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Direct interaction of resistance gene and avirulence gene products confers rice blast resistance

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Rice expressing the *Pi-ta* gene is resistant to strains of the rice blast fungus, *Magnaporthe grisea*, expressing *AVR-Pita* in a gene-for-gene relationship. *Pi-ta* encodes a putative cytoplasmic receptor with a centrally localized nucleotide-binding site and leucine-rich domain (LRD) at the C-terminus. *AVR-Pita* is predicted to encode a metalloprotease with an N-terminal secretory signal and pro-protein sequences. AVR-Pita$_{176}$ lacks the secretory and pro-protein sequences. We report here that transient expression of *AVR-Pita$_{176}$* inside plant cells results in a *Pi-ta*-dependent resistance response. AVR-Pita$_{176}$ protein is shown to bind specifically to the LRD of the Pi-ta protein, both in the yeast two-hybrid system and in an *in vitro* binding assay. Single amino acid substitutions in the Pi-ta LRD or in the AVR-Pita$_{176}$ protease motif that result in loss of resistance in the plant also disrupt the physical interaction, both in yeast and *in vitro*. These data suggest that the AVR-Pita$_{176}$ protein binds directly to the Pi-ta LRD region inside the plant cell to initiate a *Pi-ta*-mediated defense response.

Keywords: ligand/*Magnaporthe grisea*/receptor/rice/signal recognition

Introduction

Plants have evolved sophisticated multi-faceted defense mechanisms against pathogens. These defense responses include a rapid, localized cell death termed the hypersensitive response (HR), production of antimicrobial compounds, lignin formation, an oxidative burst and increased expression of pathogenesis-related genes (Cutt and Klessig, 1992; Goodman and Novarcky, 1994; Levine *et al*., 1994; Mehdy, 1994). The defense responses are often activated by the action of a host resistance (*R*) gene and a pathogen avirulence (*AVR*) gene as proposed by the gene-for-gene hypothesis (Flor, 1971). One possible explanation for the molecular basis of gene-for-gene interactions is a ligand and receptor model where the *R* gene product acts as a receptor that recognizes a ligand, or elicitor, produced directly or indirectly by the pathogen's *AVR* gene. This interaction then results in defense response activation. Structures of cloned *R* genes seem to support the ligand–receptor model because the majority of plant *R* genes contain leucine-rich repeat (LRR) domains (Bent, 1996; Hammond-Kosack and Jones, 1997). LRR proteins from animals and fungi mediate specific protein–protein interactions or ligand binding (Braun *et al*., 1991; Kobe and Deisenhofer, 1993; Jones and Jones, 1996). LRR-containing plant *R* genes fall into two classes, those with extra-cytoplasmic LRRs and those with cytoplasmic LRRs. The largest *R* gene class encodes cytoplasmic proteins with centrally localized nucleotide-binding sites (NBS) and LRRs near the C-terminus (Jones and Jones, 1996; Leister *et al*., 1996; Vos *et al*., 1998). Although the presence of the LRR domains in R proteins is consistent with their proposed receptor function, direct binding between an LRR-containing R protein and an AVR ligand has not yet been demonstrated.

In contrast to the similarities among cloned plant *R* genes, sequence analysis of cloned *AVR* genes from viral, bacterial and fungal pathogens revealed few similarities and few clues to their functions for the pathogen (for reviews see Dangl, 1994; Joosten *et al*., 1994; Rohe *et al*., 1995). A number of *AVR* genes have been shown to encode specific elicitor proteins, which induce HR either when infiltrated into the apoplastic space outside plant cells (de Wit, 1995; Knogge, 1996) or when expressed inside plant cells (Culver and Dawson, 1991; Gopalan *et al*., 1996; Leister *et al*., 1996; Van den Ackerveken *et al*., 1996; Bonas and Van den Ackerveken, 1997). The diversity among *AVR* genes is consistent with theories of evolution of gene-for-gene resistance as host plants acquired the ability specifically to recognize random molecules produced by pathogens.

Molecular characterization of several matched pairs of plant *R* genes and pathogen *AVR* genes made it possible to test the gene-for-gene receptor–ligand model. A direct interaction has been demonstrated between the tomato bacterial speck resistance gene product, Pto, and the corresponding avrPto avirulence gene product of *Pseudomonas syringae* pv. *tomato* (Scofield *et al*., 1996;Tang *et al*., 1996). Pto, a serine/threonine kinase, is a unique *R* gene product that lacks an LRR domain (Martin *et al*., 1993). Physical interaction between Pto and avrPto proteins was discovered initially with the yeast two-hybrid system (Scofield *et al*., 1996; Tang *et al*., 1996), and several lines of evidence suggest that the interaction is specific and essential for activation of the host defense response. First, the bacterial speck-susceptible cultivar Alisa Craig encodes an active Pto kinase that failed to bind avrPto in the yeast two-hybrid system (Tang *et al*., 1996; Jia *et al*., 1997). Secondly, a single amino acid residue, Thr204 of the Pto kinase, determines the

recognition specificity for avrPto in both the yeast two-hybrid system and when *Pto* is transiently expressed in plant cells using *Agrobacterium*-based DNA transfer (Frederick *et al*., 1998). Thirdly, C-terminal deletions of the avrPto protein that interact with the Pto protein in the yeast two-hybrid system also elicited HR and disease resistance in a *Pto*-dependent manner in plants (Tang *et al*., 1996).

The interaction between rice (*Oryza sativa*) and the fungal pathogen *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc.) is a well documented gene-for-gene system (Silue *et al*., 1992; Valent, 1997). The rice blast disease caused by *M.grisea* is one of the most devastating plant diseases worldwide (Valent and Chumley, 1991). This hemibiotropic pathogen penetrates directly through the plant cuticle and outer cell wall into epidermal cells of the host (Howard *et al*., 1991). The fungus grows intracellularly, filling individual plant cells with fungal mycelium before moving on to the next cell. It is unknown whether the intracellular hyphae of this pathogen are enveloped with plant plasma membrane, as is the case for haustoria produced by biotrophic fungi, or if the pathogen breaches the plasma membrane. Thus it is also unknown how *M.grisea* might deliver *AVR*-encoded proteins into the cytoplasm of plant cells.

Intense research with the *M.grisea* system has led to the isolation of the matched pair of resistance gene *Pi-ta* and avirulence gene *AVR-Pita*, previously referred to as *AVR2-YAMO* (*Pi-ta*, Bryan *et al*., 2000; *AVR-Pita*, Orbach *et al*., 2000). *Pi-ta* is a single copy gene encoding a putative cytoplasmic protein of 928 amino acids with a centrally localized NBS domain and a leucine-rich domain (LRD) at the C-terminus. Pi-ta contains 16.4% leucine within this LRD region (amino acids 586–928, http:/www.expasy.ch/), which approximately corresponds to the LRR domain of the RPM1 protein (Grant *et al*., 1995; Bryan *et al*., 2000). Although Pi-ta is most similar to R proteins of the NBS-LRR class, the Pi-ta LRD has areas of LXXLXXL motifs, but it does not contain LRRs fitting any previously reported consensus (Jones and Jones, 1996). AVR-Pita is hypothesized to be a neutral zinc metalloprotease, although direct biochemical evidence is lacking (Orbach *et al*., 2000).

We report here evidence that AVR-Pita functions as an elicitor molecule that directly binds the Pi-ta protein and triggers a signal transduction cascade leading to resistance. First, we demonstrate that AVR-Pita$_{176}$, a putative mature form of the protease containing the C-terminal 176 amino acids, is a genotype-specific elicitor, i.e. biolistic transient expression of *AVR-Pita$_{176}$* inside cells of resistant *Pi-ta* rice results in reduced activity of β-glucuronidase (GUS), an indicator of HR (Mindrinos *et al*., 1994; Gopalan *et al*., 1996). Next, we demonstrate a specific physical interaction between AVR-Pita$_{176}$ and the Pi-ta LRD region in the yeast two-hybrid system. Finally, we corroborate our two-hybrid interaction by showing that recombinant Pi-ta LRD protein binds specifically to the AVR-Pita$_{176}$ protein in far-western analysis. We suggest that AVR-Pita is translocated from the fungus into the cytoplasm of the plant cell where it binds to the Pi-ta LRD region to initiate a *Pi-ta*-mediated defense response.

