Dynamic Insertion of *Pot3* in *AvrPib* Prevailing in a Field Rice Blast Population in the Philippines Led to the High Virulence Frequency Against the Resistance Gene *Pib* in Rice

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ABSTRACT

The *Magnaporthe oryzae* avirulence gene *AvrPib* is required for the resistance mediated by its cognate resistance gene *Pib*, which has been intensively used in *indica* rice breeding programs in many Asian countries. However, the sequence diversity of *AvrPib* among geographically distinct *M. oryzae* populations was recently shown to be increasing. Here, we selected a field population consisting of 248 rice blast isolates collected from a disease hotspot in Philippine for the analysis of *AvrPib* haplotypes and their pathogenicity against *Pib*. We found that all of the isolates were virulent to *Pib* and each of them contained an insertion of *Pot3* transposon in *AvrPib*. Moreover, *Pot3* insertion was detected in different genomic positions, resulting in three different *AvrPib* haplotypes, designated *avrPib*-H1 to H3. We further conducted a genome-wide *Pot2* fingerprinting analysis by repetitive element palindromic polymerase chain reaction (PCR) and identified seven different lineages out of 47 representative isolates. The isolates

belonging to the same lineage often had the same *AvrPib* haplotype. In contrast, the isolates having the same *AvrPib* haplotypes did not always belong to the same lineages. Both mating types MAT1-1 and MAT1-2 were identified in the population in Bohol and the latter appeared dominant. On the host side, we found that 32 of 52 released rice varieties in the Philippines contained *Pib* diagnosed by PCR gene-specific primers and DNA sequencing of gene amplicons, suggesting that it was widely incorporated in different rice varieties. Our study highlights the genetic dynamics of rice blast population at both the *AvrPib* locus and the genome-wide levels, providing insight into the mechanisms of the mutations in *AvrPib* leading to the breakdown of *Pib*-mediated resistance in rice.

Keywords: *AvrPib*, haplotype, *Pib*, *Pot3*, repetitive element palindromic-PCR.

Plants are often challenged by a number of biotic stresses and, thus, have evolved two layers of immunity mediated by transmembrane pattern recognition receptors and the intracellular nucleotide-binding leucine-rich repeat (NB-LRR or NLR) proteins (Cesari et al. 2013). To break the basal plant immunity, pathogens have evolved a repertoire of effectors which interfere with the pattern recognition receptor and, thus, cause disease. In an antagonistic coevolution, plants have evolved the NLR protein to recognize pathogen effectors as a second layer of immunity (Jones and Dangl 2006) and, in return, pathogens manipulate its effector to evade recognition by the host plant. This continuous coevolution between hosts and pathogens has led to the proposition of two evolutionary models (i.e., the arms race and trench warfare)

(Kanzaki et al. 2012; Stahl et al. 1999; Woolhouse et al. 2002). Pathogens need to retain their fitness and, therefore, they evolve toward virulence and virulent strains emerge in the pathogen population. Within the pathogen population, such a virulence allele increases rapidly and, in the end, replaces the avirulent allele (Stergiopoulos et al. 2007). Conversely, the host evolves an antagonistic gene that invalidates the new virulent allele, and this corresponding process repeats over time (Kanzaki et al. 2012; Woolhouse et al. 2002).

Rice blast disease, caused by the filamentous fungal pathogen *Magnaporthe oryzae*, is the most catastrophic fungal disease in rice production worldwide (Talbot 2003). The genetic-based control strategy remains the most economic and efficient strategy to combat the disease. More than 80 rice blast resistance (*R*) genes have been identified and approximately 25 of them have been cloned (Fukuoka et al. 2012; Su et al. 2015), many of which have already been introgressed into elite rice varieties (Khush and Jena 2009). All of these *R* genes encode the highly polymorphic NB-LRR proteins, with the exception of *pi21* and *Pid2*, which encode a proline-rich protein and a B-lectine kinase protein, respectively (Chen et al. 2006, 2014; Fukuoka et al. 2009). Conversely, 11 avirulence (*Avr*) genes have been characterized to date in the rice blast pathogen, all of which encode secreted proteins of as-yet-unknown function, except *ACE1*. *ACE1* encodes a polyketide and peptide synthase (Böhner et al. 2004) and is uniquely different from other known *Avr* genes in that it is not the *ACE1* protein itself that is recognized by the host plant but the enzyme product of its secondary metabolite (Valent

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and Khang 2010). *AvrPiz-t* suppresses basal immunity by affecting flg22-mediated generation of reactive oxygen species (Park et al. 2012). It interferes with E3 ligase activity and promotes protein degradation by interacting with rice RING E3 ligases APIP6 and APIP10 (Park et al. 2012, 2016).

