The *Piks* allele of the NLR immune receptor *Pik* breaks the recognition of *AvrPik* effectors of the rice blast fungus

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Abstract

Arms race co-evolution of plant-pathogen interactions evolved sophisticated recognition mechanisms between host immune receptors and pathogen effectors. Different allelic haplotypes of an immune receptor in host mount distinct recognition against sequence or non-sequence related effectors in pathogens. We report the molecular characterization of the Piks allele of the rice immune receptor Pik against rice blast pathogen, which requires two head-to-head arrayed nucleotide binding site and leucine-rich repeat proteins. Like other Pik genes, both Piks-1 and Piks-2 are necessary and sufficient for Piks-mediated resistance. However, unlike other Pik alleles, Piks does not recognize any known AvrPik variants of M. oryzae. Sequence analysis of the genome of an avirulent isolate V86010 further revealed that its cognate avirulence (Avr) gene most likely has no significant sequence similarity to known AvrPik variants. We conclude that Piks breaks the canonical Pik/AvrPik recognition pattern. Piks-1 and Pikm-1 have only two amino acid differences within the integrated heavy metal-associated (HMA) domain. Pikm-1-HMA interact with AvrPik-A, -D and -E in vitro and in vivo, whereas Piks-1-HMA does not bind any AvrPik variants. Reciprocal exchanges of single amino acid residues between Piks-1 and Pikm-1 further reveal a dynamic recognition mechanism between Piks/Pikm alleles and their respective effectors. Piks-1E229Q/Pikm-1V261A can only activate immunity to AvrPik-D but not to other effectors, indicating that the amino acid change of E to Q at position 229 leads to its gain of a partial recognition spectrum of Pikm. By contrast, Piks-1A261V/Pikm-1Q229E confers immunity to the Piks cognate effector, indicating that the amino acid change of Q to E at position 229 leads to its shifts of the recognition from Pikm to Piks. Intriguingly, binding activities in both Y2H and analytical gel filtration assays are illustrated between Piks-1A261V/Pikm-1Q229E and AvrPik-D. However, it is unable to mount immunity against AvrPik-D, suggesting that biochemical activities based on in vitro and in vivo assays could be insufficient for sustaining biological function of receptor and effector pairs.
INTRODUCTION

Pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) are recognized by host cell surface-localized pattern-recognition receptors (PRRs) to activate the first layer of plant immunity (Jones and Dangl 2006; Zhang and Zhou 2010). Pathogens deliver a myriad of effectors into host cells to inhibit such host immune responses and/or create a favorable host cell environment for infection (Li et al. 2017). Plants have developed cytoplasmic immune receptors of the Nucleotide-binding domain leucine-rich repeat class (NBS-LRRs, known as NLRs), which directly or indirectly recognize pathogen effectors leading to intracellular immunity (Jones and Dangl 2006). This layer of immunity confers strong resistance, often associated with localised cell death known as the hypersensitive response (HR). Most plant NLRs are defined by a multi-domain architecture with central nucleotide-binding (NB-ARC) and C-terminal LRR regions, and either an N-terminal TOLL/interleukin-1 receptor (TIR) or a coiled-coil (CC) domain (Jones and Dangl 2006; Takken and Goverse 2012).

Rice blast disease, caused by the fungal pathogen Magnaporthe oryzae (M. oryzae), is the most devastating disease threatening global rice production and food security (Skamnioti and Gurr 2009; Asibi et al. 2019; Shahriar et al. 2020). The most effective and environmental-friendly approach to controlling rice blast is the deployment of rice blast resistance (R) gene. To date, more than 30 rice blast R genes have been characterized, most of which encode NLRs, with the exception of Pid2, Pi21, and Pita2/Ptr. Pid2 encodes a β-lectin receptor-like kinase (Chen et al. 2006); Pi21 encodes a proline-rich protein (Fukuoka et al. 2009); and Pita2/Ptr encodes an atypical R protein with four Armadillo repeats (Zhao et al. 2018; Meng et al. 2020). However, only a limited number of M. oryzae avirulence (Avr) genes (also known as effectors), recognized by these R genes have been cloned to date. They include AvrPita (Orbach et al. 2000), AvrPia (Yoshida et al. 2009), AvrPik and its alleles (Yoshida et al. 2009), AvrPii (Yoshida et al. 2009), AvrPiz-t (Li et al. 2009), Avr1-CO39 (Ribot et al. 2013), AvrPib (Zhang et al. 2015), and AvrPi9 (Wu et al. 2015). Based on the pattern by which rice blast R genes recognize their respective Avr genes, distinct mechanisms can be described for their function. Pairs of Pita/AvrPita and
Pib/AvrPib represent the first mode in which a single R gene recognizes a single Avr gene (Jia et al. 2000; Zhang et al. 2015). Pairs of Pi-CO39/Avr1-CO39 and Pia/AvrPia represent the second mode in which a single R gene recognizes two sequence-unrelated Avr genes (Yoshida et al. 2009; Okuyama et al. 2011; Cesari et al. 2013). Pairs of P19/AvrP19 and Piz-t/AvrPiz-t represent the third mode in which different R alleles each recognize sequence-unrelated Avr genes (Wu et al. 2015; Li et al. 2009). Pairs of Pik alleles and AvrPik variants represent the fourth mode in which different R alleles have distinct recognition specificities to different Avr variants (Yoshida et al. 2009; Kanzaki et al. 2012).

Molecular mechanisms underlying recognition of AvrPik variants by Pik alleles have been extensively characterized. Both the Pik-1/Pik-2 NLR pair and AvrPik effectors exist in an allelic series in rice and M. oryzae populations. The Pik locus is located on rice chromosome 11, where at least nine genes, Pik*, Pikm, Pikp, Pikh, Pi1, Pi7, Pik, Piks and Pike, have been identified (Ashikawa et al. 2012; Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2014; Hua et al. 2012; Campbell et al. 2004; Zhai et al. 2011; Chen et al. 2015). For AvrPik, six different variants (A–F) have been identified with 1–3 amino acid differences between pathogen isolates (Yoshida et al. 2009; Kanzaki et al. 2012; Wu et al. 2014; Longya et al. 2019). The direct interaction between Pik and AvrPik is mediated by the HMA domain of Pik-1, with this domain being integrated between the coiled-coil and nucleotide-binding (NB-ARC) domains (Maqbool et al. 2015; De la Concepcion et al. 2018, 2021). At the sequence level, Pikp-1 and Pikm-1 predominantly differ in their HMA domains, and this underpins different effector recognition specificities. Pikp only recognizes AvrPik-D, whereas Pikm can recognize AvrPik-D, AvrPik-A and AvrPik-E (Kanzaki et al. 2012; De la Concepcion et al. 2018). The interface between AvrPik effectors and the HMA domain of both Pikp and Pikm has been structurally characterized (Maqbool et al. 2015; De la Concepcion et al. 2018). The AvrPik-C and AvrPik-F effector variants are not currently recognized by any Pik alleles (Kanzaki et al. 2012; Longya et al. 2019). However, the AvrPik-C effector variant has been reported to interact with Pikh-HMA in vitro, with sufficient affinity to allow crystallization of the complex (De la Concepcion et al. 2021). Recently, the potential to engineer new-to-nature receptors
specificities, such as recognition of stealthy effector variants has been demonstrated, which has broad implications for rational design of plant NLRs (De la Concepcion et al. 2019; De la Concepcion et al. 2021; Maidment et al. 2022).

