DOI: 10.1534/genetics.108.095034

Two Adjacent Nucleotide-Binding Site–Leucine-Rich Repeat Class Genes Are Required to Confer *Pikm*-Specific Rice Blast Resistance

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Manuscript received August 9, 2008
Accepted for publication October 15, 2008

ABSTRACT

The rice blast resistance gene *Pikm* was cloned by a map-based cloning strategy. High-resolution genetic mapping and sequencing of the gene region in the *Pikm*-containing cultivar Tsuyuake narrowed down the candidate region to a 131-kb genomic interval. Sequence analysis predicted two adjacently arranged resistance-like genes, *Pikm1-TS* and *Pikm2-TS*, within this candidate region. These genes encoded proteins with a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) and were considered the most probable candidates for *Pikm*. However, genetic complementation analysis of transgenic lines individually carrying these two genes negated the possibility that either *Pikm1-TS* or *Pikm2-TS* alone was *Pikm*. Instead, it was revealed that transgenic lines carrying both of these genes expressed blast resistance. The results of the complementation analysis and an evaluation of the resistance specificity of the transgenic lines to blast isolates demonstrated that *Pikm*-specific resistance is conferred by cooperation of *Pikm1-TS* and *Pikm2-TS*. Although these two genes are not homologous with each other, they both contain all the conserved motifs necessary for an NBS–LRR class gene to function independently as a resistance gene.

PLANTS protect themselves against a wide variety of pathogens, such as viruses, bacteria, fungi, nematodes, and insects, through resistance (*R*) genes that recognize avirulence (*Avr*) genes in the pathogens. A number of *R* genes have been cloned and their structures characterized (Martin *et al.* 2003; Deyoung and Innes 2006; Takken *et al.* 2006). These *R* genes can be divided into several classes according to their structural features. The majority encode proteins belonging to the NBS–LRR class, which harbor nucleotide-binding site (NBS) and C-terminal leucine-rich repeat (LRR) motifs (Martin *et al.* 2003). The NBS domain in *R* proteins contains a number of conserved motifs, such as kinase 1a or P-loop, kinase 2, and kinase 3a, and this domain may affect *R* protein function through nucleotide binding, hydrolysis, and control of cell death. The LRR domain is generally thought to be the major determinant of recognition specificity for pathogen avirulence factors (Hulbert *et al.* 2001; Martin *et al.* 2003; Deyoung and Innes 2006; Takken *et al.* 2006). Supporting evidence has been provided, for example, by allelic comparisons and domain-swapping experiments between different alleles at the *L* and *P* loci of flax (Ellis *et al.* 1999; Dodds *et al.* 2001). However, other evidence supports the possibility that regions additional to the LRR are involved in resistance specificity. One example was provided by an analysis of the *L6* and *L7* genes for flax rust resistance (Ellis *et al.* 1999): although these two genes have distinct race-specific resistance, they contain identical NBS–LRR-encoding regions and differ only in the N-terminal Toll/interleukin-1 receptor (TIR) domain.

Rice blast, caused by *Magnaporthe grisea*, is one of the most devastating diseases of rice. There have been a number of studies identifying blast resistance genes, locating them on the rice chromosome, and (in some cases) cloning and characterizing them at the molecular level. Information obtained from these studies has been used, for example, to develop DNA markers to select blast resistant rice lines, to understand the molecular mechanisms underlying the specific host-pathogen recognition revealed by these blast resistance genes, and to learn the genome organization of *R* gene clusters and the evolution of complex *R* gene loci. To date, almost 40 rice blast resistance genes have been identified and mapped (Lin *et al.* 2007). Of these, 8 genes have been cloned: *Pib* (Wang *et al.* 1999), *Pita* (Bryan *et al.* 2000), *Pid2* (Chen *et al.* 2006), *Pi9* (Qu *et al.* 2006),

Sequence data from this article have been deposited with the EMBL/GenBank/DDBJ Data Libraries under accession nos. AB462256, AB462324, and AB462325.

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Pi2 and *Pizt* (Zhou *et al.* 2006), *Pi36* (Liu *et al.* 2007), and *Pi37* (Lin *et al.* 2007). With the exception of *Pid2*, which was reported to encode a receptor-like kinase (Chen *et al.* 2006), these cloned genes belong to the NBS–LRR class of resistance genes.

Mapping efforts have indicated that many of the blast resistance genes are allelic or closely linked. For example, at the *Piz* locus on chromosome 6, at least four genes have been identified (Liu *et al.* 2002; Hayashi *et al.* 2004) and three of them, *Pi9*, *Pi2*, and *Pizt*, have been cloned (Qu *et al.* 2006; Zhou *et al.* 2006). Structural comparisons of these cloned genes have provided information on the DNA region within these genes responsible for determining their distinct resistance specificities. In addition, analysis of sequences at the *Pi9* locus in a group of genetically distinct cultivars has revealed the complex and divergent genome organization of this locus (Zhou *et al.* 2007). Two blast resistance genes, *Pita* and *Pita2*, have been located at the *Pita* locus on chromosome 12 (Kiyosawa 1967; Rybka *et al.* 1997; Bryan *et al.* 2000). These two genes are interesting in terms of their resistance specificity: *Pita2* has a broader resistance spectrum than *Pita*. That is, no *M. grisea* isolate has been found that is avirulent toward *Pita* but virulent toward *Pita2* (Bryan *et al.* 2000). This resistance spectrum feature of *Pita* and *Pita2* suggests that *Pita2* blast specificity is conferred by a combination of *Pita* and at least one additional resistance gene (Bryan *et al.* 2000). *Pita* has been cloned (Bryan *et al.* 2000), while *Pita2* has not been cloned, and it remains to be answered whether this combination hypothesis is valid.

Another example of a major allelic blast-resistance locus is provided by the *Pik* locus on chromosome 11, where at least five genes, *Pik*, *Pikm*, *Pikh*, *Pikp*, and *Piks*, have been identified (Kiyosawa 1968, 1978; Inukai *et al.* 1994; Hayashi *et al.* 2006; Li *et al.* 2007). The genes *Pik* and *Pikm* originated in the Chinese *japonica* cultivars To-To (Kiyosawa 1968) and Hokushi Tami (Kiyosawa 1978), respectively, whereas *Pikh* and *Pikp* originated in the *indica* cultivars Te-tep (Kiyosawa 1978) and Pusur (Kiyosawa 1969a), respectively. *Piks* is thought to have originated from a Japanese *japonica* cultivar (Kiyosawa 1969b). With the exception of *Piks*, the resistance spectra of these genes to distinct blast isolates are similar; in particular, the resistance specificities of *Pik* and *Pikm* mostly overlap each other. The relationship of the resistance spectra of these two genes resembles that of *Pita* and *Pita2*: *Pikm* has a broader resistance spectrum than *Pik* (Kiyosawa and Nomura 1988). Gene cloning and the subsequent structural characterization of the *Pik* and *Pikm* genes would provide the basis for understanding the molecular features responsible for this similar, but distinct, resistance specificity of these genes.

Toward the above-mentioned goal, we have embarked on the map-based cloning of *Pik* and *Pikm*. In this article, we report the cloning and characterization of the *Pikm* gene. Precise mapping and subsequent sequencing of the gene region showed that *Pikm* resided in a highly divergent genome region, where a large deletion and an insertion between the genomes of the resistant cultivar Tsuyuake and the susceptible cultivar Nipponbare were present. Cloning of the gene revealed a unique feature of *Pikm*: the resistance provided by *Pikm* is not conferred by a single gene, but rather by a combination of two NBS–LRR class genes, both of which reside adjacently at the *Pikm* locus.

