Interestingly, none of the progenies that were susceptible to the isolate 9475-1 containing *AVR-Pik-D* showed the genotype of IRBLkp-K60 at RM224, suggesting that the resistance is tightly associated with the *Pikp* gene (Table 4). In contrast, 18 of 21 and 12 of 18 progenies that are susceptible to isolates 4029-1 and 5029-1, respectively, showed the genotype of IRBLkp-K60, suggesting that the resistance is not associated with the *Pikp* gene (Table 4). Indeed, these two isolates did not contain functional *AVR-Pik* haplotypes and they were virulent to IRBL7-M and four other IRBL containing the characterized *Pik* alleles (IRBLk-Ka, IRBLkh-K3, IRBL1-CL, and IRBLkm-Ts) (Table 3), suggesting that they were not able to activate the resistance to any *Pik*-containing rice lines. Taken together, we conclude that the virulence of these two isolates to IRBLkp-K60 is not due to the *Pikp* gene. Instead, it could be due to the existence of an additional *R* gene in IRBLkp-K60. Interestingly, these 57 isolates were found coincidentally avirulent to IRBL19-A containing the *Pi19* gene, suggesting that the presumed additional *R* gene in IRBLkp-K60 could be *Pi19* or its allele (Table 1). To validate this hypothesis, two SSR markers that were previously characterized to be linked to *Pi19* (RM27920 and RM27947) were used for the genotyping of

IRBLkp-K60, IRBLta-K1, IRBLzt-T, IRBL19-A, and LTH. PCR amplicons without a visible difference in size were obtained in four IRBL (Fig. 3). In contrast, PCR product with a distinct size was resolved in LTH at both SSR markers. Thus, we speculated that these three IRBL most likely contained the genomic fragment containing *Pi19* or its allele.

Correlation between the frequency of AVR genes and the field resistance of IRBL containing cognate *R* genes. To validate whether the frequency of *AVR* genes obtained from the characterization of a field rice blast population correlated well with the field resistance of IRBL containing the cognate *R* genes, field resistance of the IRBL was assessed in the same MET field in Bohol. All IRBL, except for IRBLta-K1 because of plant death, were scored for resistance with three replicates in the dry season of 2015. The susceptible check line, LTH, was highly susceptible, indicating that disease pressure at the field site was significant (Table 5). In total, eight IRBL, including IRBL9-W and IRBLi-F5, showed strong field resistance. In contrast, 14 IRBL were susceptible to moderately susceptible, including 6 *Pik* IRBL that each contained different alleles, IRBLa-A, and IRBLzt-T. The correlation between the frequency of *AVR* genes and the field resistance of IRBL containing cognate *R* genes was analyzed. The *AVR* genes with high frequency in the pathogen population correlated well with the strong resistance of IRBL containing cognate *R* genes; for example, *Pi9* (*AVRPiz-t* frequency: 100%) and *Pii* (*AVR-Pii* frequency: 84.9%). In contrast, the *AVR* genes with low frequency in the pathogen population correlated well with the susceptibility of IRBL containing cognate *R* genes; for example, most

TABLE 2. Primers used for the polymerase chain reaction diagnosis of avirulence (*AVR*) genes in this study

Primers	Sequence (5'–3')	T (°C) ^a	Purpose
AVR-Pi9 GF	ATGCAGTTCTCTCAGATCCTC	55	To detect <i>AVR-Pi9</i> CDS
AVR-Pi9 GR	CTACCAGTGCGTCTTTTCGAC	...	...
AVR-Pi9 CF	CCCATGTGTGCTTATCGCGTG	55	To detect <i>AVR-Pi9</i> promoter plus CDS
AVR-Pi9 CR	GACTTGAGAGAACTGCATGTC	...	...
AVR-Pii F	GGTAGATATCCGCTGACTGG	58	To detect <i>AVR-Pii</i> promoter plus CDS
AVR-Pii R	ACTGTCCGCCGCTCGTTTGG	...	...
AVR1-CO39 F	TGCCGCATTTTGCTAACCG	57	To detect <i>AVR1-CO39</i> promoter plus CDS
AVR1-CO39 R	GCGAATCCATAGACAAGAC	...	...
AVRPiz-t F	GTTGCGATTATGATCCGTCG	56	To detect <i>AVRPiz-t</i> promoter plus CDS
AVRPiz-t R	GTAATCTAGCAAACGACCGG	...	...
AVR-Pik F	TCCTGCTGCTAACTCCATTC	57	To detect <i>AVR-Pik</i> promoter plus CDS
AVR-Pik R	TCAACCAAGCGTAAACCTCG	...	...
AVR-Pia F	CAGAGAAACGGACTTGGAGG	58	To detect <i>AVR-Pia</i> promoter plus CDS
AVR-Pia R	GGTATACACGTACGGTAGGG	...	...
AVR-Pita F	CAGGCATACATTGGAGAGCC	58	To detect <i>AVR-Pita</i> promoter plus CDS
AVR-Pita R	CCCTCCATTCCAACACTAAC	...	...
RM224 F	ATCGATCGATCTTCACGAGG	58	To detect presence of <i>Pikp</i>
RM224 R	TGCTATAAAAGGCATTCGGG	...	...
RM27920 F	AAAGCGAGAAATCCGGAGATGG	58	To detect presence of <i>Pi19</i>
RM27920 R	TCCTCTCTCAAATCTCCTCGAAGC	...	...
RM27947 F	GGATGGAGGTTTCTCTCTGGATCG	58	To detect presence of <i>Pi19</i>
RM27947 R	CTGCTGTGCCGCATACAGTGG	...	...

