Recognition of the *Magnaporthe oryzae* Effector AVR-Pia by the Decoy Domain of the Rice NLR Immune Receptor RGA5

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Nucleotide binding and leucine-rich repeat proteins (NLRs) are important receptors in plant immunity that allow recognition of pathogen effectors. The rice (*Oryza sativa*) NLR RGA5 recognizes the *Magnaporthe oryzae* effector AVR-Pia through direct interaction. Here, we gained detailed insights into the molecular and structural bases of AVR-Pia-RGA5 interaction and the role of the RATX1 decoy domain of RGA5. NMR titration combined with in vitro and in vivo protein-protein interaction analyses identified the AVR-Pia interaction surface that binds to the RATX1 domain. Structure-informed AVR-Pia mutants showed that, although AVR-Pia associates with additional sites in RGA5, binding to the RATX1 domain is necessary for pathogen recognition but can be of moderate affinity. Therefore, RGA5-mediated resistance is highly resilient to mutations in the effector. We propose a model that explains such robust effector recognition as a consequence, and an advantage, of the combination of integrated decoy domains with additional independent effector-NLR interactions.

INTRODUCTION

Plant disease resistance largely relies on inducible immune responses that are triggered upon receptor-mediated recognition of pathogen molecules and that often involve a localized programmed cell death called the hypersensitive response (HR). Particularly important are NLRs, cytoplasmic nucleotide binding oligomerization domain-like receptors with a multidomain architecture composed of a C-terminal leucine-rich repeat domain and a central nucleotide binding domain (Takken and Goverse, 2012; Jacob et al., 2013; Qi and Innes, 2013). Most NLRs carry in addition an N-terminal coiled-coil or Toll/interleukin-1 receptor domain that have both been reported to mediate NLR homo- or heterodimerization (Le Roux et al., 2015; Sarris et al., 2015; Césari et al., 2013). Recent work led to the hypothesis that these highly diverse integrated domains are mimics of effector targets and can therefore be considered as integrated decoy domains (Le Roux et al., 2015; Césari et al., 2013). Recent work led to the hypothesis that these highly diverse integrated domains are mimics of effector targets and can therefore be considered as integrated decoy domains (Le Roux et al., 2015; Sarris et al., 2015; Césari et al., 2014a). Recent work led to the hypothesis that these highly diverse integrated domains are mimics of effector targets and can therefore be considered as integrated decoy domains (Le Roux et al., 2015; Sarris et al., 2015; Césari et al., 2014a). However, the molecular mechanisms of effector recognition by integrated domains and the advantages of this mode of recognition remain largely unknown.

Rice blast, caused by the fungus *Magnaporthe oryzae*, is a highly destructive crop disease and a serious threat for food security (Pennisi, 2010; Dean et al., 2012; Skamnioti and Gurr, 2010). The rice effector AVR-Pia is a homolog of the *Magnaporthe oryzae* effector AVR-Pib (Van der Hoorn et al., 2001; van der Kamoun et al., 2001). AVR-Pib is a class of effectors that has a modular architecture and mimics canonical effectors of the plant immune system. This leads to activation of a defense response in the plant that may be a guardee or a decoy, to the NLRs (Takken and Goverse, 2008). Effectors can also be recognized in a direct manner by binding, either alone, or in complex with a cofactor that may be a guardee or a decoy, to the NLRs (Takken and Goverse, 2012; Collier and Moffett, 2009). In these cases, the leucine-rich repeat domain plays a crucial role in recognition specificity and has frequently been shown to mediate direct effector binding (Ellis et al., 2007; Krasileva et al., 2010; Jia et al., 2000). Alternatively, direct effector recognition can be mediated by non-canonical domains integrated into NLRs at low frequencies (Kanzaki et al., 2012; Sarris et al., 2015; Maqbool et al., 2015; Le Roux et al., 2015; Césari et al., 2013). Recent work led to the hypothesis that these highly diverse integrated domains are mimics of effector targets and can therefore be considered as integrated decoy domains (Le Roux et al., 2015; Sarris et al., 2015; Césari et al., 2014a). However, the molecular mechanisms of effector recognition by integrated domains and the advantages of this mode of recognition remain largely unknown.

Plant NLRs recognize effectors in an indirect manner. They detect either a modification of the effector’s host target protein called a “guardian” or modifications of a host protein that mimics the effector target and is called a “decoy” (van der Hoorn and Kamoun, 2008). Effectors can also be recognized in a direct manner by binding, either alone, or in complex with a cofactor that may be a guardee or a decoy, to the NLRs (Takken and Goverse, 2012; Collier and Moffett, 2009). In these cases, the leucine-rich repeat domain plays a crucial role in recognition specificity and has frequently been shown to mediate direct effector binding (Ellis et al., 2007; Krasileva et al., 2010; Jia et al., 2000). Alternatively, direct effector recognition can be mediated by non-canonical domains integrated into NLRs at low frequencies (Kanzaki et al., 2012; Sarris et al., 2015; Maqbool et al., 2015; Le Roux et al., 2015; Césari et al., 2013). Recent work led to the hypothesis that these highly diverse integrated domains are mimics of effector targets and can therefore be considered as integrated decoy domains (Le Roux et al., 2015; Sarris et al., 2015; Césari et al., 2014a). However, the molecular mechanisms of effector recognition by integrated domains and the advantages of this mode of recognition remain largely unknown.

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2009). NLR-mediated pathogen recognition is the major mechanism in rice blast resistance. Among 25 different blast resistance genes cloned over the last 20 years, 24 code for NLRs (Liu et al., 2014). Blast resistance is frequently conferred by paired NLRs with clustered tandem organization in the genome. Among these, the NLR pair RGA4/RGA5 encoded by the Pi-CO39/Pia resistance locus has been developed as a model for molecular understanding of paired NLRs (Okuyama et al., 2011; Césari et al., 2013, 2014b). In this pair, RGA4 acts as a constitutively active disease resistance and cell death inducer that is repressed by RGA5 in the absence of pathogen (Césari et al., 2014b). In addition to its repressor function, RGA5 acts as a receptor for the M. oryzae effectors AVR1-CO39 and AVR-Pia. Direct binding of RGA5 to these effectors results in derepression of RGA4 and activation of resistance signaling. Effector binding involves the unconventional C-terminal related to ATX1 (RATX1) domain of RGA5, which is similar to a heavy metal-associated (HMA) domain protein from Saccharomyces cerevisiae that acts as a cytoplasmic copper chaperone (Césari et al., 2013). The RATX1 domain of RGA5 is dispensable for RGA4 repression and seems exclusively dedicated to effector binding (Césari et al., 2014b). Since the rice RATX1/HMA protein Pi21 is a blast susceptibility factor required for full disease development (Fukuoka et al., 2009), it has been hypothesized that AVR1-CO39 and AVR-Pia target RATX1/HMA proteins for disease development and that the RATX1 domain is an integrated decoy domain (Césari et al., 2014b, 2013).