MATERIALS AND METHODS

Plant materials and PCR primers: For mapping of *Pikm*, we used an F_2 segregating population derived from a cross between the blast-resistant cultivar Tsuyuake and the blast-susceptible line 99SL44 (Hayashi *et al.* 2006). The line 99SL44 is a Nipponbare-based chromosome substitution line in which a segment of chromosome 11 containing the *Pikm* locus is replaced by a corresponding chromosomal segment from an *indica* cultivar, Kasalath. For complementation analysis, we used the blast-susceptible cultivar Nipponbare as a host cultivar for transformation. To evaluate the resistance specificity of transgenic plants, as differential cultivars we used Tsuyuake (*Pikm*$^+$), Kanto 51 (*Pik*$^+$), K60 (*Pikp*$^+$), and three cultivars, IRBLk-Ka (*Pik*$^+$), IRBLkm-Ts (*Pikm*$^+$), and IRBLkh-K3 (*Pikh*$^+$), from Lijiangxintuanheigu monogenic lines (Tsunematsu *et al.* 2000).

We used PCR primer pairs to define the candidate genome region of *Pikm* (85H07S5, k2167, k4731, k3951, and k3952), to check the presence of transgenes in transgenic lines (Gene2TY1 and Gene1TY1), to examine expression of the transferred candidate genes in transgenic lines (RT13 and Gene1TY1), to obtain the expression profiles of the candidate genes in Tsuyuake (RRT5 and RRT17), and to perform 5′- and 3′-rapid amplification of cDNA ends (RACE) (RT30R, RT32R, RT23F, RT31F, RT21F, and RT4R). The sequences of these primers are listed in supplemental Table S1.

Construction of BAC library and sequencing: Megabase-sized rice DNA was prepared as described by Zhang *et al.* (1995) from young leaves of Tsuyuake. A bacterial artificial chromosome (BAC) library was constructed by the conventional method, through partial DNA digest by *Hin*dIII, size fractionation of high-molecular-weight DNA in pulsed-field gel electrophoresis (CHFF, Bio-Rad Laboratories, Hercules, CA), vector ligation (pIndigo BAC-5, EPICENTRE Biotechnologies, Madison, WI), and transformation of high-molecular-weight DNA into *Escherichia coli* (DH10B strain). After preparation of the library, which contained 32,706 clones with an average insert size of 153 kb, positive clones covering the *Pikm* gene region were screened by using DNA markers tightly linked to *Pikm*. The BAC clones Ts18H12, Ts69H20, and Ts50A3 were selected and their sequences determined by using a shotgun strategy (Messing *et al.* 1981) as described before (International Rice Geome Sequencing Project 2005).

Sequence annotation and computational analysis of DNA: The Rice Genome Automated Annotation System (RiceGAAS) (Sakata *et al.* 2002) was used to analyze genomic sequence data. This system integrates the programs GENSCAN, RiceHMM, FGENESH, and MZEF for finding putative gene regions and the homology-search-analysis programs Blast, HMMER, ProfileScan, and MOTIF for predicting the putative functions of genes. Pairwise comparisons between genomic or protein sequences were performed with the BLAST program (bl2seq) (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi)

and the CLUSTALW program (http://clustalw.ddbj.nig.ac.jp/top-j.html). The theoretical isoelectric point (pI) and protein molecular weight were computed as described (http://br.expacy.org/tools/pi_tool.html). The coiled-coil structures of proteins were searched for by using COILS (http://www.ch.embnet.org/software/COILS_form.html).

Candidate gene cloning and complementation analysis: Among genes predicted within the *Pikm* region from the Tsuyuake genomic sequence, we selected two putative genes, *PiKm1-TS* and *PiKm2-TS*, as candidate genes for *Pikm*. From the Tsuyuake BAC clone TS18H12, we used a high-fidelity Taq polymerase, PrimeStar (Takara, Tokyo) to amplify a 9.1-kb fragment, which contained the 1.9 kb of the *Pikm1-TS* 5′-untranslated region (UTR), the whole *Pikm1-TS* coding sequence, and the 0.9-kb *Pikm1-TS* 3′-UTR, and a 8.1-kb fragment, which contained the 2.1-kb *Pikm2-TS* 5′-UTR, the whole *Pikm2-TS* coding region, and the 2.7-kb *Pikm2-TS* 3′-UTR. The amplified products were individually inserted into the *Sma*I site of the binary vector pPZP2H-lac (Fuse *et al.* 2001) to form constructs for transformation. These constructs were validated by comparison of their insert sequences with the sequences of the corresponding regions in the BAC clone TS18H12 (accession no. AB462256).

We transformed the two constructs, each containing the *Pikm1-TS* or *Pikm2-TS* region, individually into Agrobacterium strain EHA101 and then infected Nipponbare callus with them by the method of Toki (1997). Primary transgenic plants (T_0 plants) regenerated from hygromycin-resistant calluses were grown in an isolated greenhouse. The presence of the transformed DNA fragments in the T_0 plants was checked by PCR assay with the *Pikm1-TS*-specific primer pair Gene2TY1 and the *Pikm2-TS*-specific primer pair Gene1TY1 (supplemental Table S1). The transgene copy number was evaluated by Southern hybridization analysis with an ECL Direct Labeling and Detection System (GE Healthcare, Buckinghamshire, UK). We bred the T_1 progeny through self-pollination of the T_0 plants. Some of these T_0 and T_1 plants were tested for reaction to blast infection. To develop lines harboring both the *Pikm1-TS* and *Pikm-TS2* genes, we selected T_1 plants that carried a single copy of either *Pikm1-TS* or *Pikm2-TS* and crossed a T_1 plant having *Pikm1-TS* with another T_1 plant having *Pikm2-TS*. Through these crosses, we obtained five distinct F_1 plants that harbored both of the candidate genes. We evaluated the blast resistance of F_2 plants bred from self-pollination of these F_1 plants.

Evaluation of blast resistance: The blast resistance of the transgenic lines, the parental cultivars, and the differential cultivars was evaluated by the method described by Hayashi *et al.* (2004). Briefly, we sprayed blast spores suspended in 0.02% Tween 20 onto plants and then placed these plants in a dew chamber for 22 hr at 25°. The plants were then transferred to a greenhouse and grown for 7 days before the disease reaction was examined. For the complementation analysis and gene expression analysis, we used blast isolate Ina 86-137, which is avirulent to the *Pikm* donor cultivar Tsuyuake but virulent to the transformation-recipient cultivar Nipponbare. Plants of Nipponbare and Tsuyuake were used, respectively, as susceptible and resistant controls. To evaluate the resistance specificity of transgenic plants, a set of five *M. grisea* isolates that discriminated *Pikm* from other blast-resistance genes was used.

Expression analysis: Expression of transformed *Pikm1-TS* and *Pikm2-TS* in transgenic lines was examined by a RT–PCR assay using total RNAs isolated from transgenic lines as templates and RT13R (for detecting *Pikm1-TS* RNA) and Gene1TY1 (for detecting *Pikm2-TS* RNA) as primer pairs (supplemental Table S1). Total RNA was isolated with an RNeasy plant kit (QIAGEN, Valencia, CA). To completely eliminate DNA comtaminants, we treated isolated RNA with RNase-free DNase I (QIAGEN). First-strand cDNA was synthesized from ~1 μl of total RNA by using a Superscript First-Strand synthesis system (Invitrogen, Carlsbad, CA), diluted 10 times with water, and used as a template for RT–PCR.

