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ORIGINAL PAPER

The *Pik-p* resistance to *Magnaporthe oryzae* in rice is mediated by a pair of closely linked CC-NBS-LRR genes

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Abstract The blast resistance gene *Pik-p*, mapping to the *Pik* locus on the long arm of rice chromosome 11, was isolated by map-based *in silico* cloning. Four NBS-LRR genes are present in the target region of cv. Nipponbare, and a presence/absence analysis in the *Pik-p* carrier cv. K60 excluded two of these as candidates for *Pik-p*. The other two candidates (*KP3* and *KP4*) were expressed in cv. K60. A loss-of-function experiment by RNAi showed that both *KP3* and *KP4* are required for *Pik-p* function, while a gain-of-function experiment by complementation test revealed that neither *KP3* nor *KP4* on their own can impart resistance, but that resistance was expressed when both were introduced simultaneously. Both *Pikp-1* (*KP3*) and *Pikp-2* (*KP4*) encode coiled-coil NBS-LRR proteins and share, respectively, 95 and 99% peptide identity with the two alleles, *Pikm1-TS* and *Pikm2-TS*. The *Pikp-1* and *Pikp-2* sequences share only limited homology. Their sequence allowed *Pik-p* to be distinguished from *Pik*, *Pik-s*, *Pik-m* and *Pik-h*. Both *Pikp-1* and *Pikp-2* were constitutively expressed in cv. K60 and only marginally induced by blast infection.

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B. Yuan and C. Zhai contributed equally to this work.

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Introduction

Plants have developed a multifarious defense system against their various viral, bacterial, fungal, nematode and insect pathogens. The first line of their defense is raised by pattern recognition receptors, which respond to pathogen-associated molecular patterns by initiating a basal defense response (Nurnberger et al. 2004; Zipfel and Felix 2005). This response is generally able to prevent infection by non-host pathogens. Some pathogens have evolved effector proteins and/or toxins, which inhibit this basal defense response, allowing the pathogen to colonize the plant (Jones and Dangl 2006; Zipfel 2008). Their second line of defense is activated by resistance (*R*) genes, which encode R proteins able to recognize specific effector molecules produced by the pathogen and then trigger a response within the plant cell (Da Cunha et al. 2006; Jones and Dangl 2006), R proteins typically confer race-specific resistance, and their expression is frequently associated with a hypersensitive response. Pathogen effectors able to

suppress this response are the products of avirulence (*Avr*) genes. Numerous *R* genes have been isolated from a range of plant species (Fu et al. 2009; Krattinger et al. 2009; Liu et al. 2007a; Martin et al. 1993), and their analysis has shown that they fall into several well-defined classes. The nucleotide-binding site leucine-rich repeat proteins (NBS-LRR) is the largest of these (McHale et al. 2006). Their N termini comprises either a Toll-interleukin receptor (TIR)-like domain or a coiled-coil (CC) structure, so allowing for the recognition of the TIR-NBS-LRR and the CC-NBS-LRR subtypes. The *Arabidopsis thaliana* genome includes 159 NBS-LRR genes (both the TIR and the CC type are represented), while the rice genome includes 535 exclusively CC-NBS-LRR genes (Meyers et al. 2003; Zhou et al. 2004). In both rice and *A. thaliana*, most of the NBS-LRR genes occur in clusters and some in tandem arrays. Some clusters feature a heterogeneous population of genes, while others contain groups of highly homologous sequences (Leister 2004; Richly et al. 2002). In a few cases, functionality requires the simultaneous presence of two *R* genes (Ashikawa et al. 2008; Lee et al. 2009; Loutre et al. 2009; Sinapidou et al. 2004).

Rice blast (causative pathogen *Magnaporthe oryzae*) is one of the most devastating diseases of rice (Ou 1985). Over 80 genes encoding resistance to various combinations of blast races have been documented (Ballini et al. 2008; Yang et al. 2009) and so far 13 have been isolated and characterized. Except for two (*Pi-d2* and *pi21*), all are of the NBS-LRR type. *Pi36* and *Pid3* are single copy genes (Liu et al. 2007b; Shang et al. 2009), while *Pita*, *Pib*, *Pi37* and *Pit* are members of a gene family (Bryan et al. 2000; Hayashi and Yoshida 2009; Lin et al. 2007; Wang et al. 1999). *Pi9*, *Pi2* and *Piz-t* map to a single locus, but the *Pi9* sequence is only weakly related to that of the allelic pair *Pi2*/*Piz-t* (Qu et al. 2006; Zhou et al. 2006). Rice chromosome 11 carries a large number of *R* genes, with 106 containing the protein kinase domain and 102 the NB-ARC domain (Rice Chromosomes 11 and 12 Sequencing Consortia 2005). Specifically, the chromosome carries the blast resistance genes *Pik*, *Pik-p*, *Pik-s*, *Pik-g*, *Pik-h* and *Pik-m*, along with the bacterial blight resistance genes *Xa4* and *Xa26* (Ashikawa et al. 2008; Kiyosawa 1987; Pan et al. 1998; Sun et al. 2003, 2004; Wang et al. 2009; Xu et al. 2008; Yang et al. 2003). *Pik*, *Pik-p* and *Pik-m* are probably allelic (Hayashi et al. 2006). The resistance spectrum of *Pik-m* is broader than those of *Pik*, *Pik-p* or *Pik-s* among Japanese, but not necessarily among Chinese pathogen populations (Kiyosawa 1987; Wang et al. 2009). The objective of the present study was to isolate and characterize *Pik-p*, which confers stable and strong resistance against both Japanese and Chinese isolates (Wang et al. 2009).

Materials and methods

Candidate gene identification

Pik-p has been mapped within a 126-kb interval of rice chromosome 11 plus a contig gap (Wang et al. 2009). The gene content in the equivalent interval of cv. Nipponbare was predicted using GENESCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://www.softberry.com). DNA sequence comparisons were performed by pairwise BLAST (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html) and protein sequence similarities obtained by BLASTP (Altschul et al. 1997). Transcripts of the genes present in the critical interval were sought in both the GenBank (http://www.ncbi.nih.gov/blast) and the rice full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA). The presence/absence of candidate genes was established by PCR, wherever there was a large insertion/deletion in the target region between the reference (cv. Nipponbare) and the donor (cv. K60) genomes (Figs. 1 and S1).