An HMA domain 53% identical to the RGA5 RATX1 domain is also present in another rice (Oryza sativa) NLR, Pik-1, which acts together with the NLR Pik-2 in the specific recognition of the M. oryzae effector AVR-Pik. Like in RGA5, this domain acts by directly binding the effector and is crucial for its recognition (Kanzaki et al., 2012). However, contrary to the C-terminal RATX1 domain of RGA5, the HMA domain of Pik-1 is located between the coiled-coil and nucleotide binding domains, indicating independent integration of the same domains in the two unrelated NLRs (Césari et al., 2013). Recently, the determination of the crystal structure of the AVR-Pik/Pikp-1 HMA domain complex allowed the precise identification of the AVR-Pik domain mediating binding to the Pikp-1 HMA domain (Maqbool et al., 2015).

Although AVR-Pik, AVR-Pia, and AVR1-CO39 do not share sequence similarities, they share a highly similar three-dimensional structure characterized by a six β-sandwich fold also present in two other effectors: AvrPiz-t from M. oryzae and ToxB from the wheat pathogenic fungus Pyrenophora tritici repens (Zhang et al., 2013; de Guillen et al., 2015; Maqbool et al., 2015; Nyarko et al., 2014). The corresponding, structurally related Magnaporthe Avr and ToxB effectors were termed MAX effectors. MAX effectors are present in other sometimes only distantly related phytopathogenic fungi and the MAX effector family underwent strong expansion in M. oryzae where it accounts for roughly 10% of the effectors (de Guillen et al., 2015).

In this study, we investigated the molecular and structural bases of AVR-Pia recognition by RGA5 with a focus on the role of the RATX1 domain in effector binding and recognition. We show that AVR-Pia interacts with the RGA5\textsubscript{RATX1} domain through a precise surface that shares some similarity, but also important differences, with the HMA binding surface of AVR-Pik. We demonstrate that binding to the RATX1 domain is required for effector recognition but that strong reduction in binding strength is tolerated. We also provide evidence that the RATX1 domain is not required for association of AVR-Pia with RGA5 and that it associates with additional sites in the NLR, which could explain the high tolerance of recognition to reduced AVR-Pia-RATX1 binding strength. Based on our results, we propose a model illustrating the advantages of effector recognition by integrated decoy domains as well as additional simultaneously occurring interactions with NLR receptors.

**RESULTS**

The F24S and T46N Substitutions in the Nonrecognized AVR-Pia-H3 Allele Affect Surface Properties but Not Structure

We previously described the naturally occurring AVR-Pia allele AVR-Pia-H3 that carries two nonsynonymous polymorphisms leading to the F24S and T46N substitutions (Césari et al., 2013). M. oryzae isolates carrying the AVR-Pia-H3 allele are virulent on rice varieties carrying the Pia resistance locus and AVR-Pia-H3 does not interact in yeast two-hybrid (Y2H) assays with the C-terminal part of the rice NLR immune receptor RGA5 containing the RATX1 domain (RGA5\textsubscript{C-ter}). The NMR structure of AVR-Pia showed that both the F24 and T46 residues are surface exposed and suggested that the corresponding substitutions affect only AVR-Pia surface properties without major structural rearrangements (de Guillen et al., 2015).

To test this hypothesis, the structures of AVR-Pia-H3 and the single mutants AVR-Pia\textsuperscript{F24S} or AVR-Pia\textsuperscript{T46N} were analyzed by NMR spectroscopy. We performed sequential assignments using 15N-labeled AVR-Pia samples, and the 13C\textsubscript{α} and 13C\textsubscript{β} assignments were performed using 13C-1H 2D experiments with a 13C-natural abundance sample in D\textsubscript{2}O (Supplemental Methods). When compared with AVR-Pia wild type, 1H-15N chemical shifts differed more in AVR-Pia-H3 than in AVR-Pia\textsuperscript{F24S} or AVR-Pia\textsuperscript{T46N} single mutants (Figure 1A). The NMR structure of AVR-Pia-H3 proved to be very similar to the structure of AVR-Pia (pdb code 5JHJ) (Figure 1B; Supplemental Table 1 and Supplemental Figure 1). The backbone RMSD for superposition of the AVR-Pia and AVR-Pia-H3 structures is 1.53 Å and drops to 0.93 Å when the β1-β2 loop is excluded and the superposition starts at residue R23. Like the AVR-Pia wild-type protein, AVR-Pia-H3 shows the MAX-effector topology characterized by six antiparallel β-strands (Figure 1C). The 1H-15N chemical shift data for AVR-Pia\textsuperscript{F24S} and AVR-Pia\textsuperscript{T46N} indicate that both single mutants probably also keep the MAX-effector fold (Figure 1A). We also compared 15N relaxation data between AVR-Pia and AVR-Pia-H3, by a Model-Free analysis. The order parameter (S\textsuperscript{2}) ranges from 0 for a flexible residue to 1 for a rigid one and reflects the amplitude of the fast internal motion of the H\textsubscript{N}-N bond vectors in the picoseconds-to-nanosecond time range. Our analysis indicated that both AVR-Pia\textsuperscript{F24S} and AVR-Pia-H3 have rigid structures with average S\textsuperscript{2} values of 0.8 and similar S\textsuperscript{2} profiles, indicating similar protein dynamics (Supplemental Figure 2).

The 3D structure of AVR-Pia-H3 therefore supports the conclusion that the F24S and T46N substitutions do not result in
conformational changes but rather alter AVR-Pia surface properties required for strong interaction with the RATX1 domain of RGA5 (RGA5\textsubscript{RATX1}) and disease resistance activation.

