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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

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ABSTRACT

The broad-spectrum rice blast resistance gene *Pi9* was cloned using a map-based cloning strategy. Sequencing of a 76-kb bacterial artificial chromosome (BAC) contig spanning the *Pi9* locus led to identification of six tandemly arranged resistance-like genes with a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) (*Nbs1-Pi9*–*Nbs6-Pi9*). Analysis of selected *Pi9* deletion mutants and transformation of a 45-kb fragment from the BAC contig into the susceptible rice cultivar TP309 narrowed down *Pi9* to the candidate genes *Nbs2-Pi9* and *Nbs3-Pi9*. Disease evaluation of the transgenic lines carrying the individual candidate genes confirmed that *Nbs2-Pi9* is the *Pi9* gene. Sequence comparison analysis revealed that the six paralogs at the *Pi9* locus belong to four classes and gene duplication might be one of the major evolutionary forces contributing to the formation of the NBS–LRR gene cluster. Semiquantitative reverse transcriptase (RT)–PCR analysis showed that *Pi9* was constitutively expressed in the *Pi9*-resistant plants and was not induced by blast infection. The cloned *Pi9* gene provides a starting point to elucidate the molecular basis of the broad-spectrum disease resistance and the evolutionary mechanisms of blast resistance gene clusters in rice.

DURING the last decade, >40 plant disease resistance (*R*) genes have been cloned from different plant species (Martin *et al.* 2003). Although cloned *R* genes confer resistance to a wide range of pathogens (fungi, viruses, bacteria, and nematodes), they share various conserved motifs, suggesting the existence of a common defense signal transduction pathway in different plant–microbe interaction systems (Dangl and Jones 2001; Martin *et al.* 2003). In general, the *R* genes fall into six distinct classes, the most prevalent of which is the nucleotide-binding site plus leucine-rich repeat (NBS–LRR) genes (Martin *et al.* 2003). The NBS domain of NBS–LRR proteins contains a number of conserved motifs, such as a central domain that is predicted to function as a nucleotide-binding site, kinase-1a or P-loop, kinase 2, and kinase 3a, and it may affect R protein function through nucleotide binding, hydrolysis, and the control of cell death (Martin *et al.* 2003). The LRR domains are generally thought to be involved in the interaction with avirulence (AVR) proteins and to be the major determinant of resistance specificity (Hulbert *et al.* 2001). Direct evidence of an interaction between the LRR domain of an R protein and its cognate AVR protein was provided by Jia *et al.* (2000) in experiments with the rice blast resistance protein Pi-ta and the AVR–Pi-ta from the fungal pathogen *Magnaporthe grisea*. Supporting evidence was subsequently provided 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, regions other than the LRR may also be involved in resistance specificity (Luck *et al.* 2000). For example, the proteins coded by the *L6* and *L7* genes for flax rust resistance, which have distinct race-specific resistance, are identical in the NBS–LRR region and differ at 11 residues in the N-terminal Toll/interleukin-1 receptor (TIR) domain (Ellis *et al.* 1999).

Clusters of *R* genes have been identified in diverse plant species (Islam and Shepherd 1991; Jones *et al.* 1993; Song *et al.* 1995, 1997; Kunkel 1996; Salmeron *et al.* 1996; Ellis *et al.* 1997; Meyers *et al.* 1998; Michelmore and Meyers 1998; Richter and Ronald 2000; Wei *et al.* 2002). The majority of the 149 NBS–LRR genes occurring in the Arabidopsis genome are clustered (Meyers *et al.* 2003) as are the >600 NBS–LRR genes identified in the rice genome (Bai *et al.* 2002). Moreover, the clustered *R* genes usually fall into heterogeneous

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries. The *Pi9* 76-kb contig is deposited under accession no. DQ285630.

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groups on the basis of their structural similarity, suggesting that each group may have evolved independently. Sequence analysis of a 261-kb region of the *Mla* locus in barley cv. Morex revealed that eight coiled-coil (CC)–NBS–LRR *R* gene homologs are classified into three dissimilar families, with <43% amino acid sequence similarity between families and 78–100% similarity within families (Wei *et al.* 2002). The rice *Xa21* gene, encoding a receptor-like kinase and conferring resistance to bacterial blight, is from a multigene family that contains two distinct classes of genes (Song *et al.* 1995, 1997; Wang *et al.* 1998). Four LRR receptor-like genes have recently been identified in a 67.2-kb region of the newly cloned bacterial blight *R* gene *Xa26* locus (Sun *et al.* 2004).

Rice blast is the most economically important fungal disease of rice because of its worldwide distribution (Ou 1985). Over 25 blast *R* genes have been mapped on the rice genome, many of which are allelic or closely linked (Kiyosawa 1989; Inukai *et al.* 1994; Wang *et al.* 1994; Pan *et al.* 1998; Chao *et al.* 1999). For example, 5 blast *R* genes have been identified at the *Pi-k* locus of chromosome 11 (Kiyosawa 1989; Inukai *et al.* 1994). *Pi-ta* and *Pi-ta*^{2} are allelic or at least very close to each other in the centromere region of chromosome 12 (Rybka *et al.* 1997), while *Pi5(t)* and *Pi3(t)* map at the same location on rice chromosome 5 (Inukai *et al.* 1996; Jeon *et al.* 2003). To date, only 2 blast *R* genes, *Pib* (Wang *et al.* 1999) and *Pi-ta* (Bryan *et al.* 2000), have been cloned. *Pib* belongs to the NBS–LRR class of genes and has been predicted to encode a cytoplasmic protein (Wang *et al.* 1999). *Pi-ta* differs from *Pib* and other NBS–LRR genes in that the protein predicted to be encoded by *Pi-ta* lacks a classic LRR in its C-terminal region, containing instead a highly imperfect repeating structure with 10 repeats of various lengths (from 16 to 75 amino acids), referred to as a leucine-rich domain (LRD) (Bryan *et al.* 2000).

Broad-spectrum disease resistance genes have been used for disease control in many different crops. There are two definitions for broad-spectrum disease resistance. The first one is defined as the resistance to the majority of geographically different isolates of the same pathogen. The second type is the resistance to two or more unrelated pathogens. Whether a broad-spectrum resistance gene is durable or not in multiple locations during a relatively long time is still debatable. To date, several first types of broad-spectrum *R* genes have been cloned in plants. *Xa21* is the *R* gene cloned with a high level of resistance to many *Xanthomonas oryzae* pv. *oryzae* strains (Wang *et al.* 1996). The recessive mutations (*mlo*) of the barley *Mlo* locus mediate a broad-spectrum resistance to all known isolates of powdery mildew fungus (*Blumeria graminis f.* sp. *hordei*) (Buschges *et al.* 1997). *mlo*-based disease resistance involves a spontaneous-lesion phenotype and cell-wall deposition in epidermal tissues preceding pathogen attack. Recently, the same *R* gene that was denoted *RB* by Song *et al.* (2003) and *RPI (Rpi-blb1)* by Van Der Vossen *et al.* (2003) was cloned from an NBS–LRR gene cluster. *RB/RPI* confers broad-spectrum resistance to all known races of the late blight pathogen *Phytophthora infestans*. The Arabidopsis *RPW8* gene (Xiao *et al.* 2001) belongs to the second type of broad-spectrum *R* gene and confers resistance to different isolates of the same powdery mildew pathogens as well as to different powdery mildew fungi. The protein it encodes is a small membrane protein with a putative coiled-coil domain that has limited homology to the N terminus of an NBS–LRR gene (Xiao *et al.* 2001). *RPW8* is not involved in the gene-for-gene interaction and may interact with different *AVR* genes from different pathogens.

The resistance in newly released rice cultivars to rice blast caused by *M. grisea* can be lost quickly due to the high level of instability in the genome of this fungus (Bonman *et al.* 1992). One way to overcome this problem is pyramiding of multiple *R* genes, each recognizing a unique set of *M. grisea* isolates, into a single cultivar. The deployment of rice cultivars with broad-spectrum resistance is another practical means of controlling the fungal pathogen (Bonman *et al.* 1992). The *Pi9* gene existing in the *indica* rice line 75-1-127 (Liu *et al.*, 2002) was introgressed from the wild species *Oryza minuta* (Sitch *et al.* 1989; Amante-Bordeos *et al.* 1992). Different from the rice lines carrying the blast *R* genes *Pib* and *Pi-ta*, 75-1-127 has broad-spectrum resistance to diverse *M. grisea* isolates. For example, 75-1-127 was tested with >100 Philippine *M. grisea* isolates at the International Rice Research Institute (IRRI) and no compatible isolates were identified (H. Leung, personal communication). In our previous study, 75-1-127 was highly resistant to 43 *M. grisea* isolates collected from 13 countries, and the *Pi9* locus was fine mapped on rice chromosome 6 (Liu *et al.* 2002). Here, we report the map-based cloning of the *Pi9* gene from a 76-kb region of an NBS–LRR multigene family. The application of multiple approaches, such as genomic sequencing of the targeted region, characterization of the *Pi9*-susceptible mutants, and the transformation of large-insert transformation-competent artificial chromosome (TAC) clones, facilitated our cloning effort. Functional analysis of the candidate genes and comparative analysis of the genomic structure of individual members of the *Pi9* gene cluster provide a starting point to elucidate the molecular basis of broad-spectrum disease resistance specificity and the evolutionary mechanisms of rice *R* gene clusters.

