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The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the *Magnaporthe oryzae* Effectors AVR-Pia and AVR1-CO39 by Direct Binding[W][OA]

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Resistance (R) proteins recognize pathogen avirulence (Avr) proteins by direct or indirect binding and are multidomain proteins generally carrying a nucleotide binding (NB) and a leucine-rich repeat (LRR) domain. Two NB-LRR protein-coding genes from rice (*Oryza sativa*), *RGA4* and *RGA5*, were found to be required for the recognition of the *Magnaporthe oryzae* effector AVR1-CO39. *RGA4* and *RGA5* also mediate recognition of the unrelated *M. oryzae* effector AVR-Pia, indicating that the corresponding R proteins possess dual recognition specificity. For *RGA5*, two alternative transcripts, *RGA5-A* and *RGA5-B*, were identified. Genetic analysis showed that only *RGA5-A* confers resistance, while *RGA5-B* is inactive. Yeast two-hybrid, coimmunoprecipitation, and fluorescence resonance energy transfer–fluorescence lifetime imaging experiments revealed direct binding of AVR-Pia and AVR1-CO39 to RGA5-A, providing evidence for the recognition of multiple Avr proteins by direct binding to a single R protein. Direct binding seems to be required for resistance as an inactive AVR-Pia allele did not bind RGA5-A. A small Avr interaction domain with homology to the Avr recognition domain in the rice R protein Pik-1 was identified in the C terminus of RGA5-A. This reveals a mode of Avr protein recognition through direct binding to a novel, non-LRR interaction domain.

INTRODUCTION

Plant resistance to microbial pathogens is a complex process relying on two major levels of resistance controlled by distinct types of plant receptors (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The first line of plant defense is activated by plasma membrane proteins called pattern recognition receptors, which perceive conserved microbial molecules called pathogen-associated molecular patterns (PAMPs). Adapted plant pathogens are able to bypass this PAMP-triggered immunity by producing secreted effectors that act inside or outside the host cell and manipulate key components of plant defense (Jones and Dangl, 2006). The second layer of plant immunity relies on the specific recognition of certain pathogen-derived effectors called Avirulence (Avr) proteins by so-called plant resistance (R) proteins. This effector-triggered immunity (ETI) gives rise to stronger and faster defense responses than PAMP-triggered immunity and often involves a form of localized programmed cell death called the hypersensitive response (HR) (Dodds and Rathjen, 2010). The largest class of R proteins belongs to the conserved family of NB-LRR proteins (Tameling and Takken, 2007). They contain a central nucleotide binding (NB) domain, also known as the NB-ARC (for NB adaptor shared by Apaf-1, certain R proteins, and CED-4) domain, and a C-terminal leucine-rich repeat (LRR) domain. In monocot R proteins, the LRR repeat motif is often not conserved (Bai et al., 2002) and in those cases, the domain is called leucine-rich domain (Monosi et al., 2004; Zhou et al., 2004). NB-LRR proteins are further subdivided according to their N-terminal domain into two major subclasses (Meyers et al., 1999; Pan et al., 2000). Proteins of the TIR-NB-LRR class possess an N-terminal Toll Interleukin-1 (TIR) domain, whereas CC-NB-LRR class proteins harbor a structured coiled-coil (CC) domain. Both N-terminal domains seem to be involved in R protein homodimerization and in the activation of defense signaling (Bernoux et al., 2011; Maekawa et al., 2011). In the absence of the Avr protein, R proteins are maintained in an inactive conformation to avoid inappropriate defense activation and cell death (Takken and Goverse, 2012).

Avr proteins are perceived by R proteins through direct or indirect recognition mechanisms. Direct recognition relies on

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physical binding of effectors to R proteins, and indirect recognition is based on the perception of effector-induced modifications of host proteins known as guardees, decoys, or more generally cofactors by R proteins (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008; Collier and Moffett, 2009).