AVR-Pia Binds RGA5\textsubscript{RATX1} with Intermediate Affinity

To characterize the AVR-Pia/RGA5\textsubscript{RATX1} interaction, in vitro binding assays with recombinant RGA5\textsubscript{RATX1} and AVR-Pia or AVR-Pia-H3 were performed using isothermal calorimetry (ITC). For AVR-Pia, specific and direct binding to RGA5\textsubscript{RATX1} with a one-site model and a $K_d$ of 7.8 μM was detected (Figure 2A).

For AVR-Pia-H3, no binding was detected under identical conditions, indicating that its affinity to RGA5\textsubscript{RATX1} was drastically reduced. To determine the contribution of the individual substitutions, F24S and T46N, to the reduction in binding, AVR-Pia\textsubscript{F24S} and AVR-Pia\textsubscript{T46N} single mutants were characterized for RGA5\textsubscript{RATX1} binding. AVR-Pia\textsubscript{F24S} showed no binding, while AVR-Pia\textsubscript{T46N} seemed to bind very weakly. However, the affinity was so weak that no $K_d$ value could be determined.

β-Strands 2 and 3 and Residues R23, F24, E56, and E58 Constitute a Candidate RGA5\textsubscript{RATX1} Interaction Surface in AVR-Pia

To test the hypothesis that the residues F24 and T46 are part of the AVR-Pia surface mediating direct contacts to RGA5\textsubscript{RATX1} and to identify other residues in direct contact with RGA5\textsubscript{RATX1} located in the close vicinity of the binding interface, NMR titration experiments were performed. This technique consists of recording the $^1$H-$^1$\textsuperscript{5}N-HSQC NMR spectra of $^{15}$N-labeled AVR-Pia in the presence of increasing amounts of unlabeled RGA5\textsubscript{RATX1}. When protein-protein binding occurs, it modifies the chemical environment of the
Figure 2. AVR-Pia Binds RGAS\textsubscript{RATX1} with Intermediate Affinity and a Well-Defined Interaction Surface.

(A) ITC curves for the titration of the RGAS\textsubscript{RATX1} domain by AVR-Pia\textsuperscript{wt} (squares), AVR-Pia-H3 (circles), AVR-Pia\textsuperscript{F24S} (+), and AVR-Pia\textsuperscript{T46N} (×) at 25°C. For AVR-Pia\textsuperscript{wt} the fit parameters were $n = 0.994 \pm 0.004$, $K_a = 1.28 \pm 0.04 \times 10^{-2}$ mol\textsuperscript{-1}, $\Delta H = -8179 \pm 47.95$ cal\textsuperscript{-1} mol\textsuperscript{-1}, $\Delta S = -4.06$ cal K\textsuperscript{-1} mol\textsuperscript{-1}. The red line shows a simulated curve for a 10× lower affinity ($K_a = 1.28 \times 10^{-4}$ mol\textsuperscript{-1}).

(B) NMR titration and surface mapping. Plot of the chemical shift differences ($\Delta$ppm) between unbound and bound AVR-Pia (blue) or AVR-Pia-H3 (red). Chemical shift differences were calculated as the Hamming distance (Schumann et al., 2007), $\Delta \partial$ (ppm) = |$\Delta \partial$[\textsuperscript{1H}]$_{ij}$| + 0.102 * |$\Delta \partial$[\textsuperscript{15N}]$_{ij}$|, where $\Delta \partial$[\textsuperscript{1H}]$_{ij}$ and $\Delta \partial$[\textsuperscript{15N}]$_{ij}$ are the chemical shift differences observed at $R = 0$ and $R = 2$, respectively.

(C) to (E) Structures of AVR-Pia ([C] and [D]) and AVR-Pia-H3 (E) with color-coded surfaces showing the differences in chemical shifts in the NMR titration (difference between free ($R = 0$) and RGAS\textsubscript{RATX1}-bound AVR-Pia or AVR-Pia-H3 ($R = 2$)). Surfaces of residues with chemical shift differences $\Delta \partial$[ppm] $\geq$ 0.2 are shown in dark blue (residues in white letters) and in light blue for 0.2 $>$ $\Delta \partial$[ppm] $\geq$ 0.1 ppm (residues in black letters). Surfaces of residues not observed in the AVR-Pia-RGAS\textsubscript{RATX1} complex ($R = 2$). HSQC are reported in gray (residues in red letters), and unperturbed residues are not highlighted (residues are not indicated). The view in (D) is the opposite face of (C), which has been rotated 180° from the vertical axis.
amino acids located on the binding surface. This results in a change of the chemical shift in NMR experiments. Depending on the rate of complex formation and dissociation, expressed by the exchange rate constant $k_{ex}$ and the chemical shift difference $\Delta \delta$ between the unbound and bound states ($\Delta \delta = \text{difference between the resonance frequencies of the exchanging sites}$), different exchange regimes occur. NMR titration showed that the AVR-Pia-RGA5RATX1 complex was in slow exchange with $k_e < < k_{ex}$ since separate resonances appeared for individual species (bound and unbound states) (Supplemental Figure 3A). Residues with important chemical shift changes between free AVR-Pia ($R = 0$) and AVR-Pia-bound to RGA5RATX1 (molar ratio $R = 2$) were almost exclusively surface exposed and located in a region formed essentially by β-strands 2 and 3 and including residues R23 and F24 from β-strand 1 as well as E56 and E58 from β-strand 4 (Figures 2B and 2C). No peaks were observed for residues Y27, V37, Y41, I44, and T51 in the complex. This candidate interaction surface largely overlaps with an extended, solvent-exposed patch of hydrophobic/aromatic residues formed by F24, V26, and Y28 in β1, V37, L38, and Y41 in β2, and Y85 in β6. The residues on the other side of the AVR-Pia structure were not shifted in the NMR titration and therefore seem not to be involved in the interaction with RGA5RATX1 (Figure 2D). Two exceptions were E83, which probably senses a perturbation of the residue Y41 that is close in space, and the I69 residue, which may be involved in local conformational rearrangement of the short β5 strand.