MATERIALS AND METHODS

DNA sequencing of rice BAC clones: Two BAC clones, 75-1-127BAC12 and 75-1-127BAC3, were fully sequenced using a shotgun method. Briefly, purified BAC DNA was sonicated with a sonicator. Sheared DNA fragments were size selected on an agarose gel and the 1.5- to 3-kb fragments were used in constructing a subclone library. The damaged DNA ends were

repaired using T4 DNA polymerase (Roche, Germany) and ligated to the pBluescript (KS) vector that had been previously digested with *Sma*I and treated with shrimp alkaline phosphatase (Roche, Germany). Approximately 700 individual clones from the 75-1-127BAC12 shotgun library and 450 individual clones from the 75-1-127BAC3 shotgun library were sequenced. Both ends were sequenced and all the readings were assembled with the Phred and Phrap software packages (Ewing and Green 1998; Ewing *et al.* 1998; Gordon *et al.* 1998; http://www.phred.org). At ~10 times redundancy, the sequence gaps as well as the low-quality regions of the consensus sequences were filled or resequenced and subsequently used for sequence assembly.

Computational analysis of DNA and protein sequences: For gene prediction from the rice genomic sequences, we ran GENSCAN (Burge and Karlin 1997; http://genes.mit.edu/GENSCAN.html) with the Arabidopsis organism option. The potential coding regions in the 76-kb DNA sequence were further searched against the sequences deposited in GenBank, using the BLAST programs (blastn, blastx, and blastp; Altschul *et al.* 1990, 1997; Gish and States 1993; http://www.ncbi.nlm.nih.gov/BLAST/). Pairwise comparisons between genomic sequences of the coding regions of *Pi9* candidate genes were performed using the BLAST program (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and the Matcher program (http://bioweb.pasteur.fr/seqanal/interfaces/matcher.html). Alignment of amino acid sequences was performed with the GAP program in the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, WI). Multiple alignment of DNA sequences was conducted using ClustalX version 1.83 (Thompson *et al.* 1997). On the basis of the results of ClustalX analysis, a phylogenetic tree was generated using the program TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html; Page 1996). The protein motif search was performed using the ScanProsite program (Sigrist *et al.* 2002; http://us.expasy.org/prosite/). The theoretical isoelectric point (pI) and protein molecular weight were computed as described (Wilkins *et al.* 1998; http://sosnick.uchicago.edu/pi_tool.html).

Vector construction and rice transformation: TAC vector pRTAC8 (Qu *et al.* 2003) was used for rice transformation of large genomic fragments. The DNA of 75-1-127BAC12 (Liu *et al.* 2002) was digested with *Not*I to obtain a 45-kb and a 12.5-kb genomic fragment (Figure 1A, 1–45,348 bp and 45,509–58,068 bp, respectively). The 45-kb fragment was cloned into pRTAC8 to make transformation constructs pRTAC8-45 kb (I) and pRTAC8-45 kb (II) (Figure 1B), which contained the 45-kb inserts in different orientations (Qu *et al.* 2003). pNBS4, the *Nbs4-Pi9* gene construct, was made by cloning the 12.5-kb *Not*I fragment into pRTAC8. The *Nbs5-Pi9* gene construct (Figure 1B, pNBS5) was prepared by *Hin*dIII partial digestion of 75-1-127BAC3 (Liu *et al.* 2002) and cloning of the 24.7-kb genomic fragment (Figure 1A, 49,808–74,581 bp) into the pRTAC8 *Hin*dIII site.

Transformation constructs of the individual *Nbs1-Pi9*, *Nbs2-Pi9*, and *Nbs3-Pi9* genes were constructed using the pCAMBIA1301 vector (GenBank accession no. AF234297; www.cambia.org). Both pNBS1-1 and pNBS1-2 (Figure 1B) were made for rice transformation of the *Nbs1-Pi9* gene. To construct pNBS1-1, the 10-kb *Pst*I fragment (Figure 1A, 13,613–23,605 bp) of 75-1-127BAC12 was cloned into the *Pst*I site of pCAMBIA1301. The pNBS1-2 construct was made by cloning the 6.9-kb *Sal*I fragment (Figure 1A, 12,391–19,301 bp) into the *Sal*I-digested pCAMBIA1301. pNBS2 (Figure 1B), the *Nbs2-Pi9* gene construct, was constructed by cloning the 13.5-kb *Sal*I fragment (Figure 1A, 32,363–45,848 bp) into the *Sal*I site of pCAMBIA1301. The *Nbs3-Pi9* construct, named pNBS3 (Figure 1B), was made by cloning the 14.6-kb *Hin*dIII fragment (Figure 1A, 18,395–33,070 bp) of 75-1-127BAC12 into the *Hin*dIII site of pCAMBIA1301.

Rice callus was induced from the embryos of the mature seeds of *japonica* rice cultivar TP309. Rice transformation was conducted using the Agrobacterium-mediated method (Hiei *et al.* 1994; Yin and Wang 2000; Qu *et al.* 2003).

Generation and screening of the *Pi9*-deletion mutants: The *Pi9* parental line 75-1-127 was mutated with the chemical mutagen diepoxybutane (DEB), using the method described by Wang *et al.* (2004). A total of 20,000 seeds of 75-1-127 were soaked in water overnight. The seeds were separated into two groups and treated with DEB for 4 hr, one group at 0.004% DEB and the other at 0.006% DEB. Treated seeds were washed thoroughly five times with water and sown in soil. About 60% of the seeds germinated after the 0.006% DEB treatment and 70% germinated after the 0.004% DEB treatment. M_1 plants from the DEB-induced populations were allowed to self-pollinate, and the seeds of ~12,000 M_1 plants were harvested from both treatments. The resulting M_2 seeds were harvested separately from each M_1 line. Three-week-old M_2 plants were inoculated with the *M. grisea* isolate PO6-6. Plants with visible lesions were picked out 6 days after inoculation. The susceptible phenotype in each susceptible M_2 plant was confirmed by blast inoculation of the M_3 plants. For identification of deletion mutations in the *Pi9* region, genomic DNA was isolated from the M_3 plants and PCR amplified with the primers (Table 3) specific to the individual candidate genes in the *Pi9* gene cluster.

Blast fungus inoculation: For blast inoculation, 3-week-old seedlings of parental and transgenic lines were inoculated with the *M. grisea* fungus as described (Liu *et al.* 2002). For blast inoculation of primary (T_1) transformed plants, the regenerated plants were kept in MS media and sprayed with a spore suspension (1×10^5 spores/ml) of the *M. grisea* isolate PO-6-6. The inoculated plants were placed into sealed containers to maintain humidity and grown in the dark for 24 hr. The plants were then transferred to a growth chamber and grown under a 12/12-hr (light/dark) photoperiod. Plants of the transformation recipient cultivar TP309 were used as a susceptible control. The disease reaction was examined 6–7 days after inoculation and scored as described (Liu *et al.* 2002).

Genomic DNA isolation and Southern blot hybridization: Rice genomic DNA was isolated using the CTAB method (Saghai-Maroof *et al.* 1994). Southern analysis was done according to standard methods (Sambrook *et al.* 1989). For preparation of the *Nbs2-Pi9* hybridization probe, the 928-bp genomic fragment (Figure 1A, 40,350–41,278 bp) of the *Nbs2-Pi9* gene was PCR amplified using Pi9 NBS2-O (5′-TACAACC ACCTACCATCCCAT-3′) and Pi9 NBS2-U (5′-TCTAGAACCT GCAAGTCTCG-3′) as specific primers and the pNBS2 construct DNA as template.

RNA isolation, mRNA purification, and semiquantitative RT–PCR: For full-length cDNA cloning, 3-week-old 75-1-127 plants were inoculated with *M. grisea* isolate PO6-6 (Liu *et al.* 2002). Total RNA was isolated from infected leaf tissue at 24 hr after inoculation (HAI) using Trizol (Invitrogen, San Diego). mRNA was purified with the Oligotex mRNA midi kit (catalog no. 70042; QIAGEN, Valencia, CA). cDNA was synthesized from poly(A)$^+$ RNA using the cDNA synthesis kit (Stratagene, La Jolla, CA) and used as template in two rounds of nested PCR. The primers for the first-round amplification were Pi9E (5′-CTTGAAGGGAGAGTCGAACGAA-3′) and Pi9-nbs2b (5′-TGTAATGATCAAGCAATATCTGGCT-3′). The Pi9E sequence (Figure 1A, 33,700–33,721 bp) is in the *Nbs2-Pi9* 5′-UTR region and 25 bp upstream of the *Nbs2-Pi9* start codon. The Pi9-nbs2b is complementary to the *Nbs2-Pi9* 3′–UTR sequence (Figure 1A, 43,797–43,821 bp). The forward primer of the second-round PCR was Pi9FSAL (5′-ATCGGTCGACATGGCGGAGA CGGTGCTGAG-3′) and the reverse primer was Pi9DRI (5′-CTGAGAATTCCCCTTGAGAAATTCGCCG -3′). The nested

reverse primers Pi9-nbs2b and Pi9DRI were designed in such a way that they matched perfectly with the *Pi9* sequence but had a 2-bp mismatch with *Nbs5-Pi9* at the 3′ end of each primer. Pi9FSAL contains the *Sal*I recognition sequence and 20 bp of the 5′-*Nbs2-Pi9*-coding sequence (Figure 1A, 33,725–33,744 bp). Pi9DRI contains the *Eco*RI site and the complementary sequence of 18 bp of the *Nbs2-Pi9* 3′-UTR sequence (Figure 1A, 43,639–43,656 bp). The amplified product of the first-round PCR was diluted 50 times with H_2O, and the amplicon was further used as a template for the second-round PCR. The first-round PCR was initiated by one cycle of 95° for 5 min, followed by 25 PCR cycles of 95° for 30 sec, 62° for 40 sec, and 72° for 4 min, with a final 10-min hold at 72°. The conditions of the second-round PCR were identical to those in the first-round PCR except that the annealing temperature was 63°. Platinum *Taq* DNA polymerase (Invitrogen) was used in the PCR for cloning *Pi9* full-length cDNA. The amplification was performed in a 50-μl PCR reaction volume containing 1× PCR buffer (20 mM Tris–HCl, pH 8.4; 50 mM KCl); 0.2 μM forward and reverse primers; 0.2 mM dATP, dCTP, dGTP, and dTTP; 1.5 mM $MgCl_2$; and 2.5 units of Platinum *Taq* DNA polymerase. The amplified cDNA fragment was digested with *Sal*I and *Eco*RI and cloned into pCAMBIA-1300.

