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Authors

Lee, Sang-Kyu
Song, Min-Young
Seo, Young-Su
et al.

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Rice *Pi5*-Mediated Resistance to *Magnaporthe oryzae* Requires the Presence of Two Coiled-Coil–Nucleotide-Binding–Leucine-Rich Repeat Genes

Sang-Kyu Lee,*,[1] Min-Young Song,*,[1] Young-Su Seo,[†,1] Hye-Kyung Kim,* Seho Ko,* Pei-Jian Cao,[†] Jung-Pil Suh,[‡] Gihwan Yi,[§] Jae-Hwan Roh, Sichul Lee,[††] Gynheung An,[††] Tae-Ryong Hahn,* Guo-Liang Wang,[‡‡] Pamela Ronald[†] and Jong-Seong Jeon*,[2]**

**Plant Metabolism Research Center and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Korea, [†]Department of Plant Pathology, University of California, Davis, California 95616, [‡]IRRI-Korea Office (IKO), National Institute of Crop Science, Rural Development Administration, Suwon 441-857, Korea, [§]Rice Division, Yeongnam Agricultural Research Institute, National Institute of Crop Science, Rural Development Administration, Milyang 627-803, Korea, ** Crop Environment and Biotechnology Division, National Institute of Crop Science, Rural Development Administration, Suwon 441-857, Korea, [††]Department of Life Science and National Research Laboratory of Plant Functional Genomics, Pohang University of Science and Technology, Pohang 790-784, Korea and [‡‡]Department of Plant Pathology, Ohio State University, Columbus, Ohio 43210*

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ABSTRACT

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most devastating diseases of rice. To understand the molecular basis of *Pi5*-mediated resistance to *M. oryzae*, we cloned the resistance (*R*) gene at this locus using a map-based cloning strategy. Genetic and phenotypic analyses of 2014 F_2 progeny from a mapping population derived from a cross between *IR50*, a susceptible rice cultivar, and the *RIL260* line carrying *Pi5* enabled us to narrow down the *Pi5* locus to a 130-kb interval. Sequence analysis of this genomic region identified two candidate genes, *Pi5-1* and *Pi5-2*, which encode proteins carrying three motifs characteristic of *R* genes: an N-terminal coiled-coil (CC) motif, a nucleotide-binding (NB) domain, and a leucine-rich repeat (LRR) motif. In genetic transformation experiments of a susceptible rice cultivar, neither the *Pi5-1* nor the *Pi5-2* gene was found to confer resistance to *M. oryzae*. In contrast, transgenic rice plants expressing both of these genes, generated by crossing transgenic lines carrying each gene individually, conferred *Pi5*-mediated resistance to *M. oryzae*. Gene expression analysis revealed that *Pi5-1* transcripts accumulate after pathogen challenge, whereas the *Pi5-2* gene is constitutively expressed. These results indicate that the presence of these two genes is required for rice *Pi5*-mediated resistance to *M. oryzae*.

THE innate immune response is critical to the survival of plants and animals (Asai *et al.* 2002; Martin *et al.* 2003; Nimchuk *et al.* 2003; Ausubel 2005; Lee *et al.* 2006). The response is mediated by the detection of pathogen-associated molecular patterns (PAMPs) (also referred to as microbe-associated molecular patterns) or avirulence (Avr) proteins by pathogen recognition receptors (PRRs; also called pattern recognition receptors or disease resistance proteins). In animals, a family of cytosolic PRRs that contain a nucleotide-binding oligomerization domain (NOD) mediates the apoptotic and inflammatory responses critical to protection from pathogen invasion. Plants also contain a set of intracellular PRR proteins, called nucleotide-binding and leucine-rich repeat (NB–LRR) R proteins, which are structurally similar to animal NOD proteins. These plant NB–LRR proteins are characterized by a tripartite domain architecture consisting of an N-terminal coiled-coil (CC) or Toll/interleukin-1 receptor (TIR) domain, a central NB domain, and a C-terminal LRR domain (Hammond-Kosack and Jones 1997; Martin *et al.* 2003; Ting and Davis 2005; McHale *et al.* 2006; Liu *et al.* 2007a) and typically recognize pathogen-derived Avr proteins (also called effectors) (Van der Biezen and Jones 1998; Dangl and Jones 2001; Martin *et al.* 2003; Innes 2004; Ausubel 2005; Chisholm *et al.* 2006; Jones and Dangl 2006).

In contrast to this intracellular-type recognition, plants and animals also respond to pathogen molecules present at the cell surface. In animals, the recognition of PAMPs in the extracellular compartment is largely mediated by the Toll-like receptor (TLR) family of proteins, which contain LRRs in the extracellular domain (Brennan and Anderson 2004; Ausubel 2005). The TLR proteins associate with kinases of the non-arginine-aspartic acid (non-RD) class to transduce the immune response (Dardick and Ronald 2006). On the basis of the currently available data for plants, cell surface recognition of PAMPs is mediated by receptor kinases that also fall into the non-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. EU869185 and EU869186.

[1]These authors equally contributed to this work.

[2]*Corresponding author:* Plant Metabolism Research Center and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Korea. E-mail: jjeon@khu.ac.kr

RD class of kinases (Song *et al.* 1995; Zipfel *et al.* 2004, 2006; Lee *et al.* 2006).

It has been previously hypothesized that extracellular PRRs form homo- or heterodimers to transduce their function (Ronald 1997; Wang *et al.* 1998; Torii 2000; Chinchilla *et al.* 2007). For example, the rice XA21D resistance protein, which encodes a putative secreted LRR, is predicted to interact with an intact receptor kinase to transduce the associated resistance response (Wang *et al.* 1998). Arabidopsis FLS2 forms a complex with the BRI1-associated receptor kinase to transduce the innate immune response (Chinchilla *et al.* 2007). It has also been observed that cytoplasmically located NB-LRR R proteins recruit structurally similar proteins to transduce the response (Sinapidou *et al.* 2004; Peart *et al.* 2005; Ashikawa *et al.* 2008). For example, Arabidopsis *RPP2*-mediated resistance against *Peronospora parasitica* requires two TIR–NB–LRR proteins (Sinapidou *et al.* 2004). Similarly, the tobacco TIR–NB–LRR protein N requires tobacco *N requirement gene1* (*NRG1*), encoding a CC–NB–LRR protein to mediate resistance to tobacco mosaic virus (Peart *et al.* 2005).

Rice blast is one of the most devastating diseases of rice and occurs in all areas of the world where rice is cultivated (Ou 1985). More than 70 blast *R* genes that confer resistance to geographically different sets of the rice blast pathogen *Magnaporthe oryzae* isolates have been identified to date (Ballini *et al.* 2008). For example, *Pib* confers robust resistance to a majority of the Japanese *M. oryzae* isolates (Wang *et al.* 1999). In contrast, *Pi37* confers only partial resistance to Japanese isolates but complete resistance to Chinese isolates of the same pathogen (Chen *et al.* 2005). Hence, the isolation of multiple *R* genes is required to fully understand the molecular basis of the resistance to rice blast. Such characterization of these genes will facilitate development of agronomically useful rice cultivars through marker-assisted breeding or through transgenic approaches.

To date, a total of nine rice blast resistance genes have been cloned and characterized: *Pib* (Wang *et al.* 1999), *Pita* (Bryan *et al.* 2000), *Pi9* (Qu *et al.* 2006), *Pi2* and *Piz-t* (Zhou *et al.* 2006), *Pi-d2* (Chen *et al.* 2006), *Pi36* (Liu *et al.* 2007b), *Pi37* (Lin *et al.* 2007a), and *Pikm* (Ashikawa *et al.* 2008). With the exception of *Pi-d2*, a non-RD receptor-like kinase (Chen *et al.* 2006; Dardick and Ronald 2006), these genes all encode NB–LRR-type proteins. Distinct features of these cloned rice blast resistance genes have been observed. The Pib protein contains a duplicated NB region (Wang *et al.* 1999). Pita lacks a classic LRR but contains a leucine-rich domain (LRD) consisting of imperfect repeats of various lengths. A single amino acid difference at the Pita LRD was found to distinguish resistant from susceptible alleles (Bryan *et al.* 2000). The allelic genes *Pi2* and *Piz-t* show eight amino acid differences within three consecutive LRRs, and these residues are responsible for resistance specificity (Zhou *et al.* 2006). The *Pi9* gene strongly resembles the *Pi2* and *Piz-t* genes and is located within the same region on chromosome 6 (Qu *et al.* 2006; Zhou *et al.* 2006). The *Pikm*-mediated resistance requires two adjacent NB–LRR genes, *Pikm1-TS* and *Pikm2-TS* (Ashikawa *et al.* 2008). Among these cloned *R* genes, only Pita has been observed to interact with the corresponding *M. oryzae* avirulence protein, AvrPita (Jia *et al.* 2000). Thus, defense signaling mediated by NB–LRR-type proteins remains poorly characterized in rice.

