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ORIGINAL PAPER

The isolation of *Pi1*, an allele at the *Pik* locus which confers broad spectrum resistance to rice blast

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Abstract We report the isolation of *Pi1*, a gene conferring broad-spectrum resistance to rice blast (*Magnaporthe oryzae*). Using loss- and gain-of-function approaches, we demonstrate that *Pi1* is an allele at the *Pik* locus. Like other alleles at this locus, *Pi1* consists of two genes. A functional nucleotide polymorphism (FNP) was identified that allows differentiation of *Pi1* from other *Pik* alleles and other non-*Pik* genes. A extensive germplasm survey using this FNP reveals that *Pi1* is a rare allele in germplasm collections and one that has conferred durable resistance to a broad spectrum of pathogen isolates.

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Introduction

The deployment of host resistance genes (*R* genes) is a cost-effective and environmentally sustainable means of controlling plant diseases (Khush and Jena 2009; Liu et al. 2010). Therefore, many crop breeding programs have focused on incorporating such alleles into elite cultivars (cvs). However, the majority of *R* genes do not provide long-term resistance, since most pathogens have sufficient genetic flexibility to eventually overcome them (Krattinger et al. 2009; Lee et al. 2009; Skamnioti and Gurr 2009; Liu et al. 2010). To ensure its longevity, the ideal *R* gene must combine a broad spectrum of resistance with durability. Of the several broad-spectrum *R* genes which have been characterized in some detail to date, perhaps the most renowned are the barley gene *mlo* (Büschges et al. 1997), and the rice genes *Xa21* (Wang et al. 1996) and *Pi9* (Qu et al. 2006). However, the underlying mechanisms of broad-spectrum resistance are still not well understood.

To keep pace with the diversifying pathogens, multiple alleles at a particular *R* locus are prevalent when they provide a principal fitness advantage by conferring effective disease resistance to the corresponding pathogens (Holub 2001). The basis of the race specificity expressed by *R* gene alleles can be conveniently studied by a comparative sequence analysis. A good example is the flax *L* locus, where Ellis et al. (1999) showed that the 13 known alleles shared >90 % sequence identity and that differences in the LRR domain and N-terminal region were associated with race specificity. A similar analysis of the wheat *Pm3* gene suggested that specificity was associated with a single residue in the C-terminal LRR motif (Brunner et al. 2010). Three of the five alleles at the *Pik* locus of rice (*Pik*, *Pik-p* and *Pik-m*) have been isolated and characterized (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011),

and their functionality is determined by a pair of closely linked CC-NBS-LRR genes, which contain functional nucleotide polymorphisms (FNPs) in either their N-terminal regions and/or their LRR domains (Zhai et al. 2011).

The conventional identification of alleles based on phenotype is limited by the number of pathogen isolates available. However, for *R* genes which have been isolated, it is now possible to replace phenotypes with DNA polymorphisms as the basis for defining alleles. The identification of FNPs, which include single nucleotide polymorphisms (SNPs) and insertion/deletions (InDels), which are responsible for allele specificity is particularly useful in the context of molecular breeding programs (Hausner et al. 1999; Tommasini et al. 2006; Shomura et al. 2008; Asano et al. 2011; Yuan et al. 2011; Zhai et al. 2011).

In rice, alleles at the *Pik* locus confer resistance to rice blast (incited by *Magnaporthe oryzae*). The *Pi1 R* gene, derived from the durably resistant West African cv. LAC23 (Mackill and Bonman 1992; Inukai et al. 1994), has displayed a high level of durable resistance against blast (Hittalmani et al. 2000; Fuentes et al. 2008; Yang et al. 2008; Tacconi et al. 2010) and was hypothesized to be an allele at the *Pik* locus. The objectives of the present study were to (i) isolate *Pi1* by the *in silico* map-based cloning technique (Yuan et al. 2011; Zhai et al. 2011), (ii) characterize its effectiveness against a range of isolates and (iii) determine its frequency in a range of germplasm accessions. *Pi1* proved to be a novel *Pik* allele and was distinguishable from other *Pik* alleles and *R* genes at other loci, conferring blast resistance by an FNP we identified.

Materials and methods

Blast resistance characterization

Standard carriers of *Pi1* (cv. C101LAC), *Pik* (cv. Kusabue), *Pik-p* (cv. K60), *Pik-m* (cv. Tusyuake) and *Pi21* (cv. C101A5) and a universal susceptible cv. (CO39) were challenged with 1,292 blast isolates collected over 19 cropping seasons (between 1998 and 2008) in Guangdong Province (GD), and 480 isolates originating from Fujian (FJ, 40 isolates), Hunan (HN, 40 isolates), Yunnan (YN, 43 isolates), Guizhou (GZ, 60 isolates), Sichuan (SC, 66 isolates), Liaoning (LN, 108 isolates), Jilin (JL, 60 isolates) and Heilongjiang (HLJ, 63 isolates) provinces, China. The methods for inoculation and resistance evaluation followed Pan et al. (2003).