Results

Optimization of a transient expression system in rice using biolistic bombardment

Transient gene expression systems have been used previously to investigate elicitor activity of avirulence proteins in dicot plants (Mindrinos *et al*., 1994). The assay is based on association of resistance with HR, and on reduced expression of a GUS reporter gene as an indicator of HR (Dixon *et al*., 1994; Mindrinos *et al*., 1994). We modified the transient expression system to analyze the function of the AVR-Pita protein in intact rice seedlings. We co-introduced two plasmids by particle bombardment, one expressing a GUS reporter gene and the other expressing AVR-Pita polypeptides. Resistant and susceptible seedlings were positioned side-by-side in the same Petri dish for bombardment. This was important since the efficiency of transformation varies both between bombardments and between different areas of the target tissue within a single bombardment (data not shown). To ensure efficient expression of *AVR-Pita*, we engineered a plasmid that contained a 493 bp *Adh1-6* intron behind the cauliflower mosaic virus 35S (CaMV 35S) promoter (Figure 1A; Freeling and Bennet, 1985). This construct previously has been shown to mediate high levels of expression in rice (Freeling and Bennet, 1985; S.A.McAdams, unpublished data). We also used the CaMV 35S promoter to express the GUS reporter gene *uidA* (Figure 1B). These constructs were assayed in two resistant *Pi-ta* rice cultivars (Yashiromochi and YT14), and two susceptible *pi-ta* cultivars (Nipponbare and YT16). Equal GUS activity was observed repeatedly in all four cultivars in the absence of *AVR-Pita* constructs (data not shown).

Pi-ta-containing rice plants undergo HR when they are inoculated with an avirulent *M.grisea* strain containing

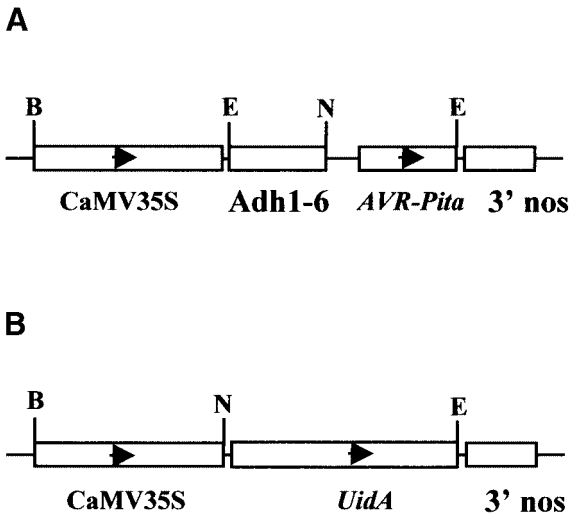

Fig. 1. Physical maps of the transgenes used for transient expression analysis. (**A**) *AVR-Pita* expression vector. The maize *Adh1-6* intron was inserted after the CaMV 35S promoter resulting in a fusion promoter for expression of constructs encoding AVR-Pita$_{223}$, AVR-Pita$_{176}$ and AVR-Pita$_{166}$. (**B**) GUS expression vector. Plasmid pML63 contained the *uidA* gene, encoding GUS, under control of the CaMV 35S promoter. The restriction endonucleases used for generating constructs are shown (B, *Bam*HI; E, *Eco*RI; N, *Nco*I). Both expression cassettes contain bacterial 3′ nos terminator sequences.

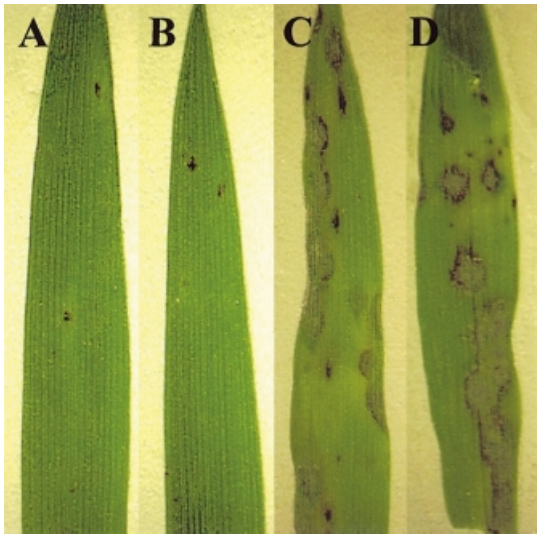

Fig. 2. Genotype-specific HR in rice seedlings induced by *M.grisea* carrying *AVR-Pita*. Sparse HR flecking is seen in *Pi-ta*-containing rice seedlings (**A**) Yashiro-mochi and (**B**) YT14, as expected. In contrast, typical symptoms of rice blast disease are seen in susceptible rice seedlings (**C**) Nipponbare and (**D**) YT16. Representative leaves are shown from rice seedlings germinated in plant nutrient medium and infected with avirulent *M.grisea* strain 4360-R-62 (see Materials and methods for details). Shown at 4 days after inoculation.

AVR-Pita. To ensure that *Pi-ta* specificity is maintained in seedlings used for transient expression analysis, we inoculated seedlings grown to the two-leaf stage on agar medium with an avirulent pathogen. Figure 2A and B shows sparse HR flecking in resistant *Pi-ta* cultivars, and Figure 2C and D shows typical leaf blast symptoms in susceptible *pi-ta* cultivars. Thus, we conclude that intact two-leaf rice seedlings are suitable for transient gene expression analysis.

AVR-Pita$_{176}$ is an elicitor that acts inside the plant cell

AVR-Pita is predicted to encode a 223 amino acid protein with similarity to fungal zinc metalloproteases. Specifically, the homology occurs in the C-terminal 176 amino acids of the proteins, a region corresponding to the mature processed version of known metalloproteases (Tatsumi *et al*., 1994; Orbach *et al*., 2000). Due to uncertainty as to the active form of the AVR-Pita protein *in vivo*, we produced constructs for expressing the full-length protein (AVR-Pita$_{223}$), the putative mature protease (AVR-Pita$_{176}$) and a protein with 10 amino acids deleted from the N-terminus of AVR-Pita$_{176}$ (AVR-Pita$_{166}$) (Figure 3A). Plasmids expressing these *AVR-Pita* constructs and plasmids expressing the GUS reporter were co-introduced into leaves of *Pi-ta* and *pi-ta* rice seedlings. GUS activity was then assayed histochemically 2 days after bombardment. No reduction of GUS activity was observed in any of the rice cultivars when *AVR-Pita*$_{223}$ was co-expressed with the GUS reporter (Figure 3A). However, we repeatedly observed reduced GUS activity in leaves of resistant *Pi-ta* varieties but not in leaves of susceptible *pi-ta* varieties when *AVR-Pita*$_{176}$ and the GUS reporter were co-expressed (Figure 3A and B). Co-expression of the GUS reporter and *AVR-Pita*$_{166}$ did not result in reduced GUS activity on either susceptible or resistant rice varieties (Figure 3A). Thus, the putative mature AVR-Pita$_{176}$ protein, but not AVR-Pita$_{223}$ or AVR-Pita$_{166}$, induces HR when produced inside cells of *Pi-ta*-containing rice.

We tested the specificity of the HR induction in the transient assay using virulent *avr-pita* alleles with single amino acid substitutions that abolish *AVR-Pita* avirulence function in whole-plant infection assays. Changing the putative protease motif active site residue (amino acid 177 in AVR-Pita$_{223}$) from glutamic acid to aspartic acid (E177D) eliminated avirulence activity in standard fungal infection assays. Co-expression of virulent *avr-pita*$_{176}^{E177D}$ with the GUS reporter gene failed to reduce GUS activity in either resistant *Pi-ta* or susceptible *pi-ta* rice cultivars (data not shown). Likewise, changing methionine to tryptophan at amino acid residue 178 (M178W) eliminated avirulence in infection assays as well as activity in the transient assay (data not shown). Thus, *AVR-Pita* mutations that abolish avirulence in fungal infection assays also abolish the ability to induce HR upon transient expression in *Pi-ta*-containing rice cells.