^a Annealing temperature.

Fig. 2. Polymerase chain reaction (PCR) detection of seven avirulence (*AVR*) genes in rice blast isolates. The isolates used as positive control are standard differential blast isolates, as described previously (Telebanco-Yanoria et al. 2008), except 2539. Two isolates, 5167-1 and 9250-2, were selected as representatives for PCR amplification. The PCR products were resolved in 1% of agarose gel.

TABLE 3. Haplotypes of different avirulence (AVR) genes in rice blast isolates and pathogenic reaction of isolates to different International Rice Research Institute-bred blast-resistant lines (IRBL)^a

Isolates	Reactions to								
	<i>Pi9</i>		Different <i>Pik</i> alleles						
	IRBL9-W	AVR hap	IRBLkp-K60	IRBL7-M	IRBLk-Ka	IRBLkh-K3	IRBLkm-Ts	IRBL1-CL	AVR hap
5008-3	R	+	R	R	R	R	R	R	D
5092-3	R	+	R	R	R	R	R	R	D
5127-1	R	+	R	R	R	R	R	R	D
5131-2	R	+	R	R	R	R	R	R	D
5167-1	R	+	R	R	R	R	R	R	D
6003-3	R	+	R	R	R	R	R	R	D
6006-1	R	+	R	R	R	R	R	R	D
6016-2	R	+	R	R	R	R	R	R	D
6050-3	R	+	R	R	R	R	R	R	D
6161-1	R	+	R	R	R	R	R	R	D
9126-1	R	+	R	R	R	R	R	R	D
9244-3	R	+	R	R	R	R	R	R	D
9406-3	R	+	R	R	R	R	R	R	D
9475-1	R	+	R	R	R	R	R	R	D
9497-3	R	+	R	R	R	R	R	R	D
9482-1	R	+	S	S	S	R	R	R	E
5029-1	R	+	R	S	S	S	S	S	F
9048-1	R	+	R	S	S	S	S	S	F
9257-1	R	+	R	S	S	S	S	S	F
BZ64-1	R	+	R	S	S	S	S	S	F
4029-1	R	+	R	S	S	S	S	S	–
5033-1	R	+	R	S	S	S	S	S	–
5047-3	R	+	R	S	S	S	S	S	–
5054-3	R	+	R	S	S	S	S	S	–
5093-3	R	+	R	S	S	S	S	S	–
5140-2	R	+	R	S	S	S	S	S	–
5156-3	R	+	R	S	S	S	S	S	–
6021-2	R	+	R	S	S	S	S	S	–
6030-3	R	+	R	S	S	S	S	S	–
6033-3	R	+	R	S	S	S	S	S	–
6054-1	R	+	R	S	S	S	S	S	–
6057-3	R	+	R	S	S	S	S	S	–
6065-2	R	+	R	S	S	S	S	S	–
6085-3	R	+	R	S	S	S	S	S	–
6116-2	R	+	R	S	S	S	S	S	–
6119-1	R	+	R	S	S	S	S	S	–
7024-1	R	+	R	S	S	S	S	S	–
7068-2	R	+	R	S	S	S	S	R	–
8036-2	R	+	R	S	S	S	S	S	–
8081-2	R	+	R	S	S	S	S	S	–
9056-1	R	+	R	S	S	S	S	S	–
9113-1	R	+	R	S	S	S	S	S	–
9113-2	R	+	R	S	S	S	S	S	–
9121-1	R	+	R	S	S	S	S	S	–
9124-1	R	+	R	S	S	S	S	S	–
9149-1	R	+	R	S	S	S	S	S	–
9157-3	R	+	R	S	S	S	S	S	–
9186-1	R	+	R	S	S	S	S	S	–
9190-3	R	+	R	S	S	S	S	S	–
9193-1	R	+	R	S	S	S	S	S	–
9202-1	R	+	R	S	S	S	S	S	–
9212-1	R	+	R	S	S	S	S	S	–
9221-3	R	+	R	S	S	S	S	S	–
9233-1	R	+	R	S	S	S	S	S	–
9245-1	R	+	R	S	S	S	S	S	–
9250-2	R	+	R	S	S	S	S	S	–
9252-1	R	+	R	S	S	S	S	S	–
9262-3	R	+	R	S	S	S	S	S	–
9264-1	R	+	R	S	S	S	S	S	–
9265-1	R	+	R	S	S	S	S	S	–
9277-3	R	+	R	S	S	S	S	S	–
9293-1	R	+	R	S	S	S	S	S	–
9304-2	R	+	R	S	S	S	S	S	–
9310-1	R	+	R	S	S	S	S	S	–
9316-1	R	+	R	S	S	S	S	S	–
9323-1	R	+	R	S	S	S	S	S	–
9324-1	R	+	R	S	S	S	S	S	–
9333-3	R	+	R	S	S	S	S	S	–
9334-3	R	+	R	S	S	S	S	S	–
9403-3	R	+	R	S	S	S	S	S	–
9440-1	R	+	R	S	S	S	S	S	–
9459-3	R	+	R	S	S	S	S	S	–
9533-1	R	+	R	S	S	S	S	S	–