It is evident that resistant varieties are usually defeated by the evolution of virulent strains shortly after deployment mainly caused by the quick evolution of the pathogen. It has been proposed that the frequent evolution toward virulence in *M. oryzae* is mainly due to mutations in *Avr* genes (Orbach et al. 2000). Rice blast fungus and many other plant pathogens use DNA modification in their *Avr* gene to suppress or evade host recognition, thereby allowing them to infect a previously resistant plant genotype (Kang et al. 2001; Zhou et al. 2007). Such DNA manipulations include frameshift mutation, insertion of transposon, and point mutations within the *Avr* gene or its complete loss (Wu et al. 2015; Yoshida et al. 2009). Beyond DNA mutations, alteration of the expression of a corresponding *Avr* gene by causing mutation in its regulatory region has also been reported as one of the mechanisms pathogens use to evade host recognition (Shan et al. 2004).

The *Pib* gene identified from the *indica* rice gene pool belongs to the NBS-LRR class of plant *R* genes encoding a polypeptide of approximately 1,251 amino acids (Wang et al. 1999). Long before *Pib* was cloned, it had been deployed by several breeding programs and introgressed into rice cultivars in Philippines, Japan, China, and many other rice-growing regions of the world to confer resistance to rice blast races with the cognate *Avr* gene (Wang et al. 1999; Zeigler et al. 1997). More recently, *Pib* together with *Pi-kh* was introgressed into elite varieties through marker-assisted back crossing due to the broad-spectrum resistance of the gene in Malaysia (Tanweer et al. 2015). Varieties carrying the *Pib* gene have been intensively used in *indica* rice breeding programs in Asia. For instance, IR 50 contains *Pib* and a number of successful Chinese cultivars, notably Teqing and Texianzhan25, carry the *Pib* *R* gene (Khush and Jena 2009; Zhang et al. 2015). Its cognate gene, *AvrPib*, has recently been isolated by a map-based cloning method in *M. oryzae*. It encodes a 75-residue protein, including a signal peptide (Zhang et al. 2015). Phenotyping and genotyping of Chinese isolates revealed four genetic events leading to its loss of function (i.e., transposable element [TE] insertion, segmental deletion, absence, and point mutation) (Zhang et al. 2015).

Identification of the molecular mechanisms of pathogen adaptation and evolution, particularly effector diversification, is important for pathogen-informed crop protection. In this study, we investigated the effectiveness of *Pib*-mediated resistance in the Philippines and the molecular basis of adaptation of Philippine isolates of *M. oryzae* to *Pib*. We analyzed the sequence variation in *AvrPib* of Philippine field isolates and determined their correlation between the *AvrPib* genotype and pathotype to the *Pib* gene. We also investigated the association between *AvrPib* haplotypes and genome-wide diversity based on DNA fingerprints. The frequency of *Pib* in released varieties was also investigated to provide insight into the host force driving the evolution of rice blast toward virulence.

MATERIALS AND METHODS

Fungal isolates, culture, rice cultivars, and pathogenicity assay. Rice-blast-infected leaf samples were collected from a rice blast hotspot in Bohol, the Philippines. In total, 250 isolates were isolated from the leaf samples. Isolates were preserved on filter paper in Petri dishes and stored in a -20°C refrigerator. Stored isolates were revived on prune agar medium and grown for 7 days in a 28°C incubator. Revived isolates were subcultured on the prune agar medium for another 7 days and mycelia were scrapped with a sterilized glass slide. Scraped fungal cultures were placed under near UV light for 3 days at 18°C to enhance sporulation. A suspension of 5×10^4 conidia/ml was made from highly sporulating

plates and used for inoculation. Tween 20 (600 μl) was added into 1 liter of autoclaved water for suspension preparation to enhance adhesion of fungal spores on plant leaves. The *Pib* rice monogenic International Rice Research Institute (IRRI)-bred Blast-resistant line (IRBL)b-B and two susceptible checks, its backcross parent Lijiangxintuanheigu (LTH) and CO39, were used for the pathogenicity assay. Pregerminated seed of IRBLb-B, LTH, and CO39 were sown at 10 seeds per line per row in a rectangular food keeper. Rice seedlings at the three- to four-leaf stage were used for the pathogenicity assay. Spraying inoculation method was employed, one food keeper per isolate. In total, 250 isolates together with the standard differential isolates were pathotyped to *Pib*. Disease reactions were evaluated for susceptible and resistant phenotypes. The disease reactions were evaluated based on the number of lesions using a standard rice blast disease evaluation scale, where 0 to 2 equals resistant and 3 to 5 equals susceptible (IRRI 2014).