The *Piks* gene was identified in the monogenic line IRBLKs-F5, and originated from the donor variety Fujisaka 5. It is responsible for the resistance against the rice blast isolate V86010 (Tsunematsu et al. 2000). However, the mechanism underlying this resistance has remained elusive, and the specificity of interaction with known *AvrPik* variants has not been deciphered. In this study, we conduct molecular characterization of *Piks* through genetic analyses and interaction assays with different *AvrPik* variants. The function of key amino acids differing between *Piks* and *Pikm* is investigated to provide insights into the evolution of distinct specificities among different *Pik* alleles.

**RESULTS**

*Piks* in IRBLKs-F5 confers novel race-specific resistance against rice blast

To verify that *Piks* identified in the monogenic line IRBLKs-F5 confers race-specific resistance against rice blast, the response of IRBLKs-F5 towards pathogen isolates V86010 and CA89 was evaluated. As shown in Figure 1A, IRBLKs-F5 is resistant to V86010, but susceptible to other isolates. Its recipient rice variety Lijiangxintuanheigu (LTH) is susceptible to all isolates. These results illustrate that *Piks* in IRBLKs-F5 determines a race specific resistance to rice blast. A set of 367 rice blast isolates from the Philippines was further used to evaluate and compare the resistance spectra conferred by different *Pik* alleles (Table S1). As Table S1 shows, the *Piks* monogenic line, IRBLKs-F5 was susceptible to all isolates except V86010, showing a 0.27% of resistance frequency to the tested isolates (Figure 1B). By contrast, other *Pik* monogenic lines showed varying resistance frequencies ranging from 42.2% for IRBLK-Ka and IRBL7-M to 73.8% for IRBLKp-K60 (Figure 1B). It is worth noting that the higher resistance frequency of IRBLKp-K60 is unlikely to be only determined by *Pikp* due to the presence of an additional *R* gene linked to the *Pi19* locus, as previously characterized (Selisana et al. 2017). These results demonstrate that *Piks* controls a much narrower resistance spectrum than other *Pik*
alleles against rice blast isolates in the Philippines.

To further investigate whether Piks resistance is dependent on the recognition of AvrPik, a set of transformed pathogen isolates, using the recipient isolate R88-002 that does not harbor AvrPik, was generated with each containing one of the six known AvrPik variants (AvrPik-A to -F) (Figure S1). Pathogenicity tests against different Pik monogenic lines were then performed. As shown in Table 1, IRBLKs-F5 was susceptible to all transformed isolates. However, Pikp, Pi7, and Pik monogenic lines were resistant to AvrPik-D transformed isolates; Pikm and Pi1 monogenic lines were avirulent to AvrPik-A, -D, and -E transformed isolates (Table 1). This R-Avr interaction assay demonstrates that Piks was unable to activate rice immunity against any of the six known AvrPik variants.

**Both Piks-1 and Piks-2 are required for Piks-mediated resistance**

To determine whether Piks is responsible for the resistance of IRBLKs-F5 against V86010, an F₂ population was developed from a cross between IRBLKs-F5 and its recipient rice variety Lijiangxintuanheigu (LTH). A total of 1387 individuals of the F₂ population were inoculated with V86010. As indicated in Table 2, the F₂ population segregated for 1053 resistant and 334 susceptible plants, showing an expected 3:1 resistance versus susceptibility ratio \( \chi^2(3:1) = 0.625 \). This result confirms that the resistance of IRBLKs-F5 against V86010 is controlled by a single gene or genetic locus.

We cloned and determined the sequences of both Piks-1 and Piks-2 in IRBLKs-F5, and these were identical to those deposited in NCBI (Genbank accession no.: HQ662329). A gene specific molecular marker RGA4-F3/R3 showing polymorphism between IRBLKs-F5 and LTH was used for the molecular analysis of the F₂ progeny (Figures 1C, S2). We found that all resistant progeny exhibited marker pattern of the resistant parent (and all susceptible progeny exhibited the susceptible parent pattern), indicating that Piks co-segregates with RGA4-F3/R3 (Figure 1C; Table 2). To determine whether the function of Piks requires co-expression of both Piks-1 and Piks-2, Piks-1/Piks-2 transgenic rice plants were generated via crossing and selfing of Piks-1 and Piks-2 individual transgenic plants. Analysis of transgenic plants showed
that only Piks-1 and Piks-2 combined transgenic lines showed resistance against V86010 (Figure 1D). By contrast, their donor transgenic lines with only either Piks-1 or Piks-2 were susceptible (Figure 1D). These results confirmed genetically that the Piks resistance requires co-expression of both Piks-1 and Piks-2. In addition, we generated three independent knockout mutants of Piks-1 and Piks-2 using CRISPR. As illustrated in Figure 2A, each of the knockout mutants Piks-KO-3, -4, and -7 had a distinct mutation pattern in either Piks-1 or Piks-2. It is noted that Piks-KO-3 and Piks-KO-7 had deletions of 6- and 30-nucleotide in Piks-1, respectively, whereas they had the same single-nucleotide insertion in Piks-2 (Figure 2A). Based on the frameshift mutations in Piks-1, Piks-2, or both, we speculated that the function of either Piks-1 or Piks-2 was likely disrupted in three mutants. The resistance phenotype of these mutants to V86010 was investigated, revealing that they were compromised (Figure 2B), further verifying that both Piks-1 and Piks-2 are required for the Piks-mediated resistance.

Piks-mediated resistance against V86010 is not determined by AvrPik

The V86010 strain contains two AvrPik variants, AvrPik-D and -E, as determined via sequencing of PCR amplicons (Figure S3). The presence of AvrPik variants was further investigated through homology search against the V86010 whole-genome shotgun (WGS) sequence (ASM210529v1). Only a single WGS contig (MWIT01000852.1) having over 99.7% sequence identity was identified, which was further found to derive from an assembly of both AvrPik-D and -E sequences due to extreme sequence similarity. Therefore, we conclude V86010 only contains two AvrPik variants. To investigate whether AvrPik-D and -E is involved in recognition of V86010 by Piks, V86010 mutant strains disrupted in both AvrPik-D and -E were generated and used in pathogenicity assays. Out of 268 hygromycin resistant transformed strains, three (#3, #4, and #38) were found to be true knockouts of both AvrPik-D and -E as they displayed none of the expected amplicons of AvrPik whereas they all contained the DNA fragment of the selectable marker (hygromycin B phosphotransferase gene) flanked by the homology arm (Figure S4). Pathogenicity tests revealed that these three mutant strains were still recognized by IRBLks-F5.
which retains a resistant phenotype (Figure 3A). In addition, they resulted in gain-of-susceptibility to other Pik monogenic lines (Figure 3A), verifying previous findings of AvrPik and Pik interactions (Kanzaki et al. 2012). Finally, these three mutant strains were used to challenge the transgenic Piks lines described above. All transgenic Piks lines harboring both Piks-1 and Piks-2 were resistant to these three mutant strains (Figure 3B). Taken together, these results suggest that V86101 contains a currently unknown Avr gene recognized by Piks, leading to activation of immunity. Moreover, this novel Avr gene cognate to Piks most likely has no significant sequence similarity to known AvrPik alleles.