(continued on next page)

^a AVR hap = haplotypes, R = resistant, S = susceptible, *aP* = *avrPita*^{191C/194H}, + = identical to the AVR gene characterized in the original avirulent isolate, and – = absent. Isolates were listed in an order based on the different haplotypes of AVR-*Pik*. The 57 isolates without functional AVR genes that displayed unexpected avirulence to three IRBL are highlighted in bold.

TABLE 3. (continued from preceding page)

[illegible]

Pik alleles (*AVR-Pik-D* and *-E* frequency: 20.5 to 21.9%), *Piz-t* (*AVRPiz-t* frequency: 2.7%), and *Pia* (*AVR-Pia* frequency: 0%). It was also observed that some IRBL showing high frequency of resistance to the isolates in the pathotype analysis did not display strong resistance in the field. For example, IRBLkp-K60 was resistant to 72 isolates in the pathotype test but was only moderately resistant in the field. In contrast, IRBL11-Zh showed a very low frequency of resistance (5.5%) based on the pathotype test but displayed strong field resistance (Table 5).

DISCUSSION

It has been documented that the effectiveness of rice blast *R* genes varies significantly because of different race composition from region to region. For example, the resistance frequency of *Pib* is high in the far northeastern region of China but low in the southern region (Zhang et al. 2015). *Pita* is effective in the south-central coast of Vietnam but not in Cambodia, based on the pathotype test of field isolates (Fukuta et al. 2014; Nguyen et al. 2015). Contrasting to the situation in those two countries, *Pita* was found to confer resistance to the major pathotypes of the rice blast pathogen population and showed stable resistance for over two decades in the United States (Jia et al. 2004). Therefore, pathogen surveillance serves as a vital step for determining the spatiotemporal effectiveness of *R* genes which, in turn, provides guidance on the efficient deployment of *R* genes in rice varieties. Several tools such as field assessments in blast nurseries and hot spots and pathotyping in the greenhouse have been described for the analysis of the virulence spectrum of a pathogen population and the evaluation of resistance levels of breeding materials (Ahn 1994; Chen et al. 1995; Correa-Victoria and Zeigler 1993; Dias Neto et al. 2010; Vasudevan et al. 2014). It is worth noting that these tools often depend on the use of differential varieties (DV); for example, LTH- and CO39-derived IRBL (Telebanco-Yanoria et al. 2008). In addition to the traditional field assessment and greenhouse pathotyping, a tool for pathogen surveillance of rice blast by analyzing the haplotypes of cloned *AVR* genes was recently introduced. It was demonstrated that *AVR*-gene-based diagnosis could be applied to the analysis of the virulence spectrum of a rice blast population and the inference of effective *R* genes without the use of DV (Imam et al. 2015; Li et al. 2012). However, this research relied solely on PCR analysis, which was unable to distinguish different haplotypes and single-nucleotide polymorphisms of some *AVR* genes. Moreover, the results derived from different tools were not validated for their accuracy and consistency.