RNAi constructs and transformation

Two *Pikp-1* cDNA fragments (1,246–1,730 bp from the start codon, within the NBS domain, and 2,526–3,275 bp corresponding to the LRR domain, see Fig. 3a) and three cDNA *Pikp-2* fragments (89–833 bp, corresponding to the CC domain, 1,163–1,748 bp, corresponding to NBS domain, and 2,546–3,061 bp, corresponding to LRR domain, see Fig. 4a) were amplified using primer sets, KP3i2 F/R, KP3i1 F/R, KP4i1 F/R, KP4i2 F/R and KP4i F/R, respectively (see Table S1). The first four of these fragments were ligated into the pDS1301 vector (Chu et al. 2006) and the fifth into the pANDA vector (Miki and Shimamoto 2004). The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation (GenePulser Xcell, Bio-Rad, Hercules, CA) and then transformed into the *Pik-p* carrier cv. K60, as described by Lin and Zhang (2005). T_0 and T_1 plants were inoculated with blast isolate CHL381, and disease reactions were scored as described by Pan et al. (2003). RT–PCR was used to verify that *Pik-p* expression was absent in susceptible T_0 plants. The T_1 progeny segregated as one resistant to three susceptibles and were randomly sampled to establish the correlation between disease phenotype and the presence of the transgene (Fig. 2a, b). Statistical testing of the silencing efficiency achieved by the various RNAi fragments was carried out using a *z* test with Excel (Table 1; Microsoft Corp., Redmond, WA).

Candidate gene cloning and transformation

Four overlapping fragments (3.5, 6.5, 5.7 and 6.4 kb; see Fig. S2) were amplified using Phusion, a high-fidelity

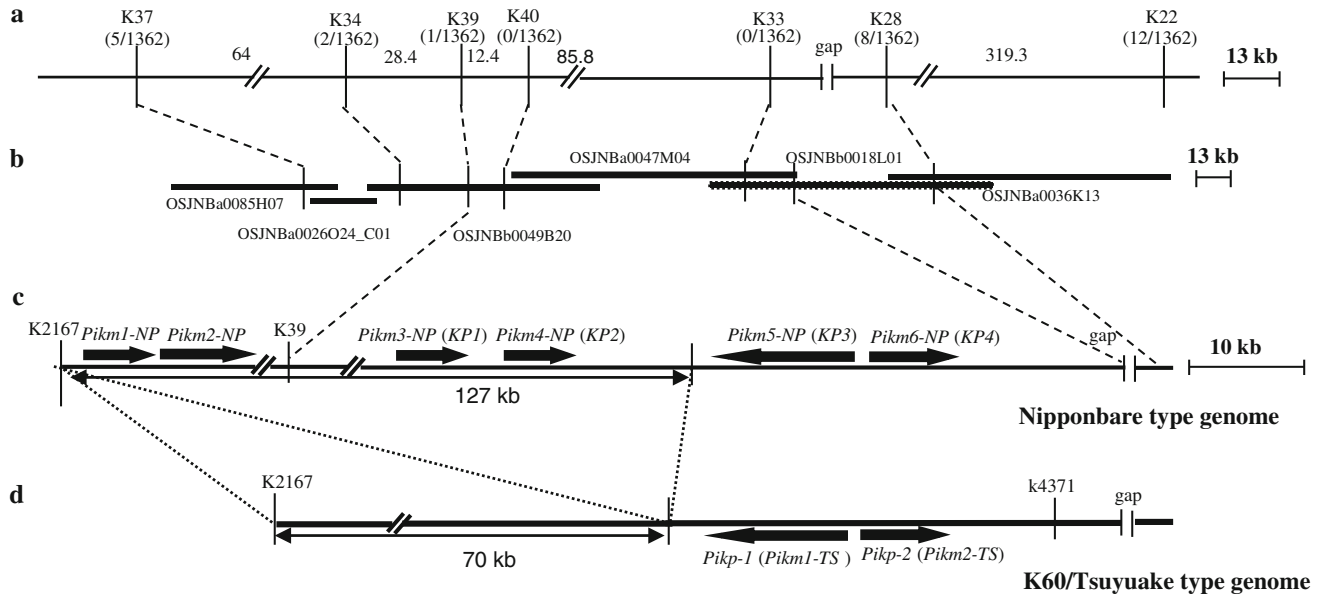


Fig. 1 Integrated physical map of the *Pik-p* and *Pik-m* loci. **a** A high-resolution physical map of *Pik-p*. The *numbers* above the map represent distances in kb, as derived from the cv. Nipponbare genome sequence. Those in *parentheses* represent the number of recombinants detected in a mapping population (Wang et al. 2009). **b** The bacterial artificial chromosome contig map spanning the *Pik-p* locus. *gap*, the sequence of the BAC clone OSJNBb0018L01 is currently unavailable from the RGP database. **c** Predicted NBS-LRR genes in the cv. Nipponbare genome sequence, as derived by FGENESH. **d** The deletion (127 kb) and insertion (70 kb) occurred in the target region between Nipponbare and K60/Tsuyuake genomes (adapted from Ashikawa et al. 2008). Both *KP1* and *KP2* are absent from cv. K60 (also see Supplementary Fig. S1)

Taq polymerase (NEB, England). After an A-tailing procedure, the 3.5-kb product was ligated into pMD20-T (TaKaRa, Dalian, China), and the other three were digested with *Asc*I and inserted into the pCAMBIA1301AscI vector. The sequences of the four resulting recombinant plasmids (KP3-3.5, KP3-6.5, KP34-5.7 and KP4-6.4) were assembled by DNAStar software (http://www.DNAStar.com). The *Kpn*I-*Mlu*I KP3-3.5, *Mlu*I-*Sal*I KP3-6.5 and *Sal*I-*Asc*I KP34-5.7 fragments were introduced in tandem into pCAMBIA1301AscI to form a full-length *Pikp-1* insert of length 12,432 bp (Fig. S2). The full-length *Pikp-2* insert (10,459 bp) comprised a combination of KP34-5.7 and KP4-6.4 (Fig. S2). The 16,919 bp *KP3+4* construct was built from the *Kpn*I–*Bam*HI (*KP3*), *Bam*HI–*Sal*I (*KP3*), *Sal*I–*Bsp*HI (*KP4*) and *Bsp*HI–*Asc*I (*KP4*) fragments ligated into pCAMBIA1301AscI (Fig. S2). The three constructs were independently transformed into the blast-susceptible cv. Q1063 as above. All T_0 plants were tested for their reaction to blast infection using isolates CHL381 and CHL346. The selected T_0 plants were then tested for the presence of both right and left border markers. T_1 progeny segregating as three resistant to one susceptible were randomly sampled and subjected to co-segregation analysis.

Analysis of full-length cDNA and predicted protein sequences

The cDNA 5′ end sequences were obtained by RACE-PCR, using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA), following the manufacturer's instructions. The *KP3* and *KP4* 5′ RACE products were amplified using nested PCR [first reaction using primers KP3-5RACE1 and KP4-5RACE1 with the universal primer A mix provided by the kit; the second PCR using KP3-5RACE2 and KP4-5RACE2 primers and the nested universal primer A (NUP) from the same kit (see Table S1; Fig. S2)]. The 3′ end sequences of *KP3* and *KP4* were obtained using a GeneRacerTM kit (Invitrogen, Groningen, The Netherlands) as described by Lin et al. (2007). Intermediate RT–PCR fragments were obtained using primer sets KP3ORF F/R and KP4ORF F/R for, respectively, *KP3* and *KP4*, which overlap the 5′ RACE and 3′ RACE fragments of each gene. The RACE and intermediate RT–PCR products were all cloned into pMD-20 (TaKaRa) for sequencing. Sequence data of *Pik-p* gene has been deposited in GenBank as accession HM035360.

The compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html; Gasteiger et al. 2005) was used to

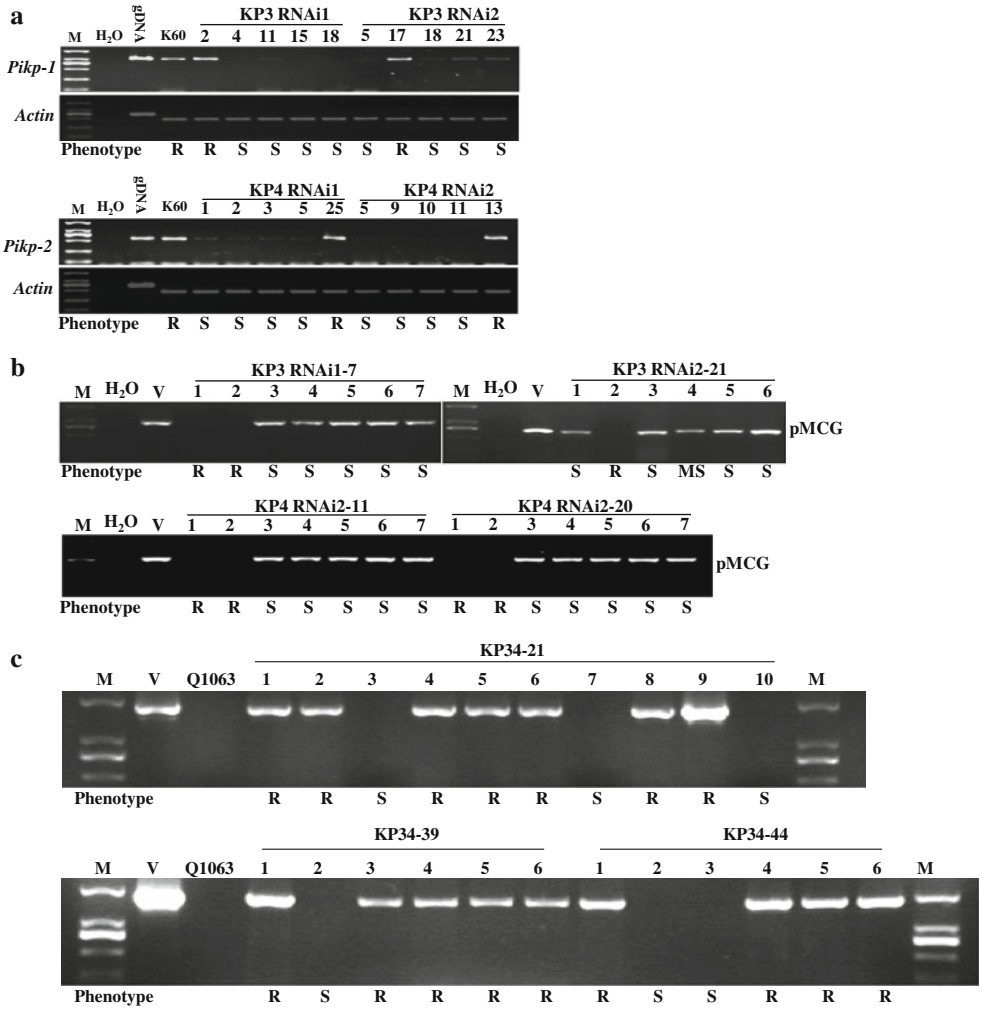


Fig. 2 Molecular characterization of transgenic plants derived from loss- and gain-of-function analyses of *Pik-p*. **a** RT–PCR-based expression analysis of *Pik-p* in the RNAi T_0 plants involving four constructs. **b** Co-segregation analysis of transgene-derived genotypes and pathogen-triggered phenotypes in four RNAi T_1 lines segregating one resistant to three susceptible using construct-specific marker. **c** Co-segregation analysis of three T_1 lines segregating three resistants to one susceptible, using the construct-specific marker, *Hpt*. Plants were inoculated with the *Pik-p*-avirulent blast isolate CHL346 or CHL381. *R* resistant, *S* susceptible, *M* size marker DL2000, *V* vector

predict the isoelectric point (pI) and molecular weight of each translation product. CC structure was predicted using either COILS (http://www.ch.embnet.org/software/COILS_form.html; Lupas et al. 1991) or Paircoil2 (http://groups.csail.mit.edu/cb/paircoil2; McDonnell et al. 2006) software.

Single nucleotide polymorphism (SNP) assay

The coding sequences of the susceptible cv. Q1063 and the *Pik-m* carrier cv. Tsuyuake, and the *Pik-p* carrier cv. K60 were aligned using Multalin (http://bioinfo.genotoul.fr/multalin/multalin.html) software. To validate the resulting putative SNP sites, primers were designed to create dCAPS markers able to distinguish *Pik-p* from *Pik*, *Pik-m*, *Pik-s* and *Pik-h*.

Characterization of the race specificity of the transgenic lines

The resistance of four T_2 lines carrying the construct KP3+4 was tested for race specificity. Five reference lines, each carrying one of *Pik*, *Pik-s*, *Pik-m*, *Pik-h* or *Pik-p* (Kobayashi et al. 2007), as well as the susceptible recipient cv. Q1063, were used as a control. Eight blast isolates were selected for the test (Table 2). Blast inoculation and disease evaluation were carried out according to Pan et al. (2003).