RGA5RATX1 titration experiments were also performed with 15N-labeled AVR-Pia-H3, which shows no binding in Y2H (Césari et al., 2013) and ITC (Figure 2A) analysis. Spectral perturbations were strongly reduced and only few and limited changes of chemical shifts occurred when titrating AVR-Pia-H3 with RGA5RATX1 (Figures 2B and 2E; Supplemental Figure 3B). Signals for the R23, S24, V42, R43, and E83 residues were still observed at the end of the titration, while they were mostly lost at a molar ratio of 0.5 in the case of AVR-Pia (Supplemental Figure 3). Similarly, signals for E58, V59, and T47 were much less perturbed. Nevertheless, the peaks for Y41, N46, and T51 were also perturbed, indicating a weak residual interaction between RGA5RATX1 and AVR-Pia-H3 (Figures 2B and 2E; Supplemental Figure 3). In summary, NMR titration identified a candidate interaction surface formed by β-strands 2 and 3 and including, in addition, residues R23, F24, E56, and E58 (Figure 2B). This surface overlaps extensively with an extended hydrophobic patch on the AVR-Pia surface that contains F24 and has T46 on its border and that may be crucial for RGA5RATX1 binding.

**Y2H Experiments with Structure-Informed AVR-Pia Mutants Confirm an Important Role of the Candidate Interaction Surface in RGA5C-ter Binding**

To test whether the AVR-Pia candidate interaction surface identified in vitro mediates binding to RGA5C-ter in vivo, we performed Y2H assays using AVR-Pia variants bearing point mutations in critical residues identified by NMR titration. Individual surface-exposed hydrophobic (M40, Y41, and Y85) or charged (R23, D29, R36, E56, and E58) amino acids, located in or at the border of the candidate interaction surface, were replaced by alanine. In addition, naturally occurring AVR-Pia polymorphisms located within the candidate interaction surface were tested: F24S and T46N from AVR-Pia-H3 and R43G from AVR-Pia-H2 identified in *M. oryzae* isolates pathogenic on rice and *Setaria* species, respectively (Supplemental Figure 4A) (Césari et al., 2013). As controls, we generated mutants where surface-exposed charged residues located outside the candidate interaction surface were replaced by alanine (D63A, K67A, K74A, and D78A; Figure 3A).

As previously reported, yeasts coexpressing BD-AVR-Pia and AD-RGA5C-ter or AD-RGAS5C-ter and BD-RGAS5C-ter grew on selective medium, indicating physical binding between AVR-Pia and RGA5C-ter and homo-interaction of the RGA5C-ter domain (Figure 3A) (Césari et al., 2013, 2014b). Yeasts coexpressing AD-RGAS5C-ter and AVR-PiaF24S, AVR-PiaR43G, or AVR-PiaE56A fused to the BD domain did not grow on selective medium, indicating that these mutations abolish binding to RGA5C-ter. Isolates expressing BD fusions of the AVR-Pia variant carrying the mutation R23A, D29A, T46N, E58A, or D63A showed reduced growth compared with wild-type BD-AVR-Pia, indicating that these mutations also affect AVR-Pia-RGA5C-ter interaction. By contrast, yeast clones expressing BD fusions of AVR-PiaY41A, AVR-PiaE56A, AVR-PiaD63A, AVR-PiaK74A, or AVR-PiaD78A showed similar growth as wild-type BD-AVR-Pia, while AVR-PiaM40A showed similar growth as wild-type BD-AVR-Pia and AVR-PiaY85A isolates showed stronger growth. All BD-AVR-Pia variants were expressed at similar levels as the wild-type BD-AVR-Pia (Figure 3B). Taken together, these Y2H data show that the replacement of all charged amino acids in the interaction surface, with the exception of E56, either abolish or reduce binding of AVR-Pia to RGA5C-ter while exchanging hydrophobic residues within the interaction surface seems to abolish the interaction (in the case of F24S) or increase the binding (in the cases of M40A and Y85A).

To rule out that reduced binding of AVR-Pia mutants to RGA5C-ter is due to major changes in protein structure, the AVR-Pia mutants R23A, D29A, R36A, R43G, and E58A were expressed in *Escherichia coli*, purified to homogeneity, and analyzed by 1H-1D-NMR experiments (Supplemental Figure 4B). All mutant proteins showed similar spectra as AVR-Pia wild type, indicating that they were well structured and only locally disturbed. Recombinant AVR-Pia-D63A could not be expressed.

Taken together, these results suggest that most residues of the AVR-Pia interaction surface identified by NMR titration play an important role in RGA5C-ter binding.

**Co-IP Experiments Identify Key Residues in the AVR-Pia Interaction Surface That Are Crucial for RGA5RATX1 Binding in Planta**

To investigate the role of the AVR-Pia interaction surface in planta binding to RGA5C-ter, coinmunoprecipitation (co-IP) experiments were performed. HA-tagged RGA5C-ter and YFP-tagged AVR-Pia mutants with reduced binding in Y2H were coexpressed in *Nicotiana benthamiana* by *Agrobacterium tumefaciens*-mediated transient transformation. We also analyzed AVR-PiaM40A as it has, according to Y2H experiments, increased affinity for RGA5C-ter. As a negative control, a YFP fusion of the cytoplasmic *M. oryzae* effector PWL2 was used (Khang et al., 2010). Immunoblotting using anti-GFP and anti-HA antibodies showed proper expression of all proteins (Figure 4). However, AVR-Pia mutants with reduced binding to RGA5C-ter in Y2H reproducibly accumulated at lower levels than AVR-Pia wild type, while AVR-PiaM40A was expressed at similar levels (Figure 4). All YFP fusion proteins were efficiently and
comparably precipitated with anti-GFP antibodies, but only AVR-PiaM40A coprecipitated RGA5C-ter as strongly as AVR-PiaWT. The other mutants showed various degrees of impairment ranging from slightly (AVR-PiaR23A, AVR-PiaE58A, and AVR-PiaD63A) to strongly (AVR-PiaD29A, AVR-PiaR36A, and AVR-PiaR43G) reduced or even completely abolished RGA5C-ter coprecipitation (AVR-PiaF24S) (Figure 4A). Since the quantities of the different AVR-Pia variants after immunoprecipitation were similar to the quantity of immunoprecipitated AVR-Pia wild type, the differences in the coprecipitation of RGA5C-ter reflect interaction strength and not differences in the expression levels of YFP-AVR-Pia variants.

The specificity of the interactions was confirmed with PWL2 that does not interact with RGA5C-ter.

It has previously been shown that the interaction of AVR-Pia with RGA5C-ter relies on interaction with the RATX1 domain (RGA5RATX1) (Césari et al., 2013). To verify that interaction specificities of the AVR-Pia mutants with RGA5C-ter correlates with their strength of interaction with RGA5RATX1, co-IP experiments were performed using HA-tagged RGA5RATX1. AVR-PiaWT and AVR-PiaM40A strongly coprecipitated HA-RGA5RATX1, while the other mutants showed reduced (R23A and D63A), strongly reduced (D29A, R36A, and E58A), or no coprecipitation of RGA5RATX1 (F24S and R43G) (Figure 4B).

