Gene Duplication and Mutation in the Emergence of a Novel Aggressive Allele of the *AVR-Pik* Effector in the Rice Blast Fungus

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Higher yield potential and greater yield stability are common targets for crop breeding programs, including those in rice. Despite these efforts, biotic and abiotic stresses continue to impact rice production. Rice blast disease, caused by *Magnaporthe oryzae*, is the most devastating disease affecting rice worldwide. In the field, resistant varieties are unstable and can become susceptible to disease within a few years of release due to the adaptive potential of the blast fungus, specifically in the effector (avirulence [*AVR*]) gene pool. Here, we analyzed genetic variation of the effector gene *AVR-Pik* in 58 rice blast isolates from Thailand and examined the interaction between *AVR-Pik* and the cognate rice resistance gene *Pik*. Our results reveal that Thai rice blast isolates are very diverse. We observe four *AVR-Pik* variants in the population, including three previously identified variants, *AVR-PikA*, *AVR-PikD*, and *AVR-PikE*, and one novel variant, which we named *AVR-PikF*. Interestingly, 28 of the isolates contained two copies of *AVR-Pik*, always in the combination of *AVR-PikD* and *AVR-PikF*. Blast isolates expressing only *AVR-PikF* show high virulence to rice cultivars encoding allelic *Pik* resistance genes, and the *AVR-PikF* protein does not interact with the integrated

heavy metal-associated domain of the *Pik* resistance protein *in vitro*, suggesting a mechanism for immune evasion.

Rice blast disease, caused by the ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), is the most devastating disease of rice production worldwide (Couch and Kohn 2002). The fungus can affect all parts of the rice plant during all stages of growth (Zhang et al. 2014). The best strategy for control of blast disease is using genetically encoded resistant rice varieties. Plants, including rice, have developed basal defense responses that are triggered by detection of conserved microbe-associated molecular patterns, usually at the cell surface (Dodds and Rathjen 2010). Pathogens secrete effectors to the plant apoplast and inside of cells, many of which interfere with basal defense signaling and can re-establish disease (Hogenhout et al. 2009; Rafiqi et al. 2012). To respond to the presence of effectors delivered inside cells, plants have evolved intracellular immune receptors of the NLR (nucleotide-binding leucine-rich repeat) superfamily. Classically, these receptors follow a ‘gene-for-gene’ relationship, in which a plant NLR recognizes a single pathogen effector. This recognition triggers the hypersensitive response and programmed cell death to block pathogen growth (Rafiqi et al. 2012). Co-evolution between pathogens and hosts has driven diversification of effectors and NLRs. For example, an effector may be deleted from a pathogen genome or acquire amino acid polymorphisms that prevent recognition and this could be selected for in the population. In the latter case, the plant may acquire mutations in NLRs that re-establish recognition and disease resistance.

More than 100 rice blast disease resistance loci have been reported in the rice genome, and 30 of these have been cloned (Liu et al. 2014; Wang et al. 2017). Identification of the pathogen effectors recognized by these resistance proteins has lagged behind, and only 12 have been cloned to date, *PW1* (Kang et al. 1995), *PW2* (Sweigard et al. 1995), *ACE1* (Fudal et al. 2005), *AVR-Pia* (Yoshida et al. 2009), *AVR-Pita* (Orbach et al. 2000), *AVR-Pik* (Yoshida et al. 2009), *AVR-Pii* (Yoshida et al. 2009), *AVR-Piz-t* (Li et al. 2009), *AVR1-CO39* (Ribot et al. 2013), *AVR-Pi9* (Wu et al. 2015), *AVR-Pib* (Zhang et al. 2015), and *AVR-Pi54* (Ray et al. 2016).

Thailand has a strong tradition of rice production. This crop is critical for the Thai economy and employs a significant portion of the labor force. Most Thai farmers elect to grow consumer-preferred rice varieties such as Khao Dawk Mali 105 (KDML105) and Kho Khor 6 (RD6) (Noenplab et al. 2006), but these are highly susceptible to rice blast disease. Rice blast

Sequence data from this article can be found in the GenBank databases under the following accession numbers: MH078516 (*AVR-PikF*), BioProject ID: PRJNA438312 (*Magnaporthe oryzae* isolate 10100), BioSample accession: SAMN08712647 (*Magnaporthe oryzae* isolate 10100), PYAN000000000 (*Magnaporthe oryzae* isolate 10100 whole-genome sequence), SRA accession: SRP152923 (*Magnaporthe oryzae* isolate 10100).

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epidemics have occurred in most provinces in the north and some in the northeast of Thailand (Disthaporn 1994; Khanthong et al. 2010). Jao Hom Nin (JHN) is a Thai nonsticky rice variety that has a long deep-purple grain (Waiyawuththanapoom et al. 2015). JHN demonstrated the highest broad-spectrum resistance against the rice blast pathogens in Thailand and the Philippines (Chaipanya et al. 2017; Sreewongchai et al. 2010). Two resistance genes in JHN were mapped. One, *Pish*, was mapped to chromosome 1 and shows a minor resistance phenotype, while the other, *Pi7* (an allele in *Pik* locus), located on chromosome 11, has a major effect (Chaipanya et al. 2017).

Rice resistance genes at the *Pik* locus encode multiple alleles (*Pik**, *Pikm*, *Pikp*, *Piks*, *Pikh*, *Pil*, and *Pi7*) with differential resistance profiles to *M. oryzae* isolates (Chaipanya et al. 2017; Kanzaki et al. 2012). *Pik* comprises two open reading frames encoding NLR proteins *Pik-1* and *Pik-2*, which are separated by approximately 2.5 kb of noncoding region and are oriented in opposite directions (Ashikawa et al. 2008). The *Pik-1* gene shows a high number of DNA sequence polymorphisms, many of which are clustered within the integrated heavy metal-associated (HMA) domain that is responsible for the direct binding of the AVR-Pik effector (De la Concepcion et al. 2018; Langner et al. 2018; Maqbool et al. 2015). Furthermore, two amino acid residues at positions 229 and 252 were proposed as diagnostic markers for the classification of *Pik* alleles (Costanzo and Jia 2010) and both of these amino acids are located within the HMA domain. In contrast, the *Pik-2* gene shows low levels of DNA polymorphism.

The *M. oryzae* AVR-Pik effector gene encodes a 113-amino acid protein with a 21-amino acid signal peptide (Yoshida et al. 2009). Five variants (alleles) of AVR-Pik (*AVR-PikA*, *AVR-PikB*, *AVR-PikC*, *AVR-PikD*, and *AVR-PikE*) are different from each other by a total of five nucleotide substitutions (Fig. 1), all of which cause amino acid changes. The *AVR-PikB* variant is rare and is only found in a Japanese isolate (Yoshida et al. 2009).

Alleles of the rice NLR *Pik* show differential recognition specificities toward different *M. oryzae* AVR-Pik effector variants (De la Concepcion et al. 2018; Kanzaki et al. 2012). Rice expressing *Pikp* and *Pi7* NLR alleles are only resistant to *M. oryzae* harboring *AVR-PikD*. Rice expressing *Pikm* is resistant to *M. oryzae* harboring *AVR-PikA*, *AVR-PikD*, or *AVR-PikE* (Chaipanya et al. 2017; Kanzaki et al. 2012), whereas rice expressing *Pik** is only resistant to *M. oryzae* harboring *AVR-PikD* and *AVR-PikE*. The molecular basis for direct recognition of AVR-Pik variants by these NLR alleles is established (De la Concepcion et al. 2018; Maqbool et al. 2015.). The effector is bound by the *Pik*-HMA domain via three predominant interaction surfaces and amino acid changes in AVR-Pik at these interfaces (both natural variants and designed mutations) affects binding in vitro and cell-death phenotypes in the model system *Nicotiana benthamiana*.