DNA preparation, polymerase chain reaction, and sequencing. Fungal isolates were cultured in liquid media at room temperature with continuous shaking for 1 week, and fungal total DNA was isolated from mycelia using a modified cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroofof et al. 1984). Mycelia were harvested by suction pressure and transferred into 2.0- μl tubes and the cell wall was broken mechanically by grinding with 1,000- μl micropipette tips in liquid nitrogen. Afterward, 2 ml of CTAB buffer was added to remove cell components other than the nucleic acids and heated at 65°C for 1 h. Chloroform (600 μl) was added to each sample, mixed thoroughly, and centrifuged at room temperature at 13,000 rpm for 10 min. Supernatant (600 μl) was transferred into 1.5- μl tubes, mixed with 400 μl of isopropanol, and centrifuged at 13,000 rpm for 5 min. The DNA pellet was washed with 500 μl of 70% ethanol, centrifuged at 13,000 rpm for 3 min, and left to dry in the flow hood for 5 h. After drying, 60 μl of polymerase chain reaction (PCR)-graded water was added and stored at -20°C . Genomic DNAs of rice varieties were isolated from leaf samples of 2-week-old plants using the CTAB method, as described above.

Each PCR was done in a 20- μl total reaction volume consisting of 2.0 μl of $10\times$ buffer (Takara Bio Inc.), 1.6 μl of dNTPs (400 mM each), 0.4 μl (10 μM) of each forward and reverse primers, 14.5 μl of distilled water, 0.1 μl of recombinant Taq DNA polymerase (Takara Bio Inc.), and 1.0 μl (25 ng) of template DNA. The PCR for the *AvrPib* was performed using the following program: 94°C for 2 min; 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s; and a final extension of 72°C for 5 min. All reactions were performed in a G-storm thermal cycler (G-Storm Biotechnology) in a 20- μl total volume for detection and 50 μl for DNA sequencing. PCR amplicon size was verified by 1% gel electrophoresis using 100 V for 30 min with 2 μl of a 1-kb Plus DNA ladder (ThermoFisher Scientific) and was documented with a digital imaging system with Quantity one software (version 4.5.1; Bio-Rad Laboratories). The PCR amplicon was subjected to direct sequencing at Invitrogen (Shanghai). To determine the position of the Pot3 element in *AvrPib*, the sequence of *AvrPib*'s amplicon using the primer pair 10583SF and 10583CR was compared with the one in the avirulent strain (GenBank accession number KM887844). The Pot3 element was identified by homology search using the BLASTN program. The primers used for PCR diagnosis of the *AvrPib* and *Pib* genes in rice blast and rice varieties, respectively, are listed in Supplementary Table S1.

Repetitive-element palindromic PCR, cluster analysis, and mating type analysis of field isolates. Genetic diversity study of the rice blast pathogen population was conducted by repetitive-element palindromic (rep)-PCR. rep-PCR was carried out using the primers of POT2-1 and POT2-2, as described previously (GenBank accession number Z33638) (George et al. 1998). Each rep-PCR contained 1.132 μM each primer pair, 20 ng of genomic DNA, 0.2 mM each of four dNTPs, and 2.5 U of Ex Taq polymerase in $10\times$ ex-buffer (Takara Bio Inc.), and was filled to 25 μl total volume with sterile nano-pure water. The reaction

mixture was amplified by initial denaturation at 95°C for 2.5 min; 4 cycles of 1 min of denaturation at 94°C, annealing at 62°C for 1 min, and extension at 65°C for 10 min; followed by 26 cycles of 30 s of denaturation at 94°C, annealing at 62°C for 1 min, and extension at 65°C for 10 min; with a final extension time of 15 min at 65°C in a G-Storm thermal cycler (G-Storm Biotechnology). The DNA fingerprint was profiled based on the patterns generated by rep-PCR. The pattern was scored based on the presence or absence of a DNA band of a particular size in all isolates tested, which was then converted into binary codes (1 and 0 for presence and absence, respectively, of a particular band size). Dice's coefficient was used to determine the similarity matrices and a cluster analysis was done by using the unweighted pair-group method with arithmetic averages method. The similarity matrices and the cluster analysis were done with BioNumerics software (Applied Math Inc.).