To confirm that *AVR-Pita*$_{176}$ was expressed in the bombarded seedlings, we extracted poly(A)$^+$ mRNA from leaves 2 days after co-bombardment and performed RNA gel blot analysis using an *AVR-Pita*$_{176}$ probe. Transcripts of *AVR-Pita*$_{176}$ were detected in susceptible *pi-ta* seedlings, but not in resistant *Pi-ta* seedlings (Figure 3C). These data demonstrate that the *AVR-Pita*$_{176}$ gene is expressed in susceptible seedlings, and that this expression has no effect on GUS activity. The absence of *AVR-Pita*$_{176}$ transcripts in *Pi-ta*-containing plant cells is consistent with the occurrence of HR-associated cell death in transformed cells. Thus, all data are consistent with the conclusion that the AVR-Pita$_{176}$ protein specifically induces a defense response when introduced into plant cells expressing *Pi-ta*.

AVR-Pita$_{176}$ binds specifically to the Pi-ta LRD region in the yeast two-hybrid system

Since the Pi-ta protein confers recognition specificity in blast resistance and AVR-Pita$_{176}$ is an elicitor of HR in a *Pi-ta*-dependent manner, we examined whether the Pi-ta protein interacts with AVR-Pita$_{176}$ using the yeast two-hybrid system (Fields and Song, 1989). We constructed a series of N-terminal deletions of the Pi-ta protein to examine the requirement for different protein domains for any physical interaction (Figure 4A). These *Pi-ta* constructs were expressed as N-terminal fusions to the GAL4 DNA-binding domain (BD). The AVR-Pita$_{176}$ protein was expressed as an N-terminal fusion to the transcriptional activation domain (AD). Physical interaction can be detected by activation of two reporter genes, *HIS3* conferring histidine prototrophy and *lacZ*, when BD and AD fusions are expressed in the same yeast cell. Expression of either the *BD-Pi-ta* construct or the *AD-AVR-Pita*$_{176}$ construct alone did not activate either reporter gene (data not shown). Positive and negative controls provided with Stratagene's two-hybrid kit gave the expected results (Figure 4A).

Only one of the four deletion constructs tested in the two-hybrid assay activated both the *HIS3* and *lacZ* reporter genes, demonstrating a physical interaction (Figure 4A). The active construct, deletion NΔ586, encodes the

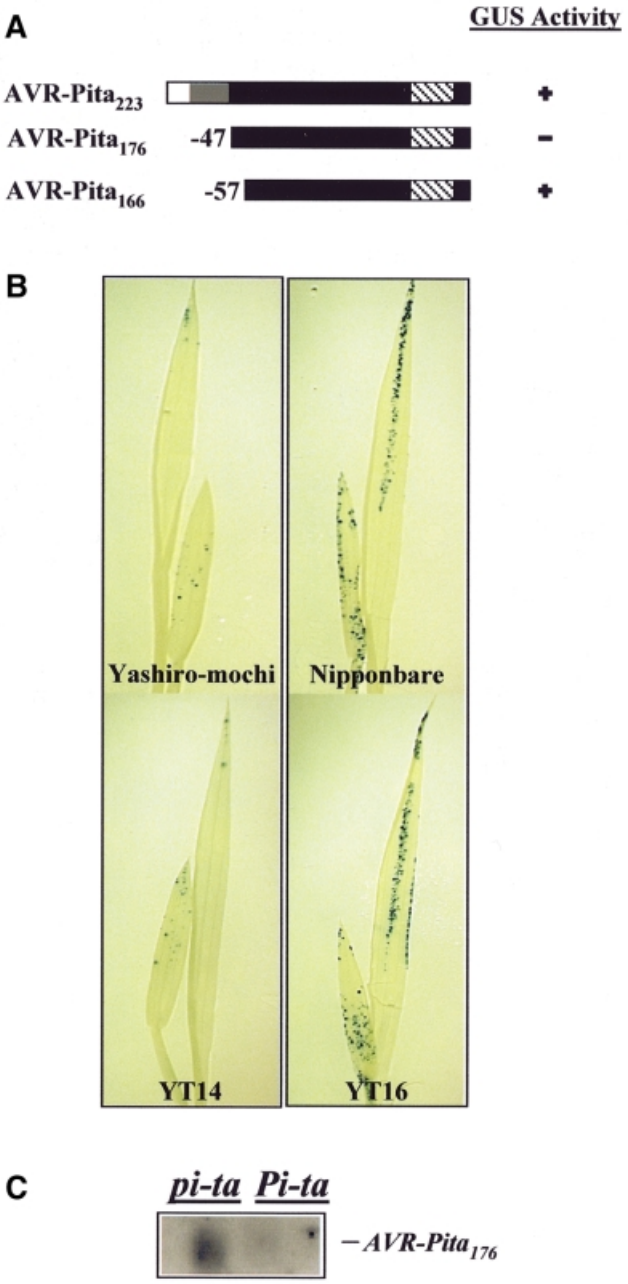

Fig. 3. AVR-Pita$_{176}$ is an elicitor. (**A**) AVR-Pita polypeptides tested in the transient assay. The white region indicates the putative secretory signal sequence, the gray region indicates the putative pro-protein domain and the hatched region indicates the putative protease motif. The black region indicates the putative mature protein. The number of amino acids missing from the N-terminus is indicated. GUS activity is indicated by '+', whereas decreased GUS activity is indicated by '–'. (**B**) Representative rice seedlings showing GUS activity. Two-leaf *Pi-ta* (Yashiro-mochi and YT14) and *pi-ta* (Nipponbare and YT16) seedlings were co-bombarded with *35S/Adh1-6::AVR-Pita$_{176}$* and *35S::uidA*. Leaves were assayed histochemically for GUS activity and cleared in 70% ethanol to visualize GUS staining. (**C**) RNA gel blot analysis of *AVR-Pita* expression in the transient assay. YT14 (*Pi-ta*) and YT16 (*pi-ta*) were co-bombarded with the *35S/Adh1-6::AVR-Pita$_{176}$* and *35S::uidA* plasmids. Leaf tissue was harvested 2 days after bombardment. Poly(A)$^+$ mRNA was then extracted, blotted to Hybond-N and hybridized with a radiolabeled *AVR-Pita$_{176}$* probe. The *AVR-Pita* transcript is indicated. Similar loading was verified before blotting by visualizing mRNA in the gel stained with ethidium bromide.

341 amino acid C-terminal portion of the Pi-ta protein that we refer to as LRD. Interestingly, the reciprocal combination of LRD in AD fusion and AVR-Pita$_{176}$ in BD fusion demonstrated an 'impaired interaction' in which the histidine biosynthetic gene was not activated, and the *lacZ* reporter gene was activated, but pigment accumulation was slowed in comparison with activation by the LRD in BD fusion (data not shown). This could result from steric constraints imposed by DNA AD and BD fusions in this particular orientation. Low levels of *lacZ* activity also indicated weak interactions between the full-length Pi-ta protein and AVR-Pita$_{176}$, and between the remaining three Pi-ta polypeptides and AVR-Pita$_{176}$ (Figure 4A).