In this study, the genetic variation of AVR-Pik from rice blast isolates in Thailand was investigated. Of the 58 Thailand isolates examined, 28 were found to encode two copies of the effector. We identify a novel AVR-Pik variant, which we name *AVR-PikF*, and show that all rice cultivars tested are susceptible to *M. oryzae* strains expressing just this effector. Further, AVR-PikF is not bound by the *Pik*-HMA domains in vitro and shows no cell-death phenotype in *N. benthamiana*, suggesting a mechanism for immune evasion by this variant. Together, this work further highlights how adaptation in pathogen effector genes can overcome disease resistance to blast in rice.

RESULTS

Diverse and novel variants of the AVR-Pik effector are found in the Thai *M. oryzae* population.

To identify genetic variation of AVR-Pik in Thai rice blast isolates, we amplified a 626-bp fragment encoding this gene

alongside reference strains Guy11 and 70-15. We were able to amplify the AVR-Pik gene from 54 of 58 Thailand isolates (Table 1). Thai rice blast isolates were very diverse. AVR-Pik variants identified to date are different from each other by a total of five DNA substitutions, all of which cause amino acid changes. We found four AVR-Pik variants in the Thai isolates studied. These include three previously identified variants, *AVR-PikA* ($n = 1$), *AVR-PikD* ($n = 22$), and *AVR-PikE* ($n = 1$), and one novel variant, which was named *AVR-PikF* ($n = 2$) (GenBank accession number MH078516). We also detected the variant *AVR-PikC*, but only in the reference isolates Guy11 and 70-15 ($n = 2$). Interestingly, sequencing chromatograms from 28 of 56 positive isolates contained double peaks at four of five variable sites. To confirm this result, all 28 of these strains were re-isolated from single conidia and were cultured on rice flour agar (RFA) before repeating DNA extraction and AVR-Pik polymerase chain reaction (PCR) amplification and sequencing. The same results of double peaks in the sequencing data were obtained, indicating no cross-contamination of blast isolates. The PCR products encoding AVR-Pik from each of the 28 strains were then ligated with the pGEM-T Easy vector (Promega Madison) and were transformed into *Escherichia coli* DH5 α cells. Five white colonies (standard blue/white screening) per isolate were selected and cultured, and the plasmid DNA was extracted and sequenced. The results revealed that all 28 rice blast isolates contained two variants of the AVR-Pik gene, specifically *AVR-PikD* and the new variant *AVR-PikF* (Table 1).

Multiple copies of AVR-Pik are present in some *M. oryzae* isolates.

Next, we used quantitative real-time PCR to investigate copy number variation in a subset of Thai rice blast isolates. This analysis showed cycle threshold (Ct) values for AVR-Pik and the identified *Magnaporthe oryzae* gene (*IDM*) (a reference gene with one copy in the rice blast genome) to be near the 20th cycles with low standard deviation values, while the melting

Fig. 1. Genetic diversity of *Magnaporthe oryzae* AVR-Pik. **A**, Schematic representation of the AVR-Pik effectors. SP indicates signal peptide. The positions of variable residues are as indicated. **B**, Amino acids of six AVR-Pik allelic products (A through F) for the five variable sites (46, 47, 48, 67, and 78).

curve results showed one peak for each gene. Following three biological replicates, the mean *AVR-Pik* gene copy number of Guy11, TH196031, B1-2, 10100, 10301, BKK55001, RBR55001, and 10459 was 1.00, 1.01, 0.69, 1.51, 1.66, 2.39, 2.38, and 2.11, respectively, relative to *IDM* (Fig. 2). This result supports the sequencing results, suggesting that some rice blast isolates encode more than one copy of *AVR-Pik*.

Table 1. Rice blast isolates, location collected, year of collection, and its *AVR-Pik* allele

No.	Isolate name	Origin	Year	<i>AVR-Pik</i> allele(s) ^a
1	10100	Srakaew	2006	D and F
2	10301	Sisaket	2006	D and F
3	10302	Sisaket	2006	D and F
4	10459	Lampang	2006	D and F
5	10551	Nakhon Ratchasima	2006	D
6	10552	Nakhon Ratchasima	2006	D
7	10576	Kalasin	2006	D and F
8	10577	Nakhon Ratchasima	2006	D and F
9	10578	Nakhon Ratchasima	2006	D and F
10	10581	Nakhon Ratchasima	2006	D and F
11	10652	Maha Sarakham	2006	D
12	10681	Roi Et	2006	D and F
13	10694	Nongkhai	2006	D
14	10732	Kamphaengphet	2006	D and F
15	10760	Kamphaengphet	2006	D and F
16	10812	Chiangrai	2006	D
17	10837	Surin	2006	D and F
18	10873	Maehongson	2006	D and F
19	10926	Phayao	2006	D and F
20	10927	Phayao	2006	D and F
21	10941	Lampang	2006	D and F
22	10971	Nan	2006	D and F
23	10985	Sisaket	2006	D
24	10993	Buriram	2006	D and F
25	11100	Udonthani	2006	D and F
26	11108	Ubon Ratchathani	2006	D and F
27	11109	Ubon Ratchathani	2006	D and F
28	BAG1.2	Phitsanulok	2006	D
29	BAG4.6	Phitsanulok	2006	D
30	40.3	—	—	A
31	SRN54001	Surin	2011	D
32	SRN54002	Surin	2011	D
33	SRN54005	Surin	2011	D
34	SRN54006	Surin	2011	D
35	SRN54007	Surin	2011	D
36	SRN54009	Surin	2011	D
37	CCO56001	Chachoengsao	2013	D and F
38	CCO56002	Chachoengsao	2013	D and F
39	CCO56003	Chachoengsao	2013	D
40	CCO56004	Chachoengsao	2013	D
41	CPM55001	Chaiyaphum	2012	D
42	CPM55002	Chaiyaphum	2012	D
43	CPM55003	Chaiyaphum	2012	D and F
44	NYK55001	Nakhon Nayok	2011	D
45	NYK55003	Nakhon Nayok	2011	D
46	BKK55001	Bangkok	2011	D and F
47	BKK55002	Bangkok	2011	D and F
48	BKK55003	Bangkok	2011	D and F
49	RBR55001	Ratchaburi	2011	D and F
50	RBR55002	Ratchaburi	2011	D
51	RBR55003	Ratchaburi	2011	D
52	B1-2	Ubon Ratchathani	2001	E
53	TH196031	Ubon Ratchathani	2001	F
54	TH196036	Ubon Ratchathani	2001	F
55	TRG1	Nongkhai	2013	None
56	TRG2	Nongkhai	2013	None
57	TRG3	Nongkhai	2013	None
58	TRG4	Nongkhai	2013	None
59	70-15	Laboratory strain	1988	C
60	Guy11	French Guyana	1988	C

^a *AVR-Pik* allele analysis by sequencing.