Direct R–Avr interaction has been detected for seven R proteins: Pi-ta (Jia et al., 2000), RRS1-R (Deslandes et al., 2003), N (Ueda et al., 2006), L5/L6 (Dodds et al., 2006), M (Catanzariti et al., 2010), RPP1 (Krasileva et al., 2010; Chou et al., 2011), and Pik/km/kp/ks/kh (Kanzaki et al., 2012). For some of them, it also has been demonstrated that physical interaction is required for resistance, validating the direct recognition model. For instance, binding specificity between the five allelic rice (*Oryza sativa*) R proteins Pik/km/kp/ks/kh-1 and the three allelic *Magnaporthe oryzae* Avr proteins AVR-Pik/km/kp determines recognition specificity (Kanzaki et al., 2012). However, molecular details of direct R–Avr interactions, such as precise knowledge on the involved R and Avr domains, and mechanisms linking R–Avr interaction with the activation of downstream resistance signaling pathways are largely unknown.

Multiple recognition specificities of R proteins are thought to be important for the perception of a broad range of pathogens with a limited R protein repertoire and have been documented for R proteins detecting multiple Avr proteins by indirect recognition (Dangl and Jones, 2001). However, direct binding of R proteins has only been demonstrated for single Avr proteins.

Rice blast, caused by the ascomycete fungus *M. oryzae*, is the most devastating rice disease (Pennisi, 2010; Dean et al., 2012). Approximately 500 NB-LRR coding genes have been predicted in the rice genome (Monosi et al., 2004; Zhou et al., 2004), 100 major rice blast *R* genes have been characterized genetically, and 19 have been cloned (Ballini et al., 2008; Sharma et al., 2012). All cloned rice blast resistance genes encode CC-NB-LRR proteins, except for *Pid-2*, which encodes a receptor-like kinase (Chen et al., 2006). Strikingly, rice blast resistance is conferred in several cases not by individual NB-LRR proteins, but by functional pairs of such proteins (Ashikawa et al., 2008; Lee et al., 2009; Okuyama et al., 2011; Yuan et al., 2011; Zhai et al., 2011). The corresponding *R* gene pairs show extremely tight physical linkage and are arranged in inverted orientation. For instance, *RGA4* and *RGA5*, located next to each other at the *Pia* locus, are necessary and sufficient to mediate *Pia* resistance and recognize the *M. oryzae* effector AVR-Pia (Okuyama et al., 2011). However, the molecular mechanism by which *R* gene pairs recognize their cognate Avr protein and in particular the role of each NB-LRR protein in recognition and resistance activation remains unclear.

Seven *Avr* genes from *M. oryzae* have been cloned. Except *ACE1* and *AVR-Pita*, which encode an enzyme involved in the synthesis of a secondary metabolite (Böhnert et al., 2004) and a putative metalloprotease (Orbach et al., 2000), respectively, *Avr* genes from the rice blast fungus encode small secreted proteins of unknown function. Experimental evidence indicates that recognition of AVR-Pita, AVR-Pia, and AVR-Pik/km/kp occurs inside host cells by their corresponding cytoplasmic R proteins (Jia et al., 2000; Yoshida et al., 2009; Kanzaki et al., 2012). Recently, we characterized molecularly the *AVR1-CO39* gene and demonstrated that it encodes a small secreted protein, expressed specifically during infection (Ribot et al., 2013). AVR1-CO39 is translocated inside the cytoplasm of rice cells where it is recognized by the product of the so far uncharacterized *Pi-CO39 R* gene (Ribot et al., 2013).

The molecular mechanism of *M. oryzae* Avr protein recognition has only been investigated in the case of AVR-Pita and AVR-Pik (Jia et al., 2000; Kanzaki et al., 2012). AVR-Pita is recognized through direct binding to the Pi-ta C-terminal LRD domain, whereas AVR-Pik specifically associates with an N-terminal domain of Pik-1, including the CC domain and additional unclassified sequences upstream of the NB domain. Hence, those examples illustrate two cases of direct recognition that seem to implicate different R protein domains and different mechanisms.