To determine the specificity of the physical interaction between the Pi-ta LRD and AVR-Pita$_{176}$, we constructed a BD fusion with the LRD region encoded by a susceptible *pi-ta* allele and tested its ability to interact with AD-AVR-Pita$_{176}$. Because all susceptible Pi-ta LRDs have a single amino acid substitution, serine for alanine at residue 918 (Bryan *et al*, 2000), we designated susceptible Pi-ta LRD as LRD^{A918S}. The impaired interaction of weak lacZ activity was observed between LRD^{A918S} and AVR-Pita$_{176}$ (Figure 4B), suggesting that substituting serine for Ala918 alters the binding specificity. As a further test to correlate the *Pi-ta* gene-for-gene interaction with physical interaction of the Pi-ta LRD and AVR-Pita$_{176}$ proteins, we co-expressed the resistant LRD in BD fusion with a virulent form of AVR-Pita$_{176}$ (avr-pita^{E177D}) in AD fusion. Similar to results with LRD^{A918S}–AVR-Pita$_{176}$ interaction, co-expression of these fusions gave weak lacZ activity, but did not activate the histidine reporter gene (Figure 4C). Finally, physical interaction of LRD with AVR-Pita$_{223}$ was also examined, and this combination failed to activate either reporter gene (data not shown). Thus, the specific physical interaction between the LRD and AVR-Pita$_{176}$ suggests that AVR-Pita$_{176}$ serves directly as a fungal signal molecule and that Pi-ta serves as a cognate receptor molecule.

Membrane-immobilized AVR-Pita$_{176}$ binds to Pi-ta and its LRD

To confirm our two-hybrid findings and test whether AVR-Pita$_{176}$ is also capable of binding to the full-length Pi-ta protein, we next used bacterially produced recombinant proteins for *in vitro* binding experiments. We used a modified far-western (Chen and Evans, 1995) procedure to conduct these protein–protein interaction experiments because bacterially produced Pi-ta protein was soluble and bacterially produced AVR-Pita proteins were insoluble. For our studies, purified AVR-Pita proteins were refolded on a nitrocellulose membrane, and incubated with total *Escherichia coli* extracts containing soluble Pi-ta protein.

To facilitate detection and purification, the Pi-ta protein was engineered with an N-terminal S tag, a synthetic peptide composed of 15 amino acids (Novagen). We then expressed the S-Pi-ta fusion protein in *E.coli* and recovered it from the soluble fraction of total proteins. Figure 5A shows that the Pi-ta protein was highly expressed. The recombinant Pi-ta protein has an estimated molecular mass of ~110 kDa as detected by S-antibody. Similarly, AVR-Pita$_{176}$ was engineered to contain an N-terminal His tag to facilitate purification. The recombi-

nant protein was found at high levels within inclusion bodies when expressed in *E.coli*. The corresponding virulent mutant protein avr-pita$_{176}^{M178W}$ was also expressed and purified from inclusion bodies for use as a control. These recombinant AVR-Pita and avr-pita proteins had an estimated molecular mass of 22 kDa (Figure 5B).

For far-western analysis, equal amounts of the purified AVR-Pita proteins were separated on a gradient SDS–polyacrylamide gel and immobilized on a nitrocellulose membrane for refolding. Immobilized, refolded AVR-Pita was then incubated with soluble extracts from *E.coli* transformants expressing S-tagged Pi-ta proteins. S-antibody was then used to detect Pi-ta protein bound to AVR-Pita on the membrane. Figure 5B shows that the Pi-ta protein bound to both AVR-Pita$_{176}$ and avr-pita$_{176}^{M178W}$. Thus, AVR-Pita$_{176}$ binds to the full-length Pi-ta protein, but the *in vivo* specificity is not retained under these conditions. Binding of full-length Pi-ta protein to avr-pita$_{176}^{M178W}$ in this instance does not correlate with ability to trigger the *Pi-ta*-mediated resistance *in vivo*.

To confirm the yeast two-hybrid interaction between LRD and AVR-Pita$_{176}$, we engineered plasmids expressing an N-terminal S tag fusion to both LRD and LRD^{A918S} polypeptides. Each fusion construct expressed a polypeptide with an estimated molecular mass of 42 kDa in the soluble fraction of *E.coli* (Figure 6A). Figure 6B shows that the LRD protein bound specifically to AVR-Pita$_{176}$, but not to avr-pita$_{176}^{M178W}$. Consistent with the results obtained from the yeast two-hybrid system, the susceptible LRD^{A918S} protein did not bind to AVR-Pita$_{176}$ under the same conditions (Figure 6C). These results *in vitro* confirm the interaction specificity of the Pi-ta LRD and AVR-Pita$_{176}$ previously demonstrated in the yeast two-hybrid assay.

Discussion

Transient expression of the predicted mature AVR-Pita$_{176}$ protease inside plant cells by particle bombardment transformation results in HR as measured by decreased GUS activity. HR occurs specifically on *Pi-ta* rice plants, not *pi-ta* plants. This result suggests that the AVR-Pita$_{176}$ protein is a race-specific elicitor that triggers *Pi-ta*-mediated resistance to the rice blast fungus. We used the yeast two-hybrid system and *in vitro* binding assays to demonstrate that the C-terminal leucine-rich region of Pi-ta protein functions as an elicitor-binding domain. Our data suggest that the Pi-ta protein is an intracellular receptor that binds directly to the AVR-Pita$_{176}$ protein inside the plant cell to initiate the defense response. These results may provide general insight into the way that the NBS class of plant *R* genes is able to detect the products of *AVR* genes.

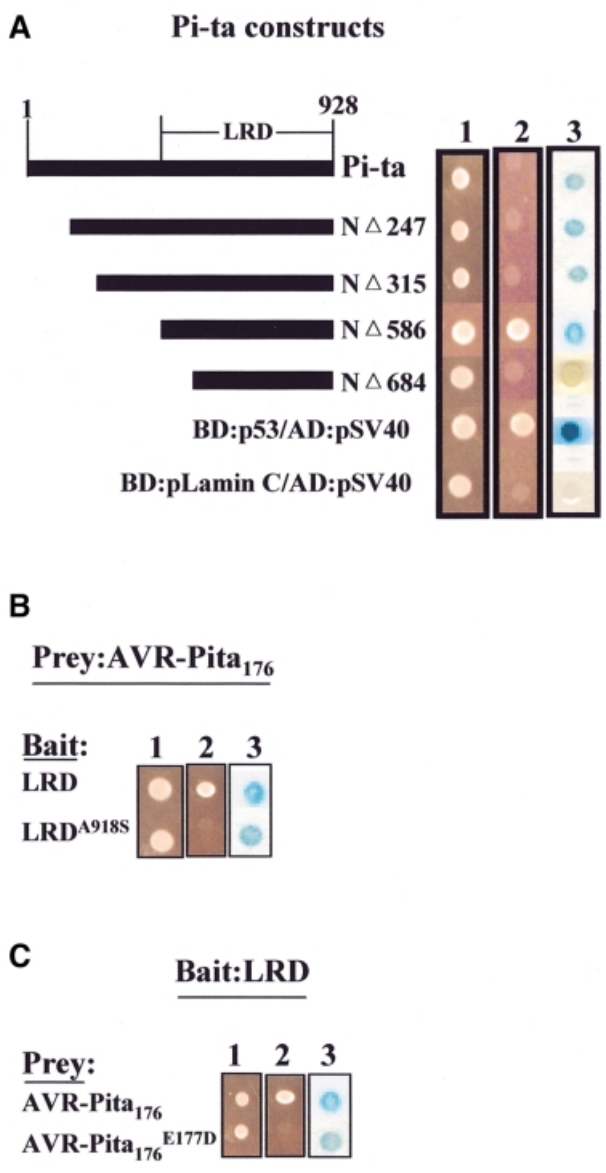

Fig. 4. AVR-Pita$_{176}$ interacts specifically with the Pi-ta LRD in the yeast two-hybrid system. (**A**) Mapping the interaction domain of the Pi-ta protein using the yeast two-hybrid system. The diagram (left) depicts the Pi-ta protein, the LRD region and a series of deletion constructs of Pi-ta. (1) YRG2 yeast cells expressing both bait and prey fusions were grown on yeast synthetic minimal (SD, Stratagene) liquid medium with omission of leucine and tryptophan (SD-LT), and (2) on yeast SD medium with omission of leucine, histidine and tryptophan for examination of *HIS3* reporter gene (–LHT). (3) Yeast cells expressing both bait and prey fusions were grown on yeast SD-LT plates and assayed for lacZ activity as described in Materials and methods. Color development is shown after 24 h. *AVR-Pita$_{176}$* was cloned as an AD fusion (in pAD-GAL4) and each *Pi-ta* deletion construct was cloned as a BD fusion (in pBD-GAL4 Cam). The positive control was YRG2 cells containing p53 and pSV40 fusion constructs, which express proteins that interact *in vivo*, and the negative control was YRG2 cells containing pLamin C and pSV40 fusion constructs, which express proteins that do not interact *in vivo* (Stratagene). (**B** and **C**) Specificity of interaction of AVR-Pita$_{176}$ with LRD polypeptide. Single amino acid substitutions that inactivate either LRD or AVR-Pita $_{176}$ *in vivo* eliminated growth in the absence of histidine and slowed color development with the *lacZ* reporter (referred to as an impaired physical interaction). LRD refers to the NΔ586 deletion of the Pi-ta protein from (A) above. LRD^{A918S} refers to LRD containing S substituted for A at position 918 of the Pi-ta protein. avr-pita$_{176}^{E177D}$ refers to the putative processed polypeptide that no longer confers avirulence due to an E to D substitution at residue 177 of AVR-Pita$_{223}$. Similar expression of each BD fusion protein in yeast was verified by western blots.

