Characterization of a *PR-10* Pathogenesis-Related Gene Family Induced in Rice During Infection with *Magnaporthe grisea*

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A partial cDNA with homology to the *PR-10* class of pathogenesis-related proteins was used to screen a rice genomic library. One 16-kb genomic clone contained three genes with *PR-10* similarity. These genes, *RPR10a*, *RPR10b*, and *RPR10c*, were arranged in tandem and separated by approximately 2.5 kb. *RPR10a* cDNA was obtained by reverse transcription-polymerase chain reaction, and sequence analysis revealed that *RPR10a* and *RPR10b* encode predicted proteins of 158 and 160 amino acids, respectively, and share 71% amino acid identity. *RPR10c* appears to be a nonfunctional pseudogene. Gene-specific probes were used to study transcript accumulations of the three *RPR10* genes in rice plants following inoculation with *Magnaporthe grisea*. *RPR10a* transcripts were induced from a low basal level within 12 h after inoculation and showed a second higher level induction at 48 h, which continued throughout the 144 h it was examined. In addition, *RPR10a* was induced strongly by salicylic and jasmonic acid applications to rice plants. Transcripts of *RPR10b* also were enhanced by *M. grisea*, but were not strongly visible until 48 h after inoculation. Tissue prints of *M. grisea*-infected rice leaves when the *RPR10a*-specific probe was used indicate that *RPR10a* is expressed most strongly in a localized fashion in response to the pathogen.

Plants respond to the presence of a perceived pathogen by activating a multicomponent defense system. This elaborate, active system can include a transient ion flux across the cell membrane, an intracellular oxidative burst of active oxygen species, localized cell necrosis and cell death at the site of infection, the production of cell wall-strengthening components and enzymes, the production of defensive proteins and other antimicrobial compounds, and in some cases, the systemic signaling through the plant phloem to secondary tissues that activates a long-lived spectrum of pathogen resistance. Although the general mechanisms of many of these plant de-

fense systems have been identified (Grant and Mansfield 1999; McDowell and Dangl 2000), numerous components and details remain to be discovered.

One area in which the details of the defense response are becoming more evident is in the plant's production of pathogen-related (PR) proteins. PR proteins have been defined as plant proteins that are induced in pathological or related situations (Antoniw et al. 1980). This term describes the collective induction of numerous plant genes that are induced in a variety of plant species in response to pathological invasion. For convenience, the major families of PR proteins have been organized into 11 different classes, primarily on the basis of their amino acid sequence identity (Van Loon et al. 1994). Although the biological and/or biochemical function of many PR proteins remains unknown, a few examples have been shown to possess antimicrobial activity. In vitro studies of chitinases (PR-3 class) and β -1,3-glucanases (PR-2 class) have shown that these proteins can inhibit fungal growth (Mauch et al. 1988; Sela-Buurlage et al. 1993; Woloshuck et al. 1991), presumably by hydrolytic degradation of fungal cell walls. In addition, transgenic studies with constitutively up-regulated expression of various PR proteins such as chitinases, β -1,3-glucanases, tobacco PR-1, and type I barley ribosome-inactivating protein (Alexander et al. 1993; Broglie et al. 1991; Jach et al. 1995; Van Den Elzen et al. 1993; Zhu et al. 1994) resulted in plants having decreased disease severity after infection by fungal pathogens. This demonstrates antimicrobial activity of at least some PR proteins and, because they are induced in response to pathogen attack, suggests that PR proteins are important for active defense against disease.

The *PR-10* class of proteins, first identified as a major pollen allergen (BetV-1) from white birch, are induced following pathogen attack in a wide variety of plant species following pathogen attack, including parsley (Somssich et al. 1986), potato (Matton and Brisson, 1989), pea (Barral and Clark, 1991), soybean (Crowell et al. 1992), asparagus (Warner et al. 1993), and sorghum (Lo et al. 1999). Studies revealed that the *PR-10* class of proteins share homology to a ribonuclease (RNase) isolated from phosphate-starved ginseng cells (Moiseyev et al. 1994), suggesting that *PR-10* proteins may possess such activity. Here, we describe the isolation and characterization of a *PR-10* gene family in rice. Members of this gene family were differentially induced in the plant following infection with *Magnaporthe grisea*. In addition, one

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gene member was induced by exogenously applied salicylic acid (SA) and jasmonic acid (JA). This same gene also was expressed in a localized fashion, with respect to the region of pathogen infection.

RESULTS

Characterization of a pathogen-induced cDNA clone and related genomic sequences.