In this work, we report the investigation of *Pi-CO39* blast resistance in rice and demonstrate that two CC-NB-LRR–coding genes, *RGA4* and *RGA5*, are required to recognize AVR1-CO39 in addition to the unrelated *M. oryzae* effector AVR-Pia. In vivo and in planta experiments show binding of RGA5 to AVR-Pia and AVR1-CO39 through a small non-LRR C-terminal domain, providing evidence for the recognition of multiple Avr proteins by direct binding to a single R protein. By exploiting natural polymorphism of *AVR-Pia*, we show that direct binding of AVR-Pia to RGA5 is correlated to *Pia*-mediated resistance. Finally, by comparing the AVR1-CO39 and AVR-Pia recognition domain in RGA5 with the AVR-Pik recognition domain in Pik-1, we identify a mode of recognition of *M. oryzae* Avr proteins by pairs of distinct rice R proteins involving a novel interaction domain.

RESULTS

The C Terminus of the CC-NB-LRR Protein RGA5 Interacts with AVR1-CO39

To identify rice proteins interacting with AVR1-CO39 and acting either as effector targets or as resistance proteins, a yeast two-hybrid screen was performed using a cDNA library generated with mRNA of the rice cultivar CO39 carrying the *Pi-CO39* resistance gene (Chauhan et al., 2002). The screening was performed with the construct *BD*:*AVR1-CO39* carrying sequences for the mature AVR1-CO39$_{22-89}$ protein deleted from its signal peptide and fused to the GAL4 DNA binding domain. We identified nine AVR1-CO39 interactors named ACI1 to ACI9 (data not shown). For *ACI1*, three independent clones called ACI1-L (970 nucleotides), ACI1-M (889 nucleotides), and ACI1-S (673 nucleotides) were identified and validated by retransformation into yeast cells (Figures 1A and 1B). BLAST analysis of *ACI1* sequences revealed that they match the 3′ end of the previously described *RGA5* gene from the rice cultivar Sasanishiki (Figure 1B) (Okuyama et al., 2011). *RGA5* encodes a CC-NB-LRR protein and confers, together with the physically linked CC-NB-LRR protein-coding gene *RGA4*, resistance to *M. oryzae* strains expressing *AVR-Pia* (Okuyama et al., 2011). Identification of *RGA5* in the two-hybrid screen for AVR1-CO39 interactors suggested that *RGA5* and *RGA4* may correspond together to the previously identified *Pi-CO39* gene (Chauhan et al., 2002). This hypothesis was further supported by the fact that genetic analysis indicates

colocalization of *Pia* with *Pi-CO39* on rice chromosome 11 and that *RGA4* and *RGA5* are present on a BAC clone containing *Pi-CO39*; this BAC clone carries a genomic DNA fragment of rice variety CO39 (Chauhan et al., 2002; Leong et al., 2004; Okuyama et al., 2011). In addition, inoculation experiments with a large panel of rice varieties showed that all rice varieties carrying *Pi-CO39* resistance are also resistant against strains carrying *AVR-Pia*, indicating that *Pia* and *Pi-CO39* resistance are linked (see Supplemental Table 1 online). Together, these results made *RGA4* and *RGA5* promising candidates for *Pi-CO39* resistance and suggested recognition of AVR1-CO39 by direct interaction with RGA5.