Two amino acids that distinguish Piks-1 from Pikm-1 are critical for resistance specificities

It is well established that Pik-1 alleles have a higher level of sequence variation than the Pik-2 alleles (Figures S5, S6). It is worth noting that the Piks-2 sequence is identical to Pikm-2 and Pik-2, suggesting that they are unlikely be the determinants of differential recognition specificities (Figure S6). Nevertheless, Piks-1 is most sequence related to Pikm-1 and Pil-5 (Figure 4A, Figure S5). There are two amino acids differing Piks-1 from Pikm-1 and Pil-5 at amino acid positions of 229 and 261. In Piks-1, position 229 is a glutamate residue (E) whilst in Pil-5 it is an aspartate (D) and in Pikm-1 a glutamine (Q). At position 261, the residues are Alanine (A) for Piks-1 and Valine (V) for both Pil-5 and Pikm-1 (Figure 4A, Figure S5). Given the amino acid sequences of Piks-2 and Pikm-2 are identical, we focused on the characterization of amino acid differences between these two haplotypes.

To enable genetic characterization in rice, two variants of Piks-1, introducing the amino acid residue at the equivalent position in Pikm-1, which are Piks-1E229Q and Piks-1A261V, were generated and used for transformation of rice cultivar Nipponbare (Nipponbare was also transformed with Piks-1/Piks-2 as a control). The resulting transgenic plants were challenged with the M. oryzae strain V86010 and V86010 knockouts in AvrPik-D/E. The Piks-1E229Q/Piks-2 transgenic plants were resistant to V86010, the same phenotype observed for the Piks-1/Piks-2 transgenic plants (Figure 3B). By contrast, we found that Piks-1E229Q/Piks-2 transgenic plants were susceptible
to all the *AvrPik*-D/E-knockout mutant strains (Figure 3B). Therefore, we deduced from this data that the switch from E to Q at position 229 leads to a loss of *Piks*-mediated resistance against the cognate (as yet unknown) *Avr* gene. To investigate which *AvrPik* haploptype is responsible to *Piks*-1^{E229Q}/*Piks*-2, the resistance phenotypes were assessed using the same set of R88-002 transformed isolates containing different *AvrPik* haplotypes described above. As shown in Table 3, *Piks*-1^{E229Q}/*Piks*-2 transgenic plants were only resistant to *AvrPik*-D containing R88-002 strains, and not other avirulent haplotypes, demonstrating a distinct spectrum compared to *Pikm* that recognizes *AvrPik*-D, -E, and -A. The *Piks*-1^{E229Q} mutation generates a protein equivalent to a *Pikm*-1^{V261A} mutation. Therefore, our data can also be interpreted as the switch from V to A at the position 261 leads to a loss of *Pikm* recognition to both *AvrPik*-E and -A, but retains the recognition of *AvrPik*-D in transgenic rice (Table 3).

We also challenged the *Piks*-1^{A261V}/*Piks*-2 transgenic Nipponbare plants against V86010 and with its knockouts of *AvrPik*-D/E and found they were resistant to all strains (Figure 3B). This result suggests that the mutation from A to V at the position 261 does not compromise the function of *Piks* to the cognate (as yet unknown) *Avr* gene. Further, they were susceptible to R88-002 transformed strains with the six *AvrPik* variants (Table 3). Likewise, *Piks*-1^{A261V} can be designated as *Pikm*-1^{Q229E}. Therefore, we deduced that the switch from Q to E at position 229 results in the loss of function of *Pikm* to *AvrPik*-D, -E, and -A.

*Piks*-1, *Pikm*-1, *Piks*-1^{E229Q}/*Pikm*-1^{V261A}, and *Piks*-1^{A261V}/*Pikm*-1^{Q229E} differentially interact with known *AvrPik* proteins

To investigate the interaction pattern of *Piks* and its alleles with known *AvrPik* variants, a protein-protein binding assay using a yeast two-hybrid (Y2H) was conducted. As shown in Figure 4B, no significant yeast growth on selective media was observed when the HMA domain of *Piks* (*Piks*-HMA) was co-transformed with each of four *AvrPik* effector variants (*AvrPik*-A, -C, -D and -E). By contrast, significant yeast growth was observed when the HMA domain of *Pikm* (*Pikm*-HMA) co-transformed with *AvrPik*-A, *AvrPik*-D, and *AvrPik*-E, but not *AvrPik*-C (Figure 4B).
β-galactosidase activity assay, resulting in the development of blue coloration, further verified the binding activity between each interacting pair. These results from Pikm-HMA are consistent with previous findings reported by different laboratories (Kanzaki et al. 2012; De la Concepcion et al. 2018). Therefore, we speculate that these two amino acid residues differentiating Pikm from Piks are critical for binding activity between Pikm and its cognate AvrPik variants.

Next, we tested the interaction of the HMA domains of Piks\textsuperscript{E229Q/Pikm\textsuperscript{V261A}} (Piks\textsuperscript{E229Q/Pikm\textsuperscript{V261A}-HMA}) and Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}} (Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}-HMA}) with each of four AvrPik effector variants (AvrPik-\textsuperscript{A}, -\textsuperscript{C}, -\textsuperscript{D} and -\textsuperscript{E}). Firstly, for the Piks\textsuperscript{E229Q/Pikm\textsuperscript{V261A}-HMA}, we only observed yeast growth and development of blue coloration of co-transformants with AvrPik-\textsuperscript{D} and not other effector variants (Figure 4B). Secondly, for the Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}} variant, we observed yeast growth and development of blue coloration of co-transformants with AvrPik-\textsuperscript{D} and -\textsuperscript{E}. However, the latter is significantly lower in growth than the former (Figure 4B). Unlike Pikm, binding activity between Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}-HMA} and AvrPik-A was not deduced based on the yeast growth (Figure 4B).