A cDNA clone, designated MAG-7, whose corresponding mRNA was highly induced in rice cultivar Co39 (*Oryza sativa* L. sub. *indica*) seedlings within 72 h after inoculation with *M. grisea*, was isolated by the differential screening of a *M. grisea*-infected rice cDNA library (Talbot et al. 1993). On the basis of sequence data, the MAG-7 clone contained a 537-bp truncated insert. A comparison of the MAG-7 sequence with sequences from various databases revealed that MAG-7 shares sequence homology with the PR-10 class of PR proteins (Van Loon et al. 1994) and 99% homology to the previously reported *PBZ1* rice cDNA (Midoh and Iwata 1996).

To obtain the genomic sequence corresponding to the MAG-7 cDNA, a genomic library was made from Co39 and screened with the 537-bp insert taken from the MAG-7 cDNA clone. One clone consisting of a 16-kb insert contained three genes that hybridized to the MAG-7 cDNA (Fig. 1). These three genes were sequenced and named *RPR10a*, *RPR10b*, and *RPR10c* (accession nos. AAF85972, AAF85973, and AF274852, respectively), in accordance to the convention suggested by Van Loon et al. (1994). The putative proteins encoded by these genes were named Rice PR-10a, Rice PR-10b, and Rice PR-10c. The *RPR10* genes are arranged in tandem and separated by approximately 2 to 2.5 kb. DNA blot analyses of Co39 genomic DNA (Fig. 2) and the 16-kb clone insert (data not shown) probed with the MAG-7 cDNA show almost identical banding-hybridization patterns, which suggests three to four copies of *RPR10*-related genes within the Co39 rice genome. This *RPR10* copy number assessment is in agreement with the estimation derived by hybridization to linearized MAG-7 cDNA (Fig. 2).

The *RPR10a* cDNA sequence was obtained with reverse transcription-polymerase chain reaction (RT-PCR) methodologies and shares 100% sequence identity to the MAG-7 cDNA. A majority of the resulting RT-PCR clones shared a common reversed-transcribed site that also was the most upstream site of any of the RT-PCR clones obtained. This site, designated the +1 nucleotide, was used to denote the putative full-length transcript. Sequence analysis of the full-length *RPR10a* cDNA revealed an 813-bp transcript that contained a 474-bp open reading frame, a 99-bp 5' untranslated region

(UTR), and a 238-bp 3' UTR. A comparison of the cDNA with the *RPR10a* genomic sequence shows that the *RPR10a* gene contains two exons (exon 1 = 175 bp; exon 2 = 302 bp) separated by an intervening sequence of 366 bp. The gene encodes a putative protein of 158 amino acids. A putative TATAA box element is located 35 bp upstream of the +1 nucleotide. In addition, three distinct DNA motifs suspected of binding nuclear factors are immediately upstream of the +1 nucleotide. These include an E-box motif (position -230, CAAATG), an A-rich element (position -202, AAAGAAAAAAAA), and a TAATNN motif (position -188, TATTAA). An AAUAAA consensus polyadenylation signal sequence (position +1,107) is located 34 bp upstream of the poly(A) addition site.

The coding region for *RPR10b* was deduced by a comparison with *RPR10a* and identifying the consensus splice sites. This predicted region contains two exons (exon 1 = 174 bp; exon 2 = 307 bp) separated by an intervening sequence of 972 bp. This gene encodes a putative protein of 160 amino acids, and the deduced *RPR10b* coding region shares 81% nucleotide

Fig. 1. *Bgl*II and *Sac*I restriction map of the genomic insert DNA, which contains the three *PRP10* genes. Scale bar = 1 kb.

Fig. 2. DNA blot analysis of rice cultivar Co39 genomic DNA probed with a 537-bp MAG-7 cDNA. DNA (15 µg) digested with *Xba*I (lane 1), *Eco*RI (lane 2), *Bgl*II (lane 3), or *Sac*I (lane 4). A linearized MAG-7 cDNA insert was used to estimate copy number in lane 6 (0.5), lane 7 (1), and lane 8 (3).

and 71% amino acid identity to *RPR10a*. The putative promoter region contains a TATAA box motif (107 bp from ATG), an E-box motif (147 bp from ATG), and an AT-rich region (249 bp from ATG).

A partial sequence of 1,591 bp was determined for the *RPR10c* gene region. On the basis of homology to the *RPR10a* genomic clone, this sequence begins 24 bp downstream of the corresponding ATG start site and continues downstream into the corresponding 3' UTR, relative to the *RPR10a* gene. The 1,591-bp *RPR10c* sequence reveals that it contains two regions that by shared homology, correspond to exons 1 and 2 of *RPR10a*. For the region related to exon 1 (positions 1 to 153), *RPR10c* shares 66% protein and 82% nucleotide homology to *RPR10a*. Within the corresponding *RPR10a* reading frame of this 153-bp segment, however, two stop codons (TAG at positions 34 and 130) are present. For the region related to exon 2

(position 982 to 1,274), *RPR10c* shares 84% protein and 86% nucleotide homology to *RPR10a*. This segment, however, contains a 10-bp deletion (position 1,205), which results in a shift in reading frame relative to *RPR10a*. In addition, a comparison of the putative intron splice sites reveals the presence of an additional nucleotide compared with *RPR10a*. This extra nucleotide also results in a reading frame shift. The presence of stop codons and frame shifts in the *RPR10c* sequence strongly suggests that this gene is nonfunctional.