Southern blot analysis was carried out to validate the *AVR-Pik* copy number in the rice blast genome. Genomic DNA of rice blast isolates was digested with *Bam*HI, five rice blast isolates that contained one copy of *AVR-Pik* (variants *AVR-PikA*, *AVR-PikC*, *AVR-PikD*, *AVR-PikE*, and *AVR-PikF*) showed one hybridization signal at 4 kb in size with the *AVR-Pik* probe (Fig. 3A). When using four rice blast isolates with a single copy of *AVR-PikD*, the result showed two different hybridization signal sizes, either 4 or 6.2 kb (Fig. 3B). Three of the five rice blast isolates containing two copies of *AVR-Pik*, namely, 10301, 11108, and 10760, showed two hybridization signals at 4 and 6.2 kb in size. The other two isolates showed only one signal at 4 kb (Fig. 3A). When using *Pst*I restriction enzyme, the result was consistent with that observed using *Bam*HI. Six rice blast isolates containing one copy of *AVR-Pik* showed one hybridization signal at 4 or 5.2 kb. Two isolates that both contained *AVR-PikD* showed two different hybridization signal sizes, one 4 kb and the other 5.2 kb. Two of four rice blast isolates (10100 and 10576) containing two copies of *AVR-Pik* showed two hybridization signals and the remaining two isolates showed a more intense signal at 5.2 kb. The rice blast isolate TRG1, which lacks *AVR-Pik* and therefore gives no PCR amplification product with *AVR-Pik*-specific primers showed no hybridization signal (Fig. 3C).

Whole-genome sequencing confirms the presence of two *AVR-Pik* variants in rice blast isolate 10100.

To confirm the results of the copy number analysis above, we re-sequenced the whole genome of rice blast isolate 10100, which our previous work suggested contained two copies of the *AVR-Pik* gene. This sequencing generated 3,159 scaffolds, approximately 39.55 Mb in total size, with N50 scaffold length = 108,598 and L50 scaffold count = 92. Scaffold %A, C, G, T, and

Fig. 2. Real-time polymerase chain reaction calculates the copy number by comparative cycle threshold (Ct) method with the *IDM* gene. Relative quantification calculates the copy number by comparative Ct method (also referred to as the $2^{-\Delta C_t}$ method) with *IDM* gene. Guy11, TH196031, and B1-2 represent *AVR-Pik* alleles C, F, and E, respectively, and isolates 10100, 10301, BKK55001, RBR55001, and 10459 contained two *AVR-Pik* alleles from sequencing. The result shows that the *AVR-Pik* gene has more than one copy in some isolates. The levels of statistical significance were represented as with one asterisk (*) for $P \leq 0.05$, two (**) for $P \leq 0.01$.

N are 24.26, 25.37, 25.41, 24.27, and 0.68, respectively (GenBank accession number PYAN00000000, SRA accession number SRP152923). Two contigs were shown to contain a copy of the *AVR-Pik* gene. Contig1790, a minus strand of 705 bp in length, had a sequence 100% identical to *AVR-PikD*, and Contig1787, a plus strand of 709 bp in length, had a sequence 100% identical to *AVR-PikF* (Supplementary Fig. S2). The two contigs share 98.59% similarity, with four indels and two polymorphic sites upstream of the *AVR-Pik* gene and four polymorphic sites within the gene that differentiate the *AVR-Pik* variants.

Multiple *AVR-Pik* variants are expressed during *M. oryzae* infection of rice.

To test whether both *AVR-Pik* variants are expressed in isolates encoding *AVR-PikD* and *AVR-PikF* during infection, we prepared complementary DNA (cDNA) from total RNA of rice leaf tissue infected with strain 10100. We then used *AVR-Pik_cds* primers to amplify the effector gene, with an expected size of 342 bp. The resulting *AVR-Pik cds* amplicon was

purified and sequenced. Chromatogram of PCR products showed a double peak at *AVR-Pik* variable sites (Supplementary Fig. S3). This result indicated that both *AVR-PikD* and *AVR-PikF* are expressed during rice infection.

Pik NLR alleles in rice do not respond to *M. oryzae* strains expressing *AVR-PikF*.

To investigate the pathogenicity of rice blast isolates expressing different *AVR-Pik* variants on Thai rice cultivars JHN and KDML105, we spray-inoculated 21-day-old rice seedlings. We find that KDML105 was susceptible to all rice blast isolates (Fig. 4). JHN was susceptible to rice blast isolates encoding *AVR-Pik* variants *AVR-PikA*, *AVR-PikC*, *AVR-PikE*, and *AVR-PikF* but showed resistance to an isolate with *AVR-PikD* and an isolate encoding two copies of the effector, *AVR-PikD* and *AVR-PikF*. The result suggested that *AVR-PikD* was specifically recognized by *Pi7* from JHN (Fig. 4).

As *Pi7* is an ortholog of the rice NLR *Pik*, we screened a panel of eight *Pik* international rice blast monogenic line (IRBL) rice near-isogenic lines (NILs) from the International Rice Research Institute (IRRI). These NILs harbor *Pik* alleles *Pik**, *Pikp*, *Pikm*, *Pikh*, *Piks*, *Pi1*, and *Pi7* (and also includes the negative control *Pik⁻*). Each of these NILs were spray-inoculated with three rice blast isolates encoding either *AVR-PikD*, *AVR-PikF*, or both *AVR-PikD* and *AVR-PikF*. The background cultivar of the NILs, Lijiang Xintuan Heigu (LTH),

Fig. 3. Genomic Southern hybridization analysis of the *AVR-Pik*. **A**, Genomic DNA was digested by *Bam*HI, and was hybridized with the *AVR-Pik* fragment; molecular sizes in kilobases are shown on the left. Lanes 1 to 5 represent rice blast isolates with different *AVR-Pik* alleles, *AVR-PikA*, *AVR-PikC*, *AVR-PikD*, *AVR-PikE*, and *AVR-PikF*, respectively. Lanes 6 to 10 show rice blast isolates with two copies of *AVR-Pik*, and lane 11 was undigested genomic DNA as a control. **B**, The four random *AVR-PikD* isolates that showed both different *AVR-Pik* signal sizes. **C**, Genomic DNA was digested by *Pst*I and was hybridized with the *AVR-Pik* fragment. Lanes 1 to 4 represent rice blast isolates with different *AVR-Pik* alleles, (*AVR-PikA*, *AVR-PikC*, *AVR-PikE*, and *AVR-PikF*, respectively). Lanes 5 to 8 show rice blast isolates with two copies of *AVR-Pik*. Lanes 9 and 10 show rice blast isolates with *AVR-PikD* and lane 11 was rice blast isolate TRG1, as negative control. Molecular sizes in kilobases are shown on the left.

Fig. 4. Pathogenicity assay showing recognition specificity of *Magnaporthe oryzae* *AVR-PikD* allele by *Pi7* resistance gene in Jao Hom Nin (JHN). Khaw Dawk Mali 105 (KDML105) was used as susceptible control, symptoms on rice cultivars 7 days after inoculation with each rice blast *AVR-Pik* isolate.

without a *Pik* gene (*Pik*⁻) and rice cultivar IRBLKS-F5 containing *Pik* genes were both susceptible to all three blast isolates. The *Pik* NILs, *Pik*^{*}, *Pikp*, *Pikm*, *Pikh*, *Pi1*, and *Pi7*, were resistant to *M. oryzae* harboring *AVR-PikD* and both *AVR-PikD* and *AVR-PikF*. Interestingly, all the rice cultivars tested were susceptible to *M. oryzae* harboring *AVR-PikF* (Fig. 5). This result identifies *AVR-PikF* is a previously undescribed aggressive variant that is not recognized by any tested *Pik* resistance allele.

AVR-PikF evades detection by *Pik* NLRs through disruption of protein interactions.