We used quantitative RT–PCR analysis to examine the expression profiles of candidate genes in Tsuyuake. We isolated total RNAs from seedling leaves of Tsuyuake collected 0, 0.5 (*i.e.*, 12 hr), 1, 3, and 5 days after inoculation (DAI) with the blast isolate Ina 86-137. For negative control inoculation, we sprayed 0.02% Tween 20 without blast spores. The primer pairs RRT5 and RRT17 (supplemental Table S1) were used for detecting transcripts from *Pikm1-TS* and *Pikm2-TS*. Primers for rice actin were used as positive RT–PCR controls (Wang *et al.* 1999). Quantitative RT–PCR analysis was done with an ABI 7500 Real Time PCR System using SYBR Premix Ex Taq (Takara). Each experiment was performed at least twice.

Rapid amplification of cDNA ends: To determine the 5′- and 3′-end sequences of the cDNAs, we performed RACE with a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA). First-strand cDNA was synthesized from total RNA isolated from Tsuyuake. The 5′ RACE product of *Pikm1-TS* was PCR-amplified by using the synthesized first-strand cDNA as a template and the gene-specific primer RT23F and the universal primer A mix in the kit; this was followed by another PCR with the nested primer RT31F (supplemental Table S1). The 5′ RACE product of *Pikm2-TS* was amplified with RT30R and the universal primer A mix, followed by another PCR with the nested primer RT32R (supplemental Table S1). The 3′ RACE products of *Pikm1-Ts* and *Pikm2-TS* were amplified with the primers RT21F and RT4R, respectively, together with the universal primer A mix. These RACE products were cloned into pCR 2.1 vector (Invitrogen) for sequencing.

RESULTS

Identification of candidate genes for *Pikm*: In our previous study, by performing linkage analysis using 2118 F_2 segregating plants bred from a cross between Tsuyuake and 99SL44, we preliminarily located the gene *Pikm* in the close vicinity of the markers k2167 and k4731 (Hayashi *et al.* 2006). In our present study, to narrow down the candidate region, we developed a number of DNA markers in the genomic region around the above two markers by using Nipponbare genome sequence data (accession no. NC 008404), and we performed linkage analysis by using the same mapping population that we used for our previous study. This analysis delimited the candidate gene region into a 180-kb interval in the Nipponbare genome between the two markers 85H07S5 and k3952. Although six plants with a chromosomal recombination in this interval were found among the F_2 plants, these recombinations occurred only around the two ends of this region: at the proximal end four recombinations were detected in a 23-kb interval next to marker 85H07S5, whereas at the distal end two recombinations were detected in a 8.3-kb interval next to marker k3952 (Figure 1A). In contrast, no recombinations were detected in the 160-kb interval flanked by the above two regions. This nonuniform distribution of the recombination sites at this locus hindered our efforts to narrow down the candidate

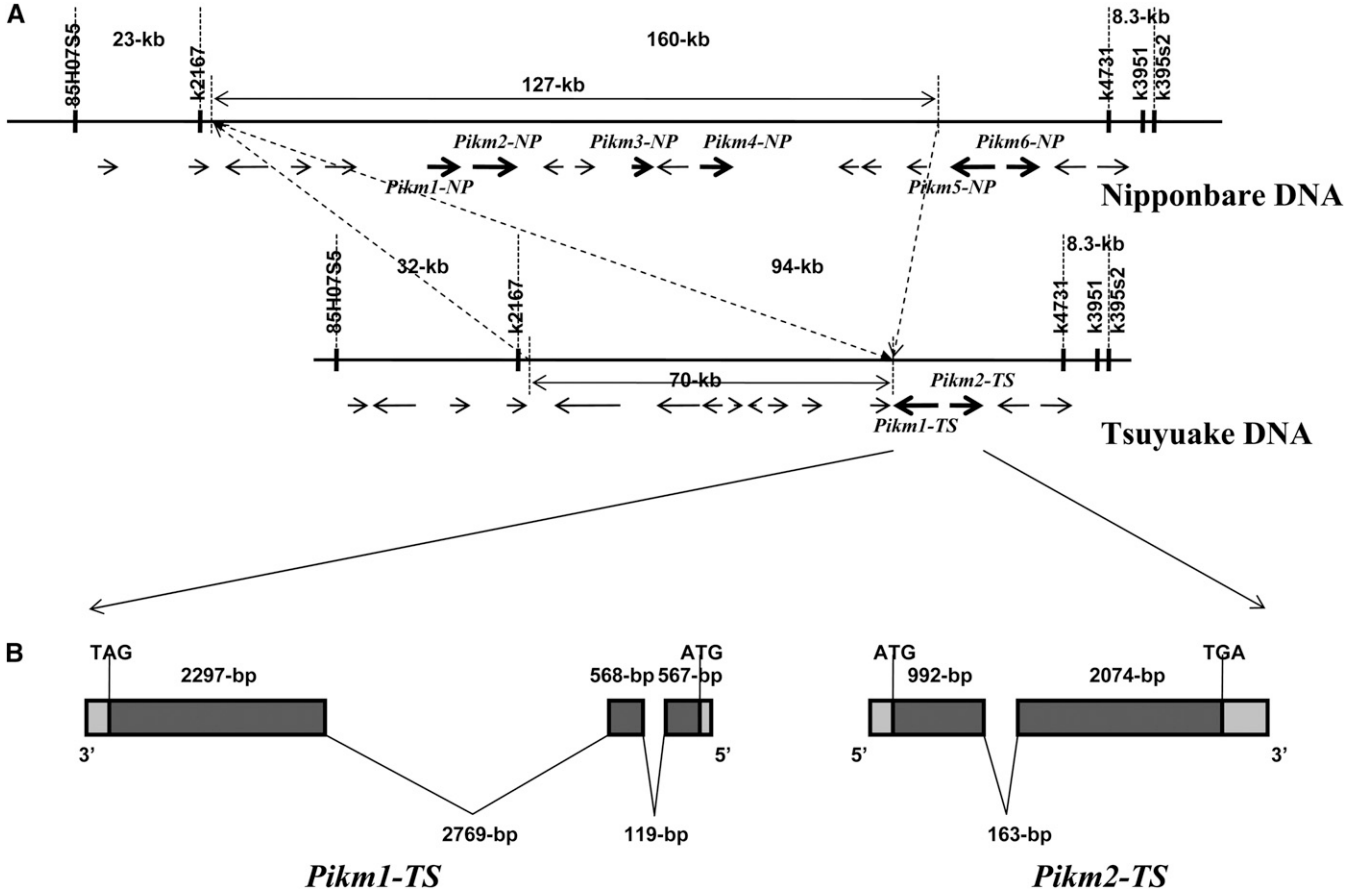

FIGURE 1.—Physical maps of the *Pikm* gene region and the structures of the two genes constituting *Pikm*. (A) Physical maps of the *Pikm* locus generated by using the Nipponbare (*Pikm*$^{-}$) genome sequence (top) and Tsuyuake (*Pikm*$^{+}$) sequence (bottom). The candidate gene region is delimited by the marker 85H07S5 from the proximal side and the marker k3951 from the distal side. The numbers above each of the maps represent distances in kilobases. The arrows below each map represent putative genes predicted by the annotation tools in RiceGAAS. The six boldface arrows on the Nipponbare map and the two boldface arrows on the Tsuyuake map represent putative *R* genes. (B) Structures of *Pikm1-TS* and *Pikm2-TS*. Dark-shaded boxes are exons, light-shaded boxes are 5′- or 3′-UTRs, and the lines are introns.

region into a smaller interval. The annotation tools accompanying the RiceGAAS database predicted the presence of 19 putative genes within this region (Figure 1 and supplemental Figure S1). Of these genes, 6 were similar to the disease *R* genes cloned from various plant species. These 6 genes encoded NBS–LRR-class proteins, and we designated them *Pikm1-NP* to *Pikm6-NP* (Figure 1A). The clustering of these putative *R* genes in this small genome region indicates that *Pikm* is located within a typical *R* gene region (BAI *et al.* 2002).