Transcript accumulation of the RPR-10 genes.

RNA blot analysis was used to determine the steady-state levels of *RPR10* transcripts in rice leaves at various times following inoculation with *M. grisea* (Fig. 3). Hybridizations with the use of a nongene-specific probe with homology to all three *RPR10* genes revealed an overall induction of *PR10*

Fig. 3. RNA blot analysis comparing the presence of different PR10 transcripts following *Magnaporthe grisea* infection. Samples (10 µg) of total RNA isolated from uninfected (0 h) or infected (12, 18, 24, 36, 48, 72, or 144 h) 3-week-old seedlings were hybridized to different *RPR10* probes. **A**, Samples hybridized with an 814-bp nonspecific probe from *RPR10a* cDNA, which shares homology to all three *RPR10* genes. **B**, Samples hybridized with a 230-bp probe derived from the 5' untranslated region (UTR) of *RPR10a*. **C**, Samples hybridized with a 198-bp probe derived from the putative 3' UTR of *RPR10b*. **D**, Samples hybridized with a 231-bp probe derived from the putative 5' UTR of *RPR10c*. Intensity of the chloroplast 23S rRNA in A–C is shown as equal loading control.

Fig. 4. Ribonuclease protection assays performed with gene-specific probes for **A**, *RPR10a*, **B**, *RPR10b*, and **C**, *RPR10c*. Samples (5 µg) of total RNA isolated from 2-week-old seedlings, inoculated with *Magnaporthe grisea* for 0, 12, 18, 24, 36, 48, 72, and 144 h (lanes 2–9), were hybridized with antisense probes specific for *RPR10a* (probe length: 307 nucleotides), *RPR10b* (probe length: 307 nucleotides), and *RPR10c* (probe length: 275 nucleotides). Following ribonuclease digestion, the expected protected fragment sizes were 230, 74, and 230 nucleotides for *RPR10a*, *RPR10b*, and *RPR10c*, respectively. In all assays for *RPR10a* and *RPR10c*, a gene-specific probe for the 28S transcript was included to assure equal loading. This 28S standard probe yielded an expected protected fragment of 118 nucleotides as well as an unexpected 50-nucleotide fragment that presumably corresponds to the chloroplast 23S rRNA transcript. The 28S standard probe was not included in most of the assays for *RPR10b* because its presence interfered in the detection of the protected 74-nucleotide fragment. Lane 1: End-labeled DNA fragments of pUC19 digested with *Sau3A1* for size estimates. Lane 10: Ribonuclease protection reaction (comparison standard) with a mixture of 36, 48, and 72 h total RNA (1.66 µg each; 5 µg total) hybridized with each of the respective gene-specific probes and including the 28S rRNA probe. Lane 11: Undigested gene-specific probe (1,000 cpm). Lane 12: Gene-specific probe hybridized with 10 µg of yeast RNA and digested with ribonucleases. Lane 13: Gene-specific probe hybridized with 10 µg of yeast RNA in buffer devoid of ribonucleases.

transcripts in infected plants within 12 h of inoculation (Fig. 3a). This induction became more pronounced at 48 h and continued to accumulate throughout the 144-h postinoculation. In order to survey the transcript accumulation of the individual *RPR10* genes, gene-specific probes were constructed and used for hybridizations. *RPR10a*-specific transcripts showed an induction pattern similar to that of the overall *PR10* transcripts, showing an induction from a constitutive low basal level within 12 h after inoculation and exhibiting an increased and prolonged induction at 48, 72, and 144 h (Fig. 3b). Transcripts of *RPR10b* also were enhanced by *M. grisea*, although *RPR10b* induction appeared later than *RPR10a* and was first strongly visible at 48 h postinoculation (Fig. 3c). Unlike *RPR10a* and *RPR10b*, *RPR10c* was not induced by *M. grisea* throughout the 144-h examination period (Fig. 3d). This lack of induction further suggests that *RPR10c* is a pseudogene.