It has been reported that *Pi5* confers resistance to many *M. oryzae* isolates collected from Korea and the Philippines (Wang *et al.* 1994; Chen *et al.* 2000; Han 2001). To gain a further understanding of the molecular basis of *Pi5*-mediated rice blast resistance, we used a map-based method to isolate the *Pi5* genomic region. We previously mapped *Pi5* to a 170-kb interval on the short arm of chromosome 9 in the *RIL260* rice cultivar (Jeon *et al.* 2003). In our study, *Pi5* was more precisely mapped to a smaller physical interval using a new mapping population derived from a cross between *RIL260* and *IR50* lacking *Pi5*. Through sequence analysis of the *Pi5* genomic region, two candidate blast resistance genes were identified on the basis of the presence of CC–NB–LRR domains in the predicted proteins. These two genes were designated *Pi5-1* and *Pi5-2*. We subsequently carried out detailed genetic analysis to determine the function of each of these genes. Surprisingly, *Pi5*-mediated resistance required the presence of both *Pi5-1* and *Pi5-2* gene products. In response to pathogen inoculation, *Pi5-1* transcripts accumulated. In contrast, the *Pi5-2* gene was constitutively expressed.

MATERIALS AND METHODS

Plant materials: The *RIL260* rice cultivar carrying the *Pi5* allele and a rice blast-susceptible cultivar, *IR50*, were used as the parental lines. The *RIL260* and *IR50* cultivars were crossed to generate a mapping population for genetic linkage analysis. Self-pollinated seeds (F_2) of the *RIL260/IR50* F_1 individuals were collected to obtain a sufficiently large mapping population. A *japonica* rice cultivar, *Dongjin*, was used as the susceptible control in the *M. oryzae* inoculation and rice transformation experiments. *RIL260* and the monogenic rice line *IRBL5-M* carrying *Pi5* (Tsunematsu *et al.* 2000; Yi *et al.* 2004) were used as the resistant control cultivars in the *M. oryzae* inoculation experiments. An additional eight monogenic rice lines, *IRBLi-F5*, *IRBL9-W*, *IRBLb-B*, *IRBLta-K1*, *IRBLz-Fu*, *IRBLks-F5*, *IRBLkm-Ts*, and *IRBLsh-S*, and the susceptible background cultivar of these monogenic lines, *Lijiangxintuanheigu* (*LTH*) (Tsunematsu *et al.* 2000; Yi *et al.* 2004), were also used in the inoculation experiments to determine the virulence pattern of *M. oryzae* isolates. Rice seedlings were grown in a greenhouse at 30° during the day and at 20° at night in a light/dark cycle of 14 hr/10 hr.

Pathogen inoculation and disease evaluation: *M. oryzae* PO6-6, a Philippine isolate, which is incompatible with the *Pi5* resistance locus, has been commonly used to detect this locus (Wang *et al.* 1994; Jeon *et al.* 2003; Yi *et al.* 2004). To analyze blast resistance in *Pi5* transgenic rice plants, an additional five different Korean *M. oryzae* isolates, KJ105a, KJ107, KJ401, KI215, and R01-1, were used. All inoculations and disease

TABLE 1

PCR primers used in this study

Marker or gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
C1454	GTATTACCTGAAATCCTAGTGGTG	AGGAACTACGGTATTACAAGGATC
JJ817	GATATGGTTGAAAAGCTAATCTCA	ATCATTGTCCTTCATATTCAGAGT
JJ803	AAGTGAGCATCCAGTGCCTAATGA	AGCCGGTGCTCATAACACGTATTA
Pi5-1	TACAAGTTGGCAGCTTTATCTGAG	TCAGAAGCACTGGATCTTTCTGCA
Pi5-2	AGTGAACTCCAAACATGTGAACAC	TCATACCTGTTGCGGTTTCTGCCT
Actin1	GGAACTGGATAGGTCAAGGC	AGTCTCATGGATACCCGCAG
PBZ1	ACCATCTACACCATGAAGCTTAAC	GTATTCCTCTTCATCTTAGGCGTA

evaluations were conducted in the greenhouse facilities at Kyung Hee University using a method that was slightly modified from Liu *et al.* (2002). Three-week-old plants of the F_3 progeny of each of the identified recombinant lines and transgenic plants were used in the inoculation experiments. *M. oryzae* was grown on oatmeal agar medium for 2 weeks at 24° in the dark. Conidia were induced 4 days prior to collection by scratching the plate surface with a sterilized loop. The inoculated plants were placed in sealed containers to maintain humidity at 24° in darkness for 24 hr and then transferred to a growth chamber at 24° and 80% humidity under a 14-hr/10-hr (light/dark) photoperiod. Disease evaluation was carried out 7 days after inoculation.

Genotypic analysis of progeny from the *RIL260/IR50* mapping population: Cleaved amplified polymorphic sequence (CAPS) markers for C1454 (Jeon *et al.* 2003) and JJ817 (Kwon *et al.* 2008) and the sequence characterized amplified region (SCAR) marker JJ803 (corresponding to the previously reported dominant marker JJ80-T3) (Yi *et al.* 2004) were used for the analysis of the *RIL260/IR50* segregating progeny (Table 1). The dominant markers JJ113-T3 and S04G03 were additionally utilized as needed (Jeon *et al.* 2003; Yi *et al.* 2004).

Genomic DNA was isolated from young leaves of rice plants using a simple miniprep method (Chen and Ronald 1999). PCR analysis was performed in a final volume of 30 μl (100 pm of each primer, 200 μm each of dNTPs, 10 mm Tris–HCl, pH 9.0, 2 mm $MgCl_2$, 50 mm KCl, 0.1% Triton X-100, and 0.5 units Taq polymerase) using 50 ng of genomic DNA as template. PCR products for the CAPS markers C1454 and JJ817 were subsequently digested with *Mlu*I and *Ase*I, respectively, and were then size-fractionated on agarose gels.

DNA sequencing and gene prediction: *RIL260* binary BAC (BIBAC) clones spanning the *Pi5* locus were selected for DNA sequencing analysis (Tsunoda *et al.* 2000; Jeon *et al.* 2003). Plasmids purified by a mini-preparation (Jeon and Ronald 2007) were partially digested with *Sau*3AI and separated by agarose gel electrophoresis. The 0.5- to 3.0-kb genomic DNA fragments were isolated using a commercial kit (gel extraction kit, Qiagen), subcloned into the *Bam*HI site of pBluescriptII SK(−) (Clontech), and then transformed into *Escherichia coli* DH10B by electroporation. For DNA sequencing of each BIBAC clone with a 25-kb average insert size, ~60 clones were selected and sequenced in one or both directions using the T3 and T7 primers.

Similarity searches against the NCBI database (http://www.ncbi.nlm.nih.gov/) were performed using BLAST (Basic Local Alignment Search Tool). To predict protein-coding gene regions, the Rice Genome Automated Annotation System (RiceGAAS) was utilized (Sakata *et al.* 2002; http://RiceGAAS.dna.affrc.go.jp/).