The cultivar Nipponbare is susceptible and hence its genome does not carry the gene *Pikm*. To identify candidate genes for *Pikm*, we attempted to obtain sequence data of the gene region from the *Pikm*-carrying cultivar Tsuyuake. First, we used the markers 85H07s5, k4731, and k3951 as probes to screen the Tsuyuake BAC library for BAC clones containing the *Pikm* region. We found that three BAC clones, Ts18H12, Ts69H20, and Ts50A13, covered the genome region delimited by the markers 85H07S5 and k3951. Comparison of the sequences of these Tsuyuake BAC clones (accession no. AB462256) with the Nipponbare sequence of the equivalent region revealed that the two cultivars had marked differences in their genome organization at this *Pikm* locus: the Tsuyuake genome lacked a 127-kb segment present in the Nipponbare genome, whereas next to this deletion there existed a 70-kb segment present only in Tsuyuake, but not in Nipponbare (Figure 1A). Owing to this insertion and deletion, the size of the *Pikm* candidate region in the Tsuyuake genome was reduced to 131 kb. Analysis of this Tsuyuake sequence by using the gene-finding programs in RiceGAAS predicted 16 genes within the region (supplemental Figure S1). Among these, two putative coding sequences, designated *Pikm1-TS* and *Pikm2-TS*, showed homology with the NBS–LRR class resistance genes. They were predicted to reside adjacent to each other, being transcribed in opposite directions. Because there were no other putative genes apart from these 2 that showed high levels of identity with the existing *R* gene, we considered *Pikm1-TS* and *Pikm2-TS* to be the most probable candidates for *Pikm*.

Blast resistance of transgenic plants harboring either *PiKm1-TS* or *PiKm2-TS*: To examine whether the *Pikm1-TS* or *Pikm2-TS* gene conferred *Pikm*-specific blast resistance, we introduced these two candidate genes individually into the blast susceptible rice cultivar Nipponbare and evaluated the resistance of the transformed plants. We evaluated the blast resistance of some of the T_0 plants. From a group of these plants, we randomly selected 12 plants to which *Pikm1-TS* had been introduced and 13 plants to which *PiKm2-TS* had been introduced. The presence of the respective transgenes in each of the tested plants was verified by PCR assay with the primers specific to *Pikm1-TS* and *Pikm2-TS*. Following inoculation with the blast isolate Ina 86-137, all of the tested plants harboring *Pikm1-TS* and most of the plants harboring *Pikm2-TS* (10 of the 13 plants) exhibited spindle-shaped lesions (data not shown), which are typically detected in blast susceptible cultivars, such as Nipponbare. This result did not support the possibility that either of the two candidates was the gene *Pikm*.

To confirm the above result, we examined the blast resistance of the T_1 plants. We used a PCR assay to select those T_1 plants that contained the transgenes. We inoculated blast spores onto a total of 21 T_1 plants having *Pikm1-TS* (derived from 4 distinct T_0 plants) and onto a total of 86 T_1 plants having *Pikm2-TS* (derived from 11 distinct T_0 plants). All of these tested transgenic lines showed no resistance to blast. To examine whether silencing of the introduced transgenes, if it was present, was responsible for this susceptible phenotype of the transgenic lines, we isolated RNA from some plants randomly selected from the tested plants and checked the expression of the integrated genes. The results of RT–PCR indicated that the two genes were actually expressed (data not shown). In light of these results in both the T_0 and the T_1 plants, we concluded that neither *Pikm1-TS* nor *Pikm2-TS* endowed blast resistance when only one of the two was introduced into the susceptible cultivar Nipponbare.

Blast resistance of transgenic plants harboring both *Pikm1-TS* and *Pikm2-TS*: We then examined the possibility that a combination of the two candidate genes conferred *Pikm*-associated blast resistance. To perform this examination, we crossed T_1 plants having *Pikm1-TS* with those having *Pikm2-TS*, and we obtained transgenic plants possessing both genes. The resultant F_1 plants were self-pollinated. We examined the blast resistance of 106 F_2 progeny. On the basis of the presence or absence of the two transgenes, these F_2 plants were classified into four groups: 59 plants with both genes, 22 with only *Pikm1-TS*, 20 with only *Pikm2-TS*, and 5 with neither. The segregation pattern of these four types of F_2 plants indicated that both of the transgenes were independently inherited. Following the inoculation with the blast isolate Ina 86-137, we observed a cosegregation between the presence of both transgenes and the resistant phenotype (Figure 2): all of the transgenic plants that carried both candidate genes were evaluated as resistant, whereas all of the plants that carried only one of the two transgenes, or neither of them, were susceptible. The group of resistant plants consisted mainly of highly resistant plants that exhibited no specks, or only several small brown specks, on their leaves (Figure 2A). The resistant group also included a small number of weakly resistant plants, which had a number of small brown specks or a small number of elliptical lesions. In contrast, like the transgenic lines that had neither of the two genes, plants with only one of the two genes were highly susceptible to blast inoculation. They developed typical spindle-shaped blast lesions on their leaves, and parts of the leaves were killed by the coalescence of lesions. This indicated that *Pikm1-TS* or *Pikm2-TS*, when present alone in a transgenic plant, did not confer partial blast resistance. Thus, we suggest that the complete resistance observed in the transgenic lines having both genes is due to cooperation of the two genes, rather than to an additive effect of partial resistance conferred by each of the two genes.

Resistance specificity of transgenic plants: To verify whether transgenic plants that had a combination of *Pikm1-TS* and *Pikm2-TS* had the same resistance specificity as *Pikm*, we inoculated the transgenic lines and differential cultivars with a set of blast isolates that enabled us to distinguish *Pikm* from other resistance genes. The transgenic lines were susceptible to the isolate 1804-4, indicating that the transgenes belonged to the *Pik* family and not to another family such as *Pib*, *Piz*, or *Pita* (Table 1). The transgenic lines were susceptible to the isolate H05-72-1 and resistant to the isolates P2-b and Kyu92-22; this indicated that the transgene was not *Pik*, *Pikp*, or *Pikh*. These results provided experimental evidence that the combination of the two genes *Pikm1-TS* and *Pikm2-TS* had the same function as the blast resistance gene *Pikm*.

Structure of *Pikm1-TS* and *Pikm2-TS*: To obtain the full-length cDNA sequences of *Pikm1-TS* and *Pikm2-TS*, we sequenced RACE products from the 5′- and 3′-ends of the transcribed regions of each gene and the overlapping intermediate RT–PCR fragments. The size and structure of both genes were determined by comparing the obtained full-length cDNA sequences with the genomic DNA sequences. *Pikm1-TS* (accession no. AB462324) contained a 3432-bp coding region, interrupted by two introns (119 and 2769 bp long) and flanked by a 62-bp 5′-UTR and a 190-bp 3′-UTR (Figure 1B). As shown in the multiple DNA sequence alignment of *Pikm1-TS* and *Pikm2-TS* (supplemental Figure S2), the structure of *Pikm2-TS* (accession no. 462325) was not homologous with that of *Pikm1-TS*: this second gene contained a 3066-bp coding region, interrupted by one intron (163 bp long) and flanked by a 109-bp 5′-UTR and a 283-bp 3′-UTR (Figure 1B).