In order to verify the gene specificity of the respective probes, RNase protection studies were carried out with total RNA from Co39 rice seedlings infected with *M. grisea* for various time periods. RNase protection assays of the *RPR10a* transcript (protected fragment: 230 nucleotides; Fig. 4a) revealed a pattern of transcript accumulation that was similar to the RNA blot analysis of the same transcript. Likewise, RNase protection of the *RPR10b* transcript (protected fragment: 74 nucleotides; Fig. 4b) yielded similar results as those found by RNA blot analysis of *RPR10b* and showed strong induction at 48 h postinoculation. RNase protection assays specific to the *RPR10c* probe (Fig. 4c) did not reveal an expected 196-nucleotide protected fragment in any of the rice RNA samples tested, which is consistent with *RPR10c* being a pseudogene. To verify that equal quantities of RNA were used in most assays, a gene-specific probe for the 28S transcript was included in all protection assays. This standard probe yielded an expected protected fragment of 118 nucleotides, which corresponds to the 28S transcript. The unexpected 50 nucleotide protected fragment presumably results from the hybridization of the standard probe to the chloroplast 23S rRNA transcript. The 28S rRNA probe was not used in most of the assays conducted for the *RPR10b* transcript because its presence interfered with the detection of the 74-nucleotide protected fragment of the *RPR10b* assay.

To determine whether SA or JA induced *RPR10* genes, RNA blot analysis was performed on rice seedlings treated with either of these compounds. Figure 5 shows the blot of total RNA from Co39 seedling leaves at 0, 12, 18, 24, 36, 48, 72, and 144 h after infection with *M. grisea* (Fig. 5, lanes 1 to 8) and 24 h following treatment with either SA or JA (Fig. 5, lanes 9 and 10) with use of the gene-specific probe to

Fig. 5. RNA blot analysis of total RNA from rice cultivar Co39 seedling leaves at 0, 12, 18, 24, 36, 48, and 144 h after infection with *Magnaporthe grisea* and after 24 h following treatment with either salicylic acid (SA) or jasmonic acid (JA) with the use of a gene-specific probe for *PR10a*. The pTRI RNA 28S antisense probe was used to assure equal loading (data not shown).

RPR10a. *RPR10a* transcript is highly induced in noninfected rice seedlings by SA and JA, and is similar in intensity to the higher level of induction of *RPR10a* transcripts in rice leaves after 48 h of infection with *M. grisea*.

To identify the location of the *RPR10a* gene expression in relation to the site of pathogen infection, tissue prints of *M. grisea*-infected Co39 leaves were made with an antisense RNA probe that was gene specific for the *RPR10a* gene. Hybridization of the *RPR10a* probe was found to occur only at the infection site (Fig. 6) and illustrates that the *RPR10a* transcript is induced in a localized fashion by *M. grisea*.

DISCUSSION

In the present study, three genes with sequence homology to the PR-10 class of PR proteins were isolated from a single 16-kb rice genomic insert. Two of these genes, *RPR10a* and *RPR10b*, are transcriptionally active and induced in the rice plant during infection with *M. grisea*. The third gene, *RPR10c*, appears to be a nonfunctional pseudogene. When gene-specific probes are used, RNA blot and RNase protection assays reveal that the two functional genes are regulated transcriptionally in slightly different manners. *RPR10a* shows an early pattern of induction at 12 h postinoculation that is increased substantially at 48 h, whereas *RPR10b* is induced only after 48 h. These findings suggest that *RPR10a* may function in early and late periods of plant defense, whereas *RPR10b* functions primarily in later periods. Additionally, the bimodal accumulation of *RPR10a* transcript may indicate the action of

Fig. 6. Tissue print illustrating localized transcription of the *RPR10a* gene during *Magnaporthe grisea* infection. A 2-week-old rice cultivar Co39 seedling inoculated for 72 h with two adjacent drops of conidial suspension was pressed onto a nylon membrane and hybridized to an antisense RNA probe specific for *RPR10a*. Inset photograph shows a 4x magnification of the infection site.

two different transcriptional signals. Although it also is possible that the second phase of *RPR10a* expression is the result of an increase of fungal biomass, Talbot et al. (1993) have estimated by DNA blot analysis that fungal biomass does not increase significantly until 72 to 96 h after inoculation with the same *M. grisea* (cv. Guy-11)-Co39 pathosystem used in the present study. Additionally, microscopic studies show that a rapid and extensive *M. grisea* colonization of plant leaf area does not occur until approximately 96 h after infection (Heath et al. 1990; Peng and Shishimay 1988).

SA and JA.

SA and JA act as important endogenous signal molecules for the induction of PR genes in a variety of plant species, including tobacco, cucumber, and Arabidopsis (Rasmussen et al. 1991; Schweizer et al. 1997; Uknes et al. 1992; Ward et al. 1991; Xu et al. 1994). That SA can induce the transcription of PR-10 genes in plants has been shown in two previous studies in which *Sam22* from soybean (Crowell et al. 1992) and *AoPR1* from asparagus (Warner et al. 1994) were induced following treatment with SA. RNA blot analysis (Fig. 5) demonstrates that the *RPR10a* gene of this study is highly induced by SA and JA and suggests a role for both compounds in plant defense in rice. In this regard, recent studies (Schweizer et al. 1997) have shown that exogenous JA application of rice plants results in a coordinate accumulation of several mRNAs, including those from at least three PR protein families. Additionally, exogenous SA has been shown to induce the transcription of other PR genes in rice such as chitinase (Nishizawa and Hibi 1991) and glucanase (Simmons et al. 1992).