A mating type analysis of isolates was performed using MAT-specific primers designed from the sequences of MAT1-1 isolate 70-15 and MAT1-2 isolate Y93-245c-2, with the accession numbers XM_003720674.1 and AB372210.1, respectively. The PCR profile used was as described above for *AvrPib* amplification.

Phylogenetic analysis. DNA sequences of several amplicons obtained with the *Pib* homolog-specific primers together with the DNA sequence of the *Pib* locus in Nipponbare and Shuhui498 were analyzed for their genetic relatedness. The sequence alignments were done with Sequencher software, and the neighbor-joining method was used to construct the phylogenetic tree in the same software with a bootstrap of 1,000 replications.

TABLE 1. *Pib* homologs in different rice lines

Rice lines	Primers	Group of <i>Pib</i> homologs	Identity to <i>Pib</i> (%)	Note
1, 5, 7, 16	PibF4/PibR7	GI	100	Identical to <i>Pib</i>
15, 42, 47	PibF4/PibR7	GII	92	...
3, 6, 14, 16, 18, 25, 47	PibF4/Pib-CO39-R5	GIII	91	...
15, 42	PibF4/Pib-CO39-R5	GIV	97	...
30	PibF4/Pib-CO39-R5	GV	92	...

RESULTS

Rice blast isolates collected in Bohol, Philippines are virulent to *Pib*. In total, 248 of 250 isolates collected from a broad range of rice varieties (Table 1) at a rice blast disease hotspot in Bohol were pathotyped against six different IRBLs. These isolates showed a varying frequency of avirulence and virulence against these six IRBLs (Fig. 1A). The virulence frequencies of these isolates against IRBL9-W (*Pi9*), IRBLi-F5 (*Pii*), IRBL5-M (*Pi5*), IRBLk-ka (*Pik*), and IRBLta-K1 (*Pita*) were 0, 2, 3, 54, and 62%, respectively, while all isolates were virulent to IRBLb-B (*Pib*). However, IRBLb-B is resistant to three *Pib* avirulent isolates (B90002, V86010, and M101-1-2-9-1) (Fig. 1B), as described previously (Telebanco-Yanoria et al. 2008), suggesting that the virulence of these 248 isolates to IRBLb-B was most likely attributable to the loss of function of *AvrPib*. Based on the pathotyping data, we concluded that the blast population in Bohol was dynamic in virulence spectrum against different IRBLs, and was highly virulent to *Pib*.

Three virulence haplotypes of *AvrPib* are present in rice blast isolates in Bohol. To investigate the mechanisms of the virulence of these isolates against *Pib*, the genomic sequence of the *AvrPib* gene covering its promoter and coding sequence (CDS) was PCR amplified. Three different PCR amplification patterns were observed in the 248 virulent isolates using different primer pairs along the *AvrPib* sequence. The first pattern was resolved by the primer pair of AvrPib-5F and AvrPib-1R covering the promoter region of *AvrPib* (Fig. 2A). A PCR amplicon >2 kb in size was obtained in 17 isolates whereas an amplicon with an expected size (376 bp in the avirulent isolates) was obtained in the remaining 231 isolates and the avirulent isolate as well (Fig. 2B; Supplementary Table S2). The second pattern was resolved by the primer pair AvrPib-2F and AvrPib-2R covering the promoter and 5' portion of CDS (Fig. 2A). Similar to the first pattern, 201 isolates produced amplicons with a larger size whereas the other 47 and the avirulent isolates produced an amplicon with an expected size (Fig. 2B). The third pattern was resolved by the primer pair AvrPib-3F and AvrPib-R covering the 3' portion of CDS (Fig. 2A). An amplicon with a larger size was obtained in 30 isolates whereas one with an expected size was

Fig. 1. Low frequency of *AvrPib* in the rice blast population in Bohol. **A**, Avirulence frequency of rice blast isolates in Bohol against different International Rice Research Institute-bred Blast-resistant lines (IRBLs). Different IRBLs were indicated with different resistance genes. **B**, Contrasting pathotype of isolates on IRBLb-B. MO15-26, MO15-2, and MO15-1 are *avrPib*H1, -H2, and -H3 isolates, respectively, whereas B90002, V86010, and M101-1-2-9-1 are *AvrPib* isolates.