RGA4 and *RGA5* Confer *Pia* and *Pi-CO39* Resistance

To assess the role of RGA4 and RGA5 in AVR1-CO39 recognition, rice lines carrying loss-of-function mutations in *RGA4* and transgenic rice lines complemented with *RGA4* and *RGA5* were analyzed. Previously, two mutant lines carrying point mutations in *RGA4* and affected in *Pia*-mediated resistance had been described (Okuyama et al., 2011). Both mutant lines and Sasanishiki wild-type plants were inoculated with transgenic *M. oryzae* Guy11 strains expressing either *AVR1-CO39* (Guy11-*AVR1-CO39*) or carrying the empty vector (Guy11-*EV*) (Ribot et al., 2013). Sasanishiki wild-type plants were highly susceptible to the Guy11-*EV* strain and developed characteristic blast disease symptoms (Figure 2A). After inoculation with the Guy11-*AVR1-CO39* strain, Sasanishiki wild-type plants never showed disease symptoms. Eventually, characteristic small and dark-brown HR lesions appeared 2 to 3 d after pathogen challenge. This demonstrates that Sasanishiki plants are fully resistant to *M. oryzae* strains carrying *AVR1-CO39*. By contrast, both *rga4* mutant lines developed disease lesions after inoculation with both the Guy11-*AVR1-CO39* and Guy11-*EV* strains (Figure 2A). In both mutants, lesion size and number were identical to Guy11-*AVR1-CO39* and Guy11-*EV*, indicating that they have completely lost AVR1-CO39–triggered resistance. These results indicate that *RGA4* is necessary for *Pi-CO39* resistance.

To further elucidate the role of *RGA4* and *RGA5* in AVR1-CO39–triggered resistance, previously generated transgenic rice lines of the susceptible cultivar Kanto51 carrying *RGA4* (nucleotides −2901 from the ATG to +3391), *RGA5* (nucleotides −2010 to +5040), *RGA4* and *RGA5*, or an empty vector (Okuyama et al., 2011) were analyzed in infection experiments with the Guy11-*AVR1-CO39* and the Guy11-*EV* strains. Transgenic rice lines carrying only *RGA4* or *RGA5* were fully susceptible to

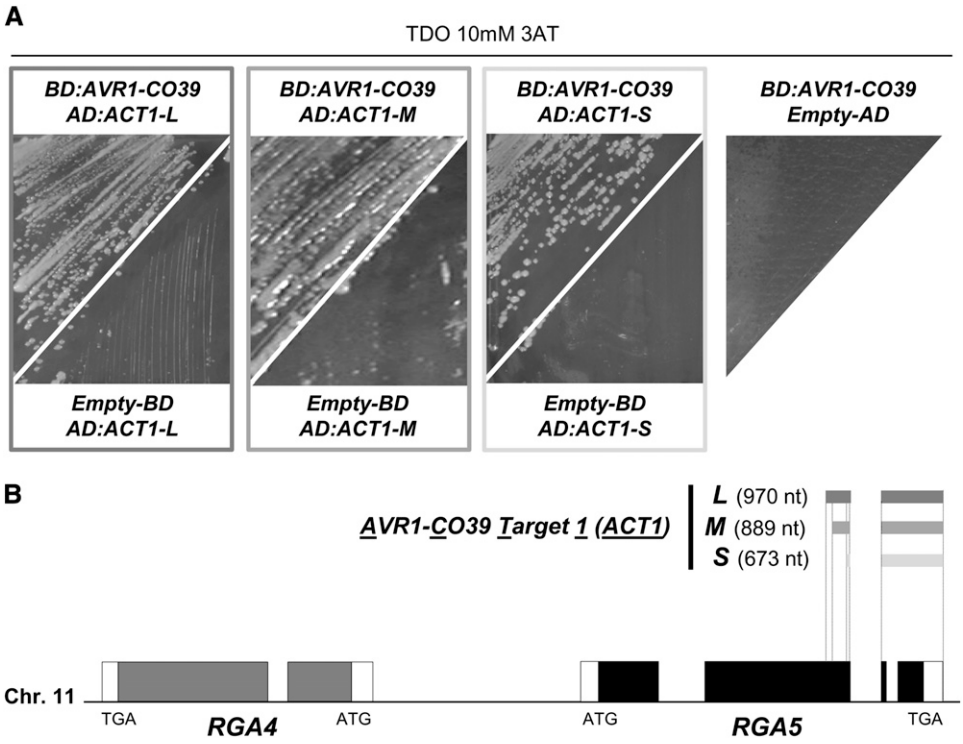

Figure 1. AVR1-CO39 Interacts Physically with RGA5.