We herein reported a case study on the pathogen surveillance of a rice blast population collected from an MET field by combining field assessment, pathotyping of purified single-spore isolates in the greenhouse, and *AVR*-gene-based diagnosis in the laboratory. Because *AVR* genes of rice blast undergo rapid evolution by different genetic events—for example, sequence mutations, insertion of transposable elements, and fragment deletion (Huang et al. 2014; Wu et al. 2014; Zhang et al. 2015)—the haplotype of different *AVR* genes was first

determined using some representative isolates by either PCR amplification or sequencing of PCR amplicons. The avirulence and virulence functionality of each haplotype of the different *AVR* genes was deduced by referring to the characterized *AVR* genes, which was further validated by pathotyping. Consistency was observed in the reactions of the isolates to most IRBL, derived from both *AVR* gene diagnosis and pathotyping using DV. The resistance of IRBL in the field hot spot correlated positively with the frequency of functional haplotypes of different *AVR* genes, suggesting that *AVR*-gene-based diagnosis is capable of determining the effectiveness of target *R* genes. However, inconsistency in the virulence reaction of some isolates to some IRBL was identified. This may be due to the existence of additional *R* genes (for example, *Pi19* or its unknown alleles) in IRBLkp-K60, IRBLta-K1, and IRBLzt-T. It is noted that Toride 1 and Yashiromochi, the donors of IRBLta-K1 and IRBLzt-T, respectively, belong to *japonica* varieties (Telebanco-Yanoria et al. 2008). Moreover, *Pi19* was found to be present widely in this varietal group (Kawasaki-Tanaka and Fukuta 2014). Thus, we speculate that the *Pi19* gene or its allele present in these IRBL was most likely inherited from their respective donors. The effect of additional genes present in IRBL could vary significantly depending on the frequency of their cognate *AVR* genes in the blast population. For example, because of the high frequency of *AVRPi19* (approximately 78%), three IRBL mentioned above displayed much higher resistance frequency determined by pathotyping than that deduced by *AVR*-gene-based diagnosis. In contrast, only 1 of 57 isolates showed unexpected virulence to IRBL1-CL, thus indicating no significant difference in the resistance frequency derived from pathotyping and *AVR*-gene-based diagnosis.

Fig. 3. Genotype analysis of different International Rice Research Institute-bred blast-resistant lines (IRBL) using two simple-sequence repeat markers associated with putative *Pi19* locus. The polymerase chain reaction (PCR) products from IRBL19-A (-19), IRBLkp-K60 (-kp), IRBLta-K1 (-ta), IRBLzt-T (-zt), and Lijiangxintuanheigu (LTH) were resolved in 4% agarose gel. The 1-kb-plus DNA ladder (Thermo Fisher Scientific) was used for the visualization of the size of PCR fragments.

TABLE 4. Genetic analysis of F₂ progeny derived from the cross between International Rice Research Institute-bred blast-resistant lines kp-K60 and Lijiangxintuanheigu (LTH)

Phenotype and genotype of F ₂ progeny ^a								
Rice blast isolates	Hap ^d	Reaction ^e	Progeny ^b		χ^2 , <i>P</i> value ^f	Susceptible progeny (<i>n</i>) ^c		
			Number			Homozygous		Heterozygous
			R	S		<i>Pikp</i> type	LTH type	
4029-1	–	Avirulence	59	21	0.067, 0.796	7	3	11
5029-1	F	Avirulence	64	18	0.407, 0.524	6	6	6
9475-1	D	Virulence	71	21	0.232, 0.630	0	21	0

^a Genotype was determined by using the simple-sequence repeat marker of RM224.
^b Number of progeny resistant (R) and susceptible (S) to isolates.
^c Number of susceptible progeny with different genotype at RM224.
^d Haplotype of *AVR-Pik*; – denotes *AVR-Pik*-free.
^e Reaction to IRBL19-A.
^f Expected 3:1 R/S ratio.