For semiquantitative RT–PCR analysis of *Pi9* expression, seeds of the *Pi9* parental line 75-1-127 were dehusked and sterilized in 40% bleach for 40 min and then germinated on 1/2 MS medium in tissue culture vessels. Twelve-day-old plants were inoculated with *M. grisea* isolate PO-6-6, and leaf tissue was collected at 0, 6, 12, 24, 48, and 72 HAI. Total RNA was extracted using the RNeasy plant mini kit (QIAGEN). One microgram of total RNA was pretreated with RNase-free DNase I (Ambion, Austin, TX) and subjected to reverse transcription using the Reverse Transcription System (Promega, Madison, WI). Semiquantitative RT–PCR with *Pi9*-specific primers NBS2-G (5′-TGCCCAACCTTTACCCACTGTA-3′) and NBS2-H (5′-AACATGAGTAGAAACAAATTAGTTTG-3′) (Figure 6A) was performed with 26, 28, and 30 cycles. The control PCR of the *Actin 1* gene (Reece *et al.* 1990) was performed using the forward primer 5′-CGTCTGCGATAATGGAACTGG-3′ and reverse primer 5′-CTGCTGGAATGTGCTGAGAGAT-3′ with 24 cycles. The RT–PCR was initiated with one cycle at 95° for 5 min, followed by 26, 28, or 30 cycles at 94° for 30 sec, 62° for 40 sec, and 72° for 1 min 10 sec, and terminated with extension at 72° for 10 min. The PCR products were resolved on 1% agarose gels.

RESULTS

An NBS–LRR gene cluster was identified by sequence analysis of a 76-kb genomic region from the *Pi9* locus: A bacterial artificial chromosome (BAC) contig had been previously constructed using the *pB8*-hybridizing BACs isolated from the BAC library of *Pi9*-carrying rice line 75-1-127 (Liu *et al.* 2002). To obtain sequence information at the *Pi9* locus, we fully sequenced two of the BAC clones, 75-1-127BAC12 and 75-1-127BAC3, using the shotgun sequencing approach. Sequence analysis revealed that the genomic fragments are 58,068 and 40,075 bp in 75-1-127BAC12 and 75-1-127BAC3, respectively, and they overlap in a 21,872-bp region, thus forming a contig of 76,272 bp (Figure 1A).

Two different approaches were used to identify the open reading frames (ORFs) of the total genomic sequence. The gene prediction program GENSCAN (Arabidposis gene model) (Burge and Karlin 1997; http://genes.mit.edu/GENSCAN.html) was used to identify the putative coding sequence (CDS) in the 76-kb region, and the BLAST program was used for homology searches to confirm the gene prediction results because cloned plant NBS–LRR *R* genes are quite conserved. Seven putative genes were identified from the 76-kb sequence. The first gene in the 76-kb region (between 10,489 and 12,966 bp), located at the SP6 end in 75-1-127BAC12, is a homolog of the maize gene that encodes a putative nitrate-induced NOI protein (GenBank accession no. AF030385). The other six genes, denoted *Nbs1-Pi9–Nbs6-Pi9* (Figure 1A), were considered to be *Pi9* candidate genes because they all have high homology to the NBS–LRR disease *R* genes cloned from various plant species (Bent 1996; Dangl and Jones 2001; Martin *et al.* 2003). The *Nbs1-Pi9* genomic region (Figure 1A) shares a high homology (2942/2984; 98% identity) with rice cDNA clone J023131G18 (GenBank accession no. AK121397.1). The gene prediction for *Nbs3-Pi9* was partially confirmed on the basis of a 2351-bp 3′-partial cDNA fragment of *Nbs3-Pi9*, which was isolated from a 75-1-127 cDNA library using an *Nbs1-Pi9* DNA fragment as the hybridization probe; the *Nbs5-Pi9* gene prediction result was confirmed by the *Nbs5-Pi9* full-length cDNA cloned by RT–PCR (data not shown). The *Nbs4-Pi9* genomic sequence (Figure 1A) has a homology (1951/2066; 94% identity) with rice cDNA clone J013122I17 (GenBank accession no. AK067966.1). *Nbs4-Pi9* appears to be a pseudogene because there are four stop codons in the coding region. *Nbs6-Pi9* is located at the 3′ region of the 76-kb sequence and appears to be incomplete because its 3′ end (LRR region) is truncated and a solo-long terminal repeat (LTR) fragment is inserted in the 5′ region (Figure 1A, 69,147–72,143 bp). This solo-LTR fragment has 94% identity with the LTR of rice *gypsy*-type retrotransposon, RIRE8 (Kumekawa *et al.* 1999). The putative coding region of *Nbs6-Pi9* (Figure 1A, 68,550–69,146 bp and 72,144–76,272 bp) shares 94% identity with rice cDNA clone J013122I17.

Sequence comparison and phylogenetic analysis revealed that the six *Pi9* NBS–LRR genes belong to four types of paralogs: Pairwise comparison between the putative coding regions of the six paralogs was performed using both the Matcher (http://bioweb.pasteur.fr/seqanal/interfaces/matcher.html) and the BLAST (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) programs (Table 1). The solo-LTR insertion sequence (Figure 1A) in *Nbs6-Pi9* was removed before performing the BLAST search, and the two cloned blast *R* genes, *Pib* and *Pi-ta*, were also included in the comparison analysis. The sequence identity derived from the Matcher program was slightly lower than that from the BLAST program. The identity between the six paralogs ranged from 63.8 to 98.6% (Matcher) and from 71.8 to 98.6% (BLAST). Interestingly, the sequence homology between *Nbs2-Pi9* and *Nbs5-Pi9* (98.6% from both