Vector construction for genetic complementation experiments: Genomic DNA regions for *Pi5-1* and *Pi5-2* were reconstituted by subcloning from BIBAC clones (Jeon *et al.* 2003). To construct a clone carrying the entire *Pi5-1* coding region, a 6.6-kb *Bam*HI–*Sac*I fragment of the JJ80 vector that includes the 0.5-kb predicted promoter was subcloned into the binary vector pC1300intC (GenBank accession no. AF294978). The resulting plasmid JJ104 was digested with *Bam*HI and *Bst*EII and fused to 7.3-kb *Hin*dIII–*Bst*EII insert of JJ106 to construct JJ105 with a 5.2-kb promoter region. The 0.5-kb *Sac*I–*Xho*I fragment was amplified by PCR using primers 5′-GTCCAAAGAGAAATGCGACAACAC-3′ and 5′-CGCTCGAG GTGGCATTTCATCCAATAGGCAAC-3′. The resulting product was inserted into the JJ105 to extend the terminator region, yielding the JJ204 construct carrying the 11,516-bp *Pi5-1* genomic region.

The *Pi5-2* gene was constructed by the multiple ligation of the following four fragments: a 4.2-kb *Eco*RI–*Bgl*II DNA fragment of JJ113, a 200-bp *Bgl*II–*Cla*I PCR product amplified using the primers 5′-GGATGATGTGATCTGCAGAGAAAC-3′ and 5′-CAGCCTCACTGAAATTGCGAAGCA-3′, a 4.2-kb *Cla*I–*Xba*I DNA fragment of JJ120, and an *Eco*RI–*Xba*I-digested pC1300intC vector fragment. In the resulting construct JJ117, the promoter region was extended by cloning the 3.7-kb *Nsi*I–*Eco*RI fragment of JJ120. Finally, by inserting a 0.9-kb extended terminator sequence into the *Eco*065I site of the JJ142 plasmid, the 13,250-bp entire genomic sequence of *Pi5-2* in JJ212 was constructed. The cloned genomic sequences in JJ204 and JJ212 were confirmed by DNA sequencing.

Production of transgenic rice plants: Genomic clones for *Pi5-1* and *Pi5-2* were transformed into *Agrobacterium tumefaciens* EHA105 or LBA4404 by electroporation and introduced into the susceptible rice cultivar *Dongjin* via Agrobacterium mediation according to an established procedure (Jeon *et al.* 2000). The transgenic plants (T_0) were self-pollinated and T_1 seeds were collected. Homozygous *Pi5-1* (*Pi5-1-63*) and *Pi5-2* (*Pi5-2-74*) transgenic lines were then selected from T_2 progeny resulting from self-pollination of the T_1 lines on the basis of the segregation patterns of the transgenes. F_1 plants carrying both *Pi5-1* and *Pi5-2* were produced from a cross between *Pi5-1-63* and *Pi5-2-74* lines and self-pollinated to produce F_2 plants.

Isolation of *Pi5-1* and *Pi5-2* cDNAs: Two preparations of total RNA were prepared from rice leaves collected at 24 and 48 hr after inoculation with *M. oryzae* PO6-6 using Trizol reagent (Invitrogen). Purified mRNAs were obtained using the PolyATtract mRNA isolation system (Promega) from each set of total RNA and mixed in a 1:1 ratio for cDNA synthesis. cDNAs larger than 0.5 kb were selected by size fractionation via gel filtration, and a cDNA library was constructed with the Uni-ZAP XR vector (Stratagene). This library was then screened via colony blot hybridizations using probes corresponding to the *Pi5-1* and *Pi5-2* coding regions, a 570-bp *Hin*dIII–*Kpn*I fragment of JJ204 and a 589-bp *Eco*RV–*Spe*I fragment of JJ212, respectively. Isolated cDNA clones were analyzed by DNA sequencing.

Phylogenetic analysis: A phylogenetic tree was constructed that included Pi5-1, Pi5-2, and other cloned rice blast re-

sistance proteins. Full-length protein sequences were aligned using Clustal W version 2.0 with default options (Larkin *et al.* 2007) and then corrected manually using the alignment editor software BioEdit Version 7.0.09 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Unrooted phylogenetic trees were generated in MEGA Version 4 (Tamura *et al.* 2007) by the neighbor-joining method, the Poisson distance method, and the pairwise deletion of gaps, with the default assumptions that the substitution patterns among lineages and substitution rates among sites were homogeneous. Because bootstrapping can provide an estimate of branch point confidence, we adopted 1000 bootstrap replicates to infer the statistical support for the tree.

RT–PCR analysis: To examine the changes in transcript accumulation in response to pathogen treatment, leaves from each of 10 *RIL260*, *IRBL5-M*, and transgenic rice plants inoculated with *M. oryzae* PO6-6 were collected at different time periods for RT–PCR analysis. Total RNA was prepared using Trizol reagent and reverse-transcribed with an oligo(dT) primer and a First Strand cDNA Synthesis kit (Roche) (Cho *et al.* 2006). First-strand cDNA was used in PCR reactions with gene-specific primers. Primers for the rice *Actin1* gene and the pathogenesis-related probenazole-inducible (*PBZ1*) gene (Ryu *et al.* 2006) were used as internal controls (Table 1). PCR conditions were as follows: 94° for 5 min followed by 28–35 cycles of 94°, 1 min; 56°, 1 min; and 72°, 1 min, with a final extension at 72° for 5 min. Three independent amplifications were performed for each primer set.

RESULTS

Genetic characterization of a 130-kb chromosomal region carrying *Pi5*: Previously, the *Pi5* resistance gene was delimited to a 170-kb interval between the two flanking markers S04G03 and C1454 on rice chromosome 9. This finding was the result of our previous analysis of two populations generated by crosses between *RIL260* carrying *Pi5* and a susceptible cultivar, *CO39*, and between *RIL260* and another susceptible cultivar, *M202* (Jeon *et al.* 2003). To further delineate the *Pi5* gene, in this study we generated a third mapping population derived from a cross between *RIL260* and another susceptible cultivar, *IR50*. Through PCR screening we found that, among the susceptible cultivars tested, only *IR50* contained the dominant marker JJ817, which was also found in the resistant cultivar *RIL260* (data not shown). In contrast, we were not able to amplify a PCR product for JJ817 in other susceptible cultivars, including *CO39* and *M202*. We selected *IR50* as a mapping parent on the basis of the similarity between the genomic regions for *RIL260* and *IR50*, which we speculated could facilitate recombination in the interval.

To identify rare recombinants within the 170-kb *Pi5* locus, a prescreening strategy using the CAPS markers JJ817 and C1454 and the SCAR marker JJ803 (Jeon *et al.* 2003; Jeon and Ronald 2007) was employed in our analysis of the *RIL260*/*IR50* F_2 population. Of the 2014 F_2 individuals analyzed, we identified eight recombinants between JJ817 and JJ803, but none between JJ803 and C1454 (Figure 1). Using the dominant markers JJ113-T3 and S04G03 (Jeon *et al.* 2003; Yi *et al.* 2004), we subsequently determined the breakage points of the eight recombinants that we isolated in their progeny (F_3) plants, which enabled us to distinguish homozygous from heterozygous genotypes. In total, all eight lines were found to harbor recombination events between JJ113-T3 and JJ817.

The disease phenotypes resulting from *M. oryzae* PO6-6 infection of these eight identified lines were then determined in the F_3 progeny in each case. These experiments further delimited the *Pi5* gene to a 130-kb interval between the markers JJ817 and C1454 (Figure 1). Our previous and current results indicated that both the JJ803 and the JJ113-T3 markers cosegregate with *Pi5*-mediated resistance (Figure 1). We were unable to further fine-map the *R* gene at the *Pi5* locus.

Genomic sequence analysis of the 130-kb chromosomal region containing the *Pi5* locus: To identify candidate *R* genes in the *Pi5* locus, seven BIBAC clones, JJ80, JJ98, JJ106, JJ110, JJ113, JJ120, and JJ123, which covered the 130-kb *Pi5* region (Jeon *et al.* 2003), were selected and sequenced. BLAST searches using these sequences against the public databases and also gene annotation analysis using the RiceGAAS program predicted a total of 18 open reading frames (ORFs) at the *Pi5* locus in the *RIL260* cultivar: seven hypothetical proteins, two NB–LRR proteins, two putative transposon proteins, a putative eukaryotic translation initiation factor, a putative GTP-binding protein, a putative tetrahydrofolate synthase, a putative aldose 1-epimerase, a putative histone H5, a putative cold-shock DEAD-box protein A, and an ankyrin-like protein (Figure 2 and supplementary Figure S1). From this genomic sequence analysis, two *Pi5* candidate genes that showed homology with NB–LRR resistance genes were identified in *RIL260* and designated *Pi5-1* and *Pi5-2*.