Protein translations of the cDNA sequences revealed that *Pikm1-TS* encoded a 1143-residue NBS–LRR-class

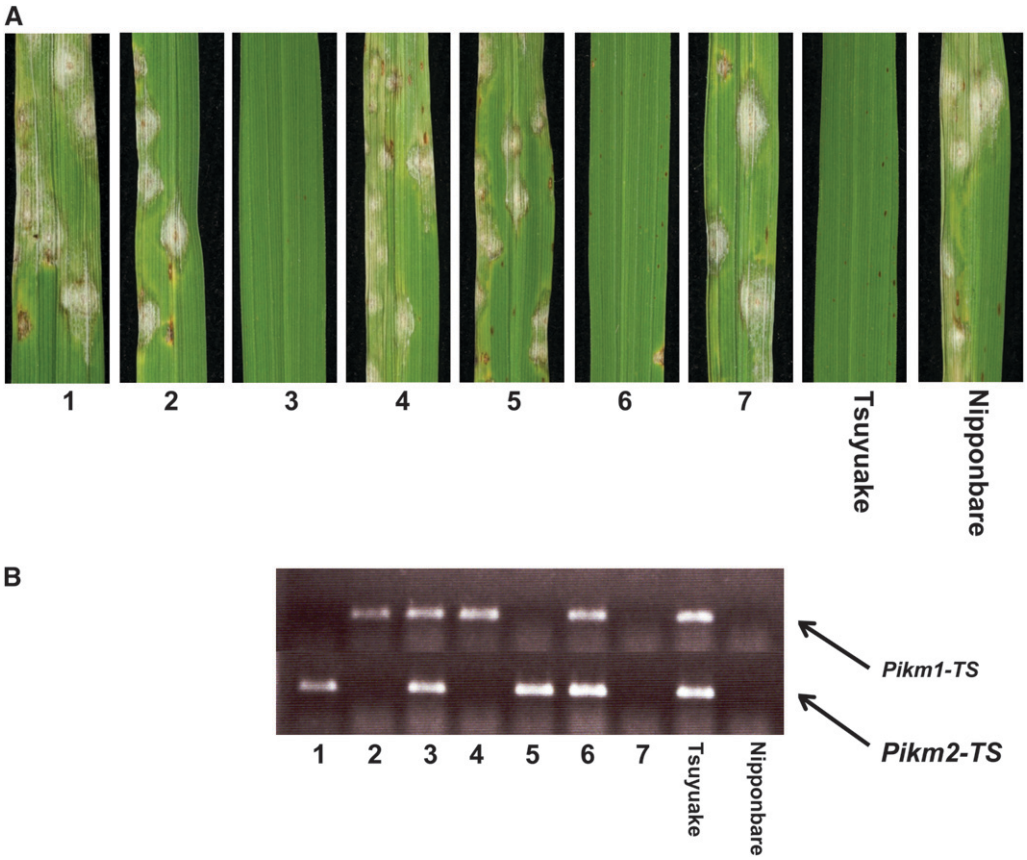

FIGURE 2.—Complementation testing and molecular analysis of the transgenic lines. (A) Reaction to inoculation with the blast isolate Ina 86-137 in Tsuyuake, Nipponbare, and a set of F_2 progeny (numbers 1–7) derived from a cross between a T_1 plant harboring *Pikm1-TS* and another T_1 plant harboring *Pikm2-TS*. The isolate Ina 86-137 is incompatible with the *Pikm*-donor cultivar Tsuyuake and compatible with the transformation-recipient cultivar Nipponbare. (B) Cosegregation of the resistance phenotype with the presence of both the *Pikm1-TS* gene and the *Pikm2-TS* gene. The presence of *Pikm1-TS* and *Pikm2-TS* in the transgenic lines is shown by the presence of a product from PCR amplification with the primers Gene2TY1 (top) and Gene1TY1 (bottom), respectively. The amplified products were separated through a 2.0% agarose gel. The transgenic lines numbered 3 and 6 contain both *Pikm1-TS* and *Pikm2-TS*, those numbered 2 and 4 contain only *Pikm1-TS*, those numbered 1 and 5 contain only *Pikm2-TS*, and that numbered 7 contains neither of the transgenes.

polypeptide (Figure 3) with a molecular weight of 126.85 kDa and a pI of 5.96. The NBS domain contained three sequence motifs characteristic to this domain, GLPGGGKTTI (beginning at residue 291), KKYLIVIDDI (beginning at residue 377), and GGRIIMTTRL (beginning at residue 405), corresponding to kinase 1a (P-loop), kinase 2, and kinase 3a, respectively. In the N-terminal region (residues 1–240), there exists a non-TIR (nT) motif of rice NBS–LRR genes (BAI *et al.* 2002), GLIAEV RELSYDLDDAVDDF (beginning at residue 74). A COILS analysis predicted the possible existence of a CC domain ($P = 0.5$) between residues 151 and 165. The interval between the kinase 1a and nT motifs (198 residues) was almost 70 residues larger than the interval of a typical rice NBS–LRR protein (~130 residues), suggesting that a polypeptide sequence was inserted in this region of the *Pikm1-TS*-encoded protein. The C-terminal domain contained ~16 imperfect LRR repeats, which were composed of ~14% leucine. This LRR region contained six repeat units that showed a good match to the consensus sequence, LxxLxxLxLxxTxxLxxLPxxLxx. Following these LRR repeats was a 103-residue non-LRR region. By comparison, *Pikm2-TS* encoded a smaller polypeptide, a 1021-residue NBS–LRR-class protein (Figure 4) with a molecular weight of 114.57 kDa and a pI of 8.64. The NBS domain of this protein also contained the three motifs, kinase 1a (beginning at residue 212), kinase 2 (beginning at residue 330), and kinase 3a (beginning at residue 359). Upstream of the NBS domain, an nT motif, WMKQIRDIAYDVECIDDF

TABLE 1

Disease reactions of transgenic plants containing *Pikm1-TS* and *Pikm2-TS* and of differential cultivars to *Magnaporthe grisea* isolates

Cultivar	Ina 86-137	Kyu 92-22	1804-4	H05-72-1	P2-b
Nipponbare	S	S	S	S	S
Transgenic plants	R	R	S	S	R
Differential cultivar[a] (*Pikm*)	R	R	S	S	R
Differential cultivar[b] (*Pik*)	R	S	S	S	R
K60 (*Pikp*)	R	S	ND	ND	S
IRBLkh-K3 (*Pikh*)	ND	R	S	R	ND

ND, resistance was not evaluated in this study; S, susceptible; R, resistant.

[a] Tsuyuake and/or IRRLkm-Ts was used.

[b] Kanto51 and/or IRRLk-Ka was used.