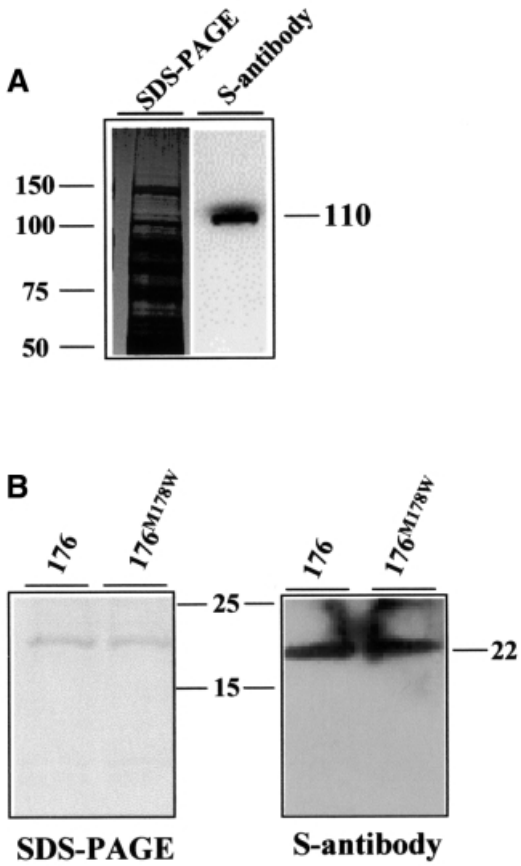

Fig. 5. The Pi-ta protein binds to AVR-Pita$_{176}$ and its mutant in far-western analysis. (**A**) Expression of the Pi-ta protein in *E.coli*. A total bacterial soluble extract expressing S-tagged Pi-ta protein was subjected to SDS–PAGE, and proteins were electroblotted onto PVDF membrane and detected by chemiluminescent visualization using monoclonal S-antibody to detect the S tag (right). The duplicate Coomassie Blue-stained gel is shown on the left. Molecular weights (kDa) were estimated using Perfect S protein markers (Novagen). (**B**) Binding of the Pi-ta protein to membrane-blotted AVR-Pita$_{176}$ proteins. Purified AVR-Pita$_{176}$ and avr-pita$_{176}^{M178W}$ proteins were subjected to SDS–PAGE, and stained by Coomassie Blue (left). A duplicate SDS–PAGE gel was electroblotted onto a nitrocellulose membrane (right), and the soluble extract expressing S-tagged-Pi-ta protein was added for binding. Bound Pi-ta protein was visualized using the monoclonal S-antibody as shown in (A). Perfect S protein markers were used.

AVR-Pita$_{176}$ is an elicitor recognized inside the host plant cell

Our transient expression assay uses intact 7-day-old rice seedlings that retain *Pi-ta*-specific responses to infection with *AVR-Pita*-containing fungus. Co-bombardment of resistant and susceptible seedlings within a single Petri dish reduced variability that occurs between bombardments, and multiple repetitions compensate for variation between different areas of the target plant during each bombardment. Results described in this report validate the transient expression assay as a robust and specific assay for inducers of *Pi-ta*-mediated rice defense responses.

We demonstrated that the putative processed protease AVR-Pita$_{176}$, but not the intact AVR-Pita$_{223}$ protein, triggered the *Pi-ta*-dependent HR when produced inside rice cells by transient expression. This interaction is very specific. Deletion of an additional 10 amino acids from the N-terminus of AVR-Pita$_{176}$ eliminated activity in this assay. The deletion removes a cysteine residue at position 53 that may be required for the protease function (Tatsumi *et al*., 1994). Indeed, *M.grisea* strains expressing an *avr-pita^{C53G}* allele substituting glycine for cysteine at residue 53 no longer exhibit full avirulence in standard infection assays (G.T.Bryan and B.Valent, unpublished). In addition, two separate mutations in the AVR-Pita protease motif that eliminate avirulence in fungal infection assays also eliminate elicitor activity in the transient expression assay. Thus, we have seen excellent correspondence between the structure and function of various AVR-Pita proteins, whether they are delivered via the normal route of fungal infection or through direct delivery of this single fungal gene into the plant cell by biolistic transformation.

The cytoplasmic site of action of AVR-Pita$_{176}$ as determined by transient expression is consistent with the structural similarity of *Pi-ta* to members of the cytoplasmic NBS-LRR receptor class of *R* genes. These results are also consistent with a lack of elicitor activity in extensive experiments when recombinant AVR-Pita$_{176}$ protein was applied to *Pi-ta* rice plants by vacuum infiltration or spray inoculation, techniques that would place these proteins in the apoplastic spaces between plant cells (G.T.Bryan and B.Valent, unpublished).

Other fungal *AVR* genes characterized so far are from pathogens that colonize the intercellular (apoplastic) spaces of their host plants, *Avr9* and *Avr4* from the tomato pathogen, *Cladosporium fulvum* (de Wit, 1995; Knogge, 1996), and *NIP1* from the barley pathogen, *Rhynchosporium secalis. Avr9* and *Avr4* encode extracellular peptides that correspond to the products of *R* genes of the extracellular LRR class. A number of bacterial pathogens have AVR proteins corresponding to members of the NBS-LRR class of *R* genes and these appear to function inside plant cells. For example, it is lethal to express *AvrB* in *RPM1 Arabidopsis* plants by either transient or stable transformation (Gopalan *et al*., 1996). Transient expression of *AvrBs3* in pepper cells using *Agrobacterium*-mediated gene delivery resulted in hypersensitive cell death specifically on *Bs3* plants (Van den Ackerveken *et al*., 1996). Our finding that AVR-Pita$_{176}$ acts inside the plant cell raises the interesting question of how *M.grisea* delivers AVR proteins into healthy plant cells. In some bacterial strains, AVR proteins are transferred into plant cells by a type III secretion system (Galan and Collmer, 1999). Perhaps hemibiotrophic and biotrophic fungi also employ specialized secretion systems for delivering proteins to the cytoplasm of their host cell without killing the cell. Details of the fungal–plant interface, such as whether *M.grisea* intracellular hyphae have breached the plasma membrane, need to be defined as a prelude to understanding how this fungus delivers proteins into the plant cytoplasm.