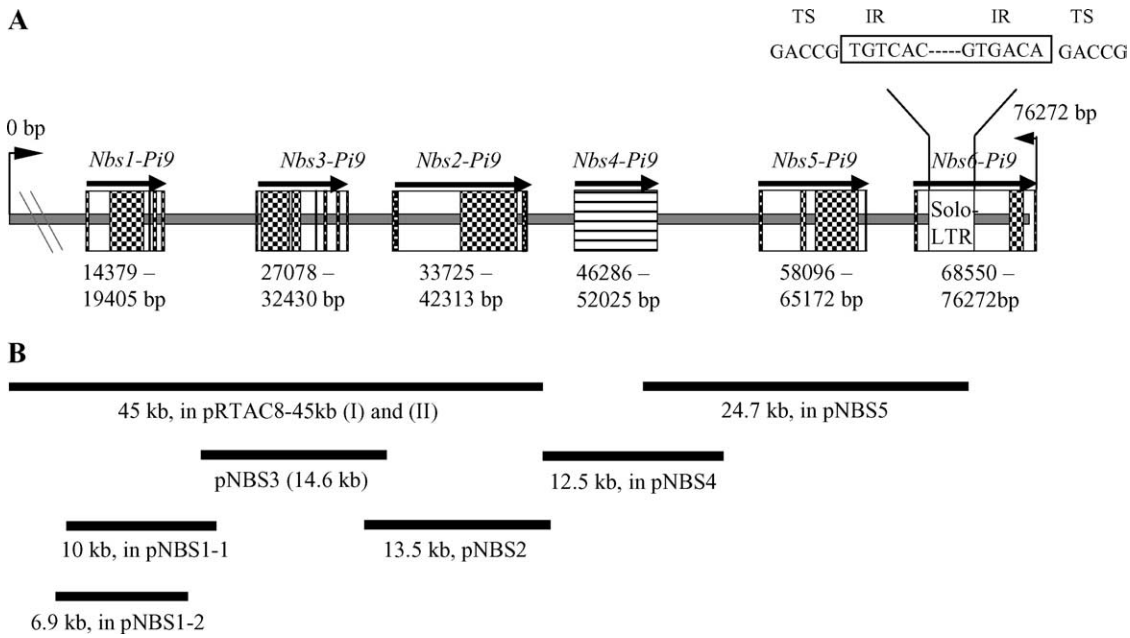

FIGURE 1.—Structure of the 76-kb genomic sequence in the *Pi9* region and the rice transformation constructs for complementation analysis of the candidate genes. (A) The six NBS–LRR genes (*Nbs1-Pi9*–*Nbs6-Pi9*) were predicted by GENSCAN and BLAST homology search; the *Nbs2-Pi9* and *Nbs5-Pi9* coding sequences were further confirmed by the *Nbs2-Pi9* and *Nbs5-Pi9* full-length cDNA sequences. The shaded line represents the whole 76-kb genomic sequence. The exons are indicated by stippled boxes and the introns by open boxes. The arrow above each NBS–LRR gene represents the transcription direction of the gene and the numbers below each gene show the start and stop sites of the coding region. No exon was identified in *Nbs4-Pi9* because four stop codons exist in the coding region; the *Nbs4-Pi9* region is shown as a box with horizontal lines. The 5′-partial region of *Nbs6-Pi9* is shown, and its coding region was disrupted by a solo-LTR retrotransposon. The direct repeats of the solo-LTR terminal sequence (TS) and the inverted repeats of the termini are shown. (B) Rice transformation constructs containing different genomic fragments from the 76-kb NBS–LRR gene cluster. The 45-kb fragment (bp 1–45,348) in pRTAC8-45 kb (I) and pRTAC8-45 kb (II) comprises the candidate genes *Nbs1-Pi9*, *Nbs2-Pi9*, and *Nbs3-Pi9* and has different cloning orientations. Construct pNBS5 carries a 24.7-kb fragment (49,808–74,581 bp) containing the *Nbs5-Pi9* gene. Construct pNBS4 carries a 12.5-kb fragment (45,509–58,068 bp) from the *Nbs4-Pi9* region. Constructs pNBS1-1 and pNBS1-2 carry a 10-kb fragment (13,613–23,605 bp) and a 6.9-kb fragment (12,391–19,301 bp), respectively, from the *Nbs1-Pi9* region. Construct pNBS2 contains a 13.5-kb fragment (32,363–45,848 bp) spanning the *Nbs2-Pi9* region. Construct pNBS3 carries 14.6 kb of the *Nbs3-Pi9* region (18,395–33,070 bp).

methods) and between *Nbs4-Pi9* and *Nbs6-Pi9* (91.9% from Matcher and 95.4% from BLAST) was much higher than that between the remaining pairs (Table 1). The promoter sequences of the two pairs were also highly homologous. These results suggest that the genomic fragment containing the *Nbs5-Pi9* and *Nbs6-Pi9* genes may be the duplication of the fragment containing the *Nbs2-Pi9* and *Nbs4-Pi9* genes.

To further investigate the relationship among the six *Pi9* candidate genes and their relationship with the other cloned blast *R* genes, the putative coding sequences of the six *Pi9* candidate genes and the rice blast *R* genes *Pib* (WANG *et al.* 1999) and *Pi-ta* (BRYAN *et al.* 2000) were compared by ClustalX multiple alignment and phylogenetic analysis (Figure 2). As shown in the phylogenetic tree, the degree of homology among these genes varies considerably, with the genes falling into five heterogeneous groups: *Nbs1-Pi9* (I), *Nbs4-Pi9*/*Nbs6-Pi9* (II), *Nbs2-Pi9*/*Nbs5-Pi9* (III), *Nbs3-Pi9* (IV), and *Pib*/*Pi-ta* (V). Therefore, the six NBS–LRR genes in the 76-kb *Pi9* multigene cluster were classified into four groups.

The protein sequences between the candidate genes were compared after the nucleotide sequences of *Nbs1-Pi9*, *Nbs2-Pi9*, *Nbs3-Pi9*, and *Nbs5-Pi9* were translated into amino acid sequences (Table 2). Neither *Nbs4-Pi9* nor *Nbs6-Pi9* was included in the analysis because neither one is likely to be an expressed gene due to the presence of four stop codons (*Nbs4-Pi9*) and a solo-LTR insertion (*Nbs6-Pi9*). The protein sequence identity among the pairs ranged from 55.2 to 98.1%, and the similarity among them ranged from 63.5 to 98.1%. Both the identity and similarity between Nbs2-Pi9 and Nbs5-Pi9 proteins were much higher than those of the other pairs (Table 2), further supporting the possibility of duplication in the *Nbs2-Pi9* and *Nbs5-Pi9* genomic regions.

Analysis of *Pi9* deletion mutants and large-insert DNA transformation localized the *Pi9* gene to the genomic region containing *Nbs2-Pi9* and *Nbs3-Pi9*: To narrow down the location of the *Pi9* gene in the gene cluster, the first approach was large-scale mutagenesis of the *Pi9* parental line 75-1-127 followed by PCR analysis of the deletions in the candidate genes in the susceptible mutants. More than 50 M_2 plants susceptible to *M. grisea*

TABLE 1

Percentage of DNA sequence identity among the six *Pi9* candidate genes

	Nbs2-Pi9	*Nbs3-Pi9*	*Nbs4-Pi9*	*Nbs5-Pi9*	*Nbs6-Pi9*	*Pib*	*Pi-ta*
Nbs1-Pi9	72.2[a]	67.1	69.1	72.4	68.4	52.5	54.0
	74.1[b]	77.1	75.2	74.5	78.0	No similarity	No similarity
Nbs2-Pi9		67.1	73.6	98.6	68.5	53.0	52.9
		75.3	87.6	98.6	76.6	No similarity	No similarity
Nbs3-Pi9			65.7	66.9	63.8	52.6	51.6
			76.1	71.8	75.1	No similarity	No similarity
Nbs4-Pi9				73.7	91.9	52.8	52.7
				87.6	95.4	No similarity	No similarity
Nbs5-Pi9					68.1	53.1	52.6
					76.6	No similarity	No similarity
Nbs6-Pi9						53.6	54.8
						No similarity	No similarity
Pib							52.1
							No similarity

The sequence comparisons were performed using the cDNA sequences of *Nbs2-Pi9*, *Nbs5-Pi9*, *Pib*, and *Pi-ta* and the putative coding sequences of *Nbs1-Pi9*, *Nbs3-Pi9*, *Nbs4-Pi9*, and *Nbs6-Pi9*.

[a] Derived from the comparison analysis using the Matcher program.

[b] Derived from the comparison analysis using the NCBI bl2seq program.

isolate PO6-6 were identified from a population of 12,000 M_1 plants. Twenty M_3 plants (Figure 3, 15 susceptible and 5 resistant) from six M_2 families that segregated for resistance to PO6-6 were analyzed by PCR using primers specific to each of the *Pi9* candidate genes (Table 3).

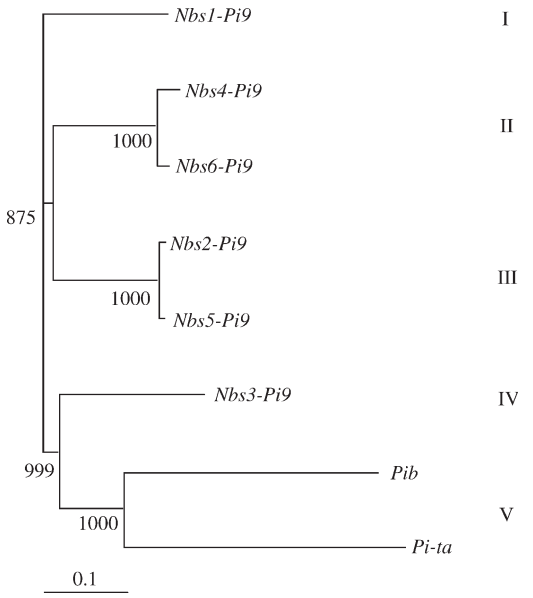

FIGURE 2.—Phylogenetic analysis of the six *Pi9* candidate genes. ClustalX version 1.83 (THOMPSON *et al.* 1997) was used for multiple alignment of the nucleotide sequences of *Nbs1-Pi9*, *Nbs2-Pi9*, *Nbs3-Pi9*, *Nbs4-Pi9*, *Nbs5-Pi9*, *Nbs6-Pi9*, *Pib* (WANG *et al.* 1999), and *Pi-ta* (BRYAN *et al.* 2000). On the basis of the ClustalX analysis results, the phylogenetic tree was further generated using the program TREEVIEW (PAGE 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Bootstrap values, corresponding to the match times of branching orders (1000 replicates), are shown at each branch point. The unit of branch length is 0.1 nucleotide substitutions per site, as indicated by a bar at the bottom left corner of the tree.

When the genomic DNA of the 20 M_3 plants was analyzed by PCR using the *Nbs2-Pi9* primers and *Nbs3-Pi9* primers, respectively, deletions in the two genes were detected in the 15 susceptible mutants (Figure 3). PCR analysis using primers specific to *Nbs1-Pi9*, *Nbs4-Pi9*, *Nbs5-Pi9*, or *Nbs6-Pi9* (Table 3) indicated that those mutants carried deletion mutations of varying sizes in the 76-kb *Pi9* region (Figure 3). The majority of the mutants had a large deletion located between *Nbs1-Pi9* and *Nbs6-Pi9*. The mutant M0566-2-5-r was resistant although a mutation was observed in the 3′ region of the *Nbs1-Pi9* gene, indicating that *Nbs1-Pi9* may not be the *Pi9* gene. In the PCR analysis of *Nbs5-Pi9*, 11 of the 15 susceptible mutants contained the *Nbs5-Pi9* region, suggesting that it also may not be the *Pi9* gene. Either *Nbs4-Pi9* or *Nbs6-Pi9* was not likely the *Pi9* gene because the *Nbs4-Pi9* region was detected in PCR of the susceptible mutant M0599-2-2-2 and the *Nbs6-Pi9* region was detected in 12 susceptible mutants (Figure 3). On the basis of all these results, we concluded that *Pi9* is located in the genomic region containing *Nbs2-Pi9* and *Nbs3-Pi9*.