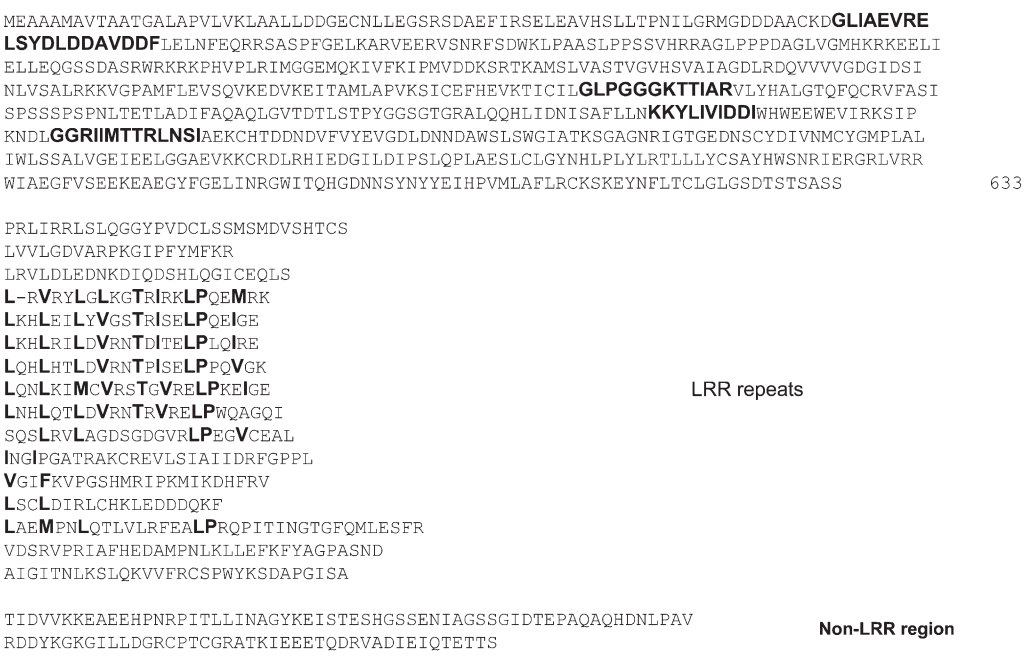

FIGURE 3.—Deduced amino acid sequence of the Pikm1-TS protein. The three conserved motifs, *i.e.*, kinase 1a, kinase 2, and kinase 3a, forming the NBS domain and the nT motif upstream of the NBS domain are in boldface type. The LRR repeat region and non-LRR region are shown detached from the rest of the sequence. The LRR region contains six repeat units that show a good match to the consensus sequence Lxx Lxx Lx Lxx-TxLxxLPxxLxx. In this LRR region, leucines and other residues matching the consensus sequence are shown in boldface type.

(beginning at residue 70), was also found, but a CC domain was not predicted. The C-terminal region was smaller than that of the *Pikm1-TS* product and contained 13 imperfect LRR repeats (residues 610–960) composed of ~17% leucine, but it did not contain a non-LRR region. The LRR repeats matching the consensus sequence were not as clearly discernible as in *Pikm1-TS*.

The susceptible cultivar Nipponbare contained the alleles of *Pikm1-TS* and *Pikm2-TS*, *i.e.*, *Pikm5-NP* (accession no. ABA95385) and *Pikm6-NP* (accession no. NP_001068487), respectively. *Pikm5-NP* and *Pikm6-NP*, respectively, encode 1125- and 1044-residue NBS–LRR-class proteins. The overall gene product architectures of *Pikm5-NP* and *Pikm6-NP* resembled those of the products of the respective Tsuyuake alleles (supplemental Figures S3 and S4): the products of both of the Nipponbare alleles contained an nT motif in the N-terminal region, three conserved motifs in the NBS region, and imperfect LRR repeats, whereas the *Pikm5-NP* product contains a C-terminal non-LRR region but *Pikm6-NP* does not. In contrast to these homologies revealed in the overall molecular design, at the amino-acid-sequence level these two proteins were markedly different from the products of their Tsuyuake alleles, especially in the N-terminal and the NBS regions (supplemental Figures S3 and S4). Through alignment of the protein sequences of these allelic genes by using the BLASTP program, the amino-acid-sequence identi-

FIGURE 4.—Deduced amino acid sequence of the Pikm2-TS protein. The three conserved motifs (*i.e.*, kinase 1a, kinase 2, and kinase 3a) forming the NBS domain and the nT motif upstream of the NBS domain are in boldface type. The C-terminal LRR is shown detached from the rest of sequence. In the LRR region, leucines and other residues matching the consensus sequence LxxLxxLxLxxTxLxxLPxxLxx are shown in boldface type.

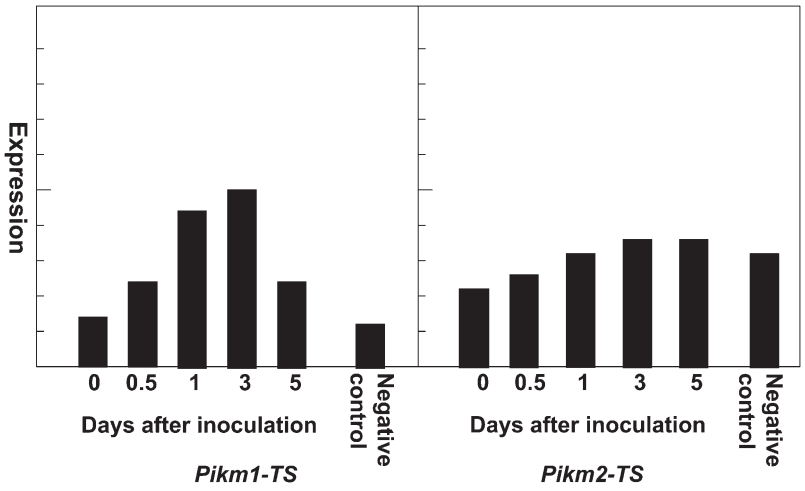

Figure 5.—Expression profiles of *Pikm1-TS* and *Pikm2-TS* in the cultivar Tsuyuake inoculated with the blast isolate Ina 86-137. Expression of these genes was examined 0, 0.5 (*i.e.*, 12 hr), 1, 3, and 5 days after inoculation, together with 5 days after inoculation without blast spores (negative control inoculation).

ties between *Pikm5-NP* and *Pikm1-TS* and between *Pikm6-NP* and *Pikm2-TS* were calculated to be as low as 59 and 76%, respectively.

Expression of *Pikm1-TS* and *Pikm2-TS in Tsuyuake*: To examine whether *Pikm1-TS* and *Pikm2-TS* were expressed in response to the challenge of blast infection, we performed quantitative RT–PCR to specifically detect each of the transcripts from the two genes in Tsuyuake at five time points (0, 0.5, 1, 3, and 5 days) after blast inoculation. Expression of both *Pikm1-TS* and *Pikm2-TS* was detected even at 0 DAI, indicating that both of the genes were constitutively expressed. Following blast inoculation, expression of *Pikm1-TS* increased from 0.5 to 3 DAI, and then declined toward the original level by 5 DAI (Figure 5). This induction of expression in *Pikm1-TS* was not detected in the negative control inoculations, indicating that the observed induction of *Pikm1-TS* expression was due to the challenge of blast infection. In contrast, although expression of *Pikm2-TS* appeared to increase slightly from 0.5 to 3 DAI, the extent of the induction was small (Figure 5).

DISCUSSION

We cloned and characterized the rice blast resistance gene *Pikm*. High-resolution mapping of this gene, followed by complementation analysis and evaluation of the resistance specificity of the transgenic plants, demonstrated that the blast resistance of *Pikm* is conferred by a combination of two genes, *Pikm1-TS* and *Pikm2-TS*. Both genes encode non-TIR NBS–LRR-class proteins. Although the products of each of the two genes contain all of the necessary structural motifs to independently function as NBS–LRR-class *R* proteins, the presence of the two genes in a plant is indispensable for the exhibition of blast resistance.