(A) Screening of a rice yeast two-hybrid cDNA library with AVR1-CO39 identified three different clones for AVR1-CO39 Interactor 1 (ACI1-L, -M, and -S). Interactions were assayed on synthetic triple dropout (TDO) medium lacking Trp, Leu, and His and supplemented with 10 mM 3-amino-1,2,4-triazole (3AT). Autoactivation of BD:AVR1-CO39 and AD:ACI1 constructs was tested using empty pGADT7-AD (Empty-AD) and empty pGBKT7-BD (Empty-BD) vectors. Photos show yeast colonies after 4 d of growth.

(B) ACI1-L, -M, and -S align to the 3′ extremity of the *RGA5* gene located adjacent to the *RGA4* gene on chromosome 11 (Chr. 11) of rice varieties Sasanishiki and CO39. nt, nucleotides.

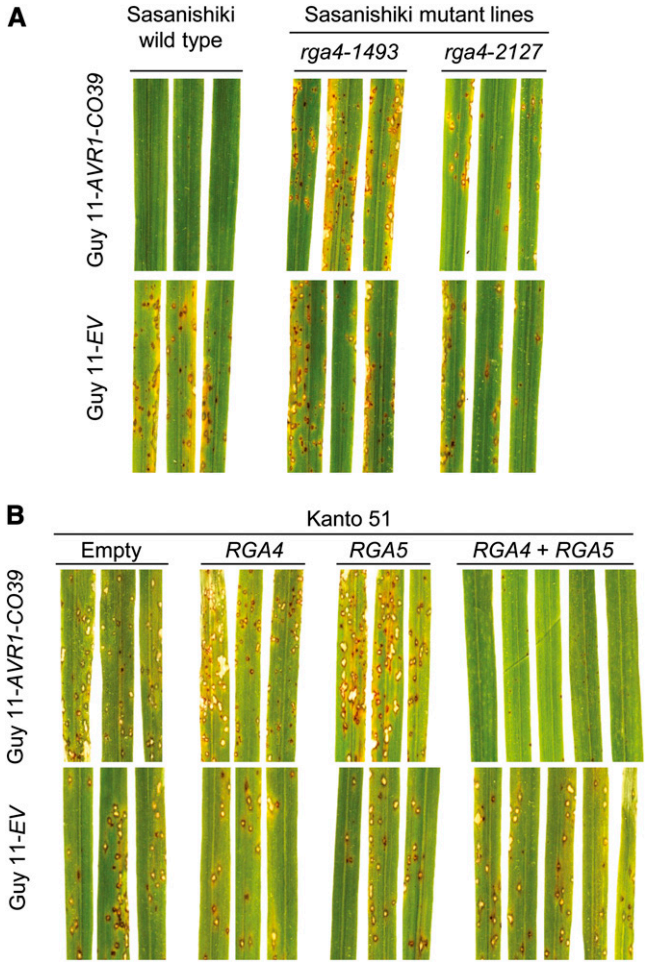

Figure 2. *RGA4* and *RGA5* Confer *Pi-CO39* Resistance.

(A) *rga4* mutant lines are compromised in *Pi-CO39* resistance. Transgenic *M. oryzae* Guy11-*AVR1-CO39* or Guy11-*EV* (empty vector) strains were spray inoculated on 3-week-old plants of the rice cultivar Sasanishiki and Sasanishiki ethyl methanesulfonate mutant lines *rga4-1493* and *rga4-2127* (Okuyama et al., 2011). Development of disease and HR symptoms was followed 7 d after inoculation to determine susceptibility or resistance. Identical results were obtained in two independent inoculation experiments using the two independent mutant lines each time. Pictures show typical symptoms at 7 d after inoculation.

(B) *RGA4* and *RGA5* are required for *Pi-CO39* resistance. Transgenic rice lines of the cultivar Kanto51 (*pi-CO39*, *pia*) carrying the empty pCambia1300 binary vector (empty), a genomic *RGA4* construct (*RGA4*), a genomic *RGA5* construct (*RGA5*), or *RGA4* and *RGA5* constructs (*RGA4* + *RGA5*) were challenged by spray inoculation with the Guy11-*AVR1-CO39* or the Guy11-*EV* strains. Only transgenic rice lines carrying both *RGA4* and *RGA5* were resistant to the *AVR1-CO39*–expressing *M. oryzae* strain. Identical results were obtained in two independent inoculation experiments. Pictures show typical symptoms at 7 d after inoculation.