We hypothesized that *AVR-PikF* evades recognition by the *Pik* NLRs in rice because the mutation Met78Lys disrupts binding to the integrated HMA domains. This mutation is unique to *AVR-PikF* and distinguishes it from the effector variant *AVR-PikA* (Fig. 1). *AVR-PikA*, *AVR-PikD*, and *AVR-PikE* bind to the *Pikm*-HMA in vitro, forming stable complexes detectable by analytical gel filtration, but *AVR-PikC* does not (De la Concepcion et al. 2018). *M. oryzae* strains expressing *AVR-PikC* are not recognized by the *Pik* NLRs *Pikp* or *Pikp*. We tested whether *AVR-PikF* could bind to either *Pikm*-HMA or *Pikp*-HMA in vitro using analytical gel filtration; the interaction of *Pikm*-HMA and *Pikp*-HMA with *AVR-PikD* was used as a positive control. We found no evidence of *AVR-PikF* binding to either the *Pikm*-HMA or *Pikp*-HMA using this assay. In contrast to *AVR-PikD*, no shift in retention volume was observed for *AVR-PikF* when the proteins were mixed, as compared with the protein alone (Fig. 6A to D).

To further support the idea that the phenotype of susceptibility in rice to *M. oryzae* strains expressing *AVR-PikF* is due

specifically to the lack of recognition of the effector by *Pik* NLRs, we performed a cell-death assay in *N. benthamiana*. This assay has proven very robust in correlating *Pik*-dependent cell death in *N. benthamiana* with immunity in rice. We find that, although the positive controls of either *Pikp* or *Pikm* with *AVR-PikD* give a robust cell-death phenotype, neither *Pikp* nor *Pikm* respond to *AVR-PikF* (Fig. 7).

DISCUSSION

The *AVR-Pik* effector escapes *Pik*-mediated plant immunity by gene substitution and gene duplication. A single amino acid change has given rise to a novel *AVR-Pik* allele, *AVR-PikF*, which shows high virulence to all rice allelic *Pik* resistance genes through perturbation of the effector-*Pik*-HMA domain binding interface. Duplication of the *AVR-Pik* gene in rice blast isolates has resulted in the presence of two different alleles in the same *M. oryzae* isolate, one avirulent allele, *AVR-PikD*, and one virulent allele, *AVR-PikF*. As *AVR-PikF* may represent an emerging *AVR-Pik* allele in pathogen populations, it is important to develop *Pik* variants that can detect *AVR-PikF* and mount an immune response.

AVR-Pik variation in rice blast isolates.

New virulent isolates of the rice blast pathogen can emerge by genetic modification of effector (*AVR*) genes by different mechanisms. Nonsynonymous point mutations in *AVR-Pita*, *AVR-Pia*, *AVR-Pii*, and *AVR-Pik* (Dai et al. 2010; Takahashi et al. 2010; Yoshida et al. 2009), transposon insertions in *ACE1*, *AVR-Piz-t*, *AVR-Pib*, and *AVR-Pita1* (Fudal et al. 2009; Li et al. 2009; Zhang et al. 2015; Zhou et al. 2007), and sexual mating and parasexual recombination (Tsujimoto Noguchi 2011) have all been shown to lead to virulent alleles. Previous reports showed that *M. oryzae* effector genes are highly diverse and are capable of rapid change in natural populations (Jia et al. 2000; Sirisathaworn et al. 2017). Yoshida et al. (2009) revealed that the DNA sequence of *AVR-Pik* is highly variable, with a nucleotide diversity of 7.1×10^3 , two orders higher than the mean value for the entire genome (8.2×10^5). Consistent with this, we found that the *AVR-Pik* gene in Thai rice blast isolates shows a high level of genetic diversity, and a novel aggressive allele, *AVR-PikF*, was identified. We speculate that selection pressure from the use of rice varieties containing the *Pik* resistance gene in Thailand may have driven the emergence of this allele.

Pathogen evolution by gene duplication and translocation.

Using resistant cultivars in breeding for blast disease resistance in rice is effective, saving labor and costs, with the ability to reduce chemical inputs in agriculture and, ultimately, increase yield. However, newly developed resistant cultivars frequently lose their ability to prevent blast disease within a few years through rapid pathogen adaptation (Kiyosawa 1982). The mechanism of adaptation is mainly by amino acid substitution and gene duplication. Two mechanisms for gene duplication in fungi are sexual mating and parasexual recombination (Tsujimoto Noguchi 2011), but sexual reproduction in the rice blast fungus has not been observed directly in the field, so parasexual recombination is most likely the major determinant cause of gene duplication in *M. oryzae* (Saleh et al. 2014). Parasexual DNA exchanges occur at a detectable frequency in the field and may provide opportunities for different isolates to exchange supernumerary chromosomes or DNA fragments carrying effector genes (Chuma et al. 2011; Zeigler et al. 1997). This causes variation in the pathogenicity of the rice blast fungus, leading to breakdown of genetically encoded resistance (Tsujimoto Noguchi 2011; Yamasaki and Niizeki 1965). *AVR-Pita* is located on supernumerary chromosomes in some

Fig. 5. Pathogenicity assay showing recognition specificity of *Magnaporthe oryzae* *AVR-Pik* alleles *AVR-PikD*, *AVR-PikF* to rice cultivars containing different *Pik* alleles. All *M. oryzae* isolates were spray inoculated into *Pik* near-isogenic lines IRBLK-KA (*Pik*^{*}), IRBLKP-K60 (*Pikp*), IRBLKM-TS (*Pikm*), IRBLKH-K3 (*Pikh*), IRBL1-CL (*Pi1*), IRBL7-M (*Pi7*), IRBLKS-F5 (*Piks*), and Lijiang Xintuan Heigu (LTH) (*Pik*⁻).

M. oryzae isolates, and this is potentially involved in AVR gene dynamics through their loss, horizontal transfer, and structural change, including gene duplications (Chuma et al. 2011). We found evidence of gene duplication of *AVR-Pik* in Thai isolates of the rice blast fungus. Of these isolates, 28 of 58 (48.28%) contained two copies of *AVR-Pik*, *AVR-PikD* and *AVR-PikF*, with both alleles expressed during rice infection. Full genome sequencing of isolate 10100 revealed two *AVR-Pik* genes located on two different contigs. The presence of rice blast isolates containing two *AVR-Pik* alleles was described previously by Yoshida et al. (2009). They found six of 16 rice blast isolates in Japan contained two copies of the *AVR-Pik* gene (*AVR-PikA* and *AVR-PikD*). Kusaba et al. (2014) also reported that rice blast isolate 84R-62B contained two copies (*AVR-PikA* and *AVR-PikD*) and has undergone translocation via supernumerary chromosomes. These findings support the hypothesis that the *AVR-Pik* gene undergoes a high level of genetic recombination (Kusaba et al. 2014; Luo et al. 2007). The pathogenicity assay of rice blast isolates with two *AVR-Pik* alleles was the same as those isolates with one *AVR-PikD* allele, which were avirulent. This result indicates that the Pik resistance proteins can recognize the *AVR-PikD* effector in the presence of *AVR-PikF* and this leads to a defense response. In contrast, rice blast isolates with only *AVR-PikF* are virulent to all *Pik* NILs. This finding supports gene duplication as a mechanism for the fungal adaptation and evolution. The 28 rice blast isolates with two copies of the *AVR-Pik* gene from our study, six rice blast isolates from Yoshida et al. (2009), and the 84R-62B isolate from Kusaba et al. (2014) each contained *AVR-PikD* and a virulent *AVR-Pik* allele. The avirulent isolates, containing only *AVR-PikD*, show reduced survival in the rice field because *AVR-PikD* can be recognized by many *Pik* alleles. The emergence of rice blast isolates with two copies of the *AVR-Pik* gene, one avirulent allele and one virulent allele, may lead to aggressive rice blast isolates by subsequent loss of *AVR-PikD*. The importance and prevalence of this mechanism of pathogen adaptation requires further study.