Genetic mapping of *Pi1* and candidate gene identification

A set of F_2 progeny generated from the cross cv. C101LAC (*Pi1*) × cv. K1 was screened for reaction to inoculation with blast isolate EHL0490. As *Pi1* was suspected to be an allele of *Pik* (Inukai et al. 1994), DNA markers known to map within the *Pik-m*/*Pik-p* region (Li et al. 2007; Wang et al. 2009) were used to genotype the F_2 progeny. The gene annotation tools RiceGAAS (http://ricegaas.dna.affrc.go.jp) and FGENSH (http://www.softberry.com) were then used to identify *Pi1* candidates within the 277-kb segment of cv. Nipponbare defined by the closest flanking markers (CRG11-7 and K28). The DNA sequence surrounding *Pik* has been described as the functional "K type" (cvs. Tsuyuake, K60, and Kusabue) or the nonfunctional "N type" (cv. Nipponbare) (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011). Therefore, the four candidates for *Pi1* within the critical insertion/deletion region were validated by a presence/absence analysis with "K" and "N" haplotype specific primer sets designed based on the BAC clone Ts18H12 of cv. Tsuyuake and the reference sequence of cv. Nipponbare, respectively. The association between SNPs within each candidate for *Pi1*- and *Pi1*-mediated resistance was examined by SNP genotyping a set of *Pi1* carrier and non-carrier cvs.

Loss- and gain-of-function tests

Conventional F_1 hybrids were made between the *Pik* allelic RNAi stock kh4i-16 (Yuan et al. 2011) and cv. C101LAC (*Pi1*), and between kh4i-11 (an alternative RNAi *Pik* knock-out line) and the *Pia* carrier cv. Aichi Asahi (*Pia* is not an allele of *Pik*), which were inoculated with blast isolate CHL381 (avirulent on both cvs. C101LAC and Aichi Asahi). A correlation between susceptibility and the presence of the RNAi construct was established using a PCR assay based on primers targeting the transgene. RT-PCR was used to verify that susceptibility was correlated with a lack of *Pi1* expression. The RT-PCR reference gene was a fragment of the rice *Actin* gene. To test for gain of function, two of the candidates for *Pi1* (*Pi1-5C* and *Pi1-6C*) were combined to form the sequence *Pi1-56C*. Based on the strategy used for *Pik-p* (Yuan et al. 2011), the *Pi1-56C* sequence was then divided into four fragments, each of which was independently amplified using Phusion™ High-Fidelity DNA Polymerase (NEB, Beijing, China) and introduced into either pMD20-T (TaKaRa, Dalian, China) or pCAMBIA 1300Asc I. The four fragments were fused in the appropriate orientation and order in pCAMBIA1300Asc I (details given in Fig. S2), which was then transformed into the blast susceptible cvs. Q1063, Longjing24 (LJ) and Nipponbare, by *Agrobacterium*-mediated transformation (Yuan et al. 2011; Zhai et al. 2011). As a control, two less-promising candidates for *Pi1* (*Pi1-3C* and *Pi1-4C*) were similarly coupled to form *Pi1-34*, and this construct was also transformed into the susceptible cv.