The region of ~90 kb, from JJ803 to JJ817, of the 130-kb *RIL260 Pi5* interval was compared with the corresponding region of the *japonica* genome represented by the sequenced cultivar *Nipponbare* (International Rice Genome Sequencing Project 2005; Figure 2). The resulting sequence analysis showed that the *Nipponbare Pi5* interval contains two NB–LRR genes, Os09g15840 (a *Pi5-1* allelic gene) and a gene that was not identified in *RIL260*, Os09g15850, denoted *Pi5-3*. In contrast, *Nipponbare* lacks the corresponding allele of *Pi5-2*. Notably, the 5′ upstream sequences of the *Pi5-1* allelic genes of *RIL260* and *Nipponbare* were very different, indicating an extreme sequence divergence within the regulatory sequences of these alleles. In addition, we did not observe significant sequence similarity in any other part of the 90-kb *Pi5* intervals in *RIL260* and *Nipponbare* (Figure 2). These results suggest that the *Pi5* resistance locus has significantly diverged between these resistant and susceptible rice cultivars.

We did not compare the *Pi5* resistance locus with that of the publicly sequenced *indica* rice cultivar *93-11* due to a large gap at this locus (Yu *et al.* 2002). In an

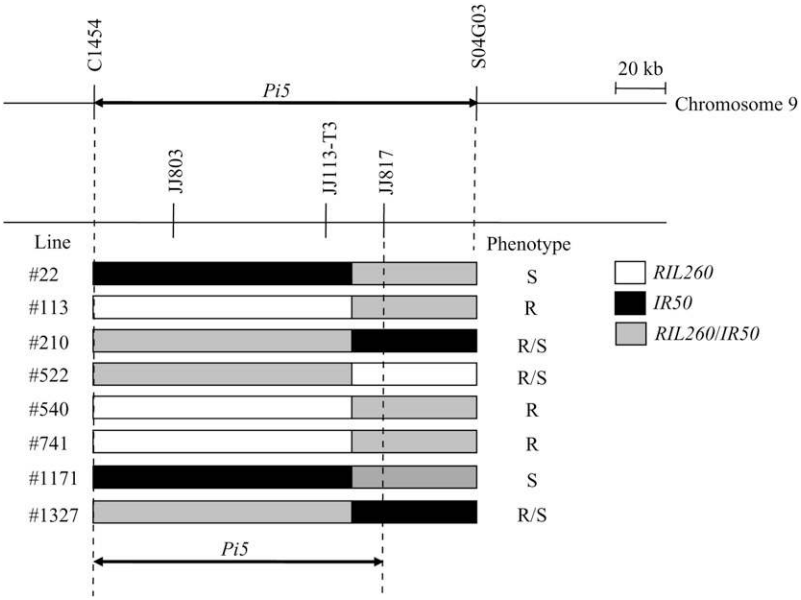


FIGURE 1.—Chromosomal location of the *Pi5* locus in the *RIL260/IR50* population. (Top) The 170-kb *Pi5* resistance genomic region is shown between the markers C1454 and S04G03 in *RIL260/CO39* and *RIL260/M202* (JEON *et al.* 2003). (Bottom) A schematic of the eight rare recombinants in the *Pi5* region identified in the *RIL260/IR50* population. Breakage points are indicated between the relevant molecular markers. Open bars indicate the presumed *RIL260* genome, solid bars indicate the *IR50* genome, and shaded bars indicate that the region is heterozygous between the two genomes. The arrow indicates the 130-kb minimal interval carrying the *Pi5* locus, delimited by analysis of the mapping population. Resistance to *M. oryzae* PO6-6 were determined in the F_3 progeny of each line. R, resistant; S, susceptible; R/S, segregating line.

inoculation experiment, we found that both *Nipponbare* and *93-11* were susceptible to *M. oryzae* PO6-6 (data not shown), indicating that neither carries the *Pi5* resistance gene.

Characterization of transgenic rice plants expressing *Pi5* candidate genes: To determine which one of the two candidate genes, *Pi5-1* and *Pi5-2*, is responsible for the *Pi5*-mediated resistance to *M. oryzae*, we used the genomic clones JJ204 and JJ212 carrying *Pi5-1* and *Pi5-2*, respectively, under the control of their native promoters to transform the susceptible *japonica* rice cultivar *Dongjin* using Agrobacterium-mediated transformation. RT–PCR analysis of the resulting transgenic lines revealed that 13 of 15 *Pi5-1* and 12 of 13 *Pi5-2* independently transformed lines expressed their transgenes upon *M. oryzae* PO6-6 inoculation (Figure 3A). The primary transgenic lines (T_0) carrying either *Pi5-1* or *Pi5-2* were inoculated with *M. oryzae* PO6-6. Surprisingly, however, none of the 13 *Pi5-1* or the 12 *Pi5-2* transgenic plants showed resistance to the *M. oryzae* isolate PO6-6 (Figure 3B). To confirm these results, we inoculated T_1 progeny from these T_0 lines and found that all progeny were susceptible to the *M. oryzae* isolate to the same extent as the wild-type control *Dongjin* cultivar. This indicates that neither *Pi5-1* nor *Pi5-2* alone confers resistance to *M. oryzae* PO6-6.

Characterization of transgenic rice plants expressing both *Pi5-1* and *Pi5-2*: Because recent reports (SINAPIDOU *et al.* 2004; PEART *et al.* 2005; ASHIKAWA *et al.* 2008) have demonstrated that the presence of two *R* genes is required for resistance to pathogen infection, we decided to test plants expressing both candidate genes for blast resistance. We therefore generated transgenic plants carrying both *Pi5-1* and *Pi5-2* by crossing the highly

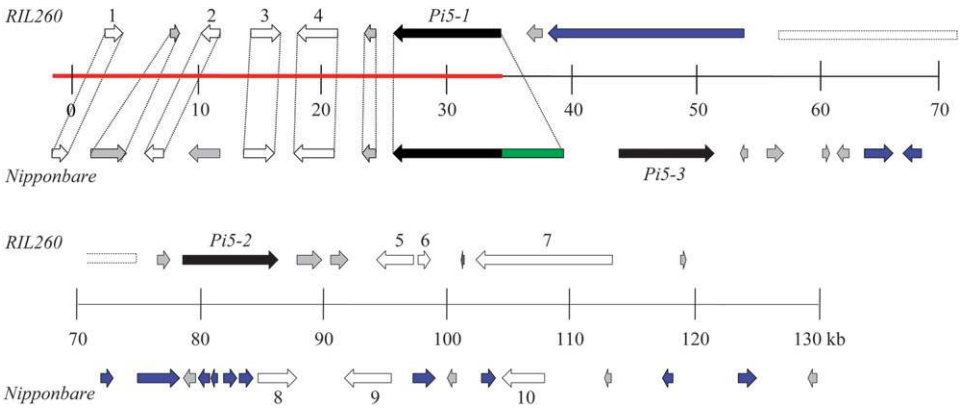


FIGURE 2.—Genomic sequence comparison at the *Pi5* loci in the *RIL260* and *Nipponbare* cultivars. Predicted ORFs determined by RiceGAAS are shown for both genomes. NB–LRR genes, *Pi5-1* alleles, and the *Pi5-2* and *Pi5-3* genes are indicated by black arrows. The N-terminal region of the *Pi5-1 Nipponbare* allele that is absent in *RIL260* is shown in green. Putative transposons and hypothetical genes are indicated by blue and gray arrows, respectively. Open arrows with numbers are predicted to encode the following proteins: 1, putative eukaryotic translation initiation factor; 2, putative GTP-binding protein; 3, putative tetrahydrofolate synthase; 4, putative aldose 1-epimerase; 5, putative histone H5; 6, putative cold-shock DEAD-box protein A; 7 and 10, ankyrin-like proteins; 8 and 9, HGWP-repeat containing proteins. The red line indicates high similarity (>90%) between the *RIL260* and *Nipponbare* ORFs. The chromosomal region that shows little or no homology is indicated by a thin line. The arrows indicate the direction of transcription. A gap in the DNA sequence in *RIL260* is indicated by the dotted box.