Transcript localization.

Tissue prints of *M. grisea*-infected Co39 rice seedlings were made to determine the location of *RPR10a* transcripts within rice leaf tissue, relative to the site of fungal infection. Hybridization of the *RPR10a* antisense RNA probe occurred primarily at the site of infection and did not appear to be systemic in nature. Other studies of PR-10 homologs in various plants also have shown localized transcription and/or expression in response to pathogen attack. For instance, in parsley, *in situ* RNA hybridization studies revealed that PR10(PR1-1) transcripts accumulate significantly around *Phytophthora megasperma* infection sites (Somssich et al. 1988) and, in sorghum, PR10 transcripts were expressed only in tissues immediately infected by pathogen (Lo et al. 1999). In transgenic tobacco plants containing fusion constructs of the *AoPR1* promoter from asparagus and the β -glucuronidase (GUS) reporter gene, GUS staining occurred predominantly at the very edge of disease lesions, with some staining in surrounding vascular tissue (Warner et al. 1993). In this regard, work by Pinto and Richardo (1995) have shown that PR-p16.5 proteins accumulate mainly above xylem tissues and in mid-vein parenchyma cells of infected *Lupinus albus* tissues.

Sequence analysis.

Examination of the *RPR10a* and *RPR10b* putative promoter regions shows the presence of distinct DNA motifs that are suspected of binding to nuclear factors in other PR-10 encoding genes. Two of these sequences, an E-box motif, which is the target sequence of helix-loop-helix factors, and an A-rich

element also are found within a 20-bp region of the potato *PR10a* gene promoter. In potato, this region is bound by the PBF-1 protein-binding factor suspected to be involved in the induction of the potato *PR10a* gene. PBF-1 is thought to be regulated positively by phosphorylation because it is phosphorylated only in tissues expressing the *PR10a* gene (Depres et al. 1995). A third element found in the *RPR10a* promoter is a TAATNN motif that is bound by various homeobox-containing proteins (Laughon 1991). This motif has been found in the parsley *PR-2* gene promoter (parsley *PR-2* is a *PR-10* homolog). In parsley, a cDNA encoding a suspected homeobox-containing protein was cloned and shown to bind to the sequence TAATTG (Korfhage et al. 1994). Mutation of this motif in the parsley *PR-2* gene reduced the elicitor responsiveness of this gene by twofold (Korfhage et al. 1994). The presence of common DNA motifs in the promoter region of *RPR10a* and other *PR10* genes suggests that they may have similar modes of regulation. Additional studies to determine the degree of similarities that exists within the promoter regions of PR10 genes will provide a better understanding of their induction and regulation and may aid in our knowledge of the induction of the general defense response in plants.

The coding region of the *RPR10a* gene shares almost 100% sequence identity (854 to 860 bp identical) to the *PBZI* rice cDNA reported by Midoh and Iwata (1996), which is likely the same gene. Comparisons of *RPR10a* and *PBZI* transcript accumulation, relative to induction by infection with virulent races of *M. grisea*, are similar, although several differences were observed. *RPR10a* and *PBZI* transcripts were present in noninfected rice seedlings at a low basal level. Unlike *RPR10a*, which showed a bimodal induction at 12 and 48 h postinoculation, transcript levels of *PBZI* did not increase from a low basal level until 60 h postinoculation. In addition, whereas *RPR10a* transcripts showed induction following foliar treatment with SA, *PBZI* transcripts did not. Several factors, including differences in varieties (Co39 versus Jikkoku) and *M. grisea* isolates (Guy-11 versus 007), differences in amounts of inoculum (1×10^6 versus 2×10^6 conidia per ml) and different periods of dark incubation following inoculation (24 versus 36 h), and differences in RNA harvest times after SA treatment (24 versus 60 h) could explain the variation between our work and the work of Midoh and Iwata (1996). Furthermore, the entire *PBZI* cDNA was used for RNA blot analysis (Midoh and Iwata 1996) and most likely hybridized to mRNA from other *RPR10* gene family members present in the rice genome. Additional studies are required to clarify these findings.