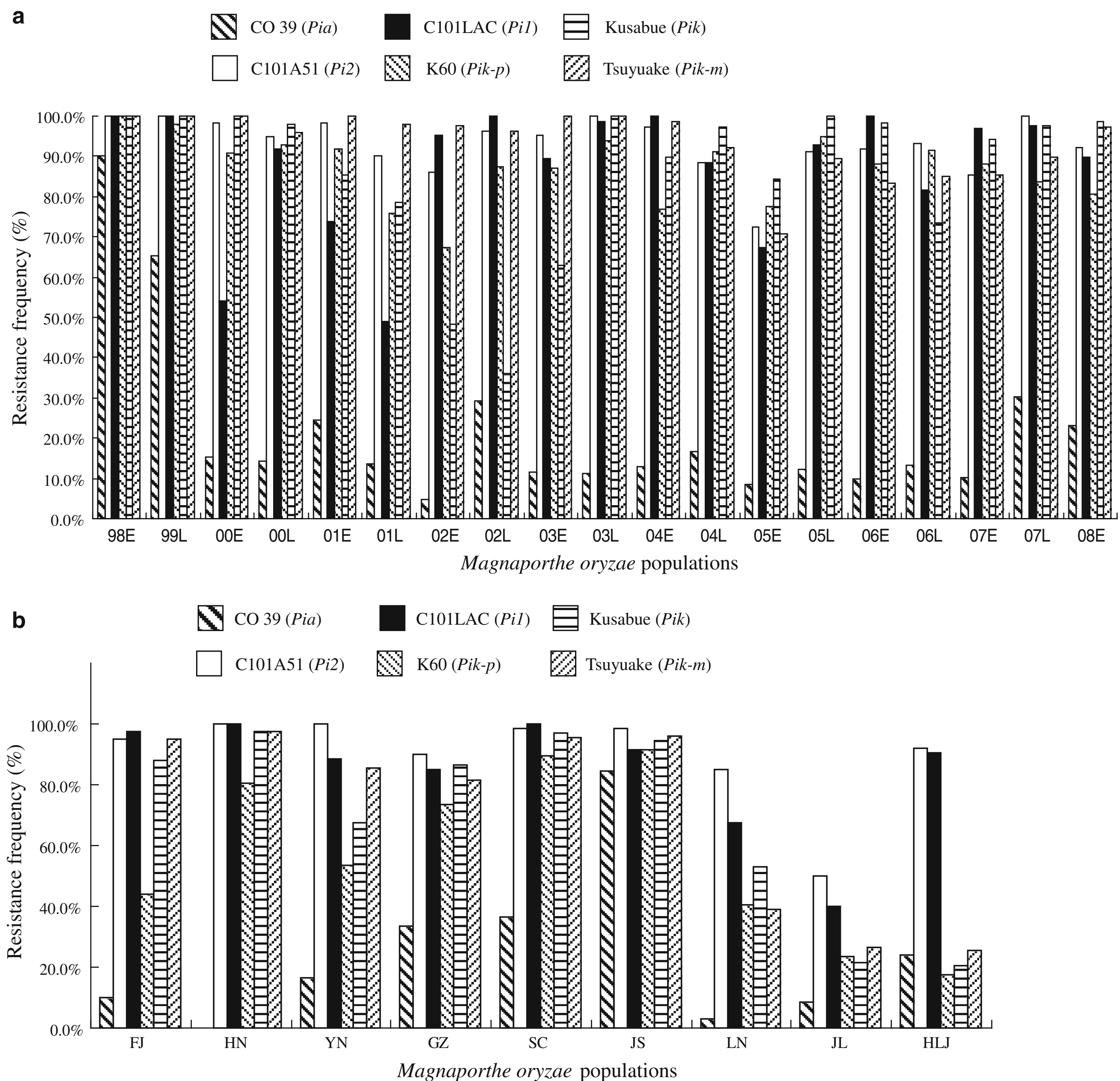

Fig. 1 Frequency of resistance among sets of blast isolates shown by three near isogenic lines (cvs. CO39, C101A51 and C101LAC) and three cvs carrying different *Pik* alleles. **a** A set of pathogen populations collected over 19 growing seasons in Guangdong Province. Codes 98E and 99L indicate populations collected, respectively, in the early season (March–July) of 1998 and the late season (July–November) of 1999, etc. **b** Populations collected from Fujian (FJ), Hunan (HN), Yunan (YN), Guizhou (GZ), Sichuan (SC), Jiangsu (JS), Liaoning (LN), Jilin (JL), Heilongjiang (HLJ) provinces in China

Q1063 in the same way. The resulting T_1 plants were challenged with the *Pi1* avirulent blast isolates CHL346 (for cvs. Q1063 and Nipponbare) or CHL357 (cv. LJ). T_2 progeny segregating in a Mendelian fashion corresponding to the isolates were used to establish the relationship between resistance and the presence of the transgene.

Sequence analysis

The entire coding sequences of *Pi1-5C* and *Pi1-6C* were amplified using the primer sets KP3ORF F/R and KP4ORF F/R, respectively (Table S1). Both 5′ and 3′-rapid amplification of cDNA ends was obtained using a GeneRacer^{TM}-kit

(Invitrogen, http://www.invitrogen.com), following the same strategy used for *Pik-p* (Fig. S2; Yuan et al. 2011). The intron–exon structure of both genes was deduced by comparing the resulting cDNA sequences with their respective gDNA sequence. Further sequence comparisons were performed by pairwise BLAST (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html), and the similarity between predicted peptide sequences by BLASTP. The prediction of the CC domain was based on COILS (http://www.ch.embnet.org/software/COILS_form.html) and Paircoil2 (http://groups.csail.mit.edu/cb/paircoil2/) analysis. The peptide sequences of the various *Pik* allele products were aligned using Multalin software (http://bioinfo.genotoul.fr/multalin/multalin.html).

SNP genotyping

A germplasm array consisting of 636 rice accessions (198 wild rice accessions, 180 *indica* landraces, 91 *japonica* landraces, 86 *indica* cultivars and 81 *japonica* cultivars) was genotyped for N and K haplotypes (Table S2), following the method described by Zhai et al. (2011). Accessions having the K type of genome were subjected to genotyping using the *Pi1*-specific FNP marker through dCAPS analysis. The haplotypes of the four *Pik* alleles (*Pi1*, *Pik*, *Pik-p* and *Pik-m*) were further subjected to allele distribution analysis.