Direct AVR-Pik-Pik-HMA interaction underpins immunity.

Amino acid polymorphisms in AVR-Pik variants correlate with evasion of immunity by disrupting direct binding of the

effector to the integrated HMA domain of *Pik* NLR alleles (De la Concepcion et al. 2018). The protein-protein interfaces formed between effector variants and *Pik*-HMA domains are well-established (De la Concepcion et al. 2018; Maqbool et al. 2015). Compared with *AVR-PikD*, *AVR-PikF* has four amino acid polymorphisms (Fig. 1). Three of these are shared with the effector variant *AVR-PikA*, at amino acids 46, 47, and 48, with the fourth being the defining mutation in *AVR-PikF*, a Met to Lys substitution at amino acid 78. *AVR-PikD* and *AVR-PikA* both bind to the HMA domain from the *Pikm* allele (*Pikm*-HMA), and this NLR responds to these effectors in both *N. benthamiana* (cell death) and rice (immunity). *AVR-PikF* escapes detection in plants by all tested *Pik* NLR alleles and does not interact with the *Pikm*-HMA or *Pikp*-HMA domains in vitro. The amino acid at position 78 is part of an extensive interface observed in the structures of *AVR-Pik* effectors in complex with *Pik*-HMA domains (termed ‘interface 3’ by De la Concepcion et al. [2018]) (Fig. 8). We speculate that as the Met78Lys mutation introduces both a larger side chain and a positive charge at this position (Fig. 8), this will disrupt contacts across this interface. This disruption results in an incompatible interaction between the effector and *Pik*-HMA domain that translates into evasion of immunity in rice.

Long term use of the *Pik* locus in Thai rice varieties.

The most effective tool to control rice blast in the field is to breed new disease-resistant rice varieties. JHN is a Thai rice variety showing broad-spectrum resistance against rice blast in Thailand (Sreewongchai et al. 2010). Two rice blast resistance genes were cloned on chromosomes 1 and 11 of JHN (Chaipanya et al. 2017). *Pi7-J* is located at the *Pik* locus on chromosome 11 and *Pish* is located on chromosome 1 (Chaipanya et al. 2017). JHN has been widely used as an elite resistant donor in conventional and molecular breeding programs against rice blast disease in Thailand (Noenplab et al. 2015). Examples of varieties from these breeding programs are RD6 (Wongsaprom et al. 2010), KDML105 (Nalampangnoenplab 2011), IR77955-24-75-284 (Kotchasetit 2013), Ban Tang (Kaewcheenchai et al. 2014), Jao Hawm Phitsanulok51 (Noenplab et al. 2015), and San Par Tong1 (Yajai and Ketsuwan 2015). From pathogenicity assays, we found that JHN is resistant to *M. oryzae* isolates expressing *AVR-PikD*, either alone or in isolates expressing

Fig. 6. Analytical gel filtration traces depicting the retention volume of *AVR-PikD*, *AVR-PikF*, *Pikm*-HMA, and *Pikp*-HMA individually and when effector and the heavy metal-associated (HMA) domain are mixed confirm that **A**, *Pikm*-HMA and **C**, *Pikp*-HMA form complexes with *AVR-PikD* but **B**, *Pikm*-HMA and **D**, *Pikp*-HMA do not form complexes with *AVR-PikF*. The arrows indicate *Pik*-HMA elution volume (does not absorb light at 280 nm). Sodium dodecyl sulfate polyacrylamide electrophoresis gels of the relevant fractions from the elution traces of the effectors. *Pik*-HMA is also shown.

both *AVR-PikD* and *AVR-PikF* (Fig. 4) but not other *AVR-Pik* effector variants. This is an identical profile to the previously described *Pikp*. Multiple sequence alignment of *Pik-HMA* domains revealed that the *Pi7-HMA* domain was identical at the amino acid level to *Pikp* (99.57% identical at the DNA level, with just a single synonymous base polymorphism). This suggests that using rice variety JHN as an elite resistant donor in

rice breeding programs in Thailand is not sustainable in the long term, as the blast fungus can overcome this resistance by deletion or mutation of *AVR-PikD*. The identification of new natural *Pik* resistance alleles with a broader recognition range for *AVR-Pik* or engineering new *Pik* alleles that can detect a wider range of *AVR-Pik* variants (including *AVR-PikF*), or both, is required.

Fig. 7. Neither *Pikp-1/Pikp-2* nor *Pikm-1/Pikm-2* trigger cell death in the presence of *AVR-PikF* in *Nicotiana benthamiana*. **A**, Representative leaf image showing cell-death response of *Pikp-1/Pikp-2* and *Pikm-1/Pikm-2* to either *AVR-PikD* or *AVR-PikF*, as autofluorescence characteristic of hypersensitive response-like cell death under UV light. Images were taken 5 days postinfiltration. **B**, Box plot summarizing the results obtained from 55 leaves in three independent experiments. Leaves were scored according to the scale by Maqbool et al. (2015). **C**, Western blots confirming expression of the proteins in a blot was probed with appropriate antibody for the tagged protein. Ponceau stain was used for control.

MATERIALS AND METHODS

Plant materials.

Rice variety JHN, a nonglutinous rice variety with long and deep purple grain, was used. JHN demonstrated broad-spectrum resistance against the rice blast pathogens in Thailand. Rice cultivar KDML105, aromatic and high-quality long grain rice, was used as susceptible rice cultivar. IRBL NILs of the rice blast-resistant gene *Pik*, including *Pik*, *Pikm*, *Pikp*, *Piks*, *Pikh*, *Pi1*, and *Pi7*, provided by IRRI, were used. Rice cultivar LTH was used as NIL background and susceptible control.

Rice blast materials.

A total of 60 rice blast isolates were used, including 58 isolates collected from Thailand rice-growing areas, representing the central, northern, and northeastern parts of Thailand. Two rice blast isolates, 70-15 and Guy11, were used as reference isolates (Table 1; Supplementary Fig S1).

Fungal culture and genomic DNA extraction.

The stock of each blast isolate in filter paper was cultured on RFA medium plates for 7 to 10 days. After mycelium growth, the mycelia were grown in potato dextrose broth medium and the cultures were incubated at 28°C for 7 days, with an orbital shaker (180 rpm). The mycelia were isolated from the medium on Whatman no. 1 filtration paper and, then, were frozen in liquid nitrogen and ground to a fine powder. Mycelial powder was extracted by the cetyltrimethylammonium bromide method (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate [SDS]). DNA quality analyzed by gel electrophoresis and DNA quantitative analyzed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.).

Genetic variation in *M. oryzae* AVR-*Pik*.

The following AVR-*Pik* gene-specific primers were designed: AVR-*Pik* F: 5'-GCGCCCATGCATTATCTTAT-3' and AVR-*Pik* R: 5'-TGAAGGTCACGGTTTGAAGA-3'. The volume of the PCR reaction mixture was 20 µl, containing 1 µl of 50-ng/µl template DNA, 0.25 µM of each primer, 1.25 mM MgCl₂, 0.1 mM dNTPs, 1× buffer A, and 0.2 µl of *Taq* DNA polymerase enzyme (Vivantis). PCR was performed under the following conditions: initial activation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, followed by a final incubation at 72°C for 5 min, using a Mastercycler Nexus (Eppendorf). PCR products were visualized by separated in 1.5% agarose gel, DNA staining with ethidium bromide and used Gel Doc XR+ with Image Lab software (Bio-Rad). The PCR products were purified and the buffer was replaced with elution buffer, using an Ambiclean kit (Vivantis). Purified PCR products were submitted for sequencing service (Macrogen) and were translated to amino acid sequences. Alleles of AVR-*Pik* were identified by a total of five DNA substitutions.