Ligand–receptor model in plant disease resistance

The ligand–receptor model suggests that a direct interaction between products of an *R* gene and an *AVR* gene is a key event in triggering resistance. So far, direct physical interaction between a cloned *R* gene product and an *AVR* gene product has only been demonstrated for *Pto–avrPto* (Scofield *et al*., 1996; Tang *et al*., 1996). Interaction

between Pto and avrPto has a functional implication in bacterial speck resistance. However, Pto is unique among other cloned race-specific *R* gene products in lacking an obvious protein interaction domain. Initiation of Pto-mediated resistance does appear to require *Prf*, an NBS-LRR *R* gene, (Salmeron *et al*., 1996) in a probable downstream role in the *Pto*-mediated signal transduction pathway (Rathjen *et al*., 1999). Thus, in the *Pto* system, an NBS-LRR protein appears involved in a manner other than as a direct receptor for the *AVR* gene product. Although AvrB seems to be recognized inside the plant cells, a direct interaction between *AvrB* and *RPM1* gene products has not been detected. Similarly, in the interaction between tomato and its fungal pathogen *C.fulvum*, the tomato *Cf9* confers resistance to *C.fulvum* expressing *Avr9*. However, *Avr9* bound to the membrane of both resistant *Cf9* and susceptible *cf9* tomato plants, suggesting that another protein may be the receptor (Kooman-Gersmann *et al*., 1996, 1998).

We demonstrate that the resistant Pi-ta LRD specifically interacts with AVR-Pita$_{176}$ both in the two-hybrid assay and in the *in vitro* binding studies. The full-length Pi-ta protein binds to AVR-Pita$_{176}$ protein that is bound to a membrane, but it shows minimal interaction, at best, in the yeast two-hybrid system. Pi-ta, with an estimated mol. wt of 105 kDa, and the GAL4 BD would form a large fusion protein that may not be suitable for the yeast two-hybrid system (Fields and Song, 1989). The lack of specificity in the binding of full-length Pi-ta to membrane-bound avr-pita$_{176}^{M178W}$ remains to be explained, and may be a property of this particular mutant protein. Overall, the high degree of specificity maintained suggests that the physical interaction has functional implications in rice blast resistance. Therefore, this system provides direct biochemical evidence for the ligand–receptor model of gene-for-gene interactions.

LRRs in plant proteins have been implicated indirectly in binding pathogen-derived signal molecules that mediate recognition specificity. Wang *et al*. (1998) suggest that the LRR domain of Xa21D is sufficient for pathogen recognition. Recent work on the *M* locus of flax suggests that alterations in the LRR domain play a significant role in the evolution of rust resistance genes and generation of novel recognition specificities (Anderson *et al*., 1997). Similarly, differences in the LRR domains of the *Cf* gene products are suggested to be responsible for ligand binding specificity (Dixon *et al*., 1996).

Pi-ta encodes a protein with the same structural organization as the cytoplasmic NBS-LRR *R* genes, and it shows significant similarity to these *R* genes in its characteristic NBS domain. Pi-ta has an LRD that is

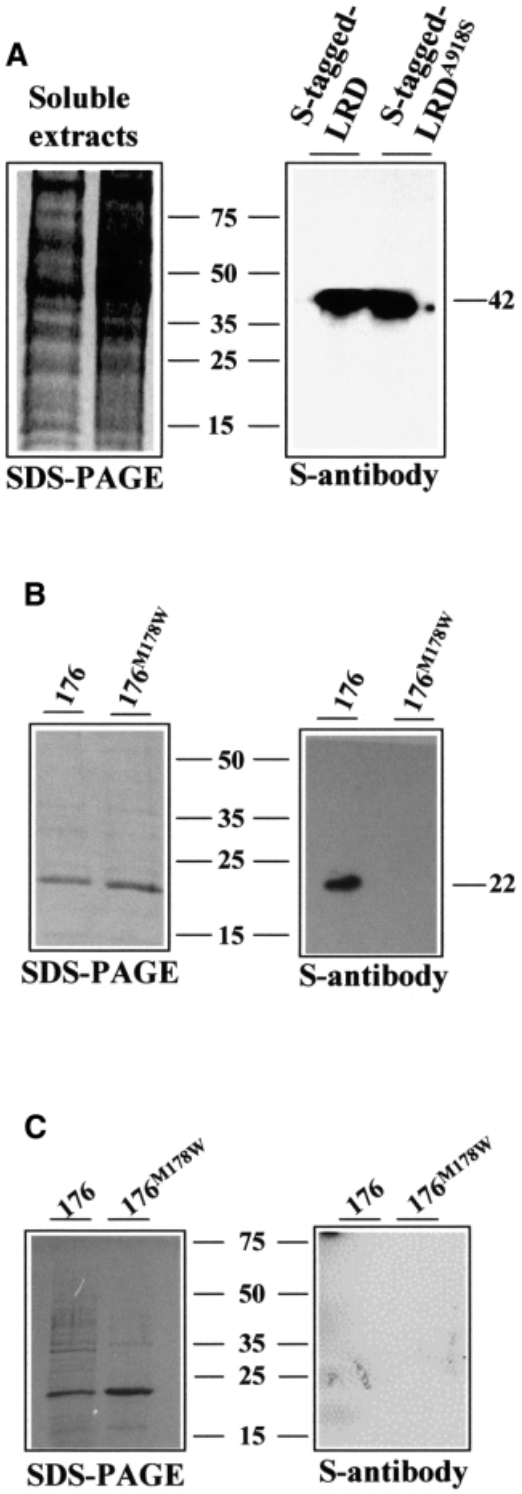

Fig. 6. AVR-Pita$_{176}$ binds specifically to the Pi-ta LRD region in far-western analysis. (**A**) Expression of Pi-ta LRD polypeptides in *E.coli*. Total bacterial soluble extracts expressing S-tagged LRD and LRD^{A918S} were subjected to SDS–PAGE, and then electroblotted onto PVDF membrane. LRD and LRD^{A918S} were detected by chemiluminescent visualization using monoclonal S-antibody (right). The duplicate Coomassie Blue-stained SDS–PAGE gel is shown (left). The molecular weight (kDa) was estimated using the perfect S protein markers (Novagen). (**B**) Binding of the LRD polypeptide to membrane-bound AVR-Pita$_{176}$. Purified AVR-Pita proteins were subjected to SDS–PAGE, and stained by Coomassie Blue (left). The duplicate SDS–PAGE gel was electroblotted onto a nitrocellulose membrane, and total soluble extract expressing S-tagged LRD was added for binding (see Materials and methods for details). The bound LRD was visualized by monoclonal S-antibody to detect S tag (right). Protein sizes (kDa) were determined as in (A). (**C**) In contrast, LRD^{A918S} polypeptide failed to bind the membrane-blotted AVR-Pita$_{176}$ proteins. Purified AVR-Pita proteins were subjected to SDS–PAGE, and stained by Coomassie Blue under identical conditions as in (B). The duplicate SDS–PAGE gel was electroblotted onto a nitrocellulose membrane, and the total soluble extract expressing S-tagged LRD^{A918S} polypeptide was added for binding. Monoclonal S-antibody was added to detect bound LRD^{A918S} (right). Protein sizes (kDa) were determined as in (A).

similar in size and location to the LRR from the *Arabidopsis RPM1* gene (Grant *et al*., 1995). The Pi-ta LRD has LXXLXXL repeats that may represent residual LRRs partially lost through evolution. Although the structure of the LRD of the Pi-ta protein differs from LRRs of other NBS-LRR proteins, the function of this LRD region from Pi-ta is likely to be similar to that of other LRRs. Thus, our data may also support the model that the LRR is the ligand-binding domain.

AVR-Pita$_{176}$ may be a zinc metalloprotease

Results described here support the hypothesis that *AVR-Pita* encodes a pre-pro-protein that is processed to a 176 amino acid active form. AVR-Pita$_{176}$, and not the intact 223 amino acid protein, is active as an HR elicitor when delivered into the plant cytoplasm. AVR-Pita$_{176}$, and not AVR-Pita$_{223}$, binds specifically to the LRD from resistant Pi-ta as determined by expression of the *HIS3* reporter gene in the two-hybrid assay. Confirming the two-hybrid analysis, AVR-Pita$_{176}$ that has been refolded on a membrane binds specifically to the LRD from resistant Pi-ta. For these *in vitro* studies, binding required addition of zinc during refolding of the AVR-Pita$_{176}$ protein on the membrane, and during incubation of the membrane with Pi-ta and LRD extracts, as would be expected if AVR-Pita$_{176}$ were a zinc metalloprotease. Mutant avr-pita proteins with conservative amino acid substitutions in the putative protease motif served as controls in our experiments. The avr-pita protein with the putative active site glutamic acid replaced with aspartic acid (E177D) failed to confer avirulence in whole-plant infection assays, failed as an HR elicitor in the transient expression assay and failed to interact with the Pi-ta LRD in the two-hybrid system. The avr-pita protein with tryptophan replacing methionine at residue 178 (M178W) in the putative protease motif failed to confer avirulence in infection assays, failed as an HR elicitor in the transient expression assay and failed to interact with the Pi-ta LRD in the *in vitro* binding studies. Our results are consistent with the hypothesis that AVR-Pita$_{176}$ is a metalloprotease. Biochemical demonstration of enzymatic activity will ultimately answer this question.