Simultaneously, large-insert complementation was carried out by transformation of a 45-kb genomic fragment containing *Nbs1-Pi9*, *Nbs3-Pi9*, and *Nbs2-Pi9* (Figure 1B). The susceptible rice cultivar TP309 was transformed with Agrobacterium containing the constructs pRTAC8-45 kb (I) and pRTAC8-45 kb (II) (Figure 1B), respectively. Among the 48 T_2 transgenic lines obtained, one line (TAC106) transformed with pRTAC8-45 kb (II) showed a segregation of resistance and susceptibility to *M. grisea* isolate PO6-6. Southern blot analysis revealed that the *Nbs1-Pi9/Nbs3-Pi9/Nbs2-Pi9* region was transformed into the resistant transgenic

TABLE 2

Amino acid identity and similarity among the Nbs1-Pi9, Nbs2-Pi9, Nbs3-Pi9, and Nbs5-Pi9 proteins

	Nbs1-Pi9	Nbs3-Pi9	Nbs2-Pi9	Nbs5-Pi9
Nbs1-Pi9		57.75[a]	62.88	62.78
		66.23[b]	69.63	69.53
Nbs3-Pi9			55.36	55.16
			63.76	63.46
Nbs2-Pi9				98.06
				98.06

The amino acid sequences of the putative NBS–LRR proteins were compared with each other using the GAP program of the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, WI).

[a] Amino acid identity.

[b] Amino acid similarity.

line (data not shown), suggesting that *Pi9* is located in the region spanning *Nbs1-Pi9*, *Nbs2-Pi9*, and *Nbs3-Pi9*. The results from the large-insert transformation experiments confirmed that *Nbs2-Pi9* and *Nbs3-Pi9* remain to be the only candidates of the *Pi9* gene.

Rice transformation with individual candidate genes determined that *Nbs2-Pi9* is *Pi9* and that the *Pi9* transgenic line has the exact resistance spectrum with the *Pi9* donor line: To pinpoint the *Pi9* gene in the genomic region, we made two genomic constructs of the *Nbs2-Pi9* and *Nbs3-Pi9* genes. The pNBS3 construct (Figure 1B) contained a 14.6-kb (Figure 1A) genomic fragment comprising the *Nbs3-Pi9* gene. T_1 plants of 19 independently transformed lines were susceptible to PO-6-6, and all of the T_2 plants from nine transgenic lines were highly susceptible to PO-6-6. For rice transformation of *Nbs2-Pi9*, we developed the pNBS2 construct (Figures 1B and 4C), which contained a 13.5-kb *Sal*I genomic fragment (Figure 1B). This 13.5-kb fragment contained 1362 bp of the *Nbs2-Pi9* 5′-UTR region (32,962–33,724 bp), the whole *Nbs2-Pi9* coding sequence, and 1804 bp of the 3′-UTR sequence (42,314–44,117 bp). Of the 19 T_1 transgenic plants we inoculated with *M. grisea* isolate PO6-6, 13 were resistant and 6 were susceptible. We subsequently evaluated T_2 plants from 3 resistant T_1 lines (nos. 10, 12, and 77) and 1 susceptible T_1 line (no. 8) for resistance to PO6-6 and observed the segregation of resistant and susceptible plants in all three resistant lines. As shown in Figure 4A, plant no. 12-2R from the no. 12 T_2 line was highly resistant to PO6-6, while plant no. 12-1S from the same line was highly susceptible.

Cosegregation of the transgene and blast resistance was investigated in the T_2 generation of two *Nbs2-Pi9* transgenic lines (nos. 12 and 77; data not shown). The T_2 plants of transgenic lines 12 and 77 were inoculated with isolate PO6-6 and examined using the β-glucuronidase (GUS) enzymatic assay (Jefferson 1987). The GUS activity observed in the transgenic plants indicated the presence of the *Nbs2-Pi9* transgene because the T-DNA of transformation construct pNBS2 (Figure 4C) carries the GUS gene. In 44 T_2 plants of line 12, 37 plants were GUS-positive and resistant to PO6-6 (GUS+/R), and 7 plants were GUS-negative and susceptible to PO6-6 (GUS−/S). Similarly, 15 GUS+/R plants and 8 GUS−/S plants were identified in 23 T_2 plants of line 77. A chi-square test showed that the ratios of 37:7 (line 12) and 15:8 (line 77) fit the Mendelian 3:1 segregation ratio (both at the 95% level), suggesting the existence of a single T-DNA copy in these transgenic plants.

Cosegregation was further confirmed in transgenic line 12 using Southern blot analyses. Genomic DNA was extracted from 10 T_2 plants of the no. 12 line, digested with *Eco*RI, and probed with a 928-bp fragment from *Nbs2-Pi9* (Figure 4C). The 928-bp fragment is located within the second exon of the putative *Nbs2-Pi9*-coding sequence (Figure 1A) and corresponds to the sequence encoding the last 151 amino acids of the NBS domain

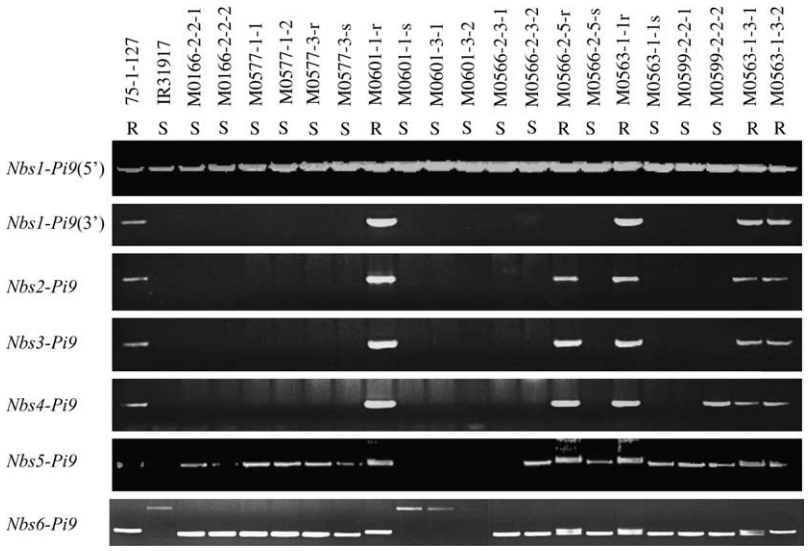

Figure 3.—PCR analysis of the *Pi9* deletion mutants. Genomic DNA from 15 susceptible (S) and five resistant (R) mutant lines was PCR amplified using primers (Table 3) specific to the *Nbs1-Pi9* 5′ or 3′ region, *Nbs2-Pi9*, *Nbs3-Pi9*, *Nbs4-Pi9*, *Nbs5-Pi9*, and *Nbs6-Pi9*. The five resistant plants analyzed in the PCR were obtained from different M_2 populations of the *Pi9*-susceptible mutants. The positive and negative controls were 75-1-127, the *Pi9* parental line, and IR31917, the recipient susceptible cultivar for the introgression of *Pi9* from *Oryza minuta* (Amante-Bordeos *et al.* 1992), respectively.

TABLE 3

PCR primers used for amplification of the *Pi9* candidate genes in the susceptible deletion mutants

Pi9 candidate gene	Forward primer	Forward primer sequence	Reverse primer	Reverse primer sequence	Product length (kb)
NBS1-Pi9 (5′)	19NBS-F_1	GTAGGTACATCA AGGACGAG	NBS/LRR-R1	AGGTGTTCGCCC CGC AGGT	1.6
NBS1-Pi9 (3′)	NBS/LRR-F_2	CACTGTTGTAGC GGAGGAGA	NBS/LRR-R2	CAGTACGCGATT TTC ATTGTTC	1.4
NBS2-Pi9	195R-1	ATGGTCCTTTATCTTTATTG	195F-1	TTGCTCCATCTCCTCTGTT	2.0
NBS3-Pi9	NBS3-F	AGATGTTAGTAGCAA GTTCC	NBS3-R	TGTCAGTTATGT CCAAAGTG	1.3
NBS4-Pi9	NBS4-F	ACTTTGTTGTGCTTGA TAAC	NBS4-R	ATGGTGAACGG TATCTGTAT	1.0
NBS5-Pi9	NBS5-F	AGAAAACTGGCTGGC TGTAG	NBS-R	TCACGTAGAGG AAAGAAAACC	0.7
Nbs6-Pi9	NBS6-F1	TCGTCACAGAATAATAATCAA	NBS6-R1	GGTTTCCCACTCTCTTACA	1.5

and the first 158 amino acids of the LRR domain (Figure 5). As shown in Figure 4B, all seven resistant T_2 plants (nos. 12-13, 12-14, 12-18, 12-20, 12-21, 12-29, and 12-30) contained the expected 11-kb *Nbs2-Pi9* band, while the three susceptible T_2 plants (nos. 12-5, 12-17, and 12-23) did not. The 11-kb hybridizing fragment was derived from the *Eco*RI digestion of the construct because the 13.5-kb *Sal*I genomic fragment had just one *Eco*RI site and the other *Eco*RI site was from the T-DNA region of the transformation vector (Figure 4C).