Copy number analysis.

Quantitative real-time PCR. Rice blast isolates with different copy numbers of the AVR-*Pik* gene were used for the analysis. The PCR amplification with AVR-*Pik* primers and detection was carried out in a MasterCycler Realplex4 thermal cycler (Eppendorf). The relative copy number of the candidate gene was calculated using the comparative Ct (2^{ΔCt}) method by normalization to the *IDM* DNA-directed RNA polymerase I subunit *RPA1* fragment gene: *IDM* F, 5'-GACCTATGCAATCACCAC-3' and *IDM* R, 5'-CGTACTCGAGTGTAATCTCG-3'. The PCR reactions (total volume 20 µl) were performed using 1 µl of each 50-ng/µl genomic DNA, 1× of QPCR Green Master Mix LRox (Biotech rabbit), and 0.2 µM of each primer.

PCR amplification was performed at 95°C for 2 min, 30 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 20 s, with three biological replications.

Southern blotting. Twenty micrograms of rice blast genomic DNA from isolates with different AVR-*Pik* alleles were digested with restriction enzymes *Bam*HI or *Pst*I (BioLabs Inc.), were fractionated through a 0.8% agarose gel in 0.5× Tris-borate-EDTA buffer, with 75-V electrophoresis for 3 h, and were processed in denaturation buffers (1.5 M NaCl, 0.5 M NaOH) for 30 min. The gel was then incubated in the neutralization buffer (1.5 M NaCl, 0.5 M TrisHCl, pH 7.4) for 15 min before transferring DNA to a nitrocellulose membrane. The DNA was cross-linked to the membrane by UV cross-linking. AVR-*Pik* fragment was used as probe, biotin-labeled probes were generated by PCR with biotin-16-dUTP (Roche Diagnostics Corp.). The membrane was hybridized overnight with biotin-labeled probes in DIG Easy Hyb solution (Roche Diagnostics Corp.) at 45°C. The hybridized membrane was washed four times by 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× SSC, 0.5× SSC, and 0.1× SSC with 0.1% SDS. Posthybridization was also performed according to the manufacturer protocol of DIG-High Prime DNA labeling and detection starter kit I (Roche Diagnostics Corp.). After hybridization, the membrane was washed and was blocked in solution containing solutions I (maleic acid buffer with 0.3% of Tween 20), II (9:1, maleic acid buffer, blocking solution [Roche Diagnostics Corp.]), antibody solution (9:1:1, maleic acid buffer, blocking solution, Anti-Dig [Roche Diagnostics Corp.]), and detection buffer, pH 9.5 (10 ml of 5 M NaCl, 6 g of Tris, 25 ml of 1 M MgCl₂), under shaker condition at room temperature for 15, 30, 30, and 5 min, respectively. The antibody solution contains anti-DIG, the chemiluminescent reaction was carried out with CDP-Star (Roche Diagnostics Corp.) chemiluminescent substrate. The membrane was exposed to X-ray film for 15 min and the film was developed and fixed in a darkroom.

Fig. 8. Schematic representation of the AVR-PikA/Pikm-HMA complex. The structure of Pikm-HMA is shown in the right helix ribbon and AVR-PikA in the left ribbon. Each protein interface in the complex was described by De la Concepcion et al. 2018. In AVR-PikF this amino acid is a lysine and due to its location, this residue likely disrupts complex formation between AVR-Pik and Pik-HMAs.

Pathogenicity assay. Rice seedlings (21 days old) were spray-inoculated with fungal spores of isolates with different *AVR-Pik* alleles (*AVR-PikA*, *AVR-PikC*, *AVR-PikD*, *AVR-PikE*, *AVR-PikF*, and two-copy isolates), approximately 10^5 spores per milliliter (about 20 ml of inoculum per experiment) adding 5 ml of 2.5% gelatin and shaking well. After inoculation, the plants were placed inside a dark room (100% humidity at 25°C for 24 h) and were then transferred to a greenhouse high humidity room. Disease evaluation was assessed at 7 days post-inoculation. The assays were repeated three times with similar results.

AVR-Pik expression. Susceptible rice cultivar KDML105 (21 days old) was spray-inoculated with three rice blast isolates, the isolate with *AVR-PikD*, the isolate with *AVR-PikF*, and the isolate with two copies of *AVR-Pik* gene. RNA was extracted, using the total RNA extraction kit (Vivatis), 72 h after inoculation by cutting an inoculated rice leaf, immediately freezing it in liquid nitrogen and grinding it to a fine powder. RNA was converted to cDNA using 200 U MMuLV reverse transcription (Biotech rabbit), 2 mM dNTP, 0.25 μ M Oligo (dT)₁₈, 1 $\times$ MMuLV reverse transcriptase buffer, 5 μ g of RNA template, total volume of 20 μ l. Mixtures were incubated at 42°C for 45 min, before inactivating the enzyme at 85°C for 10 min. The cDNA from each sample was diluted 10 times and was used as the template for *AVR-Pik* expression analysis. *AVR-Pik_cds* primers were designed from *AVR-Pik* start to stop region: *AVR-Pik_cds* F, 5'-ATGCGTGTACACTTTTAACAC-3' and *AVR-Pik_cds* R, 5'-TTAAAAGCCGGCCTTTT T-3'. The PCR reaction was performed in a 20- μ l reaction mixture containing 1 μ l of diluted cDNA, 0.25 μ M of each primer, 1.25 mM MgCl₂, 0.1 mM dNTPs, 1 $\times$ buffer A, and 0.2 μ l of *Taq* DNA polymerase enzyme (Vivantis). PCR was performed with the following conditions: initial activation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, followed by a final incubation at 72°C for 5 min, using a Mastercycler Nexus (Eppendorf). The PCR products were purified and the buffer was replaced with elution buffer, using an Ambiclean kit (Vivantis). Purified PCR products were submitted for sequencing (Macrogen) and were translated to amino acid sequences to determine the *AVR-Pik* alleles expressed.

Gel filtration. *Pikm*-HMA, *Pikp*-HMA with cleavable C-terminal 6 $\times$ His tag, and *AVR-Pik* effectors with a cleavable N-terminal maltose-binding protein tag and a noncleavable C-terminal 6 $\times$ His tag were produced in *E. coli* SHuffle cells (Biolabs, England) and were purified as previously described (De la Concepcion et al. 2018; Maqbool et al. 2015). Analytical size exclusion chromatography was performed at 4°C, using a Superdex 75 10/300 gel filtration column (GE Healthcare) pre-equilibrated in 50 mM HEPES, pH 7.5, and 150 mM NaCl. *Pik*-HMAs and *AVR-Pik* effectors were mixed in a molar ratio of 2:1 and were incubated on ice for 60 min. Individual and mixed proteins (120 μ l) were injected at a flow rate of 0.5 ml/min and 0.5-ml fractions were collected for analysis by SDS-polyacrylamide gel electrophoresis. Each experiment was repeated a minimum of three times, with similar results.