Taken together, these data indicate that AVR-PiaWT and AVR-PiaM40A strongly interact with RGA5C-ter and RGA5RATX1, while mutants affected in direct binding to RGA5C-ter in Y2H showed no interaction.
reduced association with RGA5C-ter and RGA5RATX1 in planta. Complete absence of association with RGA5RATX1 for AVR-PiaF24S and AVR-PiaR43G, both in planta and in Y2H, indicates a crucial role of these residues in the binding interface and suggests that they are pivotal for AVR-Pia recognition.

Direct Binding to the RATX1 Domain Is Required for AVR-Pia Recognition

To determine the role of the RATX1 binding surface of AVR-Pia in specific recognition by the RGA4/RGA5 pair, AVR-Pia mutants were coexpressed in N. benthamiana with RGA4/RGA5 and cell death activation was monitored. Since tagged versions of AVR-Pia proved inactive in this assay, untagged AVR-Pia mutants were used. AVR-Pia mutants with wild-type binding to RGA5RATX1 induced cell death, indicating that they are recognized by RGA5/RGA4 (Supplemental Figures 5A and 5B). Weakly or nonbinding mutants lost cell death inducing activity but were also less abundant than AVR-PiaWT or recognized AVR-Pia mutants (Supplemental Figures 5A to 5C). They could be detected only after enrichment by immunoprecipitation and showed in most cases only very low abundance (Supplemental Figure 5D). Therefore, no clear conclusions can be drawn for these mutants since lack of recognition may not only be due to reduced binding strength but also to low protein abundance or a combination of both effects. Differences in the protein level of AVR-Pia mutants were previously observed with YFP-tagged variants expressed in N. benthamiana (Figure 4) but not upon expression in E. coli or yeast (Figure 3B). Therefore, differences in the accumulation of AVR-Pia variants seem not related to an intrinsic destabilization of these proteins but rather to result from reduced stability in N. benthamiana.

Since transient heterologous experiments failed to determine the importance of the binding of AVR-Pia to RGA5RATX1 for recognition and disease resistance, the biological activity of AVR-Pia mutants was assayed in the homologous rice/M. oryzae system. Transgenic M. oryzae isolates were generated that carried the different mutant alleles under the control of the constitutive RP27 promoter (RP27p) (Bourett et al., 2002). As a control, transgenic Guy11 isolates carrying a RP27p:mRFP construct were generated and proved to be fully virulent (Figure 5; Supplemental Figure 6B). For three different PCR-validated transgenic isolates per construct, the accumulation of AVR-Pia variants was verified in culture filtrates by immunoblotting with anti-AVR-Pia antibodies (Supplemental Figure 6A). All AVR-Pia mutants were detected in at least one transgenic isolate except AVR-PiaD63A, which may be instable in M. oryzae. For AVR-PiaD29A and AVR-PiaE58A, only two and one isolate expressed the mutant protein (Supplemental Figure 6A).

The transgenic isolates were analyzed on the rice cultivars Kitaake carrying the Pia locus and Maratelli lacking Pia. All isolates were highly virulent on Maratelli, indicating that they were not affected in virulence (Supplemental Figure 6B). On Kitaake plants, the isolates expressing AVR-PiaWT, AVR-PiaR23A, AVR-PiaD29A, AVR-PiaE58A, or AVR-PiaE58A were completely avirulent and produced either no symptoms or small HR lesions characteristic of resistance (Figure 5; Supplemental Figure 6B). Consistent with the absence of protein expression, AVR-PiaD63A isolates did not induce resistance and were fully virulent on Kitaake plants. Isolates producing AVR-PiaF24S were partially virulent and formed disease lesions characterized by a gray center that were, however, smaller and less frequent than those provoked by the control mRFP isolates. Isolates expressing AVR-PiaR43G were highly virulent on Kitaake and produced large numbers of disease lesions (Figure 5; Supplemental Figure 6B).

Taken together, these results indicate that interaction of AVR-Pia with the RGA5RATX1 domain is required for recognition but that a reduction of this interaction as in AVR-PiaD29A, AVR-PiaD29A, or AVR-PiaE58A does not impair recognition. Only the R43G and F24S

Figure 4. AVR-Pia Mutants with Reduced RGA5C-ter Binding in Yeast Are Also Impaired in Binding to RGA5C-ter and RGA5RATX1 in Planta.

HA:RGA5C-ter (A) or HA:RGA5RATX1 (B) were transiently expressed with YFP:AVR-PiaWT or YFP:AVR-Pia mutants and YFP:PWL2 in N. benthamiana. Protein extracts were analyzed by immunoblotting with anti-HA (α-HA) and anti-GFP antibodies (α-GFP) (Input). Immunoprecipitation (IP) was conducted with anti-GFP beads (IP GFP) and analyzed by immunoblotting with α-GFP for the detection of immunoprecipitated AVR-Pia variants. Coprecipitated HA:RGA5C-ter (A) or HA:RGA5RATX1 (B) proteins were detected using α-HA antibody.
polymorphisms that abolished RGA5-RATX1 interaction both in planta and in yeast affected AVR-Pia recognition, with AVR-PiaF24S being completely inactive.

AVR-Pia Associates with RGA5 Outside of the RATX1 Domain

The high resilience of RGA4/RGA5-mediated AVR-Pia recognition to reduction of AVR-Pia-RGA5RATX1 interaction strength suggested that AVR-Pia might interact with additional sites in RGA5. To test this hypothesis, in planta association of the AVR-Pia mutants with the RGA5 full-length protein was assayed by co-IP. All AVR-Pia mutants, including AVR-PiaF24S and AVR-PiaR43G, coprecipitated RGA5 as efficiently as AVR-Pia wt (Figure 6A). This indicates that lack of binding to RGA5RATX1 does not abolish association with RGA5. It also further confirms that the lower expression level of some AVR-Pia variants is not limiting for interaction in co-IP experiments and that the reduced coprecipitation of RGA5RATX1 and RGA5C-ter (Figure 4) truly reflects reduced interaction strength and is not related to sometimes low expression levels of AVR-Pia variants. To test whether association of AVR-Pia with RGA5 is truly independent of the RATX1 domain, interaction of AVR-Pia with an RGA5 construct lacking the RATX1 domain (RGA5ΔRATX1) was tested by co-IP. All AVR-Pia variants coprecipitated RGA5ΔRATX1 (Figure 6B) and AVR-Pia mutants with reduced or no binding to RGA5RATX1 interacted as strongly with RGA5ΔRATX1 as AVR-Pia wt, demonstrating that the RATX1 domain is not necessary for formation of RGA5/AVR-Pia complexes. These results suggest that AVR-Pia interacts with additional sites in RGA5 outside of the RATX1 domain and that the region of AVR-Pia that mediates association with RGA5ΔRATX1 lies outside of the RATX1 binding surface.