Results

The blast resistance spectrum and durability of *Pi1*

The differential reactions to the various blast isolates showed that *Pi1* is distinct from other *Pik* alleles (*Pik*, *Pik-p* and *Pik-m*), as well as from *R* genes at the *Pia* and *Pi2* loci. *Pi1* gave a consistent level of resistance in GD throughout the period 1998–2008, suggesting that it is durable (Fig. 1a). The gene also conferred resistance to a broad range of blast isolates from across China, although it was ineffective against some blast isolates collected from the JL Province (Fig. 1b).

Genetic and physical maps of the *Pi1* locus

With respect to the reaction to inoculation with blast isolate EHL0490 (avirulent on cv. C101LAC, virulent on cv. K1), the C101LAC/K1 F_2 progeny segregated at a ratio 489 resistant to 161 susceptible, consistent with the presence of a single gene for resistance ($\chi^2_c = 0.01$, $P < 0.90$). When the markers for the *Pik-m*/*Pik-p*/*Pik* region (Table S1) were applied to the 161 blast susceptible segregants, five individuals were shown to be recombinant with respect to blast reaction and marker K37, one with respect to K28 and none with respect to K33 (Fig. 2). Of the three polymorphic CRG (Candidate Resistance Gene) markers identified from the equivalent region in cv. Nipponbare, CRG11-7 identified three recombinants, while both CRG11-5 and CRG11-6 identified none (Fig. 2). We concluded that the location of *Pi1* was confined to a region delimited by CRG11-7 and K28, equivalent to a 277-kb stretch of chromosome 11, overlapping with the location of the *Pik* locus (Fig. 2).

Candidate gene identification

The K- and N-haplotypes surrounding *Pik* differ by a large InDel (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011), so both the sequences of cv Nipponbare and BAC clones Ts50A13 and Ts18H12 from cv. Tsuyuake were used as a reference to annotate the candidate region (Fig. 2d). This produced a set of six genes having the expected *R* gene structure (Fig. 2c, d). The presence/absence analysis applied to genes *Pi1-1* to *Pi1-4* showed that *Pi1-1N* to *Pi1-4N* were all absent in cv. C101LAC, while *Pi1-1C* to *Pi1-4C* were all present (Figs. 2; S1). Thus, cv. C101LAC is the K-haplotype. A perfect association was established between SNP genotype and blast reaction among a set of *Pi1* carriers and non-carriers for both *Pi1-5C* and *Pi1-6C* (Table 1), promoting these two genes above the four others as candidates for *Pi1*. Both are orthologs of *Pik-m*/*Pik-p*/*Pik* (Fig. 2d; Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011). The *Pik-m*, *Pik-p* and *Pik* "alleles" are defined by specific sequences at two physically linked genes (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011). In the remainder of this report, we will follow the convention for describing alleles at *Pik*, where "allele" is used to define specific sequences at two genes. We hypothesize that *Pi1* also comprises two genes: *Pi1-5C* and *Pi1-6C*. This hypothesis will be tested in further experiments.

Functional characterization of the candidate genes

The three C101LAC/Kh4i-16 F_1 plants tested showed no resistance to the *Pi1/Pia*-avirulent blast isolate CHL381 (Figs. 3a; S3), while those tested from the Aichi Asahi/Kh4i-11 cross maintained resistance to the blast isolate CHL381 (Fig. 3a). Gene expression analysis revealed that the loss of *Pi1* function reflected the lack of its expression (Fig. 3b). To confirm that *Pi1* function required the presence of *Pi1-56C*, a binary construct harboring *Pi1-56C* and *Pi1-34C* was introduced into cvs. Q1063, LJ and Nipponbare (Table 2). Many of the transformants carrying the *Pi1-56C* transgene were resistant to infection by *Pi1*-avirulent blast isolates, unlike any of the *Pi1-34C* transformants (Table 2; Figs. S4–S6). An unambiguous association