Pikm1-TS and *Pikm2-TS* resided adjacently at the *Pikm* locus as a cluster (Figure 1), but their structures differed. First, they differed in the number and position of introns: both *Pikm1-TS* and *Pikm2-TS* contained an intron at the immediate N-terminal side of the sequence encoding the kinase 2 motif in the NBS domain. In addition to this, *Pikm1-TS* contained another intron upstream of the sequence encoding the NBS domain. Second, the *Pikm1-TS* product contained a C-terminal non-LRR region, whereas that of *Pikm2-TS* did not. Third, *Pikm1-TS* contained fairly well-conserved repeat units matching a consensus sequence in its LRR domain, whereas *Pikm2-TS* did not. All of the above-mentioned structural differences indicate that these two genes did not evolve from one another by a simple duplication event.

Sequence comparison showed a marked difference in the genome organization at the *Pikm* locus between Tsuyuake and Nipponbare: Tsuyuake lacked the 127-kb segment present in the Nipponbare genome, whereas it contained a 70-kb segment that was absent in Nipponbare. Thus, of the 131-kb *Pikm* candidate region in the Tsuyuake genome, only 61-kb stretches had corresponding DNA segments in the Nipponbare genome. High levels of divergence in genome structure has been reported at many *R* gene loci, such as the *Pi9* locus in rice (Zhou *et al.* 2007), the *Rp1* locus in maize (Smith *et al.* 2004), and the *Rps2* (Mauricio *et al.* 2003), *Rpp13* (Rose *et al.* 2004), and *Rpp5* (Noel *et al.* 1999) loci in Arabidopsis. It has been proposed that the dynamics of genome organization and high polymorphism characteristic of the *R* gene loci may be essential for generating new resistance specificities in the process of evolution (Meyers *et al.* 2005). In this light, a comparative study of the genome structures at the *Pikm* locus in cultivars that have other *Pik* family genes would provide insight into the origin and evolution of allelic resistance genes at this locus.

Among the *R* genes characterized to date, we consider *Pikm* to be unique, because two independent NBS–LRR class genes are required to confer *Pikm*-specific resistance. To our knowledge, two examples have been reported in which expression of resistance requires two independent genetic determinants. The first exam-

ple is the gene *RPP2* in Arabidopsis (Sinapidou *et al.* 2004). Resistance to Cala2, specified by the *RPP2* locus in Arabidopsis Col-0, requires two genes, *RPP2A* and *RPP2B*, both of which reside at the *RPP2* locus and are NBS–LRR-class genes. Although a physical interaction between the products of these two genes was proposed (Sinapidou *et al.* 2004) for the resistance response of *RPP2*, no experimental evidence supporting this idea has yet been reported. These two genes contain differences in their structures: the *RPP2B* product contains all necessary conserved motifs to function as a NBS–LRR protein, whereas the *RPP2A* product lacks the kinase 1a motif in its NBS domain and its LRR domain appears too small; thus, *RPP2A* may not independently function as an NBS–LRR gene. This is in contrast to the case of the *Pikm* genes; although the structures of *Pikm1-TS* and *Pikm2-TS* were dissimilar, the products of both genes contained all of the necessary molecular motifs required for NBS–LRR proteins to function as *R* proteins. The second example is the *Pto*-resistance complex in tomato. Although the *Pto* gene complements resistance to the bacterium *Pseudomonas syringae* in susceptible tomato (Martin *et al.* 1993), another gene, *Prf*, which lies within the *Pto* cluster, is essential for effective resistance (Salmeron *et al.* 1996). *Pto* encodes a serine-threonine protein kinase, whereas *Prf* is an NBS–LRR-class gene. Although a number of models have been proposed for the possible interaction of those two genes, the epistatic relationship between the two has not been resolved. Recently, Mucyn *et al.* (2006) showed that these two genes constitutively interact with each other. They proposed that *Prf* acts as the transduction module and *Pto* acts in the complex as a regulatory subunit of *Prf*.

So far, the number of *R* genes identified that require more than two genetic determinants for their function is very limited, and information on the mechanism underlying the cooperative function of two such distinct genes is also limited. Cloning and characterization of other examples of such classes of resistance genes and follow-up studies that investigate how the products of the two genes interact would give us a better understanding of the function of these *R* genes.

The information we obtained on *Pikm* may help us to understand the resistance specificity of the genes belonging to the *Pik* family. As described earlier, the resistance spectra of most of the *Pik* family genes are similar. *Pik* and *Pikm*, especially, show a similar resistance response to distinct blast isolates. The resistance spectrum of *Pikm* is known to be broader than that of *Pik*, and no blast isolates that are incompatible with *Pik* but compatible with *Pikm* have been reported. This suggests that *Pikm* is composed of two, or more than two, genetic factors (Kiyosawa and Nomura 1988): one is *Pik* and the other is responsible for the resistance not covered by *Pik*, and the additive effect of these genes accounts for the broader resistance spectrum of *Pikm*. Our study showed that *Pikm* is not composed of a single genetic determinant, but that two genes are involved in its function. However, neither of the two seems to function as *Pik*, because transgenic lines that contained one of the two genes were susceptible to the blast isolate Ina 86-137, with which *Pik* is known to be incompatible. This result contradicts Kiyosawa's hypothesis. To understand the molecular mechanism underlying the difference in the resistance spectra of *Pik* and *Pikm*, cloning and characterization of *Pik* are needed as a next step.

The authors thank I. Ando and H. Satoh of the National Institute of Crop Science, Japan, for their kind technical advice on blast resistance evaluation; A. Shomura of the Institute of the Society for Techno-Innovation of Agriculture, Forestry, and Fisheries, Japan, for introducing the plasmids used for transformation into Agrobacterium; and K. Hayashi of the National Agricultural Research Center, Japan, for her cooperation in the genetic mapping of *Pikm*. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Green Technology Project QT-4005).

LITERATURE CITED

Bai, J., L. A. Pennill, J. Ning, S. W. Lee, J. Ramalingam *et al.*, 2002 Diversity in nucleotide binding site-leucine-rich repeat genes in cereals. Genome Res. **12:** 1871–1884.

Bryan, G. T., K. S. Wu, L. Farrall, Y. Jia, H. P. Hershey *et al.*, 2000 A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. Plant Cell **12:** 2033–2045.

Chen, X., J. Shang, D. Chen, C. Lei, Y. Zou *et al.*, 2006 A B-lectin receptor kinase gene conferring rice blast resistance. Plant J. **46:** 794–804.

Deyoung, B. J., and R. W. Innes, 2006 Plant NBS-LRR proteins in pathogen sensing and host defense. Nat. Immunol. **7:** 1243–1249.

Dodds, P. N., G. J. Lawrence and J. G. Ellis, 2001 Six amino acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the *P* and *P2* rust resistance specificities in flax. Plant Cell **13:** 163–178.

Ellis, J. G., G. J. Lawrence, J. E. Luck and P. N. Dodds, 1999 Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell **11:** 495–506.

Fuse, T., T. Sasaki and M. Yano, 2001 Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotechnol. **18:** 219–222.

Hayashi, K., N. Hashimoto, M. Daigen and I. Ashikawa, 2004 Development of PCR-based SNP markers for rice blast resistance genes at the *Piz* locus. Theor. Appl. Genet. **108:** 1212–1220.