Guy11-*AVR1-CO39* strains (Figure 2B), indicating that on their own, neither *RGA4* nor *RGA5* is sufficient to confer resistance to AVR1-CO39. Only transgenic plants expressing both *RGA4* and *RGA5* were resistant to the Guy11-*AVR1-CO39* strain. This resistance was specific to strains containing *AVR1-CO39* as *RGA4 RGA5* plants were completely susceptible to the empty vector strain GUY11-*EV* (Figure 2B). Taken together, these results demonstrate that both *RGA4* and *RGA5* are required for *Pi-CO39* resistance, as was previously shown for *Pia* resistance. The functional pair RGA4-RGA5 thus has a double specificity for the recognition of the sequence-unrelated effectors AVR-Pia and AVR1-CO39.

Alternative Splicing of *RGA5* Transcripts Generates Two RGA5 Isoforms

Comparison of the sequences of the three independent *RGA5* cDNA clones, isolated in the yeast two-hybrid screen (ACI1-S, ACI1-M, and ACI1-L) with the cDNA sequence that had previously been described for *RGA5* (Okuyama et al., 2011), suggested alternative splicing of third intron of *RGA5* (Figure 3A). In fact, the previously described gene model derived from sequencing of a PCR product amplified from cDNA from the resistant variety Sasanishiki exhibits four exons and three introns (Okuyama et al., 2011). Here, we call the corresponding transcript *RGA5-A*. The gene model derived from the ACI1 clones only contains two introns and three exons. Intron 3 is not spliced in the corresponding transcript, which we termed *RGA5-B* (Figure 3A). Intron retention is a major phenomenon in plant alternative splicing and frequently occurs at the last intron (Ner-Gaon et al., 2004). To validate the accumulation of both transcripts, cDNA of the rice cultivar CO39 was analyzed in RT-PCR experiments with primers designed to amplify fragments of differing size for the two *RGA5* transcript variants and with primers specific for *RGA5-A* (see Supplemental Figure 1 online). These experiments confirmed production of both splice variants in CO39. In addition, a large, almost full-length, *RGA5-B* cDNA fragment was PCR amplified from CO39 cDNA with a forward primer situated in the 5′ untranslated region and a reverse primer situated in intron 3, specific for *RGA5-B*. Sequencing of this fragment confirmed that *RGA5-B* spanning exon 1, exon 2, and intron-retaining exon 3 is produced (data not shown).

Expression of the two *RGA5* transcripts is expected to lead to the synthesis of two distinct CC-NB-LRR proteins, RGA5-A and RGA5-B, of 1116 and 1071 amino acids, respectively, for the functional allele from Sasanishiki (Figure 3B). Both proteins share the CC, NB, and LRR domains and a part of the non-LRR C-terminal domain comprising residues 869 to 1024. RGA5-A and RGA5-B vary only in their very C-terminal extremities, which are 92 and 47 amino acids long, respectively, in the Sasanishiki cultivar. Interestingly, the last 123 C-terminal amino acids of RGA5-A comprise a domain of 71 residues (1000 to 1070) with features of a heavy metal–associated domain (HMA) (Okuyama et al., 2011). This HMA domain is related to the *Saccharomyces cerevisiae* copper binding protein ATX1 (Panther domain family PTHR22814), and here we call it the Related to ATX1 (RATX1) domain.