On the basis of a high sequence similarity between the RNase from ginseng (Moiseyev et al. 1994) and the PR-10 protein from parsley (Somssich et al. 1986), it has been suggested that this gene class may encode RNase. Although such enzymatic activity has not yet been demonstrated for any PR-10 class members of PR proteins, RNase activity has recently been reported with birch pollen allergen (Bufe et al. 1996; Swoboda et al. 1996). If PR-10 proteins do function as RNase, they are most likely to be specific for certain RNA substrates or require a specific activation for their enzymatic activity because their explicit activity would be detrimental to plant tissues. This specificity of RNase activity is supported by a lack of visible phenotypic abnormalities of transgenic potato plants constitutively expressing the *PR10a* gene from potato

(Constabel et al. 1993). If PR-10 proteins have such a function, an interesting parallel would be established between plant disease defense responses and pollen self-incompatibility in *Solanaceous* spp. plants because S-glycoproteins also demonstrate RNase activity (Clarke and Newbigin 1993; McClure et al. 1999). Verification of the role of PR-10 proteins as RNase and their function in plant defense responses will surely be the focus of future research.

MATERIALS AND METHODS

Construction and isolation of *M. grisea*-induced cDNA clones.

cDNA clones MAG-6 and MAG-7 were present in the pGEM-1 vector (Promega, Madison, WI, U.S.A.). Library construction, screening, and cDNA clone isolation resulted from work discussed in Talbot et al. (1993). *Xba*I and *Eco*RI were used to remove the cDNA inserts for characterization and use in DNA and RNA blot analysis.

Plant material and fungal inoculations.

Co39 seeds were obtained from the International Rice Research Institute (Los Banos, Philippines). Plants were grown in greenhouse conditions under long-day photoperiods (14 h day–10 h night). Standard procedures for the culture and storage of *M. grisea* were used (Crawford et al. 1986). Blast infections were carried out with 2-week-old Co39 rice seedlings sprayed with conidial suspensions of the compatible *M. grisea* isolate Guy-11 (0.4% gelatin, 1×10^6 conidia per ml). Inoculations and plant incubation conditions were performed as described by Valent et al. (1991). Chemical applications were performed on 2-week-old Co39 rice seedlings by saturating foliar spray with the use of either 30 mM SA or 1 mg of JA per ml (Sigma-Aldrich, St. Louis, MO, U.S.A.). Leaf tissues were harvested 24 h after treatment, frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction.

DNA and RNA blot analysis.

DNA was extracted from leaves of 1-month-old rice seedlings, following the method of McCouch et al. (1988). DNA (20- μg samples) were digested with restriction endonucleases, subjected to electrophoresis through 0.8% agarose gels, and blotted onto Hybond-N nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Prehybridization was performed for 2 to 4 h at 68°C ($5\times$ SSPE [$1\times$ SSPE is 0.18 M NaCl, 10 mM NaPO_4 , and 1 mM EDTA (pH 7.7)], $5\times$ Denhardt's solution, and 0.5% sodium dodecyl sulfate [SDS], and 20 μg of denatured salmon sperm DNA per ml). Hybridizations were performed in the same prehybridization solution at 65°C for 16 to 24 h. An *Eco*RI-*Xho*I fragment (0.54 kb) of the MAG-7 cDNA clone was used as probe. Following hybridization, membranes were washed twice (20 min each) in $2\times$ SSPE and 0.1% SDS at 68°C and once in $0.2\times$ SSPE and 0.1% SDS at 68°C for 10 min. Total RNA was isolated from Co39 rice seedlings at 0, 12, 18, 24, 36, 48, 72, and 144 h after infection with *M. grisea* in accordance with the LiCl precipitation method of DeVries et al. (1988). RNA gel blot analysis was carried out on 15 μg of RNA samples separated by electrophoresis under denaturing conditions (Talbot et al. 1993). RNA was transferred to Hybond-N, and hybridizations were carried out according to the manufacturer's suggestions.

The pTRI RNA 28S antisense probe (Ambion, Austin, TX, U.S.A.) was used as an internal loading control. This probe was found to hybridize to 28S and 23S rRNAs.

Rice genomic library construction and screening.

Genomic DNA (Co39) was isolated from leaves taken from 14-day-old seedlings by the methods of McCouch et al. (1988). DNA was partially digested with *Sau*3AI (1.11×10^{-2} units per μg of DNA at 37°C for 30 min). Partially digested DNA was cloned into GEM-11 *Xho*I half-site arms and packaged with the Packagene system, according to the manufacturer's instructions (Promega), with the use of bacterial host strain KW251. The plate lysate method was used to amplify the library (Sambrook et al. 1989). Low-density screening of a total of 250,000 PFU of the Co39 rice genomic library was carried out with the 0.54-kb MAG-7 cDNA insert as probe. Plaque lifts and hybridizations were performed with duplicate Hybond-N nylon filters according to Sambrook et al. (1989). Phage DNA was isolated from positively identified plaques by the liquid lysate method (Sambrook et al. 1989) and analyzed by restriction enzyme digestion and DNA blot analysis.

Subcloning of genomic insert fragments.