We also took advantage of the well-established methods for production of the AvrPik effectors and Pik-HMA domains in vitro to express and purify AvrPik-\textsuperscript{A}, -\textsuperscript{C}, -\textsuperscript{D}, or -\textsuperscript{E}, Pikm-HMA, Piks-HMA, Piks\textsuperscript{E229Q/Pikm\textsuperscript{V261A}-HMA} and Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}-HMA} proteins and then test for their interactions using an analytical gel filtration assay (Figure 5). Following mixing and incubation of purified proteins, this assay demonstrated complex formation between Pikm-HMA with AvrPik-\textsuperscript{A}, -\textsuperscript{D}, or -\textsuperscript{E}, but not with AvrPik-\textsuperscript{C}, consistent with previous results (De la Concepcion et al. 2018). By contrast, Piks-HMA did not form complexes with any of AvrPik proteins, which is consistent with the Y2H results (Figure 5). For Piks\textsuperscript{E229Q/Pikm\textsuperscript{V261A}-HMA} and Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}-HMA}, we found that each mutant formed complexes with AvrPik-\textsuperscript{D} but not with other AvrPik variants (Figure 5). Although weak interaction between Piks\textsuperscript{A261V/Pikm\textsuperscript{Q229E}-HMA} and AvrPik-\textsuperscript{E} was observed in the Y2H assay, it was not detected by analytical gel filtration with purified proteins.

\textit{Piks-1\textsuperscript{E229Q/Pikm-1\textsuperscript{V261A}} and Piks-1\textsuperscript{A261V/Pikm-1\textsuperscript{Q229E}} elicit weak cell death in \textit{N.}}
benthamiana when co-expressed with Piks-2 and AvrPik-D

Finally, we tested the potential of Piks, Piks-1^{E229Q}/Pikm-1^{V261} and Piks-1^{A261V}/Pikm-1^{Q229E} to elicit cell death in N. benthamiana leaves (development of necrotic tissue and accumulation of phenolic compounds) with the AvrPik variants AvrPik-A, -C, -D, or -E using Agrobacterium-mediated transformation. All constructs were co-expressed with Piks-2. Compared to the strong cell death observed in positive control (co-expression of Pikm and AvrPik-D), we observed only moderate cell death for the combinations of Piks-1^{E229Q}/Pikm-1^{V261} and Piks-1^{A261V}/Pikm-1^{Q229E} (Figure 6). For all other combinations, including Piks with all effector variants, and Piks-1^{A261V}/Pikm-1^{Q229E} and Piks-1^{A261V}/Pikm-1^{Q229E} with effector variants AvrPik-A, -C, or -E we did not observe signatures of cell death (Figure 6).

DISCUSSION

In this study, we report the characterization of the rice NLR immune receptor Piks and investigate its ability to recognize AvrPik effector variants. An extensive genetic analysis using a set of authentic isogenic rice lines and rice blast isolates demonstrates that Piks is a novel functional allele conferring a distinct resistance spectrum from other allelic variants at the Pik locus. As is well established for other Pik genes, Piks-1 and Piks-2 are necessary and sufficient for Piks-mediated resistance. However, Piks, unlike its alleles, does not recognize any known AvrPik variants in rice. Instead, our results suggest it recognizes a novel Avr gene which is most likely dissimilar to AvrPik at the sequence and/or structural level. We performed reciprocal exchange of the two amino acid residues that differ between Piks and Pikm (at positions 229 and 261), which are both contained within the HMA domain, and tested their roles in transgenic rice towards sustaining distinct specificities of Piks and Pikm. In addition to genetic characterization, the physical binding activity of Piks-HMA and Pikm-mimicking mutants towards different AvrPik variants were investigated in both Y2H and analytical gel filtration assays. Finally, cell death assay between Piks mutants and AvrPik variants was conducted in N. benthamiana. These results reveal a complex scenario of interaction outcomes between Piks mutants and AvrPik variants using
genetic and biochemical approaches.

**Piks confers a novel recognition specificity diverged from the canonical Pik NLRs**

Clustering of multiple alleles at the same R-gene locus is evident in many crop species, such as the L5/L6/L7 locus in flax (Dodds et al. 2004), the MLA1/7/9/10 locus in barley (Lu et al. 2016), and the Pi2/Piz-t/Pi9/Pi50 locus in rice (Zhou et al. 2006; Qu et al. 2006; Su et al. 2015). Notably, these allelic variants frequently share close sequence similarity. For instance, the MLA and Pi2/Piz-t/Pi9 alleles share over 90% and 96% sequence identity, respectively (Bauer et al. 2021; Zhou et al. 2007). Further, sequence variations within R-gene alleles leads to differential recognition cognate Avr genes with or without significant sequence similarity. For example, Pi9 and Piz-t activate rice immunity against distinct sets of rice blast isolates by recognizing sequence unrelated AvrPi9 and AvrPiz-t, respectively (Wu et al. 2015; Li et al. 2009).

By contrast, AvrL567-A and AvrL567-D, recognized by L5 and L6, respectively, share 92% amino acid sequence identity (Dodds et al. 2004, 2006). Different alleles of the rice R-gene Pik recognize at least one of five AvrPik variants, i.e., AvrPik-A to -E through so-called stepwise arms race co-evolution (Wu et al. 2014; Wang et al. 2017). 

Piks has been placed in the most ancestral position in the chronological order, without its functional verification at the molecular level (Wu et al. 2014). Here, we show that Piks shares characteristics of classical Pik alleles with respect to its genomic organization and sequence relatedness. However, unlike other Pik alleles, Piks does not recognize known AvrPik variants. Instead, it recognizes a novel Avr gene, which is presumably sequence unrelated to AvrPik. The finding that Piks confers an AvrPik-independent resistance specificity demonstrates a new interaction mode in addition to those previously shown between Pik alleles and AvrPik variants. This suggests that Pik variants have undergone distinct evolutionary mechanisms to recognize both sequences related and unrelated Avr effectors to activate immunity to specific pathotypes of the rice blast pathogen.

Our findings align with similar scenario illustrated in several R-gene loci which harbors multiple allelic variants, such as flax L locus (Dodds et al. 2004, 2006), barley MLA locus (Lu et al. 2016; Saur et al. 2019), Arabidopsis RPP13 locus (Hall et al.
In these R-gene loci, some allelic R-gene variants detect allelic Avr proteins, whereas others detect sequence unrelated Avr proteins, suggesting dynamic co-evolutionary mechanisms. Intriguingly, examples of sequence divergent but structurally related Avr proteins recognized by allelic R-gene variants have recently been reported. For example, five AvrPm3 variants, specifically recognized by different allelic PM3, belong to a large group of proteins with low sequence homology but predicted structural similarities (Bourras et al. 2019). Further, protein structure analysis predicted that known AVRd proteins specifically recognized by different allelic MLA variants each adopt RNase-like folds although they are sequence unrelated from one another (Bauer et al. 2021). Although the putative effector recognized by Piks is yet to be reported, and maybe sequence diverse from AvrPik, it is interesting to speculate they maybe structurally related, especially as many rice blast effectors whose structures have been determined (including all those that interact with host HMA proteins) adopt the MAX effector fold (De Guillen et al. 2015).