RGA5-A Is the Functional Isoform Mediating *Pia* and *Pi-CO39* Resistance

To investigate which RGA5 isoform is involved in *Pia* and/or *Pi-CO39* resistance, we generated transgenic rice lines specifically

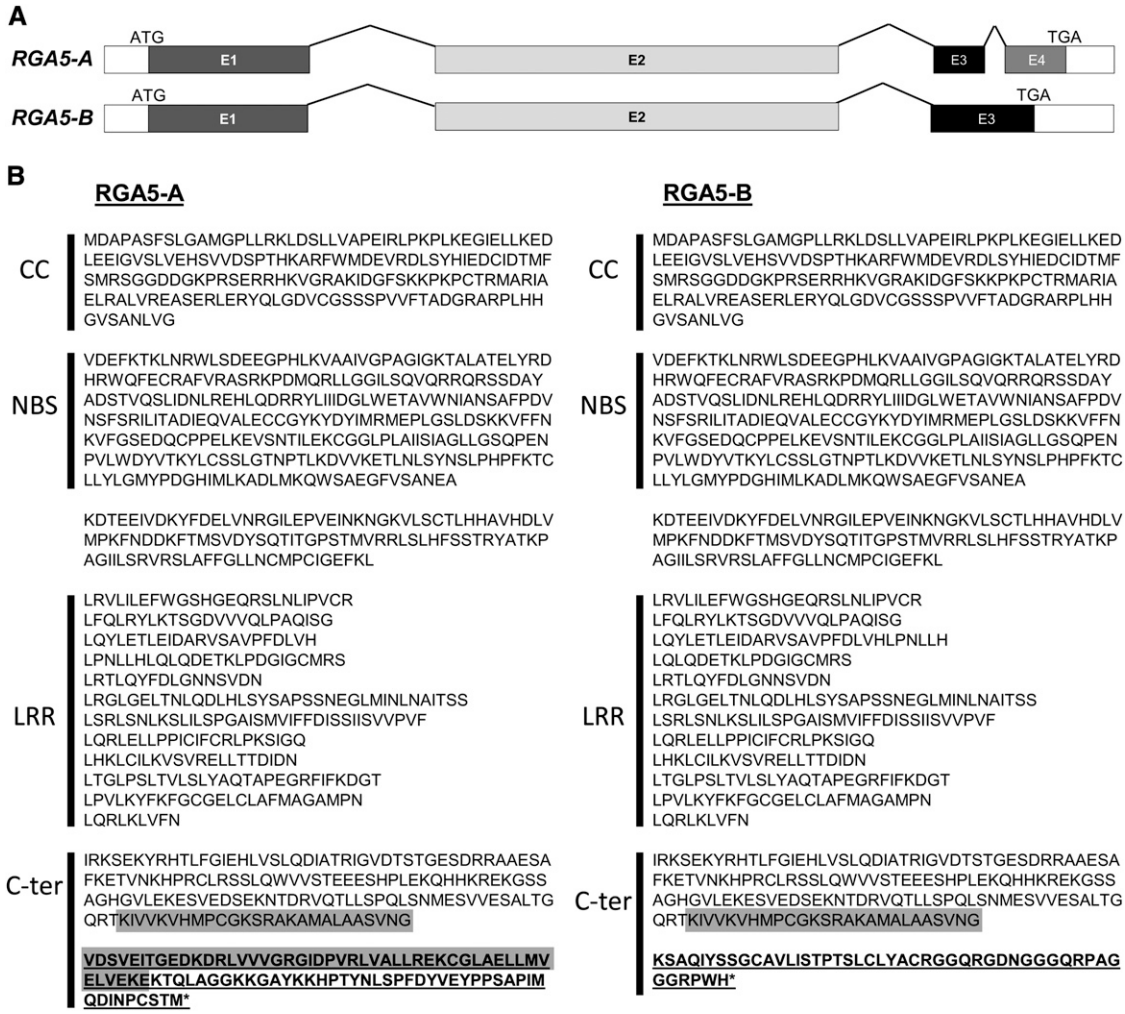

Figure 3. *RGA5* Is Subject to Alternative Splicing and Produces Two Hypothetical Protein Isoforms: RGA5-A and RGA5-B.