DNA fragments of the genomic insert, which hybridized to the MAG-7 cDNA probe and/or were of interest for sequencing, were isolated and cloned in the following manner. Phage DNA, typically 100 μg at a time, was digested to completion with either *Sac*I or *Bgl*II. Digested DNA was then electrophoresed in 1% TAE-agarose gels (Sambrook et al. 1989). Bands corresponding to 1.2-, 2.8-, 3.8-, 4.0-, and 4.1-kb *Sac*I fragments and bands corresponding to 2.7- and 4.2-kb *Bgl*II fragments were excised from the preparative TAE gels, and the DNA was isolated with Gene Clean II (Bio 101, Carlsbad, CA, U.S.A.), as described by the manufacturer. The respective fragments were subcloned into pGEM-7Zf(+) (Promega) according to the manufacturer's suggestions. Ligated plasmids containing *Sac*I inserts of 1.2, 3.8, and 4.0 kb or *Bgl*II inserts of 2.7 and 4.2 kb were transformed into XLI-Blue bacterial hosts (Stratagene, La Jolla, CA, U.S.A.) according to the supplier's protocol.

Sequencing.

Sequence data was obtained from the 1.2-, 3.8-, and 4.0-kb *Sac*I fragments with either nested sets of Exonuclease III (Erase-a-Base, Promega) deletion clones or by primer walking with oligonucleotides (Genosys Biotechnologies, The Woodlands, TX, U.S.A.). DNA sequences were determined with dATP 5'-[35S]-thiotriphosphate (Amersham Pharmacia Biotech) and the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH, U.S.A.). This procedure was modified to include the use of terminal deoxynucleotidyl transferase (United States Biochemical) to reduce sequence artifacts according to the procedure of Redston and Kern (1994). Databases at the National Center for Biological Information (NCBI, Bethesda, MD, U.S.A.) were used to compare sequence homology to other DNA sources.

RT-PCR.

RT-PCR was performed to obtain full-length cDNA sequence information for the *RPR10a* gene and attempted (un-

successfully) for the *RPR10c* gene. All reactions were carried out with the 5' Race System (Life Technologies, Gaithersburg, MD, U.S.A.) according to the manufacturer's protocols. First-strand cDNA synthesis was performed with poly(A)+ RNA isolated from Co39 rice seedlings that had been inoculated for 72 h with *M. grisea*. cDNA synthesis was primed with gene-specific antisense oligonucleotides *RPR10a*-GSP1 and *RPR10c*-GSP1 that were present in the putative 3' UTR of the respective genes. Homopolymeric (dC)-tailed cDNAs were amplified by PCR with a mixture of two primers: a nested gene-specific primer *RPR10a*-GSP2 and *RPR10c*-GSP2, which anneals 3' to GSP1, a manufacturer-supplied anchor primer. PCRs were performed in 45- μ l volumes containing 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM MgCl₂; 200 nM GSP2; 400 nM anchor primer; 200 nM dATP, dCTP, dGTP, and dTTP each; and 0.04 units of Tag DNA polymerase per μ l (PE Applied Biosystems, Foster City, CA, U.S.A.) with 2 min at 94°C, 2 min at 45°C, and 2 min at 72°C for 30 cycles. The primers used in this procedure were *RPR10a*-GSP1, 5'-TAC-TCTCAGGACTCAAACGCCA-3'; *RPR10c*-GSP1, 5'-AC-GAGAAAATTGGAGCTCGC-3'; *RPR10a*-GSP2, 5'-CAUC-AUCAUCACTGGATAGAGGC-AGTATTCC-3'; *RPR10c*-GSP2, 5'-CAUCAUCAUGCCATGGATTGTGTTGG-TTG-3'; 5' rapid amplification of cDNA ends (RACE) anchor primer, 5'-CUACUACUACUAGGCCACGCGTCGACTAG-TACGGGIIGGGIIGGG11G-3'. Following amplification, RT-PCR products were cloned into the pAMP1 plasmid by uracil DNA glycosylase (UDG) cloning with the Clone Amp1 system (Life Technologies).

Construction of gene-specific probes.