Perturbation of HMA interfaces results in alteration of binding activity and strength to AvrPik proteins

The HMA domain of Pik alleles is the key determinant of specifically for recognizing AvrPik variants through direct binding (Maqbool et al. 2015; De la Concepcion et al. 2018, 2021). There is an overall correlation between recognition specificity and binding of Pik-HMA domains by AvrPik variants (Kanzaki et al. 2012; De la Concepcion et al. 2018). Structure analyses of the complexes of Pik-HMAs and AvrPik effectors identified three interfaces in the HMA domain, dominate the interaction with AvrPik effectors (De La Concepcion et al. 2018). Pikp mainly employs interface 2 to determine its binding with AvrPik-D, whereas Pikm mediates binding through robust interaction at interfaces 2 and 3 (De La Concepcion et al. 2018). The two amino acid residues at position 229 and 261 that distinguish Piks-1 from Pikm-1 are positioned within interfaces 2 and 3, respectively, suggesting the likely importance of these two interfaces in the binding of Piks to AvrPik variants. Sequence alignment reveals that Glu229 and Ala261 (as found in Piks) are conserved in Pik-1-Ku/Ka and Pikp-1/Pi7-1/Pikh-1, respectively (Meng et al. 2021), implying
that replacement of either Glu229 or Ala261 in Piks-1 might restore binding activity between derivative HMA and AvrPik proteins. Indeed, both the mutations Piks-1^{E229Q} and Piks-1^{A261V} supported physical binding to the AvrPik-D variant as measured by Y2H and analytical gel filtration with purified proteins. But this restoration of effector binding did not extend to the variants AvrPik-E nor -A (that bind Pikm). In a recent study, Pikg, which originates from a wild rice species and differs from Pike by a single amino acid substitution (D229E) in the HMA domain, controls a distinct resistance spectrum (Meng et al. 2021). However, sequence changes at these equivalent positions in the HMA domain may not be a necessary result in the functional divergence. For example, Pikm-1 and Pi1-5 confer indistinguishable specificities although they differ from each other by a single amino acid change (Gln229Asp) at the equivalent position (Figure S5).

**Varied outcomes of interaction between Piks alleles and AvrPik variants**

In this study, we used three different approaches to characterize the function of Piks and the mutants Piks-1^{E229Q}/Pikm-1^{V261A} and Piks-1^{A261V}/Pikm-1^{Q229E}, protein-protein binding assay via Y2H and gel filtration analyses, cell death assays in *N. benthamiana*, and genetic complementation tests in rice (Table S3). The results reveal an overall consistent outcome derived from functional characterization using different approaches. Piks-1 shows no binding activity with tested AvrPik proteins in either Y2H or analytical gel filtration. It does not induce cell death in *N. benthamiana* when co-expressed with different AvrPik effectors, and cannot complement resistance in rice to isolates containing different AvrPik effectors (Table S3). Likewise, a consistent result is observed for the functional characterization of Piks-1^{E229Q}/Pikm-1^{V261A}. However, Piks-1^{A261V}/Pikm-1^{Q229E} shows a gain of binding activity to the effector variant AvrPik-D and induces cell death when co-expressed with AvrPik-D in *N. benthamiana*, but it could not activate resistance against blast isolates containing AvrPik-D. The possibility that Piks-1^{A261V}/Pikm-1^{Q229E} is not expressed properly is further excluded since the same transgenic lines retain the resistance against V86010. Therefore, we conclude that Piks-1^{A261V}/Pikm-1^{Q229E} is incapable of activating immunity against rice blast isolates with AvrPik-D although it appears functional in
both protein-protein binding and ectopic cell death assays. It is still elusive whether this similar observation could be applicable to other R-Avr functional studies. However, it provides a specific case illustrating where ectopic expression assays might blur the true function of particular R genes in recognition and immunity.

MATERIALS AND METHODS

Plant materials and growth condition
The F₂ population derived from a cross between the Piks gene monogenic line IRBLKs-F5 and its recipient rice variety Lijiangxintuanheigu (LTH) were used for mapping and co-segregation analysis. The rice cultivar Nipponbare was used as a host cultivar for gene transformation. The Pik allelic gene monogenic lines in LTH background IRBK1-CL (Pil), IRBL7-M (Pi7), IRBLK-Ka (Pik), IRBLKm-Ts (Pikm), and IRBLKp-K60 (Pikp) were used to evaluate the resistance specificity.

Rice seeds were surface-sterilized with sodium hypochlorite and plated on petri dishes covered with wet filter paper. Seeds were germinated at 37°C in a growth chamber with a 12-h/12-h light/dark cycle. Standard growth on soil in a growth chamber was under a 16h/8h light/dark cycle with 30°C/22°C, if not otherwise indicated.

M. oryzae materials and pathogenicity assay
The field M. oryzae isolates JMB8401, 5008-3, 9482-1-1, CA89 and V86010 were used in this study. To create isogenic lines of M. oryzae harboring different AvrPik alleles, we transformed R88-002 individually with expression vectors for AvrPik-A, AvrPik-B, AvrPik-C, AvrPik-D, AvrPik-E and AvrPik-F. We amplify the native promoter (~1.5kb) and ORF (~0.3kb) of AvrPik-A to -F from the M. oryzae isolates 98-06, Guy 11, V86010 and R01-1, and digested using SalI and EcoRI and connected to pCB1532-attR vector, individually.

The AvrPik gene replacement constructs pMD19-T-AvrPik-KO was generated according to the homologous recombination principle as described previously (Zhang et al. 2010). The spacers for Cas9-gRNA vectors were designed with the gRNA
designer program for best on-target scores (http://grna.ctegd.uga.edu/). The sense and
antisense oligonucleotides of the selected gRNA spacers were synthesized and
annealed to generate the corresponding gRNA spacers, and cloned between the two
BsmBI sites of pCas9-tRp-gRNA by Golden Gate cloning (NEB) (Arazoe et al. 2015) to
generate the pCas9-tRpgRNA-AvrPik constructs. Then, the gene replacement
vector pMD19-T-AvrPik-KO and its corresponding pCas9-tRpgRNA-AvrPik vector
were co-transformed into protoplasts of V86010 to obtain the AvrPik gene deletion
mutants. Hygromycin-resistant transformants were screened by PCR and selected for
phenotype analysis. Primers used in this section are listed in Table S2.
For fungal transformation, protoplasts were isolated and transformed with the PEG
method as described previously (Sweigard et al. 1995). Media were supplemented
with chlorimuron-ethyl/hygromycin B at 300 µg/ml in order to select chlorimuron-
ethyl/ hygromycin resistant transformants. All the resultant transformants were
subjected to either PCR validation using specific primers (Table S2) or Southern
hybridization to ascertain successful single-copy integration.
Rice seedlings at the 3-4 leaf stage were used for spray inoculation. The spore
suspension with 10^5 spores/ml was applied to plants using an airbrush connected to a
source of compressed air. After inoculation, plants were held in the dark room for 24 h
with 95–100% relative humidity and 24°C. Then, plants were transferred to a
greenhouse where the temperature was maintained at 25–28 °C and humidity was
keep at 70–90%. Six to Seven days after inoculation, disease symptoms were
evaluated using a standard 0–5 scale (IRRI 2002).