Gene expression

Two-week-old seedlings of cv. K60 (*Pik-p*) and cv. Tsuyuake (*Pik-m*) were inoculated with isolate CHL381 and

Table 1 Characterization of the coupled genes of *Pik-p* through loss and gain of function analyses

Candidate/ construct[a]	Recipient cultivar[b]	Expected size (bp)	Vector[c]	Reaction of T_0 plants[d]				Success ratio (%)[e]
				R	MR	MS	S	
Loss of function								
KP3 RNAi1	K60	485	pDS1301	257	4	12	138	36.5**
KP3 RNAi2	K60	750	pDS1301	341	23	45	58	22.0
KP4 RNAi	K60	745	pANDA	127	4	36	81	47.2**
KP4 RNAi1	K60	585	pDS1301	170	7	25	59	32.2
KP4 RNAi2	K60	516	pDS1301	314	28	32	29	15.1
Gain of function								
KP3	Q1063	12,432	pCAMBIA1301AscI	0	0	1	136	0
KP4	Q1063	10,459	pCAMBIA1301AscI	0	0	1	108	0
KP3+4	Q1063	16,919	pCAMBIA1301AscI	77	5	22	301	20.2

** Significant differences (at the $\alpha = 0.01$ level) in the silencing efficiency among constructs containing different fragments of the coupled genes for a χ^2

[a] Abbreviations for candidates/constructs are KP3, *Pikp-1*; KP4, *Pikp-2*; KP3+4, *Pikp-1+Pikp-2*; RNAi, RNA interference. The detailed information on the constructs are shown in Figs. 3, 4, S2

[b] Loss-of-function-constructs were transformed into the *Pik-p* carrier cultivar K60, and gain-of-function-constructs were transformed into the highly susceptible cultivar Q1063

[c] The detailed information on the primer sequences and restriction sites for constructing each vector is shown in Table S1

[d] T_0 plants derived from each construct were inoculated with the *Pik-p*-avirulent isolate, CHL381 and CHL346. *R* resistant, *MR* moderately resistant, *MS* moderately susceptible, *S* susceptible

[e] The ratios for loss- and gain-of-function analyses were calculated as (MS + S)/(R + MR + MS + S) and (R + MR)/(R + MR + MS + S), respectively

Table 2 Reactions of the five reference lines, each carrying one of the *Pik* alleles and four transgenic T_2 plants derived from the construct KP3+4, as well as the susceptible recipient cultivar Q1063 to eight isolates of *Magnaporthe oryzae*

Host	Gene	*M. oryzae* isolates							
		CHL22	CHL346	CHL42	CHL272	CHL446	CHL503	CHL508	CHL995
Reference lines									
IRBLk-Ka	*Pik*	S	R	S	S	S	R	ND	S
IRBLks-S	*Pik-s*	S	S	S	S	MS	S	R	S
IRBLkm-Ts	*Pik-m*	R	R	R	S	MR	R	R	S
IRBLkh-K3	*Pik-h*	R	R	R	S	R	R	R	S
IRBLkp-K60	*Pik-p*	R	R	S	S	S	S	S	S
Transgenic lines									
KP34-21-2	*Pikp-1+Pikp-2*	R	R	S	S	S	S	S	S
KP34-29-8	*Pikp-1+Pikp-2*	R	R	S	S	S	S	S	S
KP34-33-9	*Pikp-1+Pikp-2*	R	R	S	S	S	S	S	S
KP34-51-7	*Pikp-1+Pikp-2*	R	R	S	S	S	S	S	S
The recipient cv.									
Q1063	None	S	S	S	S	S	S	S	S

S susceptible, *R* resistant, *MS* moderately susceptible, *MR* moderately resistant, *ND* not determined

held in the dark at 25°C with 100% relative humidity for 20 h in an inoculation incubator. Inoculated leaves were sampled at the time of inoculation and then at 12, 24 and 72 h post-inoculation. Total RNA was isolated from leaves using the TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was performed in two steps: first, ~1 μg total RNA was treated with RNase-free

DNase I (Promega, Madison, WI) and reverse transcribed by M-MLV (Promega, Madison, WI). Then, a 1-μl aliquot of the reaction was used as the template for a qRT-PCR. The primer sets, RRT5 and RRT17 (Ashikawa et al. 2008; see Table S1), were employed to detect *Pikp-1* and *Pikp-2* expression. Rice *Actin1* and the pathogenesis-related probenazole-inducible *PBZ1* gene were used as internal controls (Ryu et al. 2006; Table S1). The qRT-PCR analysis was performed on a Bio-RAD CFX96 Real-Time PCR Detection System device, using SYBR Premix EX TaqTM (TaKaRa, Dalian, China).

Results

Identification of candidates for *Pik-p*

The location of *Pik-p* has been placed within the genetic interval defined by the markers K39 and K28 (Fig. 1a). In the cv. Nipponbare genome sequence, this interval is covered by the four BAC clones, OSJNBb0049B20, OSJNBa0047M04, OSJNBa0036K13 and OSJNBb0018L01 (Fig. 1b), and contains 25 predicted genes. Of these, four (*KP1*, *KP2*, *KP3* and *KP4*) are of the NBS-LRR type, and so were taken as being the most likely candidates for *Pik-p* (Fig. 1c). No ESTs corresponding to *KP1* and *KP2* could be identified, but the EST CA763104 matched the 3′ region of *KP3*, while the full-length cDNA AK073759 matched *KP4*. Thus, it appeared likely that both *KP3* and *KP4*—but neither *KP1* nor *KP2*—were expressed in cv. Nipponbare. Both *KP1* and *KP2* are absent in the *Pik-m* carrier cv. Tsuyuake (Ashikawa et al. 2008). Amplification of cv. K60 genomic DNA using PCR primer pairs targeted at *KP1* and *KP2* (sequences based on the cv. Nipponbare sequence, see Fig. 1 and Table S1) showed that neither is present in cv. K60 either, leaving *KP3* and *KP4* as the two strongest candidates for *Pik-p* (Figs. 1c, d, S1). A number of PCR primer pairs were then designed to amplify both *KP3* and *KP4* from cv. K60, but the only successful amplification was a 2.6-kb *KP4* fragment. Extension of this sequence to ~5.2 kb was achieved using hi-TAIL PCR (Liu and Chen 2007), and this sequence proved to be 96% homologous to *Pikm2-TS*, but only 48% to *Pikm6-NP* (both are alleles of *Pikp-2*; see Figs. 1, S6). Using the sequence of cv. Tsuyuake as the basis for primer design (rather than cv. Nipponbare) led to the successful amplification of both the *KP3* and *KP4* sequences from cv. K60 (see Table S1 and below).

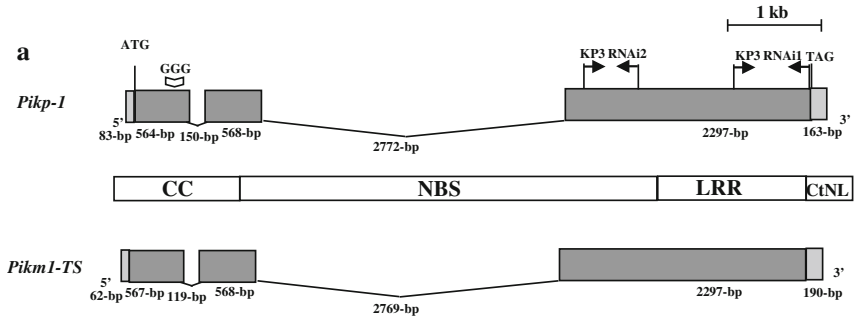
Loss- and gain-of-function analyses for the candidate genes

To determine whether *KP3* or *KP4* (or neither) was *Pik-p*, RNAi was applied to both candidates. All five RNAi constructs were properly expressed and abolished *KP3* and *KP4* expression in T_0 plants (Table 1; Figs. 2a, S3a, S3b). The presence of the transgene and the response to pathogen infection were fully correlated among the T_1 progeny (Fig. 2b). The most effective RNAi fragment was derived from the 3′ region of the gene, within its LRR domain (Table 1; Figs. 3a, 4a). Thus, both *KP3* and *KP4* appear to be required for *Pik-p* function.