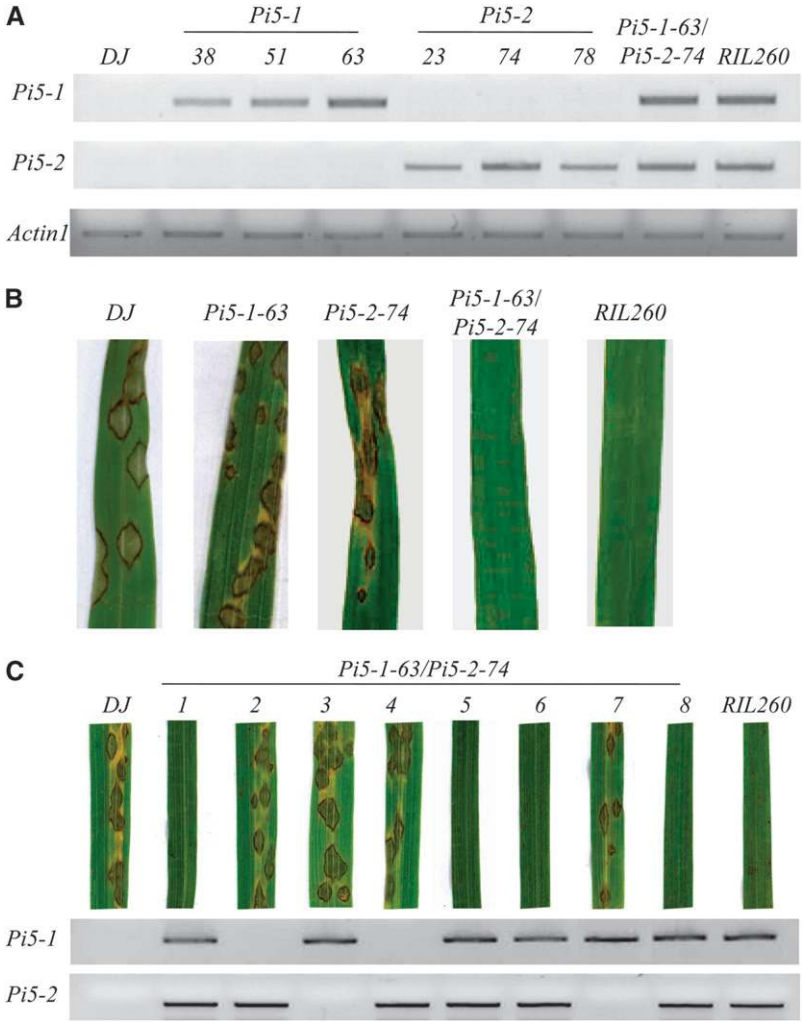


Figure 3.—Analysis of transgenic rice plants. (A) RT–PCR analysis of *Pi5-1*, *Pi5-2*, and *Pi5-1/Pi5-2* F_1 transgenic rice plants 2 days after inoculation with *M. oryzae* PO6-6. The rice *Actin1* gene was used as an internal control in these reactions. (B) Disease symptoms in *Pi5-1*, *Pi5-2*, and *Pi5-1/Pi5-2* F_1 transgenic plants 7 days after inoculation with *M. oryzae* PO6-6. (C) Genomic DNA PCR analysis and disease reaction of F_2 progeny derived from *Pi5-1-63/Pi5-2-74* F_1 transgenic plants in response to *M. oryzae* PO6-6 infection. A resistant cultivar, *RIL260* carrying *Pi5*, and a susceptible cultivar, *Dongjin* (*DJ*) lacking the *Pi5* gene, were used as controls.

susceptible homozygous *Pi5-1* line *#63* (*Pi5-1-63*) with the highly susceptible homozygous *Pi5-2* line *#74* (*Pi5-2-74*). Gene expression analysis revealed that the F_1 plants resulting from the cross expressed both the *Pi5-1* and the *Pi5-2* genes upon *M. oryzae* PO6-6 inoculation (Figure 3A). Strikingly, the 23 of *Pi5-1-63/Pi5-2-74* F_1 plants tested all displayed complete resistance to *M. oryzae* PO6-6. Transgenic lines carrying either *Pi5-1* or *Pi5-2* were susceptible as previously determined (Figure 3B).

To confirm this finding, we inoculated the F_2 progeny plants from the *Pi5-1-63/Pi5-2-74* F_1 lines with the *M. oryzae* isolate PO6-6. Of the 72 F_2 progeny tested, 37 of these carried both transgenes and conferred resistance to *M. oryzae* PO6-6. In contrast, F_2 progeny carrying either *Pi5-1* or *Pi5-2* only were susceptible (Figure 3C). RT–PCR analysis demonstrated that the *Pi5-1-63/Pi5-2-74* lines expressed their transgenes at levels that were similar to *RIL260* before and after *M. oryzae* PO6-6 inoculation (supplemental Figure S2). To test if *Pi5-1* and *Pi5-2* are required for resistance to other *M. oryzae* isolates, we inoculated the transgenic plants with four additional isolates incompatible with *Pi5*. These isolates displayed distinct virulence patterns on rice lines carrying different single *R* genes (supplemental Table S1), validating that these are indeed different *M. oryzae* isolates. We found that transgenic plants coexpressing *Pi5-1* and *Pi5-2* were resistant to all of the tested *M. oryzae* isolates. The resistance donor *RIL260* and the monogenic line *IRBL5-M* carrying *Pi5* were also resistant to these four isolates. In contrast, *Dongjin* and plants carrying either *Pi5-1* or *Pi5-2* only were susceptible to the tested *M. oryzae* isolates (Table 2). These results demonstrate that the two NB–LRR genes *Pi5-1* and *Pi5-2* are required for *Pi5*-mediated resistance to *M. oryzae* isolates.

The *Pi5* monogenic line *IRBL5-M* is susceptible to *M. oryzae* KI215 (supplemental Table S1). Genomic sequence analysis indicated that the *IRBL5-M* genomic region carrying *Pi5* is identical to that of *RIL260* (data not shown). In addition, RT–PCR analysis further demonstrated that *IRBL5-M* expresses both *Pi5-1* and *Pi5-2* at levels similar to *RIL260* either before or after *M. oryzae* PO6-6 inoculation (supplemental Figure S2). On the basis of these results, we hypothesized that transgenic plants expressing both *Pi5-1* and *Pi5-2* would also be susceptible to *M. oryzae* KI215. Indeed, our inoculation result showed that transgenic plants expressing

TABLE 2

Disease reactions of *Pi5* transgenic plants to *M. oryzae* isolates

Isolate	*Dongjin*	*RIL260*	*IRBL5-M*	*Pi5-1-63*[a]	*Pi5-2-74*[a]	*Pi5-1-63/Pi5-2-74*[a]
PO6-6	S[b]	R[b]	R	S	S	R
KJ105a	S	R	R	S	S	R
KJ107	S	R	R	S	S	R
KJ401	S	R	R	S	S	R
R01-1	S	R	R	S	S	R
KI215	S	R	S	S	S	S

[a] Transgenic lines.
[b] R, resistant; S, susceptible.

both *Pi5-1* and *Pi5-2* are susceptible to *M. oryzae* KI215. In contrast, *RIL260* was found to be resistant to *M. oryzae* KI215, indicating that it may contain an additional *R* gene that confers resistance to this isolate (Table 2).