How does the physical interaction between Pi-ta LRD and AVR-Pita$_{176}$ trigger the *Pi-ta*-mediated defense response? One straightforward possibility is that the binding of Pi-ta to AVR-Pita$_{176}$ leads to a conformational change that allows Pi-ta to interact with downstream proteins in the signal transduction pathway. Another possibility is that AVR-Pita$_{176}$ may cleave the Pi-ta protein to an active form. Alternatively, the susceptible allele of Pi-ta might be inactive because it is degraded by AVR-Pita$_{176}$. Pi-ta may be a HR inhibitor and degrading Pi-ta might activate other proteins to trigger the defense response. *Arabidopsis RPM1* is the dicot *R* gene most closely related to *Pi-ta*, and RPM1 protein appears to be degraded with the onset of the HR and resistance response (Boyes *et al*., 1998). Again, demonstration of enzymatic activity and substrate specificity of AVR-Pita, as well as crystal structures of the AVR-Pita and Pi-ta proteins will provide insight into the triggering mechanism.

The instability of *M.grisea* avirulence genes may enable the fungus to escape recognition by corresponding plant *R* genes (Valent *et al*., 1997). Thus, rapid evolution of novel resistance specificities would appear crucial for the host. We have found that the resistance specificity to *M.grisea* expressing *AVR-Pita* was altered by substitution of serine for alanine at residue 918 of the Pi-ta protein (Bryan *et al*., 2000). In the present study, we demonstrate that impaired binding to AVR-Pita$_{176}$ occurs when Ala918 in the LRD region is replaced by serine in the pi-ta protein. In the tomato Pto kinase, T204 in subdomain VIII of the Pto kinase appears to determine the ability to interact with avrPto both *in vitro* and *in vivo* (Frederick *et al*., 1998). Thus, in these instances, it appears that the inability of R protein to bind the pathogen signal molecule accounts for the loss of resistance.

In the battle between the plant and pathogen, clearly *AVR* genes in the pathogen did not evolve to prevent the pathogen from infecting its host. It is likely that plants have evolved the ability to detect pathogen molecules. For *M.grisea*, AVR-Pita is an infection-specific pathogen molecule that is highly expressed during the later stages of the infection cycle (G.T.Bryan and B.Valent, unpublished). The timing of expression correlates well with the penetration process of *M.grisea*. An exact role for AVR-Pita in pathogenesis remains to be demonstrated. However, this system represents an example where rice plants containing *Pi-ta* produce a resistance gene product in which a single amino acid difference permits binding to a pathogen protein and activation of defense responses. Further elucidation of the molecular basis of these physical interactions might allow precise manipulation of recognition specificity of plant R proteins to produce durable resistance to important plant diseases.

Materials and methods

Standard methods were used for DNA isolation and restriction enzyme digests (Ausubel *et al*., 1987). Color photo images were obtained using a CCD (charged-coupled device) camera (Optionix DEI750) coupled to a Leica MZ12 microscope. Photographs were captured using Adobe Photoshop 5.

Fungal strains, transformation and infection assays

Fungal transformation and infection assays were performed as described previously (Valent *et al*., 1991; Sweigard *et al*., 1995). Conidial suspensions (2.5×10^5 spores/ml) from fungal strain 4360-R-62 that contains *AVR-Pita* were inoculated onto 7-day-old rice seedlings growing on medium [1/2× MS (Murashige and Skoog, 1962) salt, supplemented with 100 mg/L-casein hydrolysate, pH 5.8, 0.5% agarose]. After inoculation, plates were sealed with parafilm and kept on a laboratory bench at room temperature.

Transient expression of AVR-Pita in rice seedlings

Plasmids. pGEM9Z (Promega) vectors pML63 and pML142 were gifts from Mary Locke (DuPont Agricultural Products). pML142 has an *Adh1-6* intron sequence behind the 35S promoter (Figure 1A). pML63 has a CaMV 35S promoter and bacterial 3′ nos terminator sequence for expressing GUS (Figure 1B). All AVR-Pita constructs were first cloned in the *Nco*I and *Eco*RI site of pML63. A 1434 bp fragment containing the 35S promoter of each resulting plasmid was replaced by an 1897 bp fragment containing the 35S/*Adh1-6* promoter from pML142 in the *Bam*HI–*Nco*I site. Sequences of all constructs were verified using an ABI automatic sequencer. Primer sequences and constructs (Table I) are available on request.

Plant materials, seedling preparation and bombardment. Two resistant *Pi-ta* rice cultivars, Yashiro-mochi and the doubled haploid rice line YT14, and two susceptible *pi-ta* cultivars, Nipponbare and doubled haploid line YT16, were used for leaf transient assays. YT14 and YT16 are related doubled haploid rice lines derived from a cross between Yashiro-mochi and the susceptible variety Tsuyuake. Seeds were

Table I. List of plasmids used in this study

Plasmids	Description	Source
pML63	Expression cassette for GUS	M.Locke
pML142	Expression cassette for AVR-Pita	M.Locke
pCB980	AVR-Pita ORF in *Cla*I–*Bam*HI site of pBSSK (+)	Orbach *et al.* (2000)
pCB1646	0.5 kb *Nde*I–*Not*I fragment encoding AVR-Pita$_{176}$ in pET28a (+)	this study
pCB1650	NΔ586: Pi-ta LRD in-frame fusion to *Eco*RI–*Sal*I of GAL4 DNA activation domain of pAD-GAL4	this study
pCB1657	AVR-Pita$_{176}$ in-frame fusion to *Eco*RI–*Sal*I of GAL4 DNA-binding domain of pBD-GAL4	this study
pCB1760	Pi-ta LRD^{A918S} in-frame fusion to *Eco*RI–*Sal*I of pBD-GAL4	this study
pCB1761	AVR-Pita$_{176}$ in-frame fusion to *Eco*RI–*Sal*I of pAD-GAL4	this study
pCB1896	NΔ586: Pi-ta LRD in-frame fusion to *Eco*RI–*Sal*I of pBD-GAL4	this study
pCB1900	Pi-ta LRD in-frame fusion to an S-tag in *Eco*RI–*Sal*I of modified pET 29a (+)	this study
pCB1902	AVR-Pita ORF in-frame fusion to *Eco*RI–*Sal*I of pAD-GAL4	this study
pCB1906	2.8 kb *Nco*I–*Hin*dIII of Pi-ta ORF in pSL1180	Bryan *et al.* (2000)
pCB1917	NΔ247: 2.1 kb *Eco*RI–*Hin*dIII of Pi-ta NΔ247 in pBD-GAL4	this study
pCB1921	NΔ315: 1.8 kb *Eco*RI–*Sal*I of Pi-ta in pBD-GAL4	this study
pCB1922	NΔ684: 0.7 kb *Eco*RI–*Hin*dIII of Pi-ta N684 in pBD-GAL4	this study
pCB1925	AVR-Pita$_{176}$ ^{E177D} in-frame fusion to *Eco*RI–*Sal*I of pAD-GAL4	this study
pCB1927	0.5 kb *Nde*I–*Not*I fragment encoding AVR-Pita$_{176}^{M177W}$ in pET28 (+)	this study
pCB1941	0.5 kb *Nco*I–*Kpn*I fragment encoding AVR-Pita$_{166}$ in pML142	this study
pCB1947	0.6 kb *Nco*I–*Kpn*I fragment encoding AVR-Pita$_{176}$ in pML142	this study
pCB2012	0.6 kb *Nco*I–*Kpn*I fragment encoding avr-pita$_{176}^{E177D}$ in pML142	this study
pCB2013	0.6 kb *Nco*I–*Kpn*I fragment encoding avr-pita$_{176}^{M178W}$ in pML142	this study
pCB2074	2.8 kb *Eco*RI–*Sal*I Pi-ta ORF in-frame fusion to pBD-GAL4	this study
pCB2111	0.7 kb *Kpn*I–*Spe*I fragment encoding AVR-Pita$_{223}$ in pML142	this study