To verify whether the *Pi9* transgenic plants have the same resistance spectrum with the *Pi9* donor line 75-1-127, an additional 21 rice blast isolates collected from nine countries were inoculated with both the *Pi9* donor cultivar and the transgenic line (no. 12), the transformation recipient cultivar TP309, and the susceptible control cultivar CO39 (Table 4). The inoculation showed that the *Pi9* transgenic line had the exact same resistance spectrum with its donor line 75-1-127. It is noteworthy to mention that *Pi9* is susceptible to the Korean isolate R01-1 (lesion type 4).

To rule out the presence of any other functional genes in the cluster, we also transformed the susceptible cultivar TP309 with constructs containing the *Nbs1-Pi9*,

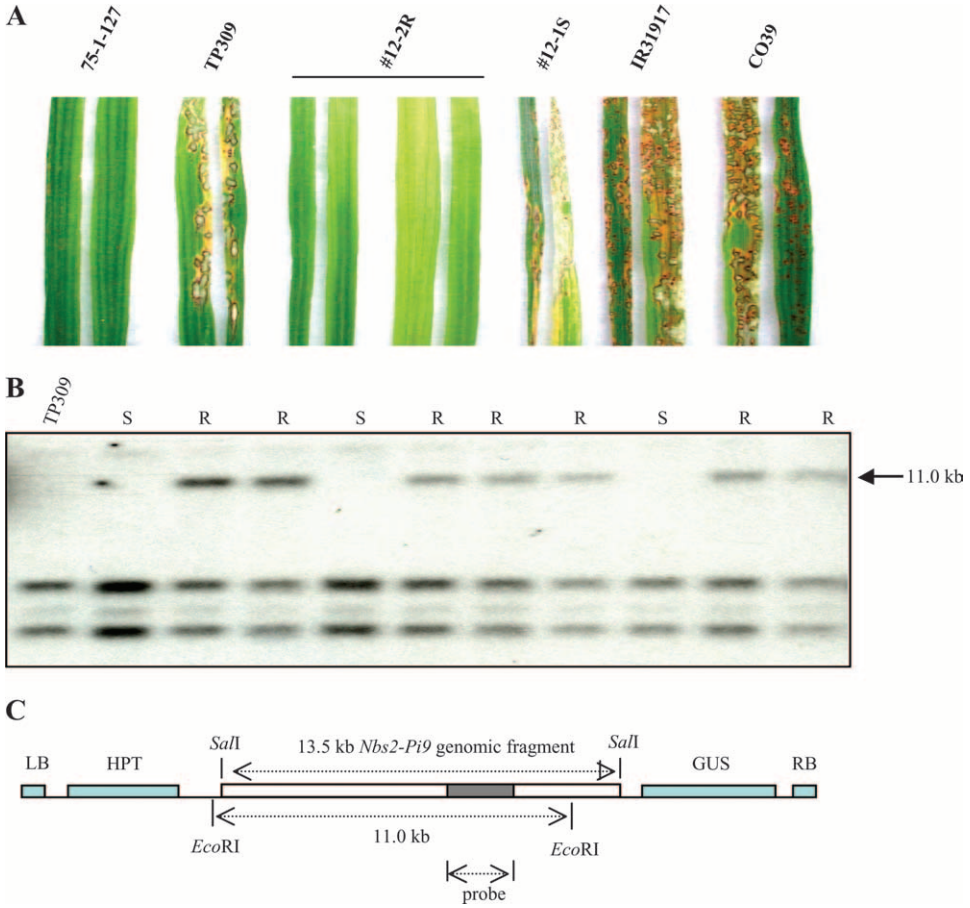

FIGURE 4.—Complementation of susceptible rice cultivar TP309 with the *Nbs2-Pi9* gene and cosegregation between the transgene and blast resistance phenotype. (A) 75-1-127 is a *Pi9*-resistant parent. IR31917 and CO39 are susceptible cultivars. Nos. 12-2R and 12-1S are resistant and susceptible plants, respectively, derived from the no. 12 T_2 line transformed with the *Nbs2-Pi9* construct pNBS2. (B) Southern blot analysis of 10 plants from the no. 12 T_2 line. Genomic DNA was digested with *Eco*RI and probed with a 928-bp *Nbs2-Pi9* fragment (Figure 1A, 40,350–41,278 bp). S and R represent susceptible and resistant T_2 plants, respectively. (C) Diagram of the pNBS2 construct and the location of the 928-bp *Nbs2-Pi9* hybridization probe.

MAETVLSMARSLVGSAISKAASAAANETSLLLGVEKDIWYIKDELKTMQAFLRAAEVMKKKDELLKV*WAE* 70
*QIRDLSYDIEDSLDEF*KVHIESQTLFRQLVKLRERHRIAIRIHNLKSRVEEVSSRNTRYNLVEPISSGTE 140
DDMDSYAEDIRNQSARNVDEAELVGFSDSKKRLLEMIDTNANDGPAKVICVVGMGGLGKTALSRKIFESE 210
EDIRKNFPCIAWITVSQSFHRIELLKDMIRQLLGPSSLDQLLQELQGKVVVQVHHLSEYLIEELKEKRYF 280
VILDDLWILHDWNWINEIAFPKNNKKGSRIVITTRNVDLAEKCATASLVYHLDFLQMNDAITLLLRKTNK 350
NHEDMESNKNMQKMVERIVNKCGRLPLAILTIGAVLATKHVSEWEKFYEQLPSELEINPSLEALRRMVTL 420
GYNHLPSHLKPCFLYLSIFPEDFEIKRNRLVGRWIAEGFVRPKVGMTTKDVGESYFNELINRSMIQRSRV 490
GIAGKIKTCRIHDIIRDITVSISRQENFVLLPMGDGSDLVQENTRHIAFHGSMSCKTGLDWSIIRSLAIF 560
GDRPKSLAHAVC 572

LDQLRMLRVLDLEDVTFLITQKDFDR 598
IALLCHLKYLSIGYSSSIYSLPRS
IGKLQGLQTLNMLRTYIAALPSE
ISKLQCLHTLRCSRKFVYDNFSLNHP
MKCITNTICLPKVFTPLVSRDDRAKQ
IAELHMATKSCWSESFGVKVPKG
IGKLRDLQVLEYVDIRRTSSRAIKE
LGHLSKLRKLGVITKGSTKEKCKILYAA
IEKLSSLQSLYVNAAL
LSDIETLECLDSISSPPPL
LRTLGLNGSLEEMPNW
IEQLTHLKKIYLLRSKLKEGKTMLI
LGALPNLMVLYLYWNAYLGEKLVFKTGAFPNLRTLR
IYELDQLREMRFEDGSSPLLEKIEISCCRLESG
IIGIIHLPRLKEISLEYKSK
VARLGQLEGEVNTHPNRP
VLRMDSDRRDHDLGAEAEG
SSIEVQTADPVPDAEGSVTVAVEATDPLPEQEGESSQSQVITLTTNDSEEIGTAQAG 1032

FIGURE 5.—Deduced amino acid sequence of the Pi9 protein. The 1032 amino acids of NBS–LRR protein encoded by the *Pi9* gene are shown. The NBS domain is between amino acids 193 and 572 and the LRR domain is between amino acids 573 and 975. In the NBS domain, the three underlined sequences, GMGGLGKT (positions 193–200), KRYFVILDDLW (positions 277–287), and GSRIVITTRNVDL (positions 307–319), correspond to kinase 1a (P-loop), kinase 2, and kinase 3a, respectively. The LRR domain is composed of 17 imperfect LRR repeats. The consensus is IXX(L)XX(L)XX(L) in which the L residues are in boldface type. The N-terminal region is the CC domain (positions 1–192). The "nT" sequence motif (*WAEQIRDLSYDIEDSLDEF*, positions 68–86) is in italics. At the C-terminal region, the last 57 amino acids (positions 976–1032) are a non-LRR sequence.

Nbs4-Pi9, and *Nbs5-Pi9* genes. For *Nbs1-Pi9*, we made two transformation constructs, pNBS1-1 and pNBS1-2 (Figure 1B), on the basis of the *Nbs1-Pi9* coding sequence predicted by GENSCAN. Forty and 28 independent transgenic lines were generated in rice transformation using the constructs pNBS1-1 and pNBS1-2, respectively. All the T_2 plants were susceptible to isolate PO-6-6. For rice transformation of *Nbs5-Pi9*, 12 independent T_1 lines were obtained by transforming the pNBS5 construct (Figure 1B). All the T_1 and T_2 plants were highly susceptible to isolate PO6-6. For *Nbs4-Pi9*, 46 independent transgenic lines were generated in rice transformation using pNBS4 (Figure 1B) and all the T_2 plants were also susceptible to isolate PO6-6. We did not make a complementation test for *Nbs6-Pi9* because our previous mapping results (LIU *et al.* 2002) suggested that *Pi9* may be upstream of the *Nbs6-Pi9* region, and importantly the *Nbs6-Pi9* itself contained a solo-LTR insertion (Figure 1A). Taken together, the inoculation tests of all transgenic lines confirmed that *Nbs2-Pi9* is the only functional gene responsible for the broad spectrum of *Pi9* at the locus.