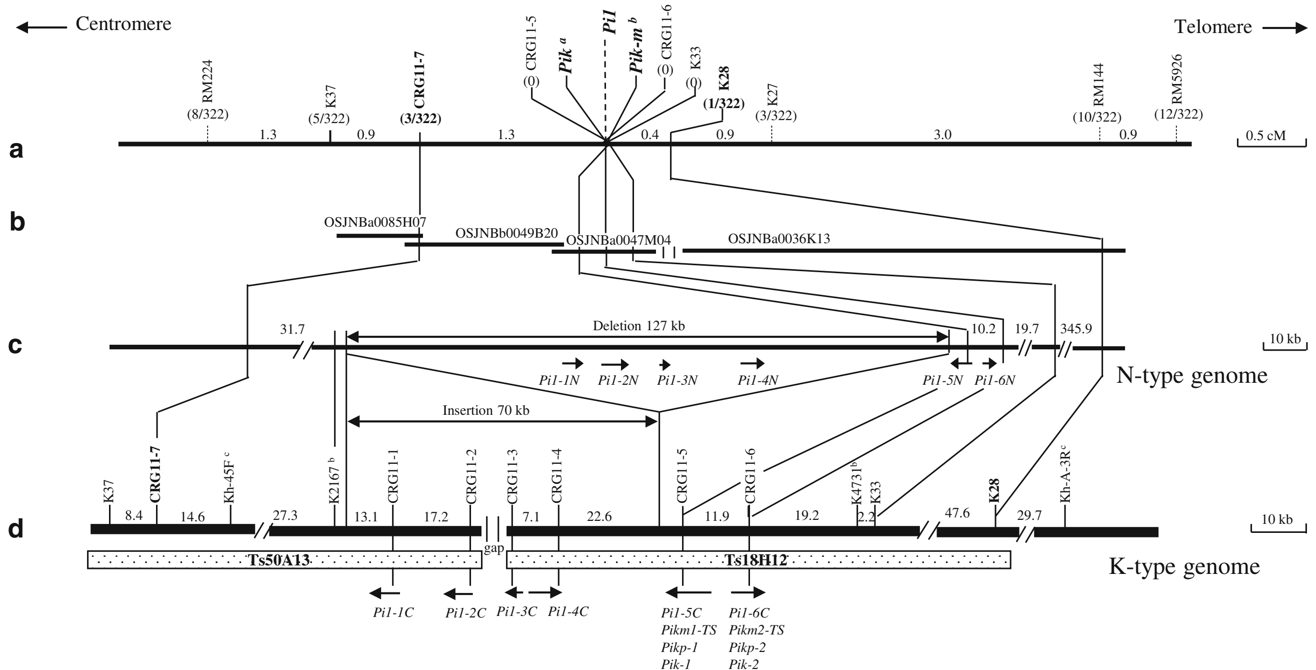

Fig. 2 Integrated genetic and physical maps of the *Pik*/*Pi1* locus. **a** Genetic map of the *Pi1* locus. The *numbers in parenthesis* are recombinants/gametes identified at the respective marker locus. **b** Contig map of the target region flanked by markers CRG11-7 and K28. **c** Physical map of the *Pi1* locus in the Nipponbare type genome, where six candidate genes with NBS-LRR structure were predicted by the gene annotation program, RiceGAAS. **d** Physical map of of the *Pi1* locus in the C101LAC type genome, where six candidate genes with the conserved *R* gene structures, i.e., *Pi1-1C* (NB-ARC), *Pi1-2C* (PK), *Pi1-3C* (NB-ARC), *Pi1-4C* (NBS), *Pi1-5C* (NBS-LRR), and *Pi1-6C* (NBS-LRR), were predicted based on the sequence of the BAC clones, Ts50A13 and Ts18H12 derived from cv Tsuyuake. A large InDel between Nipponbare and Tsuyuake/K60/C101LAC genomes was also indicated according to Ashikawa et al. (2008) (also see Fig. S1). The anchor markers were extracted from **a** Zhai et al. (2011); **b** Ashikawa et al. (2008); **c** Xu et al. (2008)

between resistance and the presence of the *Pi1-56C* transgene was established in later generations derived from the primary transformants (Fig. 4). Thus, it was clear that *Pi1-56C*, and not *Pi1-34C*, was required for *Pi1* function. *Pi1-56C* homozygous progeny shared the same race specificity as the *Pi1* standard cv. IRBL1-CL (data not shown).

The structure of *Pi1*

Pi1-5C encodes a 1,143 residue polypeptide and has the same intron/exon structure as *Pikm-1* and *Pik-1* and a very similar one to that of *Pikp-1* (Fig. S7; Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011). The Pi1-5C sequence shares, respectively, 99, 99 and 95 % peptide identity with those of Pikm-1, Pik-1 and Pikp-1. *Pi1-6C* encodes a 1,021 residue polypeptide and, apart from an extra intron in its 5′-UTR, has the same intron/exon structure as *Pikm-2* and *Pik-2*. The 5′-UTR intron is also present in *Pikp-2*, but not in either *Pik-2* or *Pikm-2* (Fig. S8; Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011). The Pi1-6C peptide sequence is 99 % identical with those of Pikm-2, Pik-2 and Pikp-2. At the nucleotide sequence level, *Pi1* can be distinguished from other *Pik* alleles with respect to two non-synonymous SNPs, namely *Pi1-5SNP* in the Pi1-5C CC domain (T1-687G) and *Pi1-6SNP* in the Pi1-6C LRR domain (T2-2261A) (Figs. S7, S8). The *Pi1* nucleotide sequence has been deposited in GenBank as accession HQ606329.