obtained in the other 218 isolates and the avirulent isolates (Fig. 2B). The nature of the amplicons with a larger size was further determined by sequencing. Interestingly, the amplicons from five randomly selected isolates representing each pattern were identical to each other (data not show), suggesting that these three patterns revealed by PCR amplification could represent three different haplotypes of *AvrPib*, designated herein as *avrPib*-H1, -H2, and -H3 (Fig. 2B). A sequence analysis further revealed that an insertion of a Pot3 element (99% in overall sequence identity to that of GenBank accession number U60989) was present at the *AvrPib* region in each of the *avrPib*-H1, -H2, and -H3 isolates. However, the sequence besides the Pot3 element was identical to the one in the wild-type sequence of *AvrPib* (GenBank accession number KM887844), explaining the size discrepancy of the PCR amplicons of these 3 *avrPib* haplotypes from the one of the avirulent isolate. Pot3 was present at the positions of -304, -125, and +169 bp in *avrPib*-H1, -H2, and -H3, respectively (Fig. 2A). It is worth noting that none of the isolates contained more than one haplotypes of *AvrPib*.

The rice blast population in Bohol displays significant genetic diversity. To investigate the mechanisms mediating the spread of *AvrPib* haplotypes in field isolates, we studied the genetic diversity of the rice blast population collected in Bohol by Pot2 fingerprinting. In all, 47 isolates consisting of three different *AvrPib* haplotypes were subjected to DNA fingerprinting by rep-PCR using inverted repeat primers of Pot2. In total, 17 patterns were resolved based on the number and size of DNA bands resolved by rep-PCR (Fig. 3). According to the definition of a lineage in which the patterns share >70% similarity to each other by Dice's coefficient analysis, these Pot2 patterns were further clustered into seven lineages (Fig. 3). Within each lineage, all isolates have the same *AvrPib* haplotypes, suggesting that the isolates having the same *AvrPib* haplotypes originated from the same progenitor. However, the isolates having the same *AvrPib* haplotypes did not always belong to the same lineages. For example, the *avrPib*-H1 and *avrPib*-H2 isolates each were scattered into three different lineages, suggesting that these isolates underwent significant sequence divergence from their progenitor after the insertion of Pot3 (Fig. 3). Moreover, we found that the isolates having the same *AvrPib* haplotypes did not always show closer lineage relationships than those having different *AvrPib* haplotypes. For example, the lineage containing MO15-16, -10, and -128 isolates having *avrPib*-H2

shared a closer lineage relationship to the one containing isolates having *avrPib*-H3 (Fig. 3). This closer lineage relationship was also observed in some isolates having *avrPib*-H1 and *avrPib*-H2 haplotypes (Fig. 3). Nonetheless, we noted that the similarity scores connecting these different lineages were relatively low, which may result in an inaccurate relationship among some lineages (Fig. 3).

MAT1-2 is dominant in the rice blast population in Bohol. To investigate the composition of MAT1-1 and -2 isolates in the rice blast population in Bohol, we conducted the mating type analysis of all 248 isolates using specific primers for MAT1-1 and -2. In total, 7 and 245 isolates were MAT1-1 and 1-2, respectively, suggesting that a predominant frequency of MAT1-2 isolates was present in the blast hotspot in Bohol. Intriguingly, four isolates (i.e., MO15-27, -28, -30, and -104) were found to have both mating types, which were further verified by the sequencing of the PCR amplicons. Three *AvrPib* haplotypes were found in MAT1-2 isolates whereas only *avrPib*-H1 and -H2 were found in MAT1-1 isolates. Moreover, four MAT1-1 isolates (i.e., MO15-27, -104, -106, and -178) selected for the rep-PCR analysis were found to form a separate lineage and showed a quite distant lineage relationship from other isolates (Fig. 3).

***Pib* is frequently present in released varieties in the Philippines.** To investigate the frequency of *Pib* in released varieties, we randomly selected 52 varieties released by IRRI and University of the Philippines, Los Baños from 1969 to 2015 (Supplementary Table S3). A sequence analysis between the *Pib* gene (GenBank accession number AB013448) and its susceptible allele in Nipponbare (position in chromosome 2: 35,102,960-35,109,965) (<http://signal.salk.edu/cgi-bin/RiceGE>) revealed that the 3' untranslated regions (3'UTR) were highly divergent from each other. Thus, we synthesized several pairs of primers from the 3' terminus and 3'UTR of *Pib* for the diagnosis of its presence in different rice varieties. Three pairs of primers were validated and used for the two-step PCR diagnosis (Fig. 4A). PCR with the primer pair *Pib*-F3/R5 produced an amplicon at the expected size (approximately 350 bp) in IRBLb-B and 32 of 52 tested rice varieties (Fig. 4B). Sequencing of this amplicon further revealed that it corresponded to the expected *Pib* fragment. Unexpectedly, an amplicon at a smaller size (approximately 250 bp) was observed in 48 tested varieties and the susceptible variety CO39. Nonetheless,