Gene-specific probes were made from the putative 3' UTR of *RPR10a* and *RPR10c* and from the putative 5' UTR of *RPR10b* by PCR cloning. The gene-specific probe for *RPR10a* was constructed with the 3.8-kb *SacI* insert fragment as template and oligonucleotide primers *RPR10a* forward primer 5'-AGGAATACTGCCTCTATCCAG-3' and *RPR10a* reverse primer 5'-GCAGAACACATTCAGACTTGC-3'. The gene-specific probe for *RPR10b* was constructed with the 2.7-kb *BglIII* insert fragment and oligonucleotide primers *RPR10b* forward primer 5'-CACAGGTCAAACCATGTGATC-3' and *RPR10b* reverse primer 5'-CTCTATCTAAGCTAGTTTCCACCTG-3'. The gene-specific probe for *RPR10c* was constructed with the 4.2-kb *BglIII* insert fragment and oligonucleotide primers *RPR10c* forward primer 5'-CTGTCTCAACCAACAATCC-3' and *RPR10c* reverse primer 5'-CACGTGTTCTAGATGTTTGTCTC-3'. PCRs were performed in 50- μ l volumes containing 1 μ g of DNA template; 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton X 100; 1 mM MgCl₂; 1 mM dATP, dTTP, dCTP, and dGTP, each; 0.4 mM forward primer; and 0.4 mM reverse primer. Template DNA was denatured by heating at 95°C for 5 min followed by the addition of 2.5 units of Taq DNA polymerase. PCR was performed at 2 min at 95°C, 2 min at 50°C, and 2 min at 72°C for 30 cycles. PCR products were electrophoresed in 1% agarose-TAE gels, purified with Gene Clean II, cloned into pT7 Blue R (Novagen, Madison, WI, U.S.A.) in accordance with manufacturer's protocol, bands were excised, and the resulting plasmids were transformed into *Escherichia coli* DH5 α cells by standard procedures (Sambrook et al. 1989). Clones containing each gene-specific segment were sequenced in their entirety and

compared to their respective template sequence. PCR products obtained for *RPR10a*, *RPR10b*, and *RPR10c* gene-specific probes were 229, 231, and 197 bp, respectively.

RNAse protection assays.

Gene-specific antisense RNA probes were synthesized with the Maxiscript II System (Ambion). An antisense RNA probe of eukaryotic 28S rRNA was additionally synthesized with the pTRI-RNA 28S antisense control template. Full-length probes were purified from 5% denaturing polyacrylamide gels as described (Sambrook et al. 1989). RNAse protection assays were performed on total RNA samples isolated from 2-week-old Co39 seedlings inoculated with *M. grisea* for 0, 12, 18, 24, 36, 48, 72, and 144 h. Assays were carried out with the RPA II System (Ambion), as suggested by the manufacturer. Five micrograms of RNA samples of each of the time points were hybridized with 2.5 fmole of high-specific activity (typically 6 to 7 $\times 10^8$ cpm per μ g) antisense probes specific for either *RPR10a*, *RPR10b*, or *RPR10c* and with 0.5 μ g of low-specific activity (typically 7 to 7.5 $\times 10^4$ cpm per μ g) 28S rRNA probe. RNAse digestions were performed with a 1:1000 dilution of the supplied RNAse cocktail (final concentration = 0.2 units RNAse A per ml and 2.0 units of RNAse T1 per ml. Reactions were subjected to electrophoresis through 5% denaturing polyacrylamide gels, wrapped in plastic wrap, and exposed to X-ray film for radiography. The expected sizes of the protected fragments with use of the various gene-specific hybridizing probes were *RPR10a*, 230 bp; *RPR10b*, 74 bp; *RPR10c*, 196 bp; and 28S rRNA, 118 bp.

Tissue printing.

Co39 seeds were sterilized and germinated in double-stacked Magenta boxes (Sigma, St. Louis, MO, U.S.A.) containing a modified MS medium (Murashige and Skoog 1962) with 1/2 strength MS salts (pH 5.70), 2% sucrose, and 0.6% agarose. Seedlings were grown aseptically for 14 days in a lighted growth chamber at 26 $\pm$ 1°C with a 16-h daily photoperiod (150 μ E m⁻² per s). Seedlings were inoculated with 20- μ l drops of *M. grisea* conidial suspension (0.4% gelatin and 50 conidia per μ l). Following inoculation, the Magenta boxes were carefully closed, placed in the dark at 26°C for 14 h, and placed into a lighted growth chamber at 26 $\pm$ 1°C with a 16-h daily photoperiod (150 μ E m⁻² per s) for an additional 58 h. Seedlings were removed from the Magenta boxes, placed carefully between one sheet of wax paper and one sheet of medium grit carborundum paper, and pressed at 10,000 psi for 1 min in a Model C Carver Laboratory Press (Fred S. Carver, Menomonee Falls, WI, U.S.A.). After pressing, the seedling was peeled carefully from the carborundum paper and placed, abaxial side down, onto Hybond-N nylon membrane, and covered with wax paper. Three pieces of Whatman 1-mm paper (Kent, U.K.) were placed on the outer side of the nylon membrane and on the outer side of the wax paper (Whatman paper provides an equalizing cushion so that the press is evenly distributed). Arranged in this manner, the seedling was again placed into the Carver Press and was subjected to 24,000 psi for an additional 10 min. The seedling was then peeled carefully from the nylon membrane. The membrane was fixed by baking at 80°C for 2 h, wrapped in plastic wrap, and stored at -20°C. Hybridizations were performed essentially the same

as they were for RNA blotting. The *RPR10a* antisense RNA was used as probe.

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