Distribution of *Pi1*

When the *Pi1-5SNP* and *Pi1-6SNP* SNP assays were applied to a large germplasm panel, it was apparent that only the latter was diagnostic for *Pi1* (Fig. 5). The survey of wild rice accessions and *indica* and *japonica* landrace and cvs (Tables S2 and S3) showed that *Pi1* was only present in two *indica* cvs (Tetep and C101LAC) and one *japonica* cv (IRBL1-CL, which was derived from cv. C101LAC). The presence of *Pi1* only in the modern cultivar populations suggested that it probably evolved later than *Pik-m*, *Pik-p* or *Pik* (Table S3). Tetep was bred in SE

Table 1 SNP genotype of the *Pi1* candidate genes and the correlation between SNP allele and blast resistance among *Pi1* differentials

Candidate gene/isolate[a]	SNP[b]	Marker type	Differentials[c]							
			C101LAC ($Pi1^{+}$)	Tetep ($Pi1^{+}$)	IRBL1-CL ($Pi1^{+}$)	CO39 ($Pi1^{-}$)	LTH ($Pi1^{-}$)	IRBLkm-Ts ($Pi1^{-}$)	IRBLkh-K3 ($Pi1^{-}$)	IRBLk-Ka ($Pi1^{-}$)
Pi1-1C	CRG11-1	CAPS	1	1	1	1	2	2	1	1
Pi1-2C	CRG11-2	CAPS	1	1	2	2	2	2	2	2
Pi1-3C	CRG11-3	d CAPS	1	1	H	1	2	2	1	2
Pi1-4C	CRG11-4	d CAPS	1	1	2	2	2	2	2	2
Pi1-5C	CRG11-5	d CAPS	**1**	**1**	**H**	2	2	2	2	2
Pi1-6C	CRG11-6	d CAPS	**1**	**1**	**H**	2	2	2	2	2
CHL22	n/a	n/a	**R**	**R**	**R**	S	S	R	R	R
CHL768	n/a	n/a	**R**	**R**	**R**	R	S	R	S	S

Bold characters indicate a perfect genotype/phenotype association

[a] Genotyping based on six candidate *R* genes lying in the region flanked by markers CRG11-7 and K28 (see Fig. 2); phenotyping reflected the plant reaction to inoculation with two blast isolates

[b] The SNP markers were based on either a CAPS or a dCAPS assay

[c] Rice cultivars/lines with/without *Pi1* selected for genotyping: 1, $Pi1^{+}$; 2, $Pi1^{-}$; H, $Pi1^{+}/Pi1^{-}$ heterozygote; –, absent; R, resistant; S, susceptible