Fig. 2. Polymerase chain reaction (PCR) genotype of field isolates. **A**, Positions of Pot3 transposon within *AvrPib* sequence (KM887844.1). Triangles denote an approximately 1.8-kb Pot3 transposon identified at the three marked positions in different isolates. *avrPib* haplotypes were identified based on size difference when amplified with locus-specific primers, as denoted in **B**. **B**, Primer pairs on each gel plane were used for PCR genotyping of field isolates. Isolates 17, 201, and 30 show sizes higher than expected when amplified with the three specific primers (i.e., H1, H2, and H3, respectively). Sequencing of randomly selected isolates from the groups show insertion of a Pot3 transposon in the amplified region. Wild-type isolate (WT) has a consistent expected amplicon size at all positions and is avirulent on *Pib* monogenic line IRBLb-B. The three haplotypes were represented by the isolates MO15-26, MO15-2, and MO15-1, respectively, and M101-1-2-9-1 as the avirulent isolate.

the sequence of this amplicon turned to be ambiguous, suggesting that the smaller amplicon could be derived from nonspecific amplification. It was found that three rice varieties (i.e., numbers 12, 23, and 39) did not produce any PCR amplicon, like the two susceptible rice varieties LTH and Nipponbare (Fig. 4). To further determine which varieties contain the *Pib* gene, two primer pairs specific to *Pib* and its allele in CO39 were used for the analysis (Fig. 4A). As expected, all 32 rice varieties producing the 350-bp fragment using Pib-F3/R5 showed the specific amplification with an expected size, as did IRBLb-B using Pib-F4/R7 (Fig. 4B).

However, four other rice varieties (numbers 15, 30, 42, and 47), negative in PCR amplification using Pib-F3/R5, produced the specific amplicon at the similar size by Pib-F4/R7 (Fig. 4B). These four amplicons were then sequenced together with four randomly selected amplicons from lines (numbers 1, 5, 7, and 16). The sequences of all of the amplicons, except the one from number 30 by Pib-F4/R7, were determined and classified into two groups, designated as Pib-GI and -GII (Table 1). It was found that Pib-GI and Pib-GII were identical and had 92% identity in sequence to *Pib*, respectively, indicating that those four varieties positive for

Fig. 3. Repetitive element palindromic polymerase chain reaction (rep-PCR) fingerprinting of Philippine isolates of *Magnaporthe oryzae*. Forty-seven isolates consisting of three *avrPib* haplotypes were fingerprinted by Pot2 using inverted repeat primers of Pot2. In total, 17 patterns marked in rectangles were identified based on the number and size of DNA bands resolved by rep-PCR. Dice's coefficient was used to calculate similarities between DNA patterns. Seven lineages marked by blue boxes which were defined based on the similarity not less than 70% between patterns were identified.

Fig. 4. Polymerase chain reaction (PCR) diagnosis of *Pib* in rice varieties in the Philippines. **A**, Schematic diagram of the diagnosis of *Pib*. The primer pair of PibF3 (1) and PibR5 (2) which worked in the *Pib* donor and CO39 but not in Nipponbare was first used to diagnose the presence of *Pib* homologs in rice varieties. The primer pair of PibF4 (3) and PibR7 (4) which worked only in the *Pib* donor but not in CO39 was then used for the diagnosis of the presence of *Pib* in rice varieties. The primer pair of PibF4 and Pib-CO39-R5 (5) was used for the diagnosis of *Pib* homologs in CO39. Exons are indicated by rectangles and arrows for the last exon. Regions sharing extremely high sequence similarity (>95% identity on average) are shadowed. The position of the primer is indicated by arrows. **B**, Three pairs of primers were used to classify the different alleles of *Pib* in Philippines varieties. The pair PibF3/PibR5 resolved amplicons at the expected size (approximately 350 bp) in IRBLb-B (number 53) and 32 of 52 tested rice lines. Meanwhile, an amplicon at a smaller size (approximately 250 bp) was resolved in 48 tested lines and the susceptible variety CO39. Two more primers specific to *Pib* and its allele in CO39 were developed. All 32 *Pib*-positive varieties with PibF3/PibR5 were also positive with PibF4/PibR7, however. Four varieties (numbers 15, 30, 42, and 47) negative in PCR amplification using PibF3/PibR5 were otherwise positive using PibF4(3)/PibR7(4). Consequently, a total of 15 lines produced specific amplicons, each with an expected size similar to the one from CO39 using PibF4/Pib-CO39-R5(5).