The three constructs *KP3*, *KP4* and *KP3+4* (Fig. S2) were then transformed into the highly susceptible *japonica* cv. Q1063 (Lin et al. 2007) to confirm *Pik-p* function by forward complementation. A total of, respectively, 137, 109 and 406 independent T_0 plants were generated. When challenged with blast isolates, CHL381 and CHL346 (avirulent on cv. K60), all the single gene transformants remained fully susceptible, but 83 of the 406 double gene transformants were resistant. This confirmed that the presence of both genes is needed to specify *Pik-p* resistance (Table 1; Fig. S4). To test the correlation between presence/absence of both genes and resistance/susceptibility, 53 of the 406 T_0 plants were scored for the presence/absence of the right and left border markers (see Table S2; Fig. S2). Of these, 39 resistant and 11 susceptible plants carried both border markers, and two susceptible ones carried one or other of the markers; only one susceptible plant lacked both markers, indicating that the majority of the T_0 plants carrying the complete construct (*KP3+4*) did not express any resistance in the recipient cultivar background when driven by their native promoters. On the other hand, the presence of the transgene and the response to pathogen infection were also fully correlated among the T_1 progeny (Fig. 2c).

Molecular characterization of *Pik-p*

Full-length *Pikp-1* (KP3) and *Pikp-2* (KP4) cDNAs were obtained by a combination of RT- and RACE-PCR (Fig. S2), and compared to their coding sequences. *Pikp-1* contains an 83-bp 5′ and a 163-bp 3′ untranslated region (UTR) and two introns (150 and 2,772 bp) within its open reading frame (ORF) (Fig. 3a). It encodes a 1,142-residue polypeptide with an estimated molecular weight of 126.7 kDa and a pI of 6.1. Four characteristic NBS family motifs are present: GLPGGGKTTVAR (beginning at residue 290), KKYLIVIDDIW (beginning at residue 376), DLGGRIIMTTRLNSI (beginning at residue 402) and EDNPCY DIVNMCYGMPLALIW (beginning at residue 461), which correspond, respectively, to kinase 1a (P-loop), kinase 2, kinase 3a and motif3 (GLPL) (Fig. 3b). COILS and Paircoil2 analyses suggested the presence of a CC domain ($P = 0.9$) between residues 146 and 177. The C-terminal region includes 16 imperfect LRR repeats, composed of ~14% leucine, while the remaining 103 residues represent the C-terminal non-LRR (CtNL) region.



b

1
MEAAAMAVTAATGALAPVLVKLAALLDDGECNLLEGSRSDAEFIRSELEAVHSLLTPNILGRMGDDDAACKDGLI
AEVRELSYDLDDAVDDFLELNFEQRRSASPFGELKARVEEHVSNRFSDWKLPAASLPPSSVHRRAGLPPP**DAELVG**
MDKRMEELTKLLEQGSNDASRWRKRKPHFPLRKTGLKQKIVIKVAMEGNNCRSKAMALVASTGGVDSVALVV
AMEGNNCRSKAMALVAST GGVDSVALVGDLRDKIEVVGYGIDPIKLISALRKKVGDAELLQVSQANKDVK
266 Kinase 1a
ETTPMLAPVKSICEFHKVKTVCIL**GLPGGGKTTVAR**ELYDALGTHFPCRVFVSVSPSSSPSPNLTKTLADIFAQAQL
Kinase 2 Kinase 3a
GVTDTLSTPYGGSGTGRALQQHLIDNISAFLLN**KKYLIVIDDIW**HWEEWEVIRKSIPKN**DLGGRIIMTTRLNSI**AEK
Motif 3 (GLPL)
CHTDDNDVFVYEVGDLDNNDALSLSWGIATKSGAGNRIGTG**EDNPCYDIVNMCYGMPLALIW**LSSALVGEIEELG
GAEVKKCRDLRHIEDGILDIPSLQPLAESLCLGYNHLPLYLRTLLLYCSAYHWSNRIERGRLVRRWIAEGFVSEEKEA
EGYFGELINRGWITQHGD NNSYNYYEIHPVMLAFLRCKSKEYNFLTCLGLGSDTSTSASS
634
PRLIRRLSLQGGYPVDCLSSMSMDVSHTCS
LVVLGDVARPKGIPFYMFKR
LR**V**L**DL**EDNK**D**I**QD**S**H**LQGICEQ**LS**
L-R**V**R**Y**L**GL**K**GT**R**IRK**LP**QEMRK
LK**H**L**E**IL**Y**V**GS**T**R**I**S**E**L**P**QEIGE
LK**H**L**R**IL**D**V**R**N**T**D**I**T**E**L**P**LQIRE
LQ**H**L**H**T**L**D**V**R**N**T**P**I**S**E**L**P**P**QVGK
LQN**L**K**I**M**C**V**R**S**T**G**V**R**E**L**P**KEIGE
LN**H**L**Q**T**L**D**V**R**N**T**R**V**R**E**L**P**WQAGQI
SQS**L**R**V**L**A**GDSGDGV**R**L**P**EG**V**CEAL
INGIPGATRAKCREVLSIAIIDRFGPPL
VGIFKVPGSHMRIPKMIKDHFRV
LS**C**L**D**IRLCHKLEDDDQKF
LAE**M**PNLQTLVLRFEA**LP**RQPITINGTGFQMLESFR
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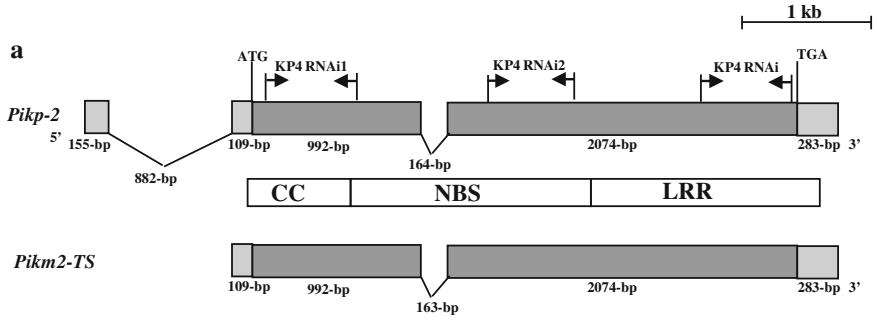
TIDVVKKEAEEHPNRPITLLINAGYKEISTESHGSSENIAGSSGIDTEPAQAQHDNLPAVRDDYKGKGILLDGRCPTCG
RATKIEEETQDRVADIEIQTETTS