TABLE 5. Field evaluation of International Rice Research Institute-bred blast-resistant lines (IRBL) in the rice blast test site in Bohol, Philippines

IRBL	Genes			Disease scale ^a				Rating ^e
	Target ^b	Additional ^c	Cognate (%) ^d	R1	R2	R3	Mean	
IRBLa-A	<i>Pia</i>	...	0	5	4	5	4.7	MS
IRBLi-F5	<i>Pii</i>	...	84.9	1	1	1	1.0	R
IRBLks-F5	<i>Piks</i>	...	...	8	8	8	8.0	S
IRBLk-Ka	<i>Pik</i>	...	D: 20.5	5	—	5	5.0	S
IRBLkp-K60	<i>Pikp</i>	<i>Pi19</i>	D: 20.5	3	—	4	3.5	MR
IRBLkh-K3	<i>Pikh</i>	...	D+E: 21.9	4	5	4	4.3	MS
IRBLz-Fu	<i>Piz</i>	...	...	1	1	1	1.0	R
IRBLz5-CA	<i>Piz5</i>	...	...	1	1	2	1.3	R
IRBLzt-T	<i>Piz-t</i>	<i>Pi19</i>	2.7	9	8	9	8.7	S
IRBLta-K1	<i>Pita</i>	<i>Pi19</i>	0	—	—	—	—	...
IRBLb-B	<i>Pib</i>	...	...	—	4	—	4.0	MS
IRBLt-K59	<i>Pit</i>	...	...	4	5	5	4.7	MS
IRBLh-S	<i>Pish</i>	...	...	1	1	1	1.0	R
IRBL1-CL	<i>Pi1</i>	...	D+E: 21.9	8	8	8	8.0	S
IRBL3-CP4	<i>Pi3</i>	...	...	3	1	3	2.3	R
IRBL5-M	<i>Pi5</i>	...	...	1	1	3	1.7	R
IRBL7-M	<i>Pi7</i>	...	D: 20.5	8	9	8	8.3	S
IRBL9-W	<i>Pi9</i>	...	100	1	1	1	1.0	R
IRBL12-M	<i>Pi12</i>	...	...	9	—	6	7.5	S
IRBL19-A	<i>Pi19</i>	...	...	6	7	—	6.5	S
IRBLkm-Ts	<i>Pikm</i>	...	D+E: 21.9	5	7	7	6.3	S
IRBL20-IR24	<i>Pi20</i>	...	...	4	4	4	4.0	MS
IRBLta2-Re	<i>Pita2</i>	...	...	8	8	8	8.0	S
IRBL11-Zh	<i>Pi11</i>	...	...	2	2	2	2.0	R
LTH ^f	No	...	...	9	9	9	9.0	S

^a Disease scale in three replicates (R1 to R3) and mean. Mean value was calculated based on three replicates; — = data missing due to no plants.

^b Target resistance (R) genes in IRBL.

^c Additional R genes diagnosed in this study.

^d Frequency of avirulence (AVR) genes cognate to target R genes.

^e Rating of resistance. The 0-to-9 disease scale method was used for the rating of field resistance (IRRI 2014), where 0 to 2.9 = resistant (R), 3 to 3.9 = moderately resistant (MR), 4 to 4.9 = moderately susceptible (MS), and 5 to 9 = susceptible (S).

^f LTH is the recurrent variety of monogenic lines which does not harbor known R genes.

It is important to have random samples representative of the population for pathogen surveillance. In this study, we observed that AVR*Pi19* strains could be overrepresented in the collection. IRBL19-A was susceptible in the same field in 2015, suggesting that the frequency of AVR*Pi19* in the field population might not be as high as 78%, as assessed by pathotyping the blast collection in 2014. Based on the analysis of the collection in 2015, we identified 90 of 246 isolates as avirulent to IRBL19-A. In addition to the improvement of sample size and representativeness, the combination of field assessment with pathotyping and AVR-gene-based diagnosis as illustrated herein can be complementary to each other to ensure precise determination of the population structure. Compared with both field assessment and pathotyping, which depend on the use of DV, the AVR-gene-based diagnosis provides a tool for R-gene-specific assessment without DV. It can also be implemented for a quantitative and scalable analysis. Moreover, it allows remote analysis by exchanging DNA samples rather than live pathogens, which is often blocked between countries. These advantages of AVR-gene-based diagnosis make it a practical and effective tool for the surveillance of rice blast pathogens.

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