TABLE 4

Disease reactions of *Pi9* transgenic plants to 21 *Magnaporthe grisea* isolates

Isolate	Country of origin	TP309	CO39	75-1-127	*Pi9* transgenic line 12
PH9	Philippines	S (5)	S (5)	R (2)	R (1)
36B23	China	S (5)	S (5)	R (0)	R (1)
86061ZE39	China	S (5)	R (2)	R (1)	R (2)
97-4-1	China	S (5)	S (5)	R (0)	R (0)
95116AZ93	China	S (5)	S (5)	R (1)	R (1)
75-49	China	S (5)	S (5)	R (1)	R (1)
97-5-1	China	S (5)	ND[a]	R (0)	R (0)
CHNOS	China	S (4)	S (5)	R (1)	R (0)
95097AZC13	China	S (5)	S (5)	MR (3)	R (0)
87088ZE3	China	S (5)	S (5)	MR (3)	R (2)
86062ZB15	China	S (5)	S (5)	R (0)	R (0)
CP16-32	Korea	S (5)	S (5)	R (0)	R (0)
R01-1	Korea	S (5)	S (5)	S (4)	S (4)
KJ201	Korea	S (5)	S (5)	R (2)	R (0)
ML25	Mali	S (5)	S (5)	R (0)	R (0)
ML8	Mali	S (5)	S (5)	R (2)	R (2)
O-249	India	S (5)	S (5)	R (0)	R (0)
DB-24	Burundi	S (5)	ND	R (0)	R (0)
IC-17	United States	S (5)	S (5)	R (1)	R (1)
GUY11	France	S (5)	S (5)	R (0)	R (1)
ES6	Spain	S (5)	S (5)	R (0)	R (0)

The number in parentheses was the highest disease score among the inoculated plants with the isolate.
[a] No data available.

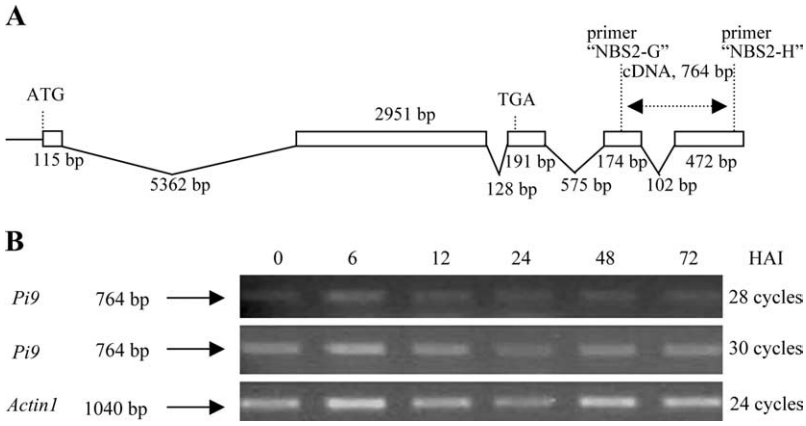

FIGURE 6.—The *Pi9* gene structure and its expression in the infected rice plants. (A) Structure of the *Pi9* gene and positions of the *Pi9*-specific primers used in semiquantitative RT–PCR analysis. Exons in the *Pi9* gene are indicated by horizontal lines and open squares. Introns are indicated by lines angled downward. The initiation (ATG) and termination (TGA) codons are also indicated as are the positions of the RT–PCR primers NBS2-G and NBS2-H. (B) Semiquantitative RT–PCR analysis of the *Pi9* expression. Total RNA was isolated from *Pi9*-resistant plants (75-1-127) at 0, 6, 12, 48, and 72 hr after inoculation (HAI). One microgram of total RNA was pretreated with RNase-free DNase I and subjected to reverse transcription. Semiquantitative PCR with 28 and 30 cycles was performed using *Pi9*-specific primers NBS2-G and NBS2-H.

***Pi9* encodes a putative NBS–LRR protein containing 1032 amino acids:** To isolate the cDNA fragment of *Pi9*, we prepared total RNA using leaf tissue from 75-1-127 collected 24 hr following blast inoculation. Two rounds of nested PCRs were performed to amplify the *Pi9* cDNA using two pairs of *Pi9*-specific primers (see MATERIALS AND METHODS). Because the *Nbs5-Pi9* gene shares high homology with *Pi9*, the first-round reverse primer and second-round reverse primer were designed in such a way that each primer sequence matched perfectly with *Pi9* but contained a 2-bp mismatch with *Nbs5-Pi9* at the 3′ end of each primer that minimized the amplification of the *Nbs5-Pi9* cDNA. Two PCR bands were amplified after the two rounds of PCR. The first band was ~4 kb, while the second PCR band was <1 kb and might originate from nonspecific amplification. Sequencing the 4-kb RT–PCR fragment indicated that it was a unique amplification product of the *Pi9* cDNA. The *Pi9* cDNA was 4009 bp, including 3099 bp of *Pi9* coding sequence and 910 bp of 3′-UTR. The sequencing result of the *Pi9* cDNA was exactly as predicted by the GENSCAN program.

Sequence alignment between the *Pi9* cDNA and its genomic sequence revealed that two introns interrupt the coding region. The length of the first intron in the coding region is 5362 bp (Figures 1A and 6A, 33,840–39,201 bp). The second intron is 128 bp (42,153–42,280 bp). We identified a 575-bp intron (Figures 1A and 6A, 42,474–43,048 bp) and a 102-bp intron (43,223–43,324 bp) in the *Pi9* 3′-UTR region. To find the transcription start site of the *Pi9* gene, we then scanned the genomic region preceding the NBS domain using the promoter prediction program available at http://www.cbs.dtu.dk/services/Promoter/ (KNUDSEN 1999). The result of the scan indicated that the transcription start site of the *Pi9* gene is at position base pair 32,962 in the 76-kb region (Figure 1A) and that the 5′-UTR is 763 bp long.

Protein translation of the cDNA sequence revealed that the *Pi9* gene encodes a predicted 1032-amino-acid polypeptide (Figure 5) with a molecular weight of 117.05 kDa and an pI of 7.55. The deduced Pi9 protein belongs to the NBS–LRR class of resistance proteins. Three sequences in the NBS domain, GMGGLGKT (Figure 5, positions 193–200), KRYFVILDDLW (positions 277–287), and GSRIVITTRNVDL (positions 307–319), correspond to kinase 1a (P-loop), kinase 2, and kinase 3a, respectively. In the CC domain (positions 1–192), the sequence motif (WAEQIRDLSYDIEDSLDEF, positions 68–86) belongs to the conserved non-TIR (nT) motif of rice NBS–LRR genes (BAI *et al.* 2002). At the C-terminal region is the LRR domain, which is composed of 17 imperfect LRR repeats.

The *Pi9* gene is constitutively expressed in the *Pi9*-resistant plants: To analyze the expression profile of the *Pi9* gene, semiquantitative reverse transcriptase (RT)-PCR was performed to specifically detect the *Pi9* transcript in *M. grisea*-infected rice plants. Following inoculation with isolate PO6-6, leaf tissue of 75-1-127 was collected at six different time points (0, 6, 12, 24, 48, and 72 HAI). Semiquantitative RT–PCR was performed using the *Pi9*-specific primer pair (NBS2-G and NBS2-H) that had been designed to amplify a 764-bp 3′-UTR sequence using 26, 28, and 30 RT–PCR cycles (Figure 6A). Only a faint band of the expected size was detected when 26 cycles were used, most likely due to the low expression level of the *Pi9* gene (data not shown). However, a stronger band was observed when 28 and 30 cycles were used (Figure 6B). The results from the three semiquantitative RT–PCRs indicated that the expression level of the *Pi9* gene in 75-1-127 before blast inoculation (zero time point) remains stable and does not change following inoculation (6–72 HAI). DNA sequencing of the RT–PCR products confirmed the true amplification of the *Pi9* cDNA fragment. These results showed that *Pi9* is constitutively expressed in the *Pi9*-carrying plants and is not induced by blast infection.

DISCUSSION

Map-based cloning of the *Pi9* gene from an *R* gene cluster: We have cloned *Pi9*, a broad-spectrum blast *R* gene in rice, using a map-based cloning strategy.

Because the *Pi9* parental line 75-1-127 was generated through a wide hybridization between cultivated rice and the wild rice *O. minuta* following repeated backcrosses (Amante-Bordeos *et al.* 1992), genetic and physical analysis of the *Pi9* locus was a challenging task. Like many other plant *R* genes (Meyers *et al.* 1998, 2003; Xiao *et al.* 2001), *Pi9* is a member of a complex *R* gene family with six candidate genes. To clone the *Pi9* gene, we employed three complementary strategies. First, we sequenced a 76-kb genomic region spanning the *Pi9* locus and identified six NBS–LRR candidate genes. This provided us with information on the genomic structure of the cluster. Second, we generated a *Pi9*-deletion population and analyzed the deletion sites within the *Pi9* gene cluster in the selected susceptible mutants, using seven pairs of gene-specific primers. PCR analysis indicated that *Pi9* is located in the genomic region containing *Nbs2-Pi9* and *Nbs3-Pi9*. At the same time, we transformed the susceptible rice cultivar TP309 with a 45-kb fragment containing *Nbs1-Pi9*, *Nbs2-Pi9*, and *Nbs3-Pi9*. On the basis of the results from both approaches, we were able to localize the *Pi9* gene in the *Nbs2-Pi9* and *Nbs3-Pi9* region. Transformation of the susceptible rice cultivar TP309 using individual constructs of *Nbs2-Pi9* and *Nbs3-Pi9* and blast inoculation tests of the transgenic plants with a total of 22 isolates finally confirmed that *Nbs2-Pi9* is the only functional gene responsible for the broad spectrum of the *Pi9* gene. Consequently, we can conclude that the application of complementary strategies such as the sequencing of BAC clones, the analysis of susceptible mutants, and large-insert transformation are useful procedures in map-based cloning of a targeted gene in a complex gene cluster.