***N. benthamiana* cell-death assays.** *Agrobacterium tumefaciens* GV3101 was transformed with the relevant binary constructs. Four-week-old *N. benthamiana* plants were infiltrated with transformed *A. tumefaciens* using a needleless syringe. Two leaves were infiltrated from each plant. The infiltration position of each NLR-effector combination was varied between replicate leaves to account for within-leaf variation in transient expression (Bashandy et al. 2015). The final optical density at 600 nm of *A. tumefaciens* cultures for infiltration was 1.5 (*Pik*-1 = 0.4, *Pik*-2 = 0.4, effector = 0.6, p19 = 0.1). Infiltrated plants were grown in a controlled environment room (22°C constant

temperature, 80% relative humidity, 16-h photoperiod). Infiltrated leaves were photographed in white (adaxial surface) and UV light (abaxial surface) 5 days postinfiltration. The presence and extent of cell death was scored on a scale of 0 to 6 as used by Maqbool et al. (2015). Box plots represent data from three independent experiments and were produced using R v3.4.0 and the ggplot2 package (Wickham 2016). The box extends from Q1 to Q3, with the central line representing the median. Outliers ($> Q3 + 1.5 \times IQR$ or $< Q1 - 1.5 \times IQR$) are indicated by crosses. Box whiskers extend to the highest or lowest nonoutlier value. The presence of each protein, as expressed in representative assays, was determined by Western blot with the appropriate antibody, anti-Flag, anti-hemagglutinin (HA), anti-Myc for *Pik*-1-Flag, *Pik*-2-HA, and *AVR-Pik*-Myc, respectively.

***M. oryzae* genome sequence.** Genomic DNA of rice blast isolate 10100 was used as a representative of isolate with two copies of *AVR-Pik* gene. Sequencing libraries were constructed using a NEB kit for Illumine Hiseq PE 150, following manufacturer recommendations (NovoGene Bioinformatics Technology Co., Ltd.). Raw reads were first cleaned by removing adaptor sequences and low quality sequences, Q-value ≤ 53 exceed the certain threshold 40% of the read length by default, eliminated reads with unknown nucleotides larger than 10% and reads that overlap with adapter over certain threshold (15 bp by default). Sanger base quality <200 bp were cut off. The clean reads were mapped to the *Magnaporthe oryzae* 70-15 reference genome (GenBank accession GCA_000002495.2) using BWA alignment. Finally, 1.6 Gb (46 $\times$ coverage) sequence reads were retained for genome assembly using two programs, *velvet* and *velvetg*, from Velvet de novo assembler (Zerbino and Birney 2008).

LITERATURE CITED

- Ashikawa, I., Hayashi, N., Yamane, H., Kanamori, H., Wu, J., Matsumoto, T., Ono, K., and Yano, M. 2008. Two adjacent nucleotide-binding site-leucine-rich repeat class genes are required to confer *Pikm*-specific rice blast resistance. *Genetics* 180:2267-2276.
- Bashandy, H., Jalkanen, S., and Teeri, T. H. 2015. Within leaf variation is the largest source of variation in agroinfiltration of *Nicotiana benthamiana*. *Plant Methods* 11:47.
- Chaipanya, C., Telebanco-Yanoria, M. J., Quime, B., Longya, A., Korinsak, S., Korinsak, S., Toojinda, T., Vanavichit, A., Jantasuriyarat, C., and Zhou, B. 2017. Dissection of broad-spectrum resistance of the Thai rice variety Jao Hom Nin conferred by two resistance genes against rice blast. *Rice (N. Y.)* 10:18.
- Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B., and Tosa, Y. 2011. Multiple translocation of the *AVR-Pita* effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. *PLoS Pathog.* 7:e1002147.
- Costanzo, S., and Jia, Y. 2010. Sequence variation at the rice blast resistance gene *Pikm* locus: Implications for the development of allele specific markers. *Plant Sci.* 178:523-530.
- Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94:683-693.
- Dai, Y., Jia, Y., Correll, J., Wang, X., and Wang, Y. 2010. Diversification and evolution of the avirulence gene *AVR-Pita1* in field isolates of *Magnaporthe oryzae*. *Fungal Genet. Biol.* 47:973-980.
- De la Concepcion, J. C., Franceschetti, M., Maqbool, A., Saitoh, H., Terauchi, R., Kamoun, S., and Banfield, M. J. 2018. Polymorphic residues in rice NLRs expand binding and response to effectors of the blast pathogen. *Nat. Plants* 4:576-585.
- Disthaporn, S. 1994. Current rice blast epidemics and their management in Thailand. Pages 333-342 in: *Rice Blast Disease*. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International, Wallingford, U.K. in association with IRRI, Manila, Philippines.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11: 539-548.