It was previously shown that RGA5ΔRATX1 inhibits RGA4-triggered cell death and, therefore, that the RATX1 domain is not required for RGA5-mediated repression of RGA4 (Césari et al., 2014b). Since AVR-Pia still associates with RGA5ΔRATX1 in planta, we tested whether AVR-Pia would be recognized by RGA5ΔRATX1/RGA4 and trigger cell death independently of the RATX1 domain. Neither coexpression of RGA4, RGA5ΔRATX1, and AVR-Pia nor expression of these three proteins together with the isolated RATX1 domain triggered cell death (Supplemental Figure 7). This finding indicates that association of AVR-Pia with regions outside of the RATX1 domain is not sufficient to release RGA5-mediated RGA4 repression and further confirms that binding of AVR-Pia to RGA5ΔRATX1 is required for derepression of RGA4. In addition, these results suggest that AVR-Pia has to interact with the RATX1 domain in the context of the full-length RGA5 protein since an isolated RATX1 domain does not complement RGA5ΔRATX1 for AVR-Pia recognition.

DISCUSSION

Identification of a RGA5ΔRATX1 Binding Surface in AVR-Pia

In this study, we provide evidence that AVR-Pia interacts with the RATX1 domain of RGA5 through a precise binding surface consisting of β-strands 2 and 3, residues R23 and F24 from β-strand 1, and residues E56 and E58 from β-strand 4 (Figure 2C). This interaction surface, identified by NMR titration experiments with recombinant AVR-Pia and the RATX1 domain, was confirmed by mutant analysis. Indeed, replacement of residues R23, F24, D29, R43, T46, or E58 strongly reduced or abolished binding to...
RGARATX1 in Y2H and co-IP experiments, while replacement of residues M40 and Y85 increased interaction in Y2H (Figures 3 and 4). Substitutions outside of the candidate interaction surface had no effect on binding, with the exception of residues R36 and D63. Residue R36 is located in the loop joining β1 and β2 and might also be involved in RATX1 binding since its mutation causes reduced association with the RGARATX1 domain. Alternatively, it may play an important role in defining the positions of β-strands 1, 2, and 6 through the salt bridge it forms with residue E83 in β6. The D63A polymorphism seems to destabilize the overall structure since AVR-PiaD63A could not be expressed in E. coli or M. oryzae. In fact, D63 seems important to the structure of the β4-β5 loop as its side-chain amid group forms a hydrogen bond with the side-chain amid group of N65. This may be required for the proper positioning of C66, which forms a disulfide bridge with C25 linking the two β sheets β1, β2, β6 and β3, β4, β5 and thereby influences global folding.

AVR-Pia and AVR-PikD Have Distinct RATX1/HMA Binding Surfaces That Are Situated at Similar Positions

The three-dimensional structure of a AVR-PikD-Pikp-1HMA complex was determined by crystallography and showed that, like the formation of the AVR-Pia-RGARATX1 complex, binding of AVR-PikD to Pikp-1HMA involves β-strands 2 and 3 (Supplemental Figure 8A) (Maqbool et al., 2015). However, in AVR-PikD, the residues of β-strand 2 that are crucial for Pikp-1HMA binding, R64 and D66, are charged and establish hydrogen bonds and salt bridge interactions, respectively. By contrast, in AVR-Pia, surfac-exposed residues of β-strand 2 are hydrophobic and probably establish hydrophobic interactions (Figure 2C). In addition, unlike AVR-Pia, AVR-PikD possesses an N-terminal extension of 32 amino acids that is crucial for physical binding to Pikp-1HMA and recognition by Pikp-1/Pikp-2 (Supplemental Figure 8B). In particular, residue H46 from this extension establishes important interactions with matching residues in Pikp-1HMA. These interactions are necessary for binding and, together with the neighboring residues P47 and G48, for matching specificities with alleles of Pikp-1 (Kanzaki et al., 2012; Maqbool et al., 2015). These residues are missing in AVR-Pia but similarly important interactions are established with the amino acid F24 from the very hydrophobic β-strand 1. Therefore, recognition of the sequence-unrelated, but structurally similar, effectors AVR-Pia and AVR-PikD seems to involve similar structural elements but relies on distinct and highly specific mechanisms.

Whether the effector interaction surfaces of the RATX1/HMA domains of RGARATX1 and Pikp-1 are similar or completely different remains an open question. Crystal structures show that Pikp-1HMA has a typical HMA α/β-sandwich fold, composed of two α-helices and a four-stranded antiparallel β-sheet, that mediates interaction with AVR-PikD (Supplemental Figure 8C) (Maqbool et al., 2015). We used molecular modeling to evaluate whether AVR-Pia-binding may involve similar regions in RGARATX1 but no consensus docking model could be generated for the AVR-Pia-RGARATX1 complex (Supplemental Figure 8C). Interestingly, none of the docking models predicted an interaction surface in RGARATX1 similar to the effector binding surface of Pikp-1HMA. This suggests that the RGARATX1-AVR-Pia complex differs significantly from the Pikp-1HMA-AVR-Pik-D complex.

Taken together, recognition of the structurally similar MAX effectors AVR-Pia and AVR-Pik by independently acquired NLR-integrated HMA domains seems to rely on distinct molecular mechanisms. Future work is required to test this hypothesis through functional studies of the Pikp-1HMA interaction surface identified by structural analysis and the identification of the surface that mediates effector binding in RGARATX1.