amplification by both Pib-F3/R5 and Pib-F4/R7 contained *Pib*. Thus, it is reasonable to speculate that all 32 varieties with expected amplicons by these two primer pairs contained *Pib*. On the other hand, a total of 15 varieties produced specific amplicons of the expected size, similar to the one from CO39 using Pib-F4/CO39-R5 (Fig. 4B). It is worth noting that only variety number 16 of the 32 varieties positive with Pib-F3/R5 produced an amplicon by Pib-F4/CO39-R5. Sequencing of the PCR amplicons by Pib-F3/CO39-R5 further resulted in the classification of 10 sequences into three groups, designated as Pib-GIII to -GV (Table 1). They shared a significant sequence similarity to *Pib*, varying from 91 to 97% (Table 1). Nonetheless, none of them were identical to the *Pib* gene, suggesting that they might be nonfunctional alleles of *Pib*.

We further investigated the genomic organization of the *Pib* locus in two rice varieties (Nipponbare and Shuhui498) that have map-based reference genome sequences. Contrasting to the genomic organization of the *Pib* locus in Nipponbare, which contained only a single gene (35,102,960-35,109,965bp on chromosome 2) (<http://signal.salk.edu/cgi-bin/RiceGE>), three *Pib* homologs designated as Pib-R498-I to III (Fig. 4) were identified at the *Pib* locus in Shuhui498 (approximately 36,868,779-36,924,798bp on chromosome 2; GenBank accession number CP018158). All of the *Pib* homologs in different rice varieties identified in this study were then used for building a phylogenetic tree (Fig. 5). In total, nine different *Pib* homologs were identified (Fig. 5). Moreover, some rice varieties such as numbers 15, 16, 42, and 47 contained more than one copy of *Pib* homologs, which was also observed in Shuhui498 (Table 1). It was found that the *Pib* allele in typical *japonica* variety Nipponbare (*Pib*-NPB), was relatively divergent from those of other *indica* rice varieties (Fig. 5). Thus, we speculate that the sequence and genomic organization of the *Pib* locus is highly complex and dynamic in different rice germplasm.

DISCUSSION

Determination of the effectiveness of a rice *R* gene based on the information derived from the frequency of its cognate *Avr* gene in the pathogen population is an informed approach for varietal improvement for resistance against rice blast. *Pib* is a major gene that was originally identified in *indica* rice germplasm and was found to confer effective resistance in different regions worldwide (Wang et al. 1999). However, the effectiveness of *Pib* was documented to be eroding due to the high frequency of virulent isolates in the rice blast population in some regions. Fukuta et al. (2014) reported that more than 60% of 122 Cambodian isolates were virulent to IRBLb-B and more than 70% in Hunan province of China (Xing et al. 2017). In this study, we determined the effectiveness of *Pib* by characterizing the virulence spectrum of a population consisting of 248 field isolates of *M. oryzae* from a hotspot based on pathotyping against the *Pib* isogenic line and genotyping of *AvrPib*. All isolates tested were virulent on IRBLb-B, suggesting that *Pib* has been defeated in the test region. It is worth noting that a blast resistance gene profile in Philippine elite varieties is sparsely known. The molecular diagnosis of 52 historic and advanced varieties showed that >60% of tested varieties contained *Pib*. We also traced the introgression of *Pib* into IRRI varieties back to as early as 1966 in IR8. Our work verified that *Pib* was extensively used in *indica* rice breeding in the Philippines since the 1960s (Khush and Jena 2009). The continuous deployment of a particular *R* gene over a period of time has been proposed to mount selection pressure on the pathogen and strains with the best fitness and reproductive success, favoring the accumulation of the virulent strains in the population and, subsequently, resulting in the loss of effectiveness of the *R* gene (Jones and Dangl 2006; Kamoun 2007). Thus, we speculate that the wide deployment of *Pib* over a period of five decades effectuated the positive selection of virulent strains in the population, therefore reducing the effectiveness of *Pib*-mediated resistance. Indeed, IRBLb-B was observed to be highly

susceptible in the screening sites in both Bohol and the IRRI experimental station in the Philippines (data not shown).