NBS
LRR
CtNL

Fig. 3 The structure of *Pikp-1* and its translation product. **a** Structures deduced from full-length cDNA sequences of *Pikp-1* (present study) and *Pikm1-TS* (Ashikawa et al. 2008). Exons are *shaded*, introns shown as a *line*, UTRs as *lightly shaded boxes*. The ATG start codon, TAG stop codon, the indel between *Pikm1-TS* (*down-arrow*) and *Pikp-1*, and the KP3 RNAi1 and KP3 RNAi2 fragments are all indicated. **b** The deduced *Pikp-1* protein sequence and its structure. The CC motif, NBS motifs (kinases 1a, 2 and 3a, motif 3 (GLPL) and xLDL LRR) are shown *underlined* and in *bold*. The allele-specific SNP is *double underlined*

Pikp-2 contains a 264-bp 5′-UTR and a 283-bp 3′-UTR, with two introns; the first is of length 882 bp and lies within the 5′UTR, ending 109 bp upstream of the ATG start codon, while the second is 164-bp long, and interrupts the ORF (Fig. 4a). Its predicted gene product is a 1,021-residue polypeptide of molecular weight 114.6 kDa and a pI of 8.6. Its NBS domain contains six conserved motifs: kinase 1a (VLSIVGFGGVGKTTIA: beginning at residue 206), kinase 2 (LEQLLAEKSYILLIDDIW, beginning at residue 323), kinase 3a (GGRIIVTTRFQAV, beginning at residue 358), GLPL (EQVPEEIWKICGGLPLAIV, beginning at residue 415), RNBS-D (CLLYLSIFPKGWK, beginning at residue 488) and MHDV (KTFQVHDMVLEYI, beginning at residue 553) (Fig. 4b). Paircoil2, but not COILS, predicted the presence of a CC domain between residues 27 and 57. The C-terminal region of the protein consists of 13 imperfect LRR repeats composed of ~17% leucine.

The levels of peptide sequence identity between *Pikp-1* and *Pikm1-TS* and *Pikm5-NP* were 95 and 59%, respectively (Fig. S5), while those between *Pikp-2* and *Pikm2-Ts* and *Pikm6-NP* were 99 and 76%, respectively (Fig. S6). The peptide sequences of *Pikp-1* and *Pikp-2* could not be aligned, as their level of similarity was lower than 23%. A comparison with the equivalent cDNA sequences amplified from the susceptible cv. Q1063 and the *Pik-m* carrier cv. Tsuyuake revealed four potential SNPs in *Pikp-1* (A222V, V230E, P251D and K261N) and three in *Pikp-2* (E230D, S434T and V627M). To determine which of these were



b

1
MELVVGASEATMKSLLGKLGNLLAQE**YALISGIRGDIQYINDELASMQAFLRDLSNV**PEGHSHGHRMKDW
MKQIRDIAYDVEDCIDDFAHRLPQDSISDAKWSFLLTKIYELWTWWPRRVIASNIAQLKVRAQQIADRRSRYGV **CC**
NNPEHLDSSSSARTRAVNYEIAEYQVTSPQIIGIKEPVG
184 Kinase 1a
MKTVMEELEVWLTNPQAENGQA**VLSIVGFGGVGKTTIA**TALYRKVSDKFQCRASVAVSQNYDQGKVLNSIL
SQVSNQEQGSSTTISEKKNLTSGAKSMLKTALSLLRGNCICQPENDGNPDNTPIRLQETTDDDQNPRK**LEQLLA**
Kinase 2 Kinase 3a
EKSYILLIDDIWSAETWESIRSILPKNNK**GGRIIVTTRFQAV**GSTCSPLETDRLHTVDFLTDDESQNLFNTSICES
Motif 3 (GLPL) **NBS**
KIRKDSNKVD**EQVPEEIWKICGGLPLAIVT**MAGLVACNPRKACCDWSKLCKSLFPEQETPLTLDGVTRILDC
RNBS-D
CYNDLPADLKT**CLLYLSIFPKGWK**ISRKRLSRRWIAEGFANEKQGLTQERVAEAYFNQLTRRNLVRPMEHGS
MHDV
NGKV**KTFQVHDMVLEYI**MSKSIEENFITVVGGHWQMTAPSNKVRRLSMQSSGSNRGSSTKG
610
LN**L**AQ**V**R**S**L**T**VFGNLNH**M**PFHSFNYG
IIQ**V**L**DL**LEDWKGLKERHMTE
ICQM**L**L**L**KYLSIRRTEISKIPSKIQK
LEY**L**E**T**L**D**IRE**T**Y**V**RD**L**P**K**S**I**VQ
LKR**I**I**S**ILGGNKNTRKG**LR**LPQEKSKKPIKNPSPQGKTKEPAKKGFLSQEKGKGAMKA
LR**V**L**S**G**I**E**I**VEESSEVAAG **LRR**
LHQ**L**TG**L**RK**L**A**I**YKLNITKGGDTFKQLQSS
IEY**L**GSCGLQTLAINDENSEFINSLGDMPAPPRY
LVA**L**E-**L**SGK**L**EK--**LP**KWITS
ITT**L**NKLTISVTVLRTETLEI
LHI**L**PS**L**FS**L**T**F**AFSLSAAKQDQDIIKDILENNKLDSDGEIVIPAEGFKSLKLLRFFAPL
VPK**L**SF**L**DKNAMPALEIIEMRFKDFEG
LFG**I**E**I**L**E**N**L**REVHLKVSDGAEAITKFLVNDLKVNTEKPKVFVDGIVTA

Fig. 4 The structure of *Pikp-2* and its translation product. **a** Structures deduced from full-length cDNA sequences of *Pikp-2* (present study) and *Pikm2-TS* (Ashikawa et al. 2008). Exons are *shaded*, introns shown as a *line* and UTRs as *lightly shaded boxes*. The ATG start codon, TAG stop codon and the KP4 RNAi, KP4 RNAi1 and KP4 RNAi2 fragments are all indicated. **b** The deduced *Pikp-2* protein sequence and its structure. The CC motif, NBS motifs (kinases 1a, 2 and 3a, motif 3 (GLPL), RNBS-D and MHDV domains and the xLDL LRR) are *underlined* and in *boldface*. The allele-specific SNP is *double underlined*

genuinely allele specific, a set of reference lines, which are known carriers of *Pik*, *Pik-p*, *Pik-m*, *Pik-s* and *Pik-h*, as well as of *Pia*, *Pii*, *Piz-t* and *Pita*, were genotyped. The outcome was that *Pik-p* could be distinguished from all the above *R* genes using a combination of the two SNPs assays, T1-783A/G at *Pikp-1* and A2-1879G at *Pikp-2* (see Figs. 3, 4, 5, S5, S6).