The structural features of the *Pi9* gene and protein: On the basis of the deduced amino acid sequence, *Pi9* belongs to the NBS–LRR class of *R* genes. In contrast to the NBS–LRR genes from dicot plants, the NBS–LRR genes from cereals often have introns in the NBS region (Bai *et al.* 2002). The *Pib* gene (Wang *et al.* 1999) has two introns (1340 and 308 bp) in its coding region, while the *Pi-ta* gene has a 1463-bp single intron in its coding region (Bryan *et al.* 2000). Although *Pi9* also has two introns in its coding region, one of the introns is much larger (5362 bp) than that of the *Pib* gene. Whether this unique feature in the *Pi9* gene has any bearing on its broad resistance spectrum will require further investigation.

NBS–LRR genes in monocot plants carry a CC structure at the N-terminal region and belong to the CC–NBS–LRR (or CNL) subgroup of *R* genes (Bai *et al.* 2002; Meyers *et al.* 2003). An analysis of the structural features of the rice NBS–LRR genes showed that their CC domains are poorly conserved in the rice genome (Bai *et al.* 2002). One conserved motif (WVxxIRELAY DIEDIVDxY), designated nT, is usually located ~130 amino acids before the P-loop in rice NBS–LRR genes (Bai *et al.* 2002). The Pi9 protein has an nT motif (WAEQIRDLSYDIEDSLDEF) that is located 107 amino acids before the P-loop. This Pi9 nT motif has 57 and 61% of identities, respectively, with the Pib nT motif (WVKQVRDTAYDVEDSLQDF; Wang *et al.* 1999) and the Pi-ta nT motif (WAKEVRELSYDVDDFLDEL; Bryan *et al.* 2000), but has no significant similarity to the Xa1 nT motif (SLGRLRGLLYDADDAVDEL), the latter of which is an NBS–LRR gene and confers resistance to the rice bacterial blight pathogen (Yoshimura *et al.* 1998).

The LRR region of most rice NBS–LRR genes contains ~15% leucine but does not have the typical LRR repeats (Bai *et al.* 2002). One example is the Pi-ta protein, whose C-terminal domain, named the leucine-rich domain, lacks the characteristic LRR motif. However, although some imperfect LRR repeats exist in the Xa1 LRR domain, Xa1 has six almost perfect repeats, each with 93 amino acids (Yoshimura *et al.* 1998). The LRR domain in Pi9 is quite similar to that of the Pib protein and consists primarily of imperfect LRR repeats. A unique structural feature of the Pi9 protein is that it contains a 57-amino-acid non-LRR region at the C terminus. In contrast, the LRRs in both Pib and Pi-ta extend to the end of the C terminus. Further research is needed to investigate whether this 57-amino-acid sequence at the C terminus of Pi9 has any special function in regulating resistance specificity to rice blast.

At the protein level, the Pi9 protein is quite different from other cloned plant R proteins with broad-spectrum resistance. The cloned potato *RB* and *RPI* genes confer broad-spectrum resistance to the oomycete pathogen *P. infestans* and encode an identical CC–NBS–LRR protein (Song *et al.* 2003; Van Der Vossen *et al.* 2003). When the amino acid sequence of the Pi9 protein was compared with that of the RB/RPI protein, the identity and similarity were found to be only 25 and 42%, respectively. The Arabidopsis *R* gene *RPW8* confers broad-spectrum resistance to powdery mildew (Erysiphe) (Xiao *et al.* 2001) and shows no homology with any of the cloned genes. No significant similarity in amino acid sequence was observed between Pi9 and RPW8.

Genomic structure at the *Pi9* locus: We sequenced a 76-kb genomic region from the *Pi9* locus and identified six NBS–LRR genes that are arranged as tandem repeats. Identification of the *Pi9* multigene family is consistent with the findings from other *R* loci where the *R* genes are commonly clustered in the genome (Michelmore and Meyers 1998; Richter and Ronald 2000; Hulbert *et al.* 2001; Bai *et al.* 2002; Wei *et al.* 2002; Sun *et al.* 2004). From the viewpoint of plant evolution, the clustering of highly homologous *R* genes at a locus provides a variety of opportunities for plants to evolve new specificities of resistance when the corresponding *AVR* gene in the pathogen has mutated. Genetically linked gene families have more possibilities for recombination than do simple loci composed of one or a few genes (Meyers *et al.* 1998; Hulbert *et al.* 2001).

On the basis of phylogenetic analysis, the six NBS–LRR genes belong to four heterogeneous types, *i.e.*, *Pi9 (Nbs2-Pi9)/Nbs5-Pi9*, *Nbs4-Pi9/Nbs6-Pi9*, *Nbs1-Pi9*, and *Nbs3-Pi9*. High homology was observed between *Nbs2-Pi9* and *Nbs5-Pi9* (98% nucleotide identity) and between *Nbs4-Pi9* and *Nbs6-Pi9* (94% nucleotide identity). The genomic region of *Nbs5-Pi9)/Nbs6-Pi9* (58,096–76,272 bp) appears to be a duplication of the *Pi9* fragment (*Nbs2-Pi9)/Nbs4-Pi9* (33,725–52,025 bp). It is conceivable that the progenitor of *Nbs4-Pi9* might be a sequence like the solo-LTR-disrupted *Nbs6-Pi9* and the *Nbs4-Pi9* pseudogene might be derived from imprecise excision of the solo-LTR retrotransposon. It remains unclear how the gene introgression occurred during the wide hybridization between *O. minuta* and IR31917. It may be difficult to identify the original *O. minuta* plant that was used for the introgression cross. However, complete sequencing of the *Pi9* locus in one of the *O. minuta* lines will provide the information on the genome structure and sequence variation among the family members in the region.

The molecular mechanism of *Pi9*'s broad-spectrum resistance to *M. grisea*: Broad-spectrum disease resistance provides a useful resource for breeding disease-resistant crops as well as for understanding the molecular basis of resistance specificity in plants. Broad-spectrum resistance can be classified into two types: resistance to many isolates of the same pathogen and resistance to two or more unrelated pathogens. Representatives of the first type are the *RB* gene (Song *et al.* 2003) and the *RPI* gene (Van Der Vossen *et al.* 2003) that confer broad-spectrum resistance to nearly all known races of the late blight pathogen *P. infestans*. A good example of the second type is the Arabidopsis *R* gene *RPW8* that is resistant to two different powdery mildew fungal pathogens, *i.e.*, *Erysiphe cruciferarum* UEA1 and *E. cichoracearum* UCSC1 (Xiao *et al.* 2001). For rice blast pathogen *M. grisea*, several *R* genes with a relatively broad spectrum of resistance to diverse isolates have been identified (Chen *et al.* 1996; Liu *et al.* 2002; Jeon *et al.* 2003). Among them, the *Pi9* parent 75-1-127 carries broad-spectrum resistance to diverse blast isolates (Liu *et al.* 2002). It is possible that the broad-spectrum blast resistance in 75-1-127 is due to the action of several NBS–LRR genes. For example, more than one functional *R* gene has been reported to be located at the rice *Xa21* locus (Wang *et al.* 1998), the tomato *Cf2* locus (Dixon *et al.* 1996), and the Arabidopsis *RPW8* locus (Xiao *et al.* 2001). In this study, each member of the *Pi9* multigene family was functionally characterized by rice transformation and inoculation tests. In the inoculation tests with an additional 21 diverse isolates from nine countries, we confirmed that the *Pi9* transgenic line had the exact resistance spectrum with the donor line, suggesting that *Nbs2-Pi9* is the sole functional member in the cluster responsible for *Pi9*'s broad-spectrum resistance to *M. grisea*. Whether the *Pi9* gene is durable or not needs more field evaluations in multiple locations in the near future.

Elucidation of the molecular basis of *Pi9*'s broad-spectrum resistance is intriguing. It is possible that Pi9 can recognize a conserved molecule in different isolates of *M. grisea* or that different molecules in diverse isolates can be recognized by Pi9. LRR domains are thought to be the major determinant of specificity in *R* genes (Hulbert *et al.* 2001). Experiments with the rice *R* gene *Pi-ta* and with the *AVR–Pita* from the pathogen *M. grisea* have provided direct evidence of an interaction between the LRR domain of an *R* gene and its cognate AVR gene (Jia *et al.* 2000), while results from flax support this notion by allelic comparisons and domain-swapping experiments between different alleles at the flax *L* and *P* loci (Ellis *et al.* 1999; Dodds *et al.* 2001). Isolation of the AVR–Pi9 protein or of a conserved effector molecule(s) from *M. grisea* that interacts with Pi9 in rice cells will be an important step forward in our understanding of the molecular basis of *Pi9* broad-spectrum resistance. It is also possible that the Pi9 protein interacts with a RIN4-like protein that is the target of the AVR–Pi9 protein as demonstrated in Arabidopsis RPM1 and the AVRRpm1/AVRB system (Mackey *et al.* 2002).

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