**DNA extraction, PCR primers and sequencing analysis**
Genomic DNA of all the plant materials was extracted from fresh leaves using CTAB
method. For fungal DNA extractions, mycelia were harvested from 3-day-old cultures
grown in liquid CM at 28°C with shaking at 150 rpm. General molecular biology
techniques for nucleic acid analysis were performed according to standard protocols.
We used PCR primer pair RGA4-F3/RGA4-R3 to genotype the F2 population for co-
segregation analysis. The primer pairs Piks-1-F/Piks-1-R and Piks-2-F/ Piks-2-R were
used to define the candidate genome region of Piks, to check the presence of
transgenes in transgenic lines (Table S2). For AvrPik gene haplotype analysis in wild
M. oryzae isolates, the primer AvrPik-F/AvrPik-R was use for PCR amplification, and
selected PCR products were subjected to Sanger sequencing. The sequence of the
primers were list at Table S1.

Candidate gene cloning and complementation analysis
As reported previously, the two genes of Pik (Pik-1 and Pik-2) alleles working
together to trigger Pik mediated resistance. From the genomic DNA of IRBLKs-F5,
we used a high-fidelity Taq (Phanta Super-Fidelity DNA Plomerase, Vazyme) to
amplify a 10.5-kb fragment, which contain the whole Piks-1 coding sequence, 5’-
untranslated region (UTR) and 3’-UTR. The whole Piks-2 coding sequence, 5’-
untranslated region (UTR) and 3’-UTR, which was 8.9-kb were also amplified using
the same method. The two amplified products were individually inserted into the Ascl
and PacI site of the vector pCambia 1305.2 to form constructs for transformation.
These constructs were validated by comparison of their insert sequences with the
sequence published at NCBI (HQ662329). Mutagenesis of Piks-1 was performed
using the Mut Express MultiS Fast Mutagenesis Kit V2 (VAZYME, Nanjing, China).
The two mutant Piks-1E229Q/Pikm-1V261A and Piks-1V261V/Pikm-1Q229E were confirmed
successful gene mutation by select the plasmids for sequencing.
The four constructs each containing the Piks-1, Piks-1E229Q/Pikm-1V261A, Piks-
1A261V/Pikm-1Q229E and Piks-2, individually, were transferred into Nipponbare callus
by Agrobacterium-mediated transformation. The Primary transgenic plants (T0 plants)
regenerated from hygromycin-resistant calluses were grown in an isolated greenhouse.
The T0 plants of Piks-1, Piks-1E229Q/Pikm-1V261A and Piks-1A261V/Pikm-1Q229E were
checked by PCR assay with the Piks-1 specific primer pair Piks-1-F/Piks-1-R and the
Piks-2 T0 plants were checked by specific primer pair Piks-2-F/ Piks-2-R (Table S2).

Gene cloning: heterologous protein production, Y2H and in planta expression
For in vitro studies, Piks-HMA, Piks-1E229Q/Pikm-1V261A-HMA and Piks-1V261V/Pikm-
1Q229E -HMA (residues Gly186 to Asp264, codon optimized for expression in E. coli)
was generated by introducing the Glu229Gln and Ala261Val in Piks-HMA by site-
directed mutagenesis, and followed by cloning into the vector pOPIN-M (Maqbool et al. 2015; De la Concepcion et al. 2018). The expression constructs of Pikm-HMA and AvrPik used in this study were described previously (Maqbool et al. 2015; De la Concepcion et al. 2018).

For yeast-2-hybrid analyses, the Pikm-HMA, Piks-HMA, Piks-1^{E229Q}/Pikm-1^{V261A}-HMA and Piks-1^{A261V}/Pikm-1^{Q229E}-HMA (residues Gly186 to Ile300) was synthesized and supplied in the pGBKKT7, the AvrPik-A, -C, -D and -E (residues Glu22 to Phe113, lacking the signal peptide) was synthesized (Sangon Biotech) and supplied in the pGADT7.

For protein expression in planta, the Pikm-HMA, Piks-HMA, Piks-1^{E229Q}/Pikm-1^{V261A}-HMA and Piks-1^{A261V}/Pikm-1^{Q229E}-HMA domain was assembled into a full-length NLR construct using Golden Gate cloning (Maqbool et al. 2015; De la Concepcion et al. 2018) and then into the plasmid pICH47742 with a C-terminal 6xHis/3xFLAG tag. The expression was driven by the A. tumefaciens Mas promoter and terminator.

Expression and purification of proteins for in vitro binding studies.

The Pikm-HMA, Piks-HMA, Piks-1^{E229Q}/Pikm-1^{V261A}-HMA and Piks-1^{A261V}/Pikm-1^{Q229E}-HMA with the 6xHis-MBP-tag were produced in E. coli SHuffle cells using the protocol described previously (Maqbool et al. 2015; De la Concepcion et al. 2018). Cell cultures were incubated in auto induction media at 30°C for 5-7 hours and then at 16°C overnight. Cells were harvested by centrifugation and resuspended in the medium used previously (Maqbool et al. 2015; De la Concepcion et al. 2018). The suspended Cells were sonicated and then centrifugated at 40000 x g for 30min, the supernatant lysate was applied to a Ni^{2+}-NTA column connected to an AKTA Xpress purification system (GE Healthcare) (Maqbool et al. 2015; De la Concepcion et al. 2018). Proteins were step-eluted with elution buffer and directly injected onto a Superdex 75 26/600 gel filtration column pre-equilibrated 20mM HEPES pH 7.5, 150 mM NaCl. Purification tags were then removed by incubating with 3C protease (10 μg/mg fusion protein) overnight at 4°C followed by passing through tandem Ni^{2+}-NTA and MBP Trap HP columns (GE Healthcare). The flow-through was concentrated as appropriate and loaded on a Superdex 75 26/600 gel filtration column.
for final purification and buffer exchange into 20 mM HEPES pH 7.5, 150 mM NaCl (Maqbool et al. 2015; De la Concepcion et al. 2018). The AvrPik effectors, with a 3C protease-cleavable N-terminal SUMO tag and a non-cleavable C-terminal 6xHis tag, were produced in and purified from E. coli SHuffle cells as previously described (Maqbool et al. 2015; De la Concepcion et al. 2018). All protein concentrations were determined using a Direct Detect Infrared Spectrometer (Merck).

**Analytical gel filtration**

Analytical size exclusion chromatography was performed at 4°C using a Superdex 75 10/300 gel filtration column (GE Healthcare) pre-equilibrated in 20 mM HEPES pH 7.5 and 150 mM NaCl (Maqbool et al. 2015; De la Concepcion et al. 2018). Samples were centrifuged before loading. A 100 μl of the sample was injected at a flow rate of 0.8 ml/min and 0.5 ml fractions were collected for analysis by SDS-PAGE gels. Proteins were mixed and incubated on ice for 60 min before loading for study complex formation (Maqbool et al. 2015; De la Concepcion et al. 2018).