Race specificity of resistance in the transgenic lines

The five reference lines for the *Pik* alleles, *Pik*, *Pik-s*, *Pik-m*, *Pik-h* and *Pik-p*, reacted differentially to infection by the eight blast isolates (Table 2). This showed that these isolates were able to successfully distinguish between the various *Pik* alleles. Since the disease reaction of the four T_2 lines carrying both *Pikp-1* and *Pikp-2* was identical to that of IRBLkp-K60 (*Pik-p*), the presence of *Pikp-1* and *Pikp-2* clearly imparts the same phenotype as that of *Pik-p*.

Pikp-1 and *Pikp-2* expression in cv. K60

The transcription level of both genes was assessed in cv. K60 at four time points after blast inoculation. Both *Pikp-1* and *Pikp-2* transcripts were detected prior to exposure to the pathogen (Fig. 6). The effects of mock- and pathogen inoculation were to reduce the transcription level over the first 24 h, implying a response to stresses resulting from both inoculation and incubation in a humidity chamber (Fig. 6). Expression of *Pikp-1* and *Pikp-2* appeared to increase only marginally during the 72 h following either

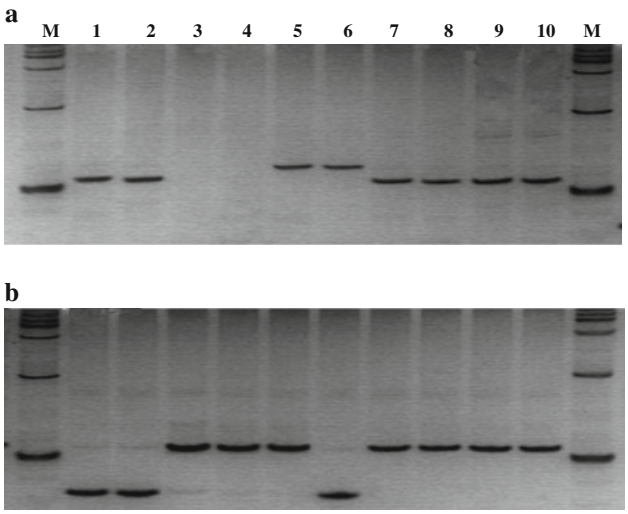


Fig. 5 *Pik-p*-specific SNP assay. SNP assay for **a** T1-783A/G and **b** T2-1879G. *Lane 1* cv. K60 (*Pik-p*), *lane 2* IRBLkp-K60 (*Pik-p* reference line), *lane 3* IRBLkm-Ts (*Pik-m* reference line), *lane 4* IRBLk-Ka (*Pik* reference line), *lane 5* IRBLks-S (*Pik-s* reference line), *lane 6* IRBLkh-K3 (*Pik-h* reference line), *lane 7* IRBLa-A (*Pia* reference line), *lane 8* IRBLi-F5 (*Pii* reference line), *lane 9* IRBLzt-T (*Piz-t* reference line) and *lane 10* IRBLta-K1 (*Pita* reference line). *M* Size marker DL2000

mock or genuine inoculation. The expression patterns of both *Pikm1-TS* and *Pikm2-TS* (the alleles present in cv. Tsuyuake) were similar to those in cv. K60, but in contrast that of the pathogen-inducible gene, *PBZ1* (Ryu et al. 2006), in cv. Tsuyuake differed in several respects (Fig. 6).

Discussion

Twenty-one *R* genes have been mapped to date onto rice chromosome 11, including the six rice blast resistance genes *Pik*, *Pik-s*, *Pik-m*, *Pik-p*, *Pik-h* and *Pik-g*, all of which have been shown by classical genetic analysis to be allelic to one another (Ballini et al. 2008; Yang et al. 2009). With regard to the race specificities and/or resistance spectra of those alleles, Kiyosawa (1987) found that there are "stair-type" resistances among the alleles in the Japanese blast pathogen population, and ranked the strength of these alleles in the order *Pik-m* > *Pik* > *Pik-p* > *Pik-s*. This, in turn, indicated that *Pik*, *Pik-p* and *Pik-s* all form part of the larger or stronger allele, *Pik-m* (Kiyosawa 1987). Among Chinese isolates, the same ranking was found in Fujian, Yunnan, Jiangsu and Heilongjiang, but not in Guandong,