Different genetic events leading to the loss of function of *AvrPib* were identified from 300 isolates collected in five different rice-growing areas in China, out of which TE insertion in various positions accounted for approximately 80% in the virulent isolates (Zhang et al. 2015). In this study, we found that the *AvrPib* sequence was disrupted exclusively by a Pot3 element in all isolates, reiterating the important role of TEs in mediating the loss of function of *Avr* genes in rice blast (Li et al. 2009; Wu et al. 2015). Moreover, three events of Pot3 insertion in different positions resulting in the generation of different *AvrPib* haplotypes were identified, which are unique from those described previously (Zhang et al. 2015) in the Philippine isolates, suggesting that TE insertion in the sequence of *AvrPib* could be relatively random and more dynamic. We reason that mutant isolates mediated by the insertion of Pot3 individually at the different positions of *AvrPib* emerged and gained the virulence to *Pib*, and were further selected by the *Pib*-harboring varieties and dominated in the population. Given that the isolates dropped within the same lineage were found to have the same *AvrPib* haplotype, we speculate that clonal reproduction posterior to the different progenitors, each with a unique insertion position of Pot3, could contribute significantly to the build up of the population, as documented in the field rice blast population previously (Levy et al. 1991, 1993). Nonetheless, genetic divergence among isolates having the same *AvrPib* haplotypes, particularly in *avrPib*-H1 and -H2 isolates (Fig. 3), indicates the occurrence of genetic exchange among the isolates, reiterating the finding that *M. oryzae* is not as exclusively clonal as many other fungal species (Chuma et al. 2011; Zeigler et al. 1997).

It is a matter of debate about the mechanisms underlying the genetic recombination in *M. oryzae*. The evidence of parasexual recombination of DNA identified in the rice blast population suggests that parasexual recombination takes place in the field (Zeigler et al. 1997). Chuma et al. (2011) demonstrated the horizontal gene transfer mediated by TEs in asexual clones of the rice blast pathogen. Nevertheless, the extent to which the asexual recombination contributes to the genetic divergence of the rice blast pathogen population is still elusive. Moreover, the sexual cycle was observed in a contemporary population of *M. oryzae*, demonstrating that the rice blast pathogen also reproduces sexually in nature, at least in some regions in Southeast Asia, the center of its origin (Saleh et al. 2012). In this study, we found that both *avrPib*-H1 and -H2 isolates contain two mating types, although MAT1-1 is disproportionately low in frequency. Moreover, the MAT1-1 isolates having *avrPib*-H1 form a separate lineage (Fig. 3), suggesting that genetic recombination took place prior to its separation from other

Fig. 5. Phylogenetic relatedness of *Pib* homologs in different rice varieties. Polymerase chain reaction amplicons in selected rice lines using different primer pairs were sequenced and used for the phylogenetic analysis. The *Pib* homologs from CO39, Nipponbare, and Shuhui498 (*Pib*-R498-I to -III) were included in the phylogenetic analysis. Bootstrap values are indicated.

isolates having the same *AvrPib* haplotype. Both the presence of two mating types and genetic recombination between isolates imply that sexual recombination could be also involved in the genetic exchange in the rice blast population.

A Southern blotting analysis revealed that both resistant and susceptible rice varieties contained multiple *Pib* homologs (Wang et al. 1999). The sequence analysis of the reference genomes of Nipponbare and Shuhui498 revealed that they contain one and three copies of *Pib* homologs, respectively (Fig. 5). Moreover, nine *Pib* homologs were identified in different rice varieties which share significant sequence similarity to each other, indicating that the *Pib* locus is highly dynamic with respect to its genomic organization. The complexity of the *Pib* locus could blur the diagnosis of the existence of *Pib* in different rice varieties by PCR amplification using gene-specific primers. Instead, we herein introduced a method similar to the so-called targeted amplicon sequencing (<https://www.illumina.com/>) by combining PCR amplification and amplicon sequencing for the diagnosis of *Pib* in different rice varieties. Given the fact that many *R* genes are distributed as multiple alleles or haplotypes with a high sequence similarity in different rice varieties, haplotype sequencing is believed to provide a more reliable and precise method for the diagnosis of known *R* genes from their functional and nonfunctional alleles in different rice varieties. The distribution of *Pib* in >60% of released varieties in the Philippines since the late 1960s could be associated with the high selection pressure to the rice blast pathogen for the depletion of *AvrPib*-containing isolates in the population, which, to some extent, can explain the low resistance frequency of *Pib* against the contemporary blast population.

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