**Yeast two-hybrid assay**

We use the Matchmaker Gold Yeast Two-Hybrid System (Takara Bio USA) to detect protein–protein interactions between Pik-HMAs and AvrPik effectors. The DNA encoding the Piks-HMAs in pGBK7 was co-transformed with either the individual AvrPik variants in pGADT7 into chemically competent Saccharomyces cerevisiae Y2HGold cells (Takara Bio USA). The single colonies grown on selection plates were inoculated in SD-Leu-Trp plate and grown at 30 °C for overnight. Saturated culture was then used to make serial dilutions of optical density at 600 nm (OD600) 1, 1⁻¹, 1⁻² and 1⁻³, respectively. Five microlitre of each dilution was spotted on a SD-Leu-Trp plate as a growth control and also on a SD-Leu-Trp-Ade-His plate containing X-α-gal and aureobasidine, as detailed in the user manual. After incubated at 30 °C for 60–72 h, the plates were imaged. Each experiment was repeated three times, with similar results.
**N. benthamiana cell death assays.**

For agroinfiltration in *N. benthamiana*, Agrobacterium tumefaciens GV3101 was transformed with the relevant T-DNA constructs. Leaves of 4-week-old *N. benthamiana* plants grown at 22–25 °C with high light intensity were agroinfiltrated using a needleless syringe (Maqbool et al. 2015; De la Concepcion et al. 2018). *Piks-1 (Piks-1'E229Q/Pikm-1'V261A-1* and *Piks-1'A261V/Pikm-1'O229E-1), Piks-2, AvrPik* and P19 were mixed at OD600 0.4, 0.4, 0.6 and 0.1, respectively (Maqbool et al. 2015; De la Concepcion et al. 2018). Detached leaves were imaged at 5 dpi from the adaxial side of the leaves for white light image and abaxial side of the leaves for UV images. Pictures are representative of three independent experiments, with internal repeats. The cell death index used for scoring is as presented previously (Maqbool et al. 2015; De la Concepcion et al. 2018). The scoring for all replicas is presented as box plots, which were generated using R v3.4.3 (https://www.r-project.org/) and the GraphPad Prism 9.0 (https://www.graphpad-prism.cn/). The centre line represents the median, the box limits are the upper and lower quartiles, the whiskers are the 1.5× interquartile range and all of the data points are represented as dots (Maqbool et al. 2015; De la Concepcion et al. 2018).

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**AUTHOR CONTRIBUTIONS**

G.X., J.W. and B.Z. conceived and designed the experiments. G.X., W.W., M.L., Y.L., carried out the experiments. G.X., J.L., M.F. and Z.Y. analyzed the data. X.Z., Z.Z. and G.L. provided technical assistance. G.X., M.B., J.W. and BZ wrote the manuscript. All authors read and approved the final manuscript.
SUPPORTING INFORMATION

Figure S1. Detection of the rice blast strain R88-002 transformed with six *AvrPik* variants (*AvrPik*-A to –F) by PCR assay.

Figure S2. Gene specific marker which can distinguishes *Piks* and *Pikm* from LTH (Lijiangxintuuanheigu) and other five *Pik* alleles. Lane 1, IRBLks-F5 (*Piks*); Lane 2, LTH; Lane 3, IRBLkm-TS (*Pikm*); Lane 4 IRBLkh-K3 (*Pikh*); Lane 5, IRBLkp-K60 (*Pikp*); Lane 6, IRBLK-Ka (*Pik*); Lane 7, IRBL1-CL (*Pi1*); Lane 8, IRBL7-M (*Pi7*); L, DNA Ladder.

Figure S3. The rice blast isolate V86010 contain both *AvrPik*-D and *AvrPik*-E.

(A) Sequence alignment of *AvrPik* in V86010, *AvrPik*-D and *AvrPik*-E. (B) Sequencing results of *AvrPik* PCR amplicon. The red triangle marked out the location of sequence difference and double peak of the sequencing results.

Figure S4. PCR amplification results of the three *AvrPik* knock out mutant strains of V86010. *AvrPik*-In-F/R primer pair cover the coding region of *AvrPik* while the primer pair *AvrPik*-out-F/Hoh-R amplified the replacement fragment and promoter of the *AvrPik*.

Figure S5. Alignment of amino acid sequence encoded by *Pik*-1 alleles.

Figure S6. Amino acid residue polymorphisms among *Pik*-2 alleles. Dots represent residues identical to those in *Piks*-2.
Figure S7. Representative SDS-PAGE gels of relevant fractions. (A) SDS-PAGE gel of AvrPik-D protein. (B) SDS-PAGE gel of Pikm and AvrPik-D protein complex.

Table S1. Pathogenicity of different M. oryzae isolates toward Pik allelic monogenic lines in Lijiangxintuanheigu (LTH).

Table S2. Primers used in this study.

Table S3. The various interactions and phenotypes between Piks-1, Piks mutants and AvrPik variants in this study.

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resistance in rice. New Phytol 206: 1463–1475


Table 1. Disease reactions of different Pik alleles in the near isogenic lines to *M. oryzae* R88-002 isolate expressing six AvrPik variants.

<table>
<thead>
<tr>
<th>Strains</th>
<th>AvrPik variants</th>
<th>Differential near isogenic lines and respective Pik alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IRBLKs-F5</td>
</tr>
<tr>
<td>R88-002&lt;sup&gt;AvrPik-A&lt;/sup&gt;-1</td>
<td>AvrPik-A</td>
<td>S</td>
</tr>
<tr>
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<td>S</td>
</tr>
<tr>
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<td>AvrPik-B</td>
<td>S</td>
</tr>
<tr>
<td>R88-002&lt;sup&gt;AvrPik-B&lt;/sup&gt;-4</td>
<td>AvrPik-B</td>
<td>S</td>
</tr>
<tr>
<td>R88-002&lt;sup&gt;AvrPik-C&lt;/sup&gt;-3</td>
<td>AvrPik-C</td>
<td>S</td>
</tr>
<tr>
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<td>S</td>
</tr>
<tr>
<td>R88-002&lt;sup&gt;AvrPik-D&lt;/sup&gt;-4</td>
<td>AvrPik-D</td>
<td>S</td>
</tr>
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<td>AvrPik-D</td>
<td>S</td>
</tr>
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<td>AvrPik-F</td>
<td>S</td>
</tr>
<tr>
<td>R88-002</td>
<td>None</td>
<td>S</td>
</tr>
</tbody>
</table>