Binding of AVR-Pia to the Integrated RATX1 Domain Is Required for Recognition but Is of Moderate Affinity

The mutants AVR-PiaF24S and AVR-PiaR43A showed drastically reduced RGARATX1 binding and triggered no or reduced resistance,
respectively, indicating that the AVR-Pia-RGA5\textsubscript{RATX1} interaction is required for RGA4/RGA5-mediated recognition. The presence of these polymorphisms in naturally occurring AVR-Pia alleles (Ribot et al., 2013) suggests that in rice isolates of \textit{M. oryzae}, AVR-Pia is undergoing selection for mutations in the RATX1-interaction surface and escape from RGA4/RGA5-mediated recognition. These results therefore provide further support for a crucial role of nonconventional, integrated decoy domains in effector recognition and NLR specificity.

However, we also found high resilience of AVR-Pia recognition to a reduction in RGA5\textsubscript{RATX1} binding strength since the weakly binding AVR-Pia mutants AVR-Pia\textsubscript{R23A}, AVR-Pia\textsubscript{E58A}, AVR-Pia\textsubscript{D29A}, and AVR-Pia\textsubscript{R36A} were still able to trigger resistance. Similar observations were made regarding AVR-PikD, the only other example where the affinity of an effector to the integrated decoy domain of its NLR receptor has been determined (Maqbool et al., 2013). Indeed, AVR-PikDA67D and AVR-PikDP47A G48D mutants showed drastically reduced binding to Pikp-1\textsubscript{HMA} but were nevertheless perfectly well recognized by Pik-1/Pik-2.

A possible explanation for this tolerance to a reduction in the affinity between effectors and integrated decoys could be that effectors interact with multiple independent sites in NLR receptors. Indeed, our study suggests that, besides the RATX1 domain, AVR-Pia interacts with other, not yet defined, regions in RGA5. In the simplest case, this interaction relies on direct physical binding, but since it was solely detected by co-IP experiments, the possibility that the binding is indirect and involves additional cofactors cannot be excluded. This interaction seems mediated by other AVR-Pia surfaces than those involved in RGA5\textsubscript{RATX1} binding since mutants with reduced binding to RGA5\textsubscript{RATX1} are not affected in interaction with RGA5\textsubscript{RATX1}. As the RATX1 domain is covalently linked to the rest of the RGA5 receptor, AVR-Pia binding to these other sites has the potential to increase the overall effector binding affinity to RGA5 despite the low affinity binding to RGA5\textsubscript{RATX1} ($K_D = 7 \, \mu M$). In this context, further mutation-induced reduction of AVR-Pia affinity toward the RATX1 domain may not have a dramatic effect unless it completely abolishes AVR-Pia/ RGA5\textsubscript{RATX1} interaction. This situation highlights an advantage of the integration of the decoy domain into the NLR receptor over a situation where the decoy is a separate molecule and has to bind to the effector before subsequent binding to the NLR receptor. In the latter case, low affinity of the effector-decoy interaction would lead to drastically reduced receptor occupancy and render the corresponding resistance more vulnerable to effector mutations affecting decoy binding.

**Interaction of Effectors with Multiple Independent Sites Is a Hallmark of NLR Receptor Activation**

Effector recognition by RGA4/RGA5 differs from other well-studied NLR models. Indeed, RGA5 has no inherent signaling activity and functions, on the one hand, by repressing RGA4 signaling activity and, on the other, by releasing repression upon AVR-Pia binding (Césari et al., 2014a). Interestingly, the RATX1 domain is required only for derepression and not for repression (Supplemental Figure 7) (Césari et al., 2014b). Providing the RATX1 domain separately in the presence of RGA5\textsubscript{RATX1} and AVR-Pia does not relieve repression despite the fact that AVR-Pia interacts with the separate partners, RGA5\textsubscript{RATX1} and RGA5\textsubscript{RATX1} (Supplemental Figure 7). To explain this result, we propose the hypothesis that simultaneous binding of AVR-Pia to different sites in RGA5, including the RATX1 domain, is required to trap RGA5 in a conformation unable to repress RGA4 (Figure 7).

That effectors have to establish simultaneously several independent interactions with NLRs or NLRs and cofactors to be recognized and trigger resistance has been frequently observed with effectors from various origins (Collier and Moffett, 2009). In the \textit{Pseudomonas syringae} effector AvrRPS4, two different surface areas on opposite and distant sites of the molecule are required for recognition by the RRS1/RPS4 pair (Sohn et al., 2012). One of these sites is crucial for binding to the integrated WRKY decoy domain of RRS1, while the other seems to interact with other not yet identified regions in RRS1 (Sarris et al., 2015). Similarly, recognition of the \textit{Hyaloperonospora arabidopsidis} effector ATR1-EMOY2 by the NLRs RPP1-NdA or RPP1-WsB from Arabidopsis relies on two different surface areas from two

![Figure 7. Model of AVR-Pia Recognition by the RGA4/RGA5 Receptor Complex.](image-url)
different domains and on opposite sides of the molecule, suggesting simultaneous interaction with independent binding sides in RPP1-NdA and RPP1WbS (Chou et al., 2011; Steinbrenner et al., 2015). Also in NLRs that recognize effector-cofactor complexes, simultaneous binding of these complexes to different parts of the NLR, generally involving the N terminus and the leucine-rich repeat have been frequently described (Collier and Moffett, 2009). Therefore, we propose the hypothesis that effectors or effector-cofactor complexes forcing or trapping NLRs in an activated state by simultaneously binding to multiple binding sites and inducing or stabilizing by this major conformational changes is a widespread mechanism in NLR activation and particularly in NLRs with integrated domains. Future structural and functional analysis will be necessary to test this model and elucidate in more detail how activation occurs at the molecular level.

METHODS

Growth Conditions of Plants and Fungi and Infection Assays

Nicotiana benthamiana plants were grown in a growth chamber at 22°C under fluorescent light (Radium; fluorescent lamp Spectralux Plus NL-T8 58W/865/G13) with a 16-h light period. Rice plants (Oryza sativa) were grown as described (Faireve-Rampant et al., 2008). Transgenic Magnaporthe oryzae GUY11 strains were grown at 25°C during 5 d on rice flour agar for spore production (Berruyer et al., 2003) and in Tanaka complete culture medium (Villalba et al., 2008) agitated at 60 rpm and 25°C during 5 d for liquid culture.

For the analysis of interaction phenotypes, a suspension of M. oryzae conidiospores in water with 0.1% of gelatin and adjusted to 5 x 10^4 spores mL^(-1) was sprayed on the leaves of 3-week-old rice plants (Berruyer et al., 2003). Symptoms were analyzed 7 d after inoculation on the youngest leaf.