Hayashi, K., H. Yoshida and I. Ashikawa, 2006 Development of PCR-based allele specific and InDel marker sets for nine rice blast resistance genes. Theor. Appl. Genet. **113:** 251–260.

Hulbert, S. H., C. A. Webb, S. M. Smith and Q. Sun, 2001 Resistance gene complexes: evolution and utilization. Annu. Rev. Phytopathol. **39:** 285–312.

International Rice Genome Sequencing Progect, 2005 The map-based sequence of the rice genome. Nature **436:** 793–800.

Inukai, T., R. J. Nelson, R. S. Zeigler, S. Sarkarung, D. J. Mackill *et al.*, 1994 Allelism of blast resistance genes in near-isogenic lines of rice. Phytopathology **84:** 1278–1283.

Kiyosawa, S., 1967 Inheritance of resistance of the rice variety Pi No. 4 to blast. Jpn. J. Breed. **17:** 165–172.

Kiyosawa, S., 1968 Inheritance of blast-resistance in some Chinese rice varieties and their derivatives. Jpn. J. Breed. **18:** 193–204.

Kiyosawa, S., 1969a Inheritance of resistance of rice varieties to a Philippine fungus strain of *Pyricularia oryzae*. Jpn. J. Breed. **19:** 61–73.

Kiyosawa, S., 1969b Inheritance of blast-resistance in west Pakistani rice variety. Pusur. Jpn. J. Breed. **19:** 121–128.

Kiyosawa, S., 1978 Identification of blast-resistance genes in some rice varieties. Jpn. J. Breed. **28:** 287–296.

Kiyosawa, S, and K. Nomura, 1988 Disease resistance in crop plants and mutational breeding. Gamma Field Symposia No. 27. Institute of Radiation Breeding, NIAR, MAFF, Ibaraki, Japan.

Li, L. Y., L. Wang, J. X. Jing, Z. Q. Li, F. Lin *et al.*, 2007 The *Pik-m* gene, conferring stable resistance to isolates of *Magnaporthe oryzae*, was finely mapped in a crossover-cold region on rice chromosome 11. Mol. Breed. **20:** 179–188.

Lin, F., S. Chen, Z. Que, L. Wang, X. Liu *et al.*, 2007 The blast resistance gene *Pi37* encodes a nucleotide binding site-leucine-rich repeat protein and is a member of a resistance gene cluster on rice chromosome 1. Genetics **177:** 1871–1880.

Liu, G., G. Lu, L. Zeng and G. L. Wang, 2002 Two broad-spectrum blast resistance genes, *Pi9(t)* and *Pi2(t)*, are physically linked on rice chromosome 6. Mol. Genet. Genomics **267:** 472–480.

Liu, X., F. Lin, L. Wang and Q. Pan, 2007 The *in silico* map-based cloning of *Pi36*, a rice coiled-coil-nucleotide-binding site-leucine-rich repeat gene that confers race-specific resistance to the blast fungus. Genetics **176:** 2541–2549.

Martin, G. B., S. H. Brommonschenkel, J. Chunwongse, A. Frary, M. W. Ganal *et al.*, 1993 Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science **262:** 1432–1436.

Martin, G. B., A. J. Bogdanove and G. Sessa, 2003 Understanding the function of plant disease resistance proteins. Annu. Rev. Plant Biol. **54:** 23–61.

Mauricio, R., E. A. Stahl, T. Korves, D. Tian, M. Kreitman *et al.*, 2003 Natural selection for polymorphism in the disease resistance gene *Rps2* of *Arabidopsis thaliana*. Genetics **163:** 735–746.

Messing, J., R. Crea and P. H. Seebung, 1981 A system for shotgun DNA sequencing. Nucleic Acids Res. **9:** 309–321.

Meyers, B. C., S. Kaushik and R. S. Nandety, 2005 Evolving disease resistance genes. Curr. Opin. Plant Biol. **8:** 129–134.

Mucyn, T. S., A. Clemente, V. M. E. Andriotis, A. L. Balmuth, G. E. D. Oldroyd *et al.*, 2006 The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. Plant Cell **18:** 2792–2806.

Noel, L., T. L. Moores, E. A. Van Der Biezen, M. Parniske, M. J. Daniels *et al.*, 1999 Pronounced intraspecific haplotype divergence at the *RPP5* complex disease resistance locus of Arabidopsis. Plant Cell **11:** 2099–2111.

Qu, S., G. Liu, B. Zhou, M. Bellizzi, L. Zeng *et al.*, 2006 The broad-spectrum blast resistance gene *Pi9* encodes a nucleotide-binding site-leucine-rich repeat protein and is a member of a multigene family in rice. Genetics **172:** 1901–1914.

Rose, L.E., P. D. Bittner-Eddy, C. H. Langley, E. B. Holub, R. W. Michelmore *et al.*, 2004 The maintenance of extreme amino acid diversity at the disease resistance gene, *RPP13*, in *Arabidopsis thaliana*. Genetics **166:** 1517–1527.

Rybka, K., M. Miyamoto, I. Ando, A. Saito and S. Kawasaki, 1997 High resolution mapping of the *indica*-derived rice blast resistance genes II. *Pita-2* and *Pita* and a consideration of their origin. Mol. Plant Microbe Interact. **10:** 517–524.

Sakata, K., Y. Nagamura, H. Numa, B. A. Antonio, H. Nagasaki *et al.*, 2002 RiceGAAS: an automated annotation system and database for rice genome sequence. Nucleic Acids Res. **30:** 98–102.

Salmeron, J. M., G. E. D. Oldroyd, C. M. T. Rommens, S. R. Scofield, H. S. Kim *et al.*, 1996 Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell **86:** 123–133.

Sinapidou, E., K. Williams, L. Nott, S. Bahkt, M. Tor *et al.*, 2004 Two TIR:NB:LRR genes are required to specify resistance to *Peronospora parasitica* isolate Cala2 in Arabidopsis. Plant J. **38:** 898–909.

Smith, S. M., A. J. Pryor and S. H. Hulbert, 2004 Allelic and haplotypic diversity at the *Rp1* rust resistance locus of maize. Genetics **167:** 1939–1947.

Takken, F. L. W., M. Albrecht and W. I. L. Tameling, 2006 Resistance proteins: molecular switches of plant defense. Curr. Opin. Plant Biol. **9:** 383–390.

Toki, S., 1997 Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol. Biol. Rep. **15:** 16–21.

Tsunematsu, H., M. J. T. Yanoria, L. A. Ebron, N. Hayashi, I. Ando *et al.*, 2000 Development of monogenic lines of rice for blast resistance. Breed. Sci. **50:** 229–234.

Wang, Z. X., M. Yano, U. Yamanouchi, M. Iwamoto, L. Monna *et al.*, 1999 The *Pib* gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. Plant J. **19:** 55–64.

Zhang, H. B., X. Zhao, X. Ding, A. H. Paterson and R. A. Wing, 1995 Preparation of megabase-size DNA from plant nuclei. Plant J. **7:** 175–184.

Zhou, B., S. Qu, G. Liu, M. Dolan, H. Sakai *et al.*, 2006 The eight amino-acid differences within three leucine-rich repeats between Pi2 and Piz-t resistance proteins determine the resistance specificity to *Magnaporthe grisea*. Mol. Plant Microbe Interact. **19:** 1216–1228.

Zhou, B., M. Dolan, H. Sakai and G. L. Wang, 2007 The genomic dynamics and evolutionary mechanism of the *Pi2/9* locus in rice. Mol. Plant Microbe Interact. **20:** 63–71.

Communicating editor: A. H. Paterson