germinated on agarose medium, 1/2× MS salt with casein hydrolysate, and grown for 1 week in an incubator at 25°C in a 12 h photoperiod with 100 μE^{-1} of cool white light. At the two-leaf stage, seedlings were removed from the agarose medium, labeled and placed in a Petri dish containing a pre-wetted filter paper. Biolistic bombardment was performed using a Bio-Rad PDS-1000/He apparatus and 1150 p.s.i. rupture disks. Gold particles, 0.6 μm in diameter, were prepared according to the instructions provided by the manufacturer (Bio-Rad). For each co-bombardment, 0.5 μg of gold particles were coated with 0.5 μg of *35S::uidA* and 1 μg of *AVR-Pita* expression plasmid. After bombardment, seedlings were maintained at 25°C for 48 h in Petri dishes containing pre-wetted filter paper in the above seedling conditions. Histochemical GUS staining was done using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (Biosynth AG) as a substrate (Seki *et al*., 1991).

RNA isolation and northern blot analysis

RNA isolation and northern blot analysis were done as previously described (Jia and Martin, 1999). The DNA probe for *AVR-Pita* was a PCR product amplified from *AVR-Pita* cDNA pCB980 (Orbach *et al*., 2000) using primers YL29 (5′-GGCGGGCTCCATGGGAACGCTA-TTC-3′) and YL33 (5′-CCCCCATGGCACAATATTTATAACGT-3′). Hybridization was performed at 42°C using a standard procedure (Ausubel, 1987). Hybridized filters were washed in 1× SSC, 0.1% SDS at 59°C for 20 min.

Two-hybrid plasmid construction, interactions and immunoblot analysis of bait protein in yeast

Mutant alleles encoding various regions of the Pi-ta protein and different *AVR-Pita* coding sequences were generated by PCR with *Pfu* DNA polymerase (Stratagene). Oligonucleotides containing 5′ *Eco*RI and 3′ *Sal*I sites were used to amplify sequences from *Pi-ta* genomic clone pCB1641 and from cDNA clone pCB1906 (Bryan *et al*., 2000) for bait fusions. Similarly, oligonucleotides containing 5′ *Eco*RI and 3′ *Sal*I were used to amplify *AVR-Pita* cDNA clone pCB980 (Orbach *et al*., 2000) for prey fusions. All amplified products were digested with *Eco*RI and *Sal*I and cloned into the corresponding sites in two-hybrid bait pBD-GAL4 Cam phagemid vector and prey vector pAD-GAL4 phagemid (Stratagene). Each in-frame fusion was verified by sequencing. Primer sequences and constructs (Table I) are available on request.

Each BD and AD fusion presented in Figure 4 was co-transformed into yeast strain YRG2 using competent cells for examination of interaction (Stratagene) according to the manufacturer's instruction. Briefly, transformed YRG2 cells were plated on synthetic minimal medium (SD) lacking leucine and tryptophan for expression of BD and AD fusions at 30°C for 3 days. The *HIS3* reporter was examined by plating YGR2 cells on SD medium lacking histidine, leucine and tryptophan for 3 days. LacZ activity was examined by growing YRG2 cells containing both bait and prey fusions on SD lacking leucine and tryptophan at 30°C for 2 days. The yeast colonies were then blotted onto filter papers (VWR Scientific Products), and the filter papers were frozen rapidly in liquid nitrogen and air-dried. Each air-dried filter paper was then placed on top of a filter paper pre-wetted with Z buffer containing X-gal (98 ml of Z buffer, 0.27 ml of β-mercaptoethanol, 1.67 ml of X-gal stock solution/l). LacZ activities were recorded at 24 h after incubation at room temperature.

Levels of expression of all bait constructs in the GAL4 BD were determined by western blots. Briefly, total proteins were extracted and analyzed as described in the instructions with the two-hybrid kit (Clontech). The protein profile was visualized by Coomassie Blue staining. Equal amounts of proteins were electrophoresed on 10–15% gradient polyacrylamide gels and transferred to a nitrocellulose membrane (Novex) by electroblotting according to the manufacturer's recommendations (Bio-Rad). GAL4 fusion proteins were detected by ECL (ECL kit, Pierce) using a monoclonal antibody to the GAL4 BD (Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions.

Expression of Pi-ta and LRD polypeptides in E.coli

A 2800 bp *Nco*I–*Hin*dIII DNA fragment encoding the open reading frame of the *Pi-ta* gene was isolated from pCB1906. The 1036 bp *Eco*RI–*Sal*I DNA fragments encoding LRD and LRD^{A918S} were isolated and purified from the two-hybrid bait vector. Each DNA fragment was then cloned into the corresponding site of a modified version of Novagen vector pET29a (+) (kindly provided by Dr Qun Zhu, DuPont CR&D) to introduce an N-terminal S tag (15 amino acids, Novagen). Bacterial host cells BL21 (DE3, Novagen) for expressing the fusion proteins were grown at 30°C with shaking at 250 r.p.m. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration) to the culture for 3 h. The bacterial pellets were suspended in buffer ST (50 mM Tris–HCl pH 8.0, 150 mM NaCl) with 1 mg/ml lysozyme (Sigma) and sonicated briefly on ice. Triton X-100 (Sigma) was added to 1% final concentration. Extracts were cleared by centrifugation at 13 000 r.p.m. for 10 min.

Modified far-western analysis

In vitro binding studies were performed with modification of the methods described by Chen and Evans (1995). Briefly, both AVR-Pita$_{176}$ and virulent avr-pita$_{176}^{M178W}$ were purified using an Ni-NTA column (Qiagen) and separated on 10–20% gradient SDS–polyacrylamide gels

without previously boiling to denature, and blotted onto nitrocellulose membrane (Millipore). Non-specific binding to membranes was first blocked using 1% non-fat milk (Sigma), and then the AVR-Pita polypeptides were subjected to conditions for refolding. Membranes were first soaked for 30 min in 6 M urea. They were then soaked in 3 M urea for 15 min, in 1.5 M urea for 15 min, in 0.75 M urea (1 mM $ZnCl_2$) for 15 min and subsequently in each half reduction of molarity of the solution for 15 min until the final solution was 0.05 M urea (1 mM $ZnCl_2$). The final solution was replaced with blocking buffer (25 mM HEPES–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM $ZnCl_2$ and 0.05% NP-40) containing total *E.coli* extracts expressing S-tagged-Pi-ta and LRD proteins. The membranes were rinsed briefly with 1× TBST (1× TBST = 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20). Immunodetection was done by a modification of methods described by Novagen. Briefly, membranes were incubated with a 1:5000 dilution of an S-antibody–horseradish peroxidase (HRP) conjugate (Novagen) in TBST for 15 min at room temperature, and membranes were washed thoroughly three times (1–2 min each time) with 25 ml of TBST at room temperature. The resulting image was visualized by detecting HRP chemiluminescence (ECL kit, Pierce) with X-ray film (Kodak). S protein perfect marker (Novagen) was used for molecular weight standards.

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Support for the ligand–receptor model to explain gene-for-gene specificity in plant disease resistance was obtained through transient expression of the *Arabidopsis thaliana* resistance gene *RPS2* and its corresponding avirulence gene *avrRpt2* in leaf mesophyll protoplasts, followed by co-immunoprecipitation of an *in vivo* complex containing the RPS2 and AvrRpt2 proteins.

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