Constructs

Plasmids were generated by Gateway cloning (Thermo Fisher), restriction/ligation, site-directed mutagenesis using the QuickChange Lightning kit (Agilent), or gap-repair cloning in yeast (Bruno et al., 2004). Gateway entry clones were generated using the pDONR207 plasmid (Thermo Fisher). Gateway destination vectors were modified pBIN19 plasmids for expression of tagged proteins in N. benthamiana (Césari et al. 2013) or modified pGAD-T7 or pGBK-T7 plasmids (Clontech) for yeast two-hybrid experiments (Bermonu et al., 2011). For protein expression, the pET-15b vector (Merck-Millipore) was used. For M. oryzae transformation, constructs were based on the pDL02 plasmid (Bruno et al., 2004). For details on PCR and mutagenesis primers and generation of plasmids, refer to Supplemental Tables 2 and 3.

NMR Spectroscopy and Structure Determination

Spectra were acquired on a 700 MHz Avance Bruker spectrometer equipped with triple-resonance (1H, 15N, 13C) z-gradient cryoprobe at 305K. All spectra are referenced to the internal reference DSS for the 1H dimension and indirectly referenced for the 15N and 13C dimensions (Wishart et al., 1995).

Spectra were processed using Topspin (version 3.2) and analyzed using strip-plots with Cindy in house software and CCPN (Franken et al., 2005) (analysis v 2.3). The 1H, 15N, and 13C assignments were derived by analogy from the assignments of AVR-Pia wild type without the need to prepare a 13C-labeled sample and the details are given in Supplemental Methods.

For the interaction of AVR-Pia variants and RGA5 with respect to H3 (50 μM) and various concentrations of unlabeled RATX1 (ratios 2:1, 1:1, 0.5:1, 0.25:1, and 0.1:1 for the reference) were prepared. HSQC spectra were recorded at 305K on a Bruker Avance 700 MHz spectrometer. Chemical shift differences were measured from the HSQC spectra of AVR-Pia or AVR-H3 alone and the AVR-RATX1 complex at R = 2. They are reported as Hamming distance weighted by the magnetogoric ratios (Schumm et al., 2007).

NMR Titration

For the assignments, protein samples (1 mM) in 20 mM potassium-sodium phosphate, pH 5.4, and 150 mM NaCl were used. For the titrations of 15N-labeled AVR-Pia proteins, different samples with constant concentrations of AVR-Pia wild type or H3 (50 μM) and various concentrations of unlabeled RATX1 (ratios 2:1, 1:1, 0.5:1, 0.25:1, and 0.1:1 for the reference) were prepared. HSQC spectra were recorded at 305K on a Bruker Avance 700 MHz spectrometer. Chemical shift differences were measured from the HSQC spectra of AVR-Pia or AVR-H3 alone and the AVR-RATX1 complex at R = 2. They are reported as Hamming distance weighted by the magnetogoric ratios (Schumm et al., 2007).

Co-IP and Y2H Interaction Assays

Protein-protein interaction analyses by coimmunoprecipitation were performed with protein extracts from N. benthamiana leaf discs harvested 2 d after Agrobacterium infiltration (Césari et al., 2013). For the interaction of AVR-Pia variants with RGA5 and RGA5RATX1, five leaf disks per sample were homogenized in extraction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 0.1% IEGPAL CA-630 [Nonidet P-40]), supplemented with complete protease inhibitor cocktail (Roche) and polyvinylpyrrolidone (PVP; 0.5%). After two centrifugations (30 min, 15,000g), 5 μL magnetic GFP-trap M beads (Chromotek) per sample washed twice with protein extraction buffer (without PVP) were added to 500 μL protein extract and incubated with gentle rotation for 2 h at 4°C. Beads were separated and washed three times with 600 μL protein extraction buffer (without PVP).

For the interaction of AVR-Pia variants and RGA5 or RGA5RATX1, a modified protein expression buffer was used (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 1.0% IGE-PAL CA-630 [Nonidet P-40], 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with a complete protease inhibitor cocktail [Roche] and 0.5% PVP). Co-IP was performed with 8 μL agarose GFP-trap M beads (Chromotek) per sample washed two times with protein extraction buffer (without PVP).

Bound proteins were eluted by boiling for 10 min at 70°C in 50 μL NuPAGE sample buffer, separated by polyacrylamide gel electrophoresis using NuPAGE 4 to 12% gels (Invitrogen), transferred to nitrocellulose membrane (Millipore), and analyzed by immunoblotting. For immunodetection of proteins, rat anti-HA-horseradish peroxidase (clone 3F10; Sigma-Aldrich) or mouse anti-FLAG (clones 7.1 and 13.1, Sigma-Aldrich) and goat anti-mouse horseradish peroxidase (Sigma-Aldrich) were used in combination with the Immobilon western kit (Millipore).

Binding domain (BD) fusions of AVR-Pia variants in pGBKTT7-S3 and activation domain (AD) fusions of RGA5RATX1 in pGAD77 were transformed in gold and Y187 yeast strain, respectively. Interactions assays were performed according to the Matchmaker Gold yeast two-hybrid system protocol (Clontech).

Transient Protein Expression and HR Assays in N. benthamiana

For agroinfiltration in N. benthamiana, pBIN19 binary vectors containing either AVR-Pia, PWL2, or RGA5 variants were transformed into Agrobacterium strain.
GV3101 by electroporation. Individual clones were selected and grown in Luria-Bertani liquid medium containing 50 mg mL\(^{-1}\) rifampicin, 15 mg mL\(^{-1}\) gentamycin, and 50 mg mL\(^{-1}\) kanamycin at 28°C for 24 h before agroinfiltration. Coinoculation mixtures adjusted to an OD\(_{600}\) of 1.0 were infiltrated in 4-week-old \(N.\) benthamiana plants. The infiltrated plants were incubated for 48 or 96 h in growth chambers under controlled conditions for coimmunoprecipitations or cell death assays, respectively. Three days after infiltration, \(N.\) benthamiana leaves were scanned using a Typhoon FLA9000 fluorescence scanner (GE Healthcare) with excitation at 635 nm and a long-pass red filter (LPR-665 nm) to evaluate the HR response as a lack of red chlorophyll fluorescence.

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Recognition of the *Magnaporthe oryzae* Effector AVR-Pia by the Decoy Domain of the Rice NLR Immune Receptor RGA5

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