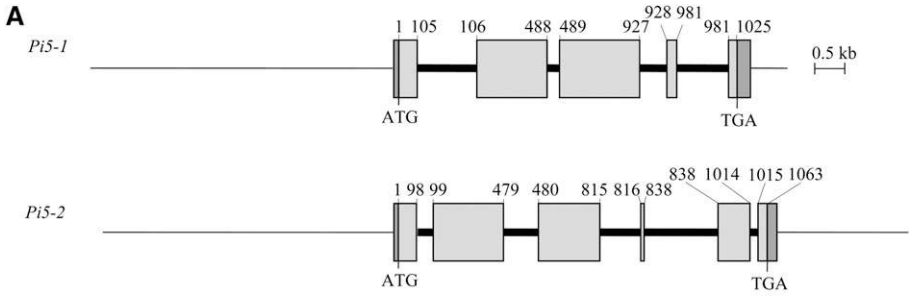
Characterization and phylogenetic analysis of the proteins encoded by *Pi5-1* and *Pi5-2*: To isolate the cDNA clones corresponding to both *Pi5* genes under study, a cDNA library for *RIL260* was constructed with the Uni-ZAP XR vector using mRNA isolated from rice leaves collected at 24 and 48 hr after inoculation with *M. oryzae* PO6-6. This library was screened using a colony hybridization methodology using the gene-specific regions of *Pi5-1* and *Pi5-2* as probes. We identified seven and five cDNA clones for *Pi5-1* and *Pi5-2*, respectively. Sequence analysis further revealed that three of the *Pi5-1* cDNA clones contained an entire ORF, whereas the others lacked an N terminus encompassing an ATG translation initiation codon. Among the three full ORF clones, the longest clone (#1-7) was fully sequenced. These experiments revealed that *Pi5-1* encodes a protein of 1025 amino acids and that the ORF is flanked by 5′- and 3′-untranslated regions of 70 and 220 bp, respectively (GenBank accession no. EU869185; Figure 4, A and B). Sequence analysis of the *Pi5-2* clones revealed that three of the five clones contained an entire ORF. Among these, the longest clone (#2-4) was further characterized by sequencing. This analysis indicated that *Pi5-2* encodes an ORF of 1063 amino acids and that this ORF is flanked by 5′- and 3′-untranslated regions of 73 and 164 bp, respectively (GenBank accession no. EU869186; Figure 4, A and C).

Comparison of their deduced amino acid sequences revealed that both *Pi5-1* and *Pi5-2* encode an N-terminal CC, a centrally located NB and LRR, and also C-terminal regions (Figure 4, B and C). A conserved domain search using the Pfam and SMART databases predicted that residue 109–576 of Pi5-1 and 109-567 of Pi5-2 contain an NB domain, which is a signaling motif shared by plant *R*-gene products (Hammond-Kosack and Jones 1997; Dangl and Jones 2001; Martin *et al.* 2003; Belkhadir *et al.* 2004; Liu *et al.* 2007a). The conserved internal domains characteristic of NB-containing *R*-gene products were also identified in Pi5-1 and Pi5-2, including the P-loop, kinase-2, RNBS-B, GLPL, RNBS-D, and MHDV domains (Meyers *et al.* 2003). Additional analysis using the Paircoil2 program (http://groups.csail.mit.edu/cb/paircoil2/) predicted a potential CC domain with a threshold of 0.1 between amino acids 31 and 67 in Pi5-1 and 26 and 87 in Pi5-2 (McDonnell *et al.* 2006), indicating that these proteins belong to the CC subset of the NB–LRR resistance proteins.

The LRR regions of Pi5-1 and Pi5-2 consist of 24.3 and 22.6% leucine residues, respectively, and contain a series of imperfect repeats (10–12) of various lengths (Figure 4, B and C). Of note, a few repeats of the Pi5-1 and Pi5-2 proteins matched the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx observed in other cytoplasmic R proteins (Jones and Jones 1997). The first and third repeat regions of Pi5-1 and the first, third, and sixth repeat regions of Pi5-2 contained the xLDL motif that is conserved in the third LRR of many NB–LRR proteins (Axtell *et al.* 2001; Meyers *et al.* 2003; Figure 4, B and C). Notably also, the Pi5-1 and Pi5-2 proteins harbor a unique C terminus that is distinct from those of other NB–LRR proteins (Dodds *et al.* 2001) and that does not match any known protein motif.

Sequence comparisons between the cDNA and genomic sequences for these *R* genes revealed that *Pi5-1* and *Pi5-2* carry five and six exons, respectively (Figure 4A). The *Pi5* genes have a larger number of introns within their coding regions compared with other cloned rice *R* genes that confer resistance to *M. oryzae* (Bryan *et al.* 2000; Qu *et al.* 2006; Zhou *et al.* 2006; Lin *et al.* 2007a; Liu *et al.* 2007b). Furthermore, the *Pi5-1* and *Pi5-2* genes contain an intron in both RNBS-D and MHDV domains.

Among the known plant NB–LRR proteins, the Pi5-1 and Pi5-2 proteins show relatively high levels of similarity with the wild potato species *Solanum bulbocastanum* gene *Rpi-blb1*, which confers broad-spectrum resistance to the oomycete pathogen *Phytophthora infestans*, the causal agent of late blight (supplemental Figure S3) (Van der Vossen *et al.* 2003). To further analyze the evolutionary relationship between the *Pi5* genes and other rice NB–LRR genes, a phylogenetic tree was constructed for both the Pi5 proteins under study and the other



B

1
MVGAEMLVAAAVSQVARKINDIVGVAQGEV*KLCCNFSDDLEGIKDTLVYLETLLKNAENNSFGSDRA*NLRHWLGQ
IKSLAYDIEDIVDGYYSSKEQFDGGSYAQKGSL

CC

109
FCSLSNPMLLKGSMVYKMKSKREMLQQSQQLPNQYHFLSYINSAVHYFEEKQTTSYRNTDIAIVGRDADLDHLMD

P-loop
LLMQNSAEELCI**IPIVGPVGFGKTSLAQLVF**NDTRTEVFSFRIWVHVSMGNINLEKIGRDIVSQTTEKIEGNMQL

kinase-2 RNBS-B
QSIKNAVQRVLNKY**SCLIIIDSLW**GKDE**EVNELKQMLLTGRHTESKIIVTTHSN**KVAKLISTVPLYKLAALSEDD

GLPL
CLKIFSQRAMTGPGDPLFREYGE**EIVRRCEGTPLVANFL**GSVVNAQRQRREIWQAAKDEEMWKIEEDYPQDKISP

RNBS-D
LFPSFKIIYYNMPHELRL**CFVYCSIFPKGTVIEKKKLIQQWIALDMI**ESKHGTLPLDVTAEKYIDELKAIYFLQV

MHDV
LERSQNDAERSSASEEMLR**MHNLAHDLARSVA**GEDILVILDAENERNARYCNYRYAQVSASSLESIDRKAWPSKA
RSLIFKNSGVDFEHVSEV

NB

577
LSVNKYLRVLDLSGCCVQDIPSP
IFQLKQLRYLDVSSLSITALPLQ
ISSFHKLQMLDLSETELTELPPFISN
LKGLNYLNLQGCQKLQRLNSLHL
LHDLHYLNLSCCPEVTSFPESIEN
LTKLRFLNLSGCSKLSTLPIRFLESFAS
LCSLVDLNLSGFEFQMLPDFFGN
IYSLQYLNLSKCLKLEVLPQSFGQ
LAYLKSLNLSYCSDLKLLESFEC
LTSLRFLNLSNCSRLEYLPSCFDK
LNNLESLNLSQCLGLKALPES
LQNLKNLQLDVSGCQDCIVQSFSLST

LRR

865
RSSQSCQRSEKAEQVRSRNSEISEITYEEPAEIELLKNNPSKDLASISHLNEDRIEEPEVVTEPSATRGMVQQIP
GNQLSSPSSHLSSFASSSAPFASSSSDTSTSEHPVPNEEAAALTVPRSKEKCDNTPMPVKDGLISEDDAPVHLHQ
KPLQATAMAAI