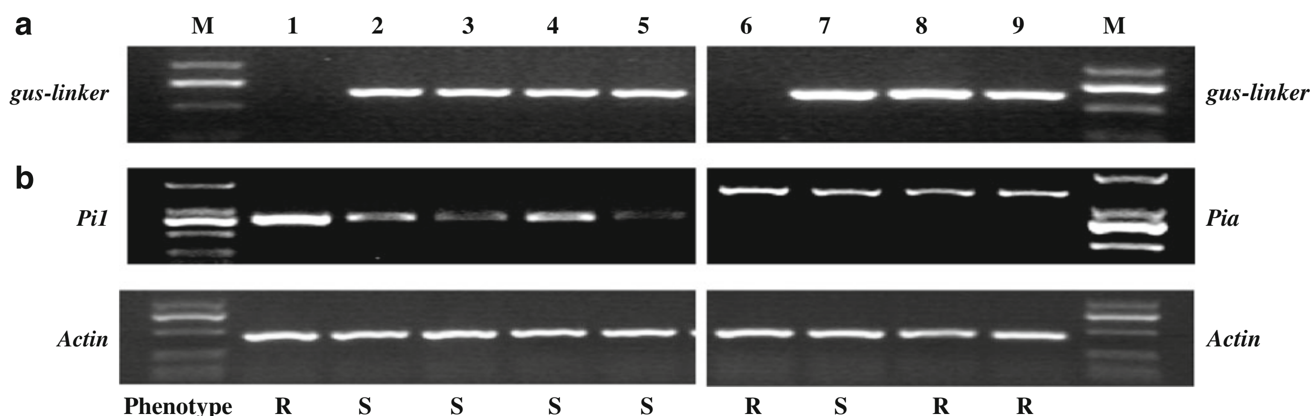

Fig. 3 Molecular characterization of hybrid F_1 plants derived from the conventional crosses for loss-of-function analysis of *Pi1*. **a** The amplification of a fragment of the *gus-linker* sequence indicates the presence of the RNAi construct targeting *Pik-p*. **b** Expression analysis of *Pi1* (*lanes 1–5*) or *Pia* (*6–9*). The *lower panel* shows the expression of the reference gene *actin*. *Lane 1* cv. C101LAC (*Pi1*), *lane 2* kh4i-16 (*Pik-p*-RNAi construct), *lanes 3–5* C101LAC/kh4i-16 F_1 plants, *lane 6* cv. Aichi Asahi (*Pia*), *lane 7* kh4i-11 (*Pik-p*-RNAi construct), *lanes 8–9* Aichi Asahi/kh4i-11 F_1 plants. *M* DNA ladder. Plants inoculated with the blast isolate CHL381 (avirulent on all *Pik* alleles including *Pi1*, as well as on *Pia*). The F_1 plants in *lanes 3–5* were susceptible (S), and those in *lanes 8–9* were resistant

Asia (Vietnam) and LAC23 (the *Pi1* donor to C101LAC and IRBL1-CL) originated in W Africa (Liberia).

Discussion

We have shown that *Pi1* is a novel and rare allele at the *Pik* locus. *Pi1* is now the fourth cloned and characterized allele at this complex locus. The *Pik* locus has proven valuable to rice breeders concerned with blast resistance, as most alleles impart broad spectrum and durable resistance (Kiyosawa 1987; Ashikawa et al. 2008; Xu et al. 2008; Wang et al. 2009; Yuan et al. 2011; Zhai et al. 2011). *Pi1* has been used in a number of rice breeding programs. For example, it was combined with *Pi2* and *Pita* by IRRI (Hittalmani et al. 2000) for use in both the Philippines and India; by Fuentes et al. (2008) in Latin America; in Europe by Tacconi et al. (2010), and in China it is regarded as having great potential for achieving broad spectrum and durable resistance (Li et al. 2005; Yang et al. 2008; Hu et al. 2010; Zhang et al. 2010). Our results confirm that the *Pi1* allele is effective against many, but not all, isolates collected from the broad range of cropping seasons and regions.

Table 2 Complementation test for *Pi1* candidates

Candidate/construct[a]	Insert size (bp)	Recipient cultivar	Reaction of T_1 plants[b]					Success ratio (%)[c]
			R	MR	MS	S	Total	
Pi1-34C	8,909	Q1063	0	0	0	0	74	0.0
Pi1-56C	17,722	Q1063	58	11	5	249	323	21.4
Pi1-56C	17,722	Longjing 24 (LJ)	33	3	3	1	40	90.0
Pi1-56C	17,722	Nipponbare	93	2	7	60	162	58.6

[a] Constructs *Pi1-34C* and *Pi1-56C* represent fusions between, respectively, *Pi1-3C* and *Pi1-4C*, and *Pi1-5C* and *Pi1-6C* (see Table 1; Fig. 2)

[b] T_1 plants were inoculated with the *Pi1*-avirulent blast isolate CHL346 or CHL357. *R* resistant, *MR* moderately resistant, *MS* moderately susceptible, *S* susceptible

[c] Genetic transformation success ratio calculated from (R + MR)/(R + MR + MS + S)