R88-002 is the recipient rice blast isolate; R, resistance; S, susceptible
Table 2. Genetic and co-segregation analysis of the LTH/IRBLKs-F5 F₂ population inoculated with the rice blast isolate V86010

| R  | S  | Total | $\chi^2$ (3:1) | $P$ value | Marker | R/AA | R/Aa | S/aa | $\chi^2$ (1:2:1) | $P$ value |
|----|----|-------|----------------|-----------|--------|-------|------|------|------|----------------|-----------|
| 1053 | 334 | 1387  | 0.625 | 0.429 | RGA4F3/R3 | 360   | 693  | 334  | 0.975 | 0.614 |

R, resistance; S, susceptible
Table 3. Disease reactions of Piks gene and two Piks mutants \( \text{Piks}^{E229Q/\text{Pikm}^{V261A}} \) and \( \text{Piks}^{A261V/\text{Pikm}^{Q229E}} \) transgenic lines to \( \text{M. oryzae} \) R88-002 isolate expressing six AvrPik variants.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Gene</th>
<th>( \text{M. oryzae} ) isolate R88-002 transformed with different AvrPik variants</th>
<th>R88-002</th>
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<tr>
<td></td>
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<td>Pikm</td>
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<td>Transgenic lines in NIP background</td>
<td>Piks(^{E229Q/\text{Pikm}^{V261A}-1+\text{Piks}-2})</td>
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<td>L300-5</td>
<td>Piks(^{E229Q/\text{Pikm}^{V261A}-1+\text{Piks}-2})</td>
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<td>S</td>
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<td>S</td>
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<td>L303-29</td>
<td>Piks-1+Piks-2</td>
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<td>L303-31</td>
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</tr>
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<td>LTH</td>
<td>None</td>
<td>S</td>
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R, Resistance; S, Susceptible; NIP, Nipponbare; LTH, Lijiangxintuanheigu
Figure 1. Characterization and cloning of Piks. (A) Identification of a *M. oryzae* isolate avirulence to Piks gene near isogenic line IRBLKs-F5. V86010 and CA89 are two *M. oryzae* isolates used for inoculation. (B) Resistance spectrum of Piks and other Pik allelic genes. LTH (Lijiangxintuanheigu) is the susceptible control for inoculation and also the recipient cultivar for the development of monogenic line IRBLKs-F5 F2 progeny. PCR amplification assay of the parental lines and F2 progeny using primer pair RGA4-F3/RGA4-R3. Enzymatic digestion results of the PCR products using PstI. R1 to R5 means the resistance progeny 1 to 5, S1 to S5 means the susceptible progeny 1 to 5. AA homozygous resistant, Aa heterozygote, aa homozygous susceptible. (D) Complementation testing and molecular analysis of the transgenic plants. Reaction of IRBLKs-F5, Nipponbare, and a set of F2 progeny (numbers 1–12) derived from a cross between T1 plants harboring Piks-1 and Piks-2, individually, to the blast isolate V86010. The isolate V86010 is avirulence to the Piks donor cultivar IRBLKs-F5 and virulence to the transformation recipient cultivar Nipponbare. Co-segregation of the resistance phenotype with the presence of both the Piks-1 gene and the Piks-2 gene. The transgenic lines numbered 1 to 3 harbor only Piks-1, those numbered 4 to 6 contain only Piks-2, number 7-9 contain both Piks-1 and Piks-2, and that numbered 10-12 harbor neither of the transgenes.
**Figure 2.** Genotype and phenotypes of *Piks* loss-of-function mutants created by CRISPR technology. (A) Schematic description of the CRISPR mutant of *Piks*-1 and *Piks*-2. Mutations detected in genomic DNA of *Piks*-KO-3, *Piks*-KO-4 and *Piks*-KO-7 mutants are shown by SANGER sequencing chromatography. (B) Phenotype of *Piks* loss-of-function mutants. Rice blast isolate V86010 is used for inoculation. LTH (Lijiangxintuanheigu) and IRBLKs-F5 are used as control.
<table>
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<tr>
<th>Rice materials</th>
<th>M. Oryzae V86010</th>
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<td></td>
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<td>LTH WT</td>
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<td>S</td>
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| Piks-1+Piks-2 | L303-29 | R | R | R |
|               | L303-31 | R | R | R |
|               | L303-32 | R | R | R |
| PiksE229Q/PikmV261A-1+Piks-2 | L300-5 | R | R | R |
|               | L300-11 | R | R | R |
|               | L300-24 | R | R | R |
| PiksA261V/PikmQ229K-1+Piks-2 | L301-2 | R | R | R |
|               | L301-3 | R | R | R |
|               | L301-12 | R | R | R |

Figure 3. The avirulence of V86010 to Piks do not rely on AvrPik. (A) Disease reaction of the Pik allelic gene monogenic lines inoculated with V86010 and three AvrPik-D/-E knock-out mutant strains. (B) Disease reaction of the Piks and Piks single amino acid substitution mutant (PiksE229Q/PikmV261A and PiksA261V/PikmQ229K) transgenic plants challenged with blast isolate V86010 and V86010 knock-out of AvrPik-D/-E strains. NIP is the recipient variety of the transgenes.
**Figure 4.** Yeast two hybrid assay demonstrate the binding of *AvrPik* effector variants to the HMA domain of *Piks*, *Pikm* and *Piks* single amino acid substitution mutants (*Piks* E229Q/*Pikm* V261A and *Piks* A261V/*Pikm* Q229E). (A) Amino acid sequence alignment of *Piks*-1 and *Pikm*-1 HMA domains. (B) *Piks*-HMA does not interaction with any *AvrPik* variants as assayed by Y2H. The control plate for yeast growth is on the left, with the selective plate on the right. Each experiment was repeated a minimum of three times, with similar results.
Figure 5. Pikm-HMA can not binding to any AvrPik variants. Analytical Gel Filtration traces depicting the retention volume of AvrPik variants (AvrPik-A, AvrPik-C, AvrPik-D and AvrPik-E) and Pik-HMA (Piks-HMA, Piks^{E229Q}/Piks^{V261A}–HMA and Piks^{E261V}/Piks^{Q229E}–HMA) and the complex. Pikm-HMA and the complex are used as the control. Representative SDS-PAGE gels of AvrPik-D variants and AvrPik-D/Pikm protein complex were shown in Figure S7. Each experiment was repeated a minimum of three times, with similar results.
Figure 6. The Piks and Piks single amino acid substitution mutants mediated cell death response to AvrPik effector variants in *N. benthamiana*. (A-C). Representative leaf image showing Piks, Piks$^{E229Q}$/Pikm$^{V261A}$ and Piks$^{A261V}$/Pikm$^{Q229E}$ mediated cell death to AvrPik variants; Images showing autofluorescence are horizontally flipped to present the same leaf orientation as white light images; Pikm-mediated cell death with AvrPik-D is included as a positive control. (D) Box plots showing repeats of the cell death assay. For each sample, the number of repeats was 90. The centre line represents the median, the box limits are the upper and lower quartiles, the whiskers are the 1.5× interquartile range and all of the data points are represented as dots. The cell death scoring scale used is shown in Maqbool et al., 2015.