CT

C

1
MATAGAAVDRLLRRLASGAGRLELP*SSIDEDMAHVKRTLARLQDVLLTVEGKYFKMGAEVQEWMRKIKQIAYGIQ*
*DLLDEFEDSSGT*GSQRNGSRISEGTLSCSSAPF

CC

109
FCHLSRSQRIRVLKRKLDQSTKDTSVFSLLQHSLSNLDKSNEQEVLLHRTEIIGRDTDKENIKNLLLQNDVDKLP

P-loop
I**IPIVGLAGLGKTAVAKLIF**HEQGEGWNFDQRIWVHLDKKLDLNKIANSIISQVNQSVDTTKNQIQNNLQFKRNC

kinase-2 RNBS-B
LQEVLCD**QSSLIVLDDLF**STEEN**QIAELKEMLRGTKKGTKIIVTTSSE**ISAELIHTVPPYKLGPLSEGDCSTIFC

GLPL
QRAFGDGHENSSLTEIAK**QIVKRCEGIPAVAYSL**GSLVRNKNKEAWLYARDKEIWELPTLFPNGFELLASFSEMY

RNBS-D
ICMPSALKS**CFAYLSTIPKGTIIDREKLIEQWIALDMV**GSKHGTLPAYVQGEMFIQQLLSISFLQVRNKPSATRI

MHDV
RDTNQSKELR**IHNLVHDFAMYVA**RDDLIILDGGEKASSLRKNIHVFYGVVNNDIGQSALRKGLLSSARAVHFKNC
KSEKLLVEA

NB

568
FSVLNHLRVLDLSGCCIVELPDF
ITNLRHLRYLDVSYSRILSLSTQ
LTSLSNLEVLDLSETSLELLPSSIGS
FEKLKYLNLQGCDKLVNLPPFVCD
LKRLENLNLSYCYGITMLPPN
LWKLHELRILDLSSCTDLQEMPYL
FGNLASLENLNMSKCSKLEQLPES
LGDLCYLRSFNLSGCSGLKMLPESLKN
LTNLEYINLSNIGESIDFNQ
IQQLRHILKKTFFSGDIGGSELQ

LRR

803
TCEHAADSADSKKEITMDFSANLHGNITLPPKCSTEEKSGENSERFLSAAVREDSSSTDVSTYVKPVVSSLIGVL
RRPTRLDVPAGAMASQVGLAQMPSSNNGKAGPHPTMAAAQTPEIDQPVHKRVRWDDIIDYSRPPNSKPARSASLV
QSTDLSTPKKSYKKIHSMPVVYSSIPKGSSGGTYLMPAKAIASSYRRYSPQRWEQHIGYQGTDEDELMVVPPFGE
WDQSPTLRKSDFRYEKVFAKLTEEKMSGQRQKPQQV

CT

FIGURE 4.—Genomic structure of the *Pi5-1* and *Pi5-2* genes and their gene products. (A) Gene structure of *Pi5-1* and *Pi5-2*. Exons are indicated by lightly shaded boxes and introns are indicated by thick lines. The 5′- and 3′-untranslated regions are indicated by darkly shaded boxes. ATG and TGA denote the translation initiation and stop codons, respectively, and the numbers indicate the amino acid positions. (B) Pi5-1 protein. (C) Pi5-2 protein. Both resistance proteins contain a CC, NB, LRR, and C-terminal region (CT). Amino acids 31–67 of Pi5-1 and 26–87 of Pi5-2, shown in underlined italics, contain CC motifs. The conserved internal motifs characteristic of NB proteins, namely the P-loop, kinase-2, RNBS-B, GLPL, RNBS-D, and MHDV domains, are underlined and in boldface type. A conserved xLDL motif found in the LRR of many NB–LRR proteins is also underlined.

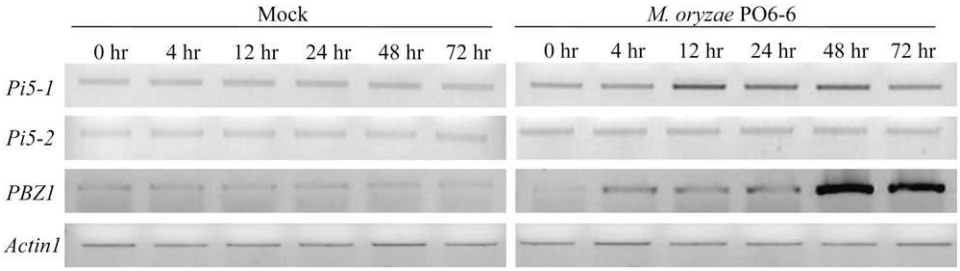


FIGURE 5.—RT–PCR analysis of the *Pi5* genes and the *PBZ1* gene in the *RIL260* cultivar inoculated with *M. oryzae* PO6-6. cDNAs prepared from the leaf tissue of *RIL260* at 0, 4, 12, 24, 48, and 72 hr after pathogen inoculation were used in the experiment. The rice *Actin1* gene was used as an internal control.

cloned rice blast resistance proteins (supplemental Figure S3). The degree of similarity among these proteins was found to vary considerably and two heterogeneous groups could be recognized, indicating an early divergence in the evolution of rice blast resistance genes. *Pi5* genes formed a clade with *Pi37*, which was separated from another clade containing the blast resistance genes *Pib*, *Pi2/Piz-t*, *Pi9*, *Pita*, *Pi36*, and *Pikm*. In addition, our results showed that *Pi5-1* has a relatively close evolutionary relationship with *Pi5-2*.

Expression analysis of the *Pi5-1* and *Pi5-2* genes: To examine whether the expression of the two identified *R* genes was altered upon pathogen treatment, we performed RT–PCR analysis of these two genes in *RIL260*, *IRBL5-M*, and *Pi5-1-63/Pi5-2-74* transgenic plants infected with *M. oryzae* PO6-6 (Figure 5 and supplemental Figure S2). Total RNAs isolated from the leaves of 3-week-old plants harvested at different time points after *M. oryzae* PO6-6 inoculation were used for this purpose. The results revealed that *Pi5-1* expression increased 12 hr after pathogen challenge, whereas the *Pi5-2* gene is constitutively expressed at a low level in *RIL260* both before and after infection (Figure 5). The *IRBL5-M* and *Pi5-1-63/Pi5-2-74* lines also exhibited similar expression patterns of the *Pi5* genes (supplemental Figure S2). These findings indicated that both *Pi5-1* and *Pi5*-2 are expressed during pathogen infection, suggesting that the encoded proteins are also coexpressed. Transcripts of *PBZ1*, a pathogen-inducible gene, accumulated to high levels in *M. oryzae*-treated leaves (Figure 5).

DISCUSSION

Characterization of the *Pi5* resistance locus: From a total of 2014 F_2 plants derived from an *RIL260/IR50* cross generated in our study, we identified eight lines that had undergone recombination events in the 130-kb chromosomal region carrying *Pi5*. Each of these recombination events occurred close to the JJ817 marker, which shares similarity with the *RIL260* and *IR50* rice cultivars (Figure 1). In a previous study, we were unable to detect any recombination in the same *Pi5* interval from >2100 segregating plants in the *RIL260/CO39* and *RIL260/M202* populations (JEON *et al.* 2003). This suggests that the similarity in the genomic regions between *RIL260* and *IR50* reduces the suppression of recombination observed in the *RIL260/CO39* and *RIL260/M202* crosses.

Given that each screened recombinant was selected from 4028 meiotic events (2014 individuals), the eight recombination events in the 130-kb interval correspond to a genetic distance of ~0.2 cM, giving a ratio of 650 kb/cM. This is much higher than the average physical/genetic ratio of 260–280 kb/cM estimated for the rice genome (WU and TANKSLEY 1993). The most likely explanation for this is the lack of pairing and also subsequent strand-exchange events between homologous parental genomes at the *Pi5* locus, which is supported by the significant differences between the DNA sequences of resistant and susceptible rice genomes (Figure 2). In further support of this hypothesis, the results of DNA gel-blot analysis confirmed the presence of *Pi5-2* in the resistant *RIL260* cultivar and the absence of the corresponding allele in the susceptible *Nipponbare* cultivar. Conversely, *Pi5-3* hybridized with genomic DNA in *Nipponbare* but not in *RIL260* (Figure 2; data not shown). Together with our finding that *Pi5-1* is also polymorphic in resistant and susceptible rice cultivars, these data indicate that the *Pi5* locus is highly divergent among rice cultivars that are resistant and susceptible to *M. oryzae*.