- Fudal, I., Böhnert, H. U., Tharreau, D., and Lebrun, M. H. 2005. Transposition of MINE, a composite retrotransposon, in the avirulence gene *ACE1* of the rice blast fungus *Magnaporthe grisea*. *Fungal Genet. Biol.* 42:761-772.
- Fudal, I., Ross, S., Brun, H., Besnard, A. L., Ermel, M., Kuhn, M. L., Balesdent, M. H., and Rouxel, T. 2009. Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in *Leptosphaeria maculans*. *Mol. Plant-Microbe Interact* 22:932-941.
- Hogenhout, S. A., Van der Hoorn, R. A. L., Terauchi, R., and Kamoun, S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Mol. Plant-Microbe Interact* 22:115-122.
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* 19:4004-4014.
- Kaewchenchai, R., Sriwisut, S., Noenplab, A., Nalampangnoenplab, A., Ruksopa, K., Dungsoongnern, P., Prommart, U., and Rasidee, N. 2014. Improving blast resistance in a rice variety 'Bahng Taen' by using marker-assisted backcrossing. *Proceeding of the 32nd Rice and Temperate Cereal Crop Annual Conference*, Petchaburi, Thailand. 158-170.
- Kang, S., Sweigard, J. A., and Valent, B. 1995. The *PWL* host specificity gene family in the blast fungus *Magnaporthe grisea*. *Mol. Plant-Microbe Interact* 8:939-948.
- Kanzaki, H., Yoshida, K., Saitoh, H., Fujisaki, K., Hirabuchi, A., Alaux, L., Fournier, E., Tharreau, D., and Terauchi, R. 2012. Arms race co-evolution of *Magnaporthe oryzae* *AVR-Pik* and rice *Pik* genes driven by their physical interactions. *Plant J.* 72:894-907.
- Khanthong, S., Jantasuriyarat, C., and Katengam, S. 2010. Blast disease and rice breeding for blast disease resistance through marker-assisted selection. *Thai J. Genet.* 3:106-119.
- Kiyosawa, S. 1982. Genetics and epidemiological modelling of breakdown of plant disease resistance. *Annu. Rev. Phytopathol.* 20:93-117.
- Kotchasetit, A. 2013. An early maturing, non-glutinous promising rice line resistant to blast. *Proceedings of the 30th Rice and Temperate Cereal Crops Annual Conference 2013*, Bangkok, Thailand. 56-71.
- Kusaba, M., Mochida, T., Naridomi, T., Fujita, Y., Chuma, I., and Tosa, Y. 2014. Loss of a 1.6 Mb chromosome in *Pyricularia oryzae* harboring two alleles of *AvrPik* leads to acquisition of virulence to rice cultivars containing resistance alleles at the *Pik* locus. *Curr. Genet.* 60:315-325.
- Langner, T., Bialas, A., and Kamoun, S. 2018. The blast fungus decoded: Genomes in flux. *MBio* 9:e00571-18.
- Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z., Zhao, Q., Feng, Q., Zhang, H., Wang, Z., Wang, G., Han, B., Wang, Z., and Zhou, B. 2009. The *Magnaporthe oryzae* avirulence gene *AvrPiz-t* encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene *Piz-t*. *Mol. Plant-Microbe Interact* 22:411-420.
- Liu, W., Liu, J., Triplett, L., Leach, J. E., and Wang, G. L. 2014. Novel insights into rice innate immunity against bacterial and fungal pathogens. *Annu. Rev. Phytopathol.* 52:213-241.
- Luo, C. X., Yin, L. F., Ohtaka, K., and Kusaba, M. 2007. The 1.6Mb chromosome carrying the avirulence gene *AvrPik* in *Magnaporthe oryzae* isolate 84R-62B is a chimera containing chromosome 1 sequences. *Mycol. Res.* 111:232-239.
- Maqbool, A., Saitoh, H., Franceschetti, M., Stevenson, C. E. M., Uemura, A., Kanzaki, H., Kamoun, S., Terauchi, R., and Banfield, M. J. 2015. Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor. *eLife* 4:e08709.
- Nalampangnoenplab, A. 2011. Minimization of rice blast severity by means of multilines in the lower north. Pages 225-241 in: *Proceedings of Rice Research Symposium 2011*. Rice Research Center Groups in Upper and Lower Northern Region, Phrae, Thailand.
- Noenplab, A., Nalumpangnernplab, A., and Palawisut, S. 2015. Increasing blast resistance in HPSL1 using marker assisted selection. Pages 3-16 in: *Proceeding of the 8th Rice Research Conference 2015*. Chiang Rai, Thailand. Rice Research Center Groups in Upper and Lower Northern Region, Phrae, Thailand.
- Noenplab, A., Vanavichit, A., Toojinda, T., Sirithunya, P., Tragoonrungs, S., Sriprakhon, S., and Vongsaprom, C. 2006. QTL mapping for leaf and neck blast resistance in Khao Dawk Mali 105 and Jao Horm Nin recombinant inbred lines. *Sci. Asia* 32:133-142.
- Orbach, M. J., Farrall, L., Sweigard, J. A., Chumley, F. G., and Valent, B. 2000. A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. *Plant Cell* 12:2019-2032.
- Rafiqi, M., Ellis, J. G., Ludowici, V. A., Hardham, A. R., and Dodds, P. N. 2012. Challenges and progress towards understanding the role of effectors in plant-fungal interactions. *Curr. Opin. Plant Biol.* 15:477-482.
- Ray, S., Singh, P. K., Gupta, D. K., Mahato, A. K., Sarkar, C., Rathour, R., Singh, N. K., and Sharma, T. R. 2016. Analysis of *Magnaporthe oryzae* genome reveals a fungal effector, which is able to induce resistance response in transgenic rice line containing resistance gene, *Pi54*. *Front. Plant Sci.* 7:1140.
- Ribot, C., Césari, S., Abidi, I., Chalvon, V., Bournaud, C., Vallet, J., Lebrun, M. H., Morel, J. B., and Kroj, T. 2013. The *Magnaporthe oryzae* effector *AVR1-CO39* is translocated into rice cells independently of a fungal-derived machinery. *Plant J.* 74:1-12.
- Saleh, D., Milazzo, J., Adreit, H., Fournier, E., and Tharreau, D. 2014. South-east Asia is the center of origin, diversity and dispersion of the rice blast fungus, *Magnaporthe oryzae*. *New Phytol.* 201:1440-1456.
- Sirisathaworn, T., Srirat, T., Longya, A., and Jantasuriyarat, C. 2017. Evaluation of mating type distribution and genetic diversity of three *Magnaporthe oryzae* avirulence genes, *PWL-2*, *AVR-Pii* and *Avr-Piz-t*, in Thailand rice blast isolates. *ANRES* 51:7-14.
- Sreewongchai, T., Toojinda, T., Thanintorn, N., Kosawang, C., Vanavichit, A., Tharreau, D., and Sirithunya, P. 2010. Development of elite indica rice lines with wide spectrum of resistance to Thai blast isolates by pyramiding multiple resistance QTLs. *Plant Breed.* 129:176-180.
- Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7:1221-1233.
- Takahashi, M., Ashizawa, T., Hirayae, K., Moriwaki, J., Sone, T., Sonoda, R., Noguchi, M. T., Nagashima, S., Ishikawa, K., and Arai, M. 2010. One of two major paralogs of *AVR-Pita1* is functional in Japanese rice blast isolates. *Phytopathology* 100:612-618.
- Tsujimoto Noguchi, M. 2011. Parasexual recombination in *Magnaporthe oryzae*. *Jpn. Agric. Res. Q.* 45:39-45.
- Waiyawuththanapoom, P., Waiyawuththanapoom, W., and Tirastittam, P. 2015. Social media as a distribution channel for Thailand's rice berry product. *World Acad. Sci. Eng. Technol.* 9:194-197.
- Wang, B., Ebbole, D. J., and Wang, Z. 2017. The arms race between *Magnaporthe oryzae* and rice: Diversity and interaction of *Avr* and *R* genes. *J. Integr. Agric.* 16:2746-2760.
- Wickham, H. 2016. *ggplot2: Elegant graphics for data analysis*. Springer-Verlag, New York.
- Wongsaprom, C., Sirithunya, P., Vanavichit, A., Pantuwan, G., Jongdee, B., Sidhiwong, N., Lanceras-Siangliw, J., and Toojinda, T. 2010. Two introgressed quantitative trait loci confer a broad-spectrum resistance to blast disease in the genetic background of the cultivar RD6 a Thai glutinous jasmine rice. *Field Crops Res.* 119:245-251.
- Wu, J., Kou, Y., Bao, J., Li, Y., Tang, M., Zhu, X., Ponaya, A., Xiao, G., Li, J., Li, C., Song, M. Y., Cumagun, C. J. R., Deng, Q., Lu, G., Jeon, J. S., Naqvi, N. I., and Zhou, B. 2015. Comparative genomics identifies the *Magnaporthe oryzae* avirulence effector *AvrPi9* that triggers *Pi9*-mediated blast resistance in rice. *New Phytol.* 206:1463-1475.
- Yajai, P., and Ketsuwan, K. 2015. The use of anther culture for developing rice blast resistant true breeding lines. Pages 47-52 in: *Proceedings of the 8th Rice Research Conference 2015*. Rice Research Center Groups in Upper and Lower Northern Region, Chiang Rai, Thailand.
- Yamasaki, Y., and Niizeki, H. 1965. Studies on variation of the rice blast fungus, *Pyricularia oryzae* Cav. I. Karyological and genetic studies on variation. *Bull. Natl. Inst. Agric. Sci. Jpn.* 13:231-273.
- Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Yoshida, K., Tosa, Y., Chuma, I., Takano, Y., Win, J., Kamoun, S., and Terauchi, R. 2009. Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. *Plant Cell* 21:1573-1591.
- Zeigler, R. S., Scott, R. P., Leung, H., Bordeos, A. A., Kumar, J., and Nelson, R. J. 1997. Evidence of parasexual exchange of DNA in the rice blast fungus challenges its exclusive clonality. *Phytopathology* 87:284-294.
- Zerbino, D. R., and Birney, E. 2008. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* 18:821-829.
- Zhang, H., Wu, Z., Wang, C., Li, Y., and Xu, J. R. 2014. Germination and infectivity of microconidia in the rice blast fungus *Magnaporthe oryzae*. *Nat. Commun.* 5:4518.
- Zhang, S., Wang, L., Wu, W., He, L., Yang, X., and Pan, Q. 2015. Function and evolution of *Magnaporthe oryzae* avirulence gene *AvrPib* responding to the rice blast resistance gene *Pib*. *Sci. Rep.* 5:11642.
- Zhou, E., Jia, Y., Singh, P., Correll, J. C., and Lee, F. N. 2007. Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence. *Fungal Genet. Biol.* 44:1024-1034.