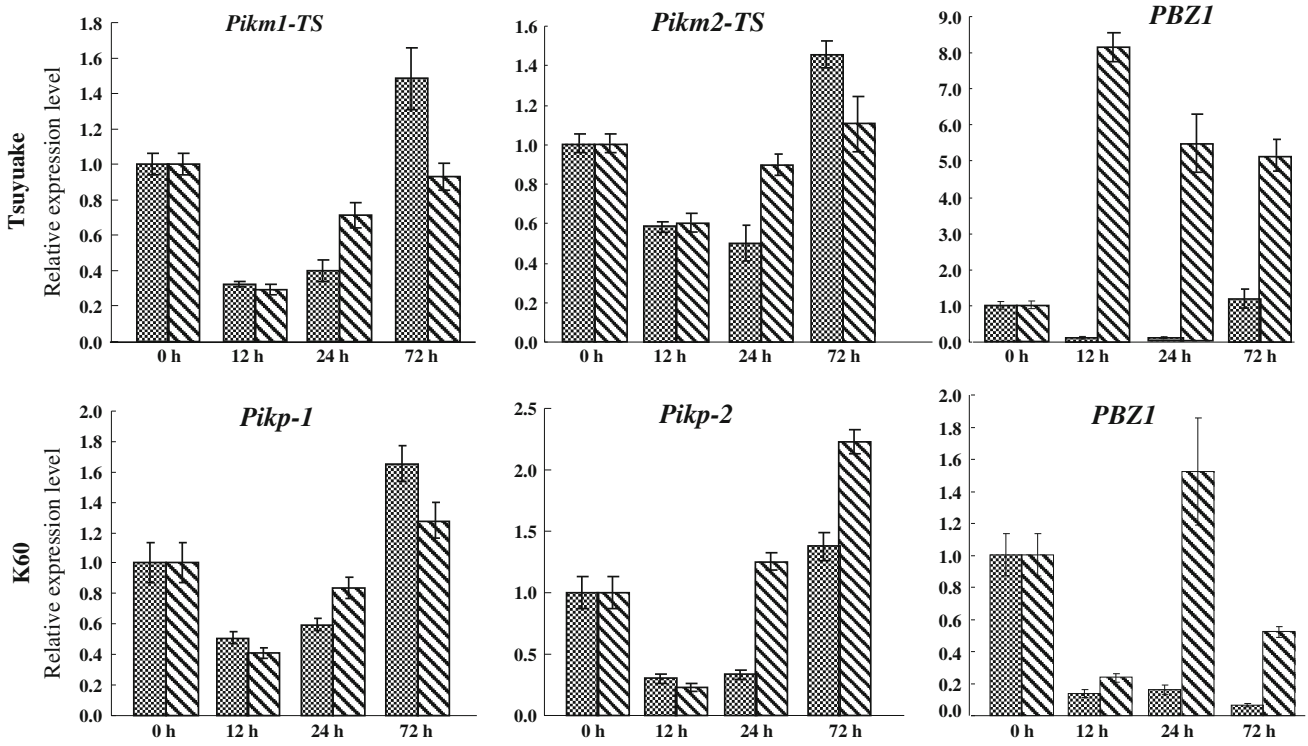


Fig. 6 Expression analysis of *Pik* alleles. Transcription of *Pik-m* in cv. Tsuyuake and *Pik-p* in cv. K60 as compared by qRT-PCR. Leaf RNA was sampled before inoculation and then at 12, 24 and 72 h after inoculation with either blast isolate CHL381 or water. The specific RNA content of each sample was estimated from the mean of three replicate qRT-PCRs. Outcomes of mock inoculations are shown as *black bars* and those of pathogen inoculations as *hatched* ones

Hunan, Guizhou, Sichuan, Jiangsu, Liaoning or Jiling. In the latter regions, many isolates that were virulent against *Pik-m* were avirulent against *Pik* and *Pik-p*, which has been taken to indicate that the *Pik* alleles *Pik-m*, *Pik* and *Pik-p* (and perhaps other *Pik* alleles as well) are, indeed, independent *R* genes able to condition differential reactions against various isolates (Wang et al. 2009). The isolation of *Pik-m* and *Pik-p* has revealed that they are allelic and independent. They can be distinguished from one another on the basis of both their gene structure and their sequence. Thus, *Pikp-1* and *Pikm1-TS* differ from one another with respect to the length of their introns and a three base pair indel (Fig. 3), while *Pikp-2* carries an intron in its 5′ UTR, which is not present in *Pikm2-TS* (Fig. 4). Furthermore, *Pik-p* could be distinguished from other alleles, as well as from *R* genes at other loci with a combination of two SNP assays (Fig. 5).

Pikp-1 and *Pikp-2* are closely linked to one another, but their sequence and structure are quite distinct. The presence of a CC domain in the *Pikp-1* product was identified by both COILS and Paircoil2 software, but this was less certain for the *Pikp-2* protein. Secondly, the *Pikp-2* NBS domain carries the motifs RNBS-D and MHDV, which are absent in *Pikp-1*. Thirdly, the *Pikp-1* protein has a CtNL region, which *Pikp-2* lacks. Fourthly, although in common with most rice NBS-LRR genes, both *Pikp-1* and *Pikp-2* contain an intron in their NBS kinase 2 motif (see Figs. 3, 4; Bai et al. 2002), and a gene-specific intron is present in the *Pikp-1* CC domain (Fig. 3) and in the *Pikp-2* 5′UTR (Fig. 4). Thus these two genes, although physically closely linked with one another, may well be functionally different and certainly are evolutionarily distant from one another (Ashikawa et al. 2008; Bai et al. 2002; Sinapidou et al. 2004). The *Pikp-1* alleles appear to be more polymorphic than the *Pikp-2* ones (Figs. S5, S6), suggesting that *Pikp-1* may be under greater pathogen selection pressure than *Pikp-2*. As the CC and NBS regions of *Pik-p* and *Pik-m* are so divergent (unlike their LRR region) (Figs. S5, S6), the indication is that race specificity is probably determined at the CC and/or NBS, rather than at the LRR, a situation which is reproduced at the wheat *Lr10* gene (Loutre et al. 2009).

The reverse genetics approach RNAi has been widely used to test for loss of function (Chu et al. 2006; Miki et al. 2005; Peart et al. 2005; Peng et al. 2009). To work successfully, it is necessary for the RNAi sequence to both effectively and specifically inhibit the expression of the target gene. A major effect on silencing efficiency has been shown by a range of sequences directed at various sites along a single mRNA, but the 3′ mRNA cleavage acts as the most effective siRNAs (Holen et al. 2002). Here, we tested two *Pikp-1* and three *Pikp-2* fragments for their efficacy as RNAi sequences (Figs. 3, 4). The most effective fragment in each gene was also identified at the 3’ ORF region, corresponding to the LRR domain (Table 1; Figs. 3, 4). These results are of general interest in furthering the understanding of the function and specificity of the NBS-LRR *R* genes.

Already, five *R* genes have turned out to be in fact a coupled pair. An effective allele of the *A. thaliana RPP2* gene requires the presence of both *RPP2A* and *RPP2B*, separated from one another by ~5 kb and arrayed in the same orientation (Sinapidou et al. 2004). *RPP2A* is unusual in that it has only a short LRR domain at its C-terminus, preceded by two incomplete TIR-NBS domains, whereas *RPP2B* has all the components expected for a full TIR-NBS-LRR class *R* gene. In both tetraploid and hexaploid wheat, the presence of the two adjacent, but distinct, CC-NBS-LRR genes *Lr10* and *RGA2* is required to confer leaf rust resistance (Loutre et al. 2009). Whereas *Lr10* is a normal CC-NBS-LRR gene showing a measurable degree of sequence diversity in its CC domains, *RGA2* is similar with *RPP2A* in its possession of two NBS domains and relative paucity of sequence diversity In rice, *Pi5-1* and *Pi5-2* are both necessary to confer race-specific blast resistance in the line RIL260 (Lee et al. 2009). They are arrayed in opposite orientations, separated from one another by some 15 kb of sequence, and their sequences are highly divergent from one another. The last two examples relate to *Pikm1-TS*/*Pikm2-TS* and *Pikp-1*/*Pikp-2*, where, in both cases, the pair is arrayed in opposite orientation and separated by only ~1 kb. This small separation suggests that both genes may be under the control of a common, bidirectional promoter, such as the one driving *A. thaliana cab1* and *cab2* (Mitra et al. 2009). The evidence, such as it is from a small number of examples, is that coupled *R* genes consist of sequences that are divergent from one another, although both may well belong to the NBS-LRR class. It appears unlikely that these gene pairs can have evolved from one another following an initial duplication event (Sinapidou et al. 2004).

The allelism of *Pik-p* with *Pik-m* raises a number of questions regarding the *Pik* locus. Are *Pik*, *Pik-h* also allelic to *Pik-p*/*Pik-m*? Do they, like *Pik-p*/*Pik-m*, consist of a pair of coupled genes? How do these coupled genes evolve, particularly with respect to generating their allele-specific resistance? And how do they interact with one another, and then with their equivalent pathogen *Avr* genes?

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