Structure of the *Pi5-1* and *Pi5-2* genes: *Pi5-1* and *Pi5-2* belong to a family of CC–NB–LRR *R* genes and contain unique C-terminal regions consisting of 161 and 280 amino acids, respectively. Some plant NB–LRR genes such as *RRS-1R* (DESLANDES *et al.* 2002), *P2* (DODDS *et al.* 2001), *RPS4* (GASSMANN *et al.* 1999), *RPP1-WsA*, *RPP1-WsB*, and *RPP1-WsC* (BOTELLA *et al.* 1998) encode proteins with additional domains after the LRR in their C terminus. For example, RRS-1R contains a WRKY motif in its C-terminal region whereas the other proteins listed contain a C-terminal non-LRR domain. The C termini of the Pi5-1 and Pi5-2 proteins did not match either of these known domains, nor have these domains been previously characterized. Future characterization of the functional role(s) of these novel C-terminal regions will provide valuable insights into the mechanism of *Pi5*-mediated resistance.

Our phylogenetic analysis indicated that the *Pi5* genes form a distinct clade that can be separated from another clade containing cloned rice blast resistance genes (supplemental Figure S3). We examined the intron positions in the NB domain of the *Pi5* genes to better interpret the phylogenetic relationship between *Pi5* and other cloned rice blast resistance genes. Notably, *Pi5-1* and *Pi5-2* harbor an intron between their RNBS-D and

MHDV domains. *Pita* (Bryan *et al.* 2000), *Pi36* (Liu *et al.* 2007b), and *Pikm* (Ashikawa *et al.* 2008) carry an intron at the immediate N-terminal side of the kinase-2 motif, which is the most common intron position in cereals (Bai *et al.* 2002). *Pib* carries an intron between its RNBS-B and GLPL domains (Wang *et al.* 1999). *Pi9*, *Pi2/Piz-t*, and *Pi37* do not contain any introns within the conserved NB motif region (Qu *et al.* 2006; Zhou *et al.* 2006; Lin *et al.* 2007a). In addition, *Pi5-1* and *Pi5-2* appear to have relatively many introns, four and five, respectively, compared with other identified rice blast resistance genes except for *Pi36*, which carries four introns in its coding regions (Liu *et al.* 2007b). *Pi37* carries no intron within its coding region (Lin *et al.* 2007a). *Pita* and *Pikm2-TS* contain a single intron (Bryan *et al.* 2000; Ashikawa *et al.* 2008) and *Pib*, *Pi9*, *Pi2/Piz-t*, and *Pikm1-TS* have two introns in their coding regions (Wang *et al.* 1999; Qu *et al.* 2006; Zhou *et al.* 2006; Ashikawa *et al.* 2008). The distinctive number of introns and the genomic positions of *Pi5-1* and *Pi5-2* are thus consistent with the results of our phylogenetic analysis (supplemental Figure S3), indicating that they indeed belong to the same clade and are distinct from other NB–LRR genes.

Previous genetic studies have indicated that *Pi5* and *Pii* are allelic (Inukai *et al.* 1996; Yi *et al.* 2004) and that *Pi15* is located at the same locus (Lin *et al.* 2007b). Sequence analysis of the corresponding genomic DNA fragments in *Pii*- and *Pi15*-carrying cultivars and functional characterization of the candidate genes of *Pii* and *Pi15* are underway in our laboratory to address whether these genes are allelic or not.

Disease resistance to *M. oryzae* requires the presence of both the *Pi5-1* and *Pi5-2* genes: To further elucidate the mechanism of *Pi5*-mediated resistance in rice, we investigated the expression patterns of our two identified *Pi5* genes by RT–PCR. There have been some previous reports on induced *R*-gene expression in response to pathogen inoculation in rice. For example, transcripts of rice *Xa1* are detected in leaves 5 days after wounding or inoculation with compatible or incompatible strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Yoshimura *et al.* 1998). *Pib* transcripts accumulate in response to *M. oryzae* infection and also in response to altered temperature and darkness (Wang *et al.* 1999). In contrast, other cloned *R* genes, including *Pita*, *Pi2/Piz-t*, *Pi9*, *Pi36*, and *Pi37*, are expressed constitutively in the absence of pathogen challenges (Bryan *et al.* 2000; Chen *et al.* 2006; Qu *et al.* 2006; Zhou *et al.* 2006; Lin *et al.* 2007a; Liu *et al.* 2007b). *Pi5-1* transcripts accumulate in response to pathogen infection, whereas *Pi5-2* expression is constitutive (Figure 5). These results suggest that the increased expression of *Pi5-1* in response to pathogen challenge in conjunction with the constitutive expression of *Pi5-2* is an important aspect of *Pi5*-mediated resistance in rice.

Our present data indicate that *Pi5*-mediated resistance to rice blast is conferred by two CC–NB–LRR genes, *Pi5-1* and *Pi5-2*. It was previously reported in a study using genetic complementation experiments in Arabidopsis that two adjacent TIR–NB–LRR genes, *RPP2A* and *RPP2B*, are essential for the resistance to the *P. parasitica* isolate Cala2 (Sinapidou *et al.* 2004). *RPP2A* has an unusual structure as it harbors two incomplete TIR–NB domains and a short LRR motif, whereas *RPP2B* has a complete TIR–NB–LRR structure. Similarly, using a virus-induced gene-silencing system, the CC–NB–LRR protein NRG1 was found to be an essential component of *N*-mediated resistance against tobacco mosaic virus (Peart *et al.* 2005). In the absence of *NRG1*, *N*-mediated resistance is affected both in the *N* transgenic *Nicotiana benthamiana* plants and in *N. edwardsonii* carrying the *N* gene. It has been recently found that rice *Pikm*-mediated resistance is also conferred by cooperation of two independent proteins, Pikm1-TS, a CC–NB–LRR protein, and Pikm2-TS, an NB–LRR protein lacking a CC domain (Ashikawa *et al.* 2008). These earlier results together with the data presented here suggest that a requirement for the presence of two NB–LRR proteins is more common than has been previously recognized.

The gene-for-gene hypothesis predicts that a single plant *R*-gene product recognizes a single bacterial *Avr* gene product (Flor 1971). In support of this hypothesis, it has been shown that the *R*-gene products rice Pita and flax L directly bind to their cognate Avr proteins (Jia *et al.* 2000; Dodds *et al.* 2006; Ellis *et al.* 2007). In contrast, in several other species it has been shown that R proteins do not directly interact with *Avr* gene products (Gabriel and Rolfe 1990; Van der Biezen and Jones 1998; Innes 2004; Jones and Dangl 2006). For example, in Arabidopsis, the NB–LRR R proteins RPM1 and RPS2 do not appear to interact directly with the cognate *Pseudomonas syringae* Avr proteins, AvrRpm1 and AvrB, and AvrRpt2, respectively. Instead, RPM1 and RPS2 are hypothesized to guard the host protein RIN4. Upon pathogen attack, these effectors modify RIN4 (Mackey *et al.* 2002, 2003; Axtell and Staskawicz 2003; Kim *et al.* 2005; Jones and Dangl 2006). Similarly, Arabidopsis RPS5 appears to guard the serine/threonine kinase protein PBS1, which is targeted for proteolysis by AvrRphB (Ade *et al.* 2007). Moreover, the silencing of *NRG1* does not block the N-protein oligomerization that has been observed as an early response to the p50 elicitor, suggesting that NRG1 may not be necessary for elicitor recognition.

We speculate that it is unlikely that *Pi5-1* and *Pi5-2* control independent resistant pathways that act in an additive fashion because our current data clearly demonstrate that transgenic plants carrying either *Pi5-1* or *Pi5-2* remain highly susceptible to *M. oryzae*. We instead propose several possible models for the mechanism underlying *Pi5-1*-mediated resistance. First, Pi5-1 and Pi5-2 may interact with each other directly or indirectly. In this scenario, it is possible that both Pi5-1 and Pi5-2 proteins interact with the corresponding Avr effector. Alternatively, the presence of the corresponding Avr

effector may be required to trigger their interaction. Second, either Pi5-1 or Pi5-2 alone may interact with the Avr effector while the other serves as a "guard." Third, both the Pi5-1 and Pi5-2 proteins may guard a third host protein, which is targeted by the Avr effector.

Our findings demonstrate that two CC–NB–LRR genes, called *Pi5-1* and *Pi5-2*, are required to confer *Pi5*-mediated resistance to *M. oryzae*. The future successful cloning of the *AvrPi5* effector and investigations of Pi5-1/Pi5-2 interactions will contribute to a more complete understanding of the mechanism of rice *Pi5*-mediated resistance.

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