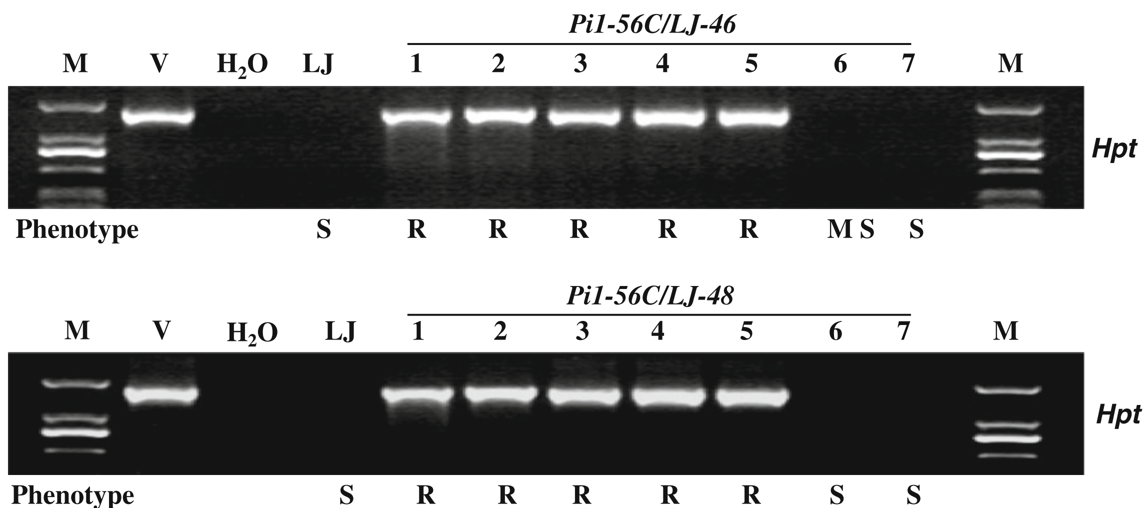

Fig. 4 Co-segregation of resistance with the presence of *Pi1-56C* in segregating transgenic progeny. *M* DNA ladder, *V* vector, *LJ* cv Longjing 24, *R* resistant, *MS* moderately susceptible, *S* susceptible (also see Table 2). The codes of *Pi1-56C/LJ-46* and *Pi1-56C/LJ-48* indicate #46 and #48 T2 lines generated from transformation of *Pi1-56C*

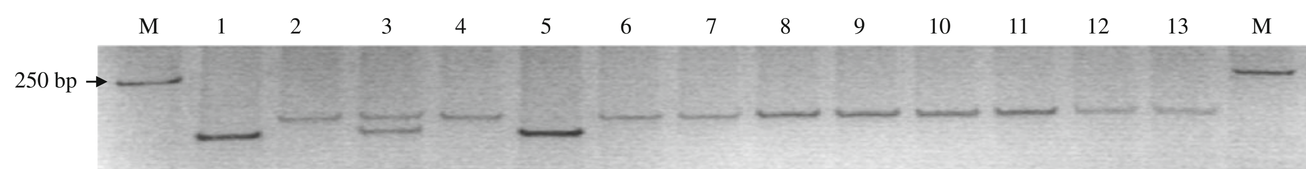

Fig. 5 The *Pi1* allele-specific FNP distinguishes it from other blast *R* genes including the five *Pik* alleles. *Lane 1* cv. C101LAC (*Pi1*), *Lane 2* cv. CO39 (susceptible parent of cv. C101LAC), *lane 3* IRBL1-CL (*Pi1*), *lane 4* cv. LTH, *lane 5* cv. Tetep (*Pi1*), *lane 6* IRBLks-S (*Pik-s*), *lane 7* IRBLk-Ka (*Pik*), *lane 8* IRBLkp-K60 (*Pik*), *lane 9* IRBLkh-K3 (*Pik-h*), *lane 10* IRBLKM-TS (*Pik-m*), *lane 11* IRBLz-CA (*Pi2*), *lane 12* IRBL7-M (*Pi7*), *lane 13* IRBL9-W (*Pi9*). *M* DNA ladder

R genes are characteristically organized into clusters, and even as alleles at the particular locus (Bai et al. 2002; Wang et al. 2009). Before the development of marker technology, the effective characterization of *R* genes depended entirely on testing with a panel of race-specific pathogen isolates. This approach is problematic whenever no matching race is available which is virulent against a carrier of a novel allele. The advent of DNA markers has encouraged the implementation of the *R* gene pyramiding concept, which in principle avoids the need for inoculation (Huang et al. 1997; Hayashi et al. 2004). The requirement for this strategy is that markers for all genes in the pyramid are both diagnostic and robust (Tommasini et al. 2006; Hayashi et al. 2010; Perumalsamy et al. 2010). Ideally, each marker needs to be gene (or allele) specific, as is an FNP. The bread wheat *R* locus *Pm3* provides an example of

the development of FNPs (Tommasini et al. 2006), in which seven distinct alleles have been independently marked. Not only can these FNPs be used to search for novel alleles in large germplasm sets and as effective selection tools for resistance breeding, but they also have the potential to allow the parallel introduction of multiple alleles into a single individual plant via a transgenic approach. The current situation with the *Pik* locus is that four allele-specific FNPs (*Pik*, *Pik-p*, *Pik-m*, and *Pi1*) are now available (Fig. 5; Yuan et al. 2011; Zhai et al. 2011). These FNP markers can be widely used to stack the *Pik* alleles with other *R* genes in elite cvs via MAS and transgenic approaches.

Only 2 of 636 germplasm accessions surveyed carry *Pi1*. One accession (LAC23) is from W Africa and the other (Tetep) in SE Asia. The presence of *Pi1* only in both modern cultivars suggests that it evolved relatively recently, unlike *Pik-p* which pre-dates the domestication of rice, and *Pik* and *Pik-m*, both of which evolved somewhat after rice domestication (Zhai et al. 2011). The geographic dispersal of *Pi1* may trace to the introduction of rice to Africa in the sixteenth century (Linares 2002). Alternatively, active selection for blast resistance could easily have promoted the incorporation of the rare *Pi1* into local cultivars in both SE Asia and W Africa (Gross et al. 2010). It is testimony to the effectiveness of plant breeding that such a valuable and potentially durable allele would occur at such low frequency in germplasm collections and at a higher frequency in contemporary breeding programs.

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