(A) Structure of *RGA5-A* and *RGA5-B* transcripts. *RGA5-A*, as described by Okuyama et al. (2011), is produced by splicing of three introns from the primary *RGA5* transcript. *RGA5-B* is produced by splicing of the first two introns and retention of intron 3. E1, exon 1; E2, exon 2; E3, exon 3; E4, exon 4.
(B) RGA5-A and RGA5-B differ only in their C terminus. Intron retention in RGA5-B leads to a divergent C terminus (underlined) and disruption of the RATX1 domain present only in RGA5-A (marked in gray). In the rest of the proteins, including CC, NB, and LRR domains, as well as the first half of C-terminal non-LRR sequences, RGA5-A and RGA5-B are identical.

expressing *RGA5-A* or *RGA5-B* in combination with *RGA4*. To this aim, a genomic *pRGA5:RGA5-A* construct was engineered where the third intron of *RGA5* was deleted from its genomic sequence, thus preventing *RGA5-B* production (see Supplemental Figure 2A online). A *pRGA5*:*RGA5-B* construct was obtained by introducing a point mutation in the donor splice site of the third intron to prevent its splicing and generate only intron 3–retaining *RGA5-B* mRNAs (see Supplemental Figure 2A online). To determine whether these constructs actually lead to specific expression of the expected *RGA5* transcript variants, RT-PCR experiments were conducted on rice protoplasts of the Nipponbare variety (*rga5*) transformed with the different *RGA5* constructs. These experiments showed that protoplasts transfected with *pRGA5*:*RGA5-A* or *pRGA5*:*RGA5-B* produced *RGA5-A* or *RGA5-B*, respectively (see Supplemental Figure 2B online). As positive controls, Nipponbare protoplasts transfected with the *pRGA5*:*RGA5* wild-type construct and untransformed protoplasts of the variety Kitaake, carrying *Pia*, were analyzed. As expected, *RGA5-A* and *RGA5-B* transcripts were detected in both positive controls. By contrast, in Nipponbare protoplasts transformed with the empty vector, which were analyzed as negative controls, no *RGA5* transcripts were detected (see Supplemental Figure 2B online). The validated mutant constructs were introduced into the pCambia2300 plasmid to transform rice plants of the Kanto51 variety and transgenic Kanto51 lines carrying a genomic *pRGA4*:*RGA4* construct. As controls, transgenic lines carrying the empty pCambia2300 plasmid and lines expressing both splice variants from a wild-type genomic construct (*pRGA5*:*RGA5*) were generated. Two independent transformation experiments were performed, and at least 20

independent rice lines per construct were obtained in each experiment.

At least 15 independent transgenic lines per construct were inoculated with the field isolate INA72, which carries *AVR-Pia* (Yoshida et al., 2009), and with the transgenic isolates Guy11-*AVR1-CO39* and Guy11-*EV*. For this, regenerated T0 plants with at least three tillers were split in three plantlets, of which each was inoculated with another strain. Interestingly, only the rice lines carrying the *RGA5-A* construct in combination with *RGA4* were fully resistant to the *AVR1-CO39*– and *AVR-Pia*–carrying strains Guy11-*AVR1-CO39* and INA72 (Figure 4; see Supplemental Figure 3 online). Conversely, lines expressing *RGA5-B* in combination with *RGA4* were fully susceptible, suggesting that only RGA5-A has the capacity to recognize both Avr proteins and/or to activate signaling upon recognition. The positive control lines carrying the wild-type *pRGA5*:*RGA5* construct together with *RGA4* were, as expected, resistant to Guy11-*AVR1-CO39* and INA72, while all other transgenic lines, including those that either carried only *RGA4* or carried *RGA5* variants but lacked *RGA4*, were fully susceptible. All analyzed lines were fully susceptible to the Guy11-*EV* strain, demonstrating that resistance responses are due to specific Avr recognition. These results indicate that the production of RGA5-A in combination with RGA4 is necessary and sufficient to mediate recognition of both AVR-Pia and AVR1-CO39 in rice (Figure 4; see Supplemental Figure 3 online). RGA5-A is thus the functional RGA5 isoform, and RGA5-B is not active in *Pi-CO39* or *Pia* resistance despite its direct interaction with AVR1-CO39 in the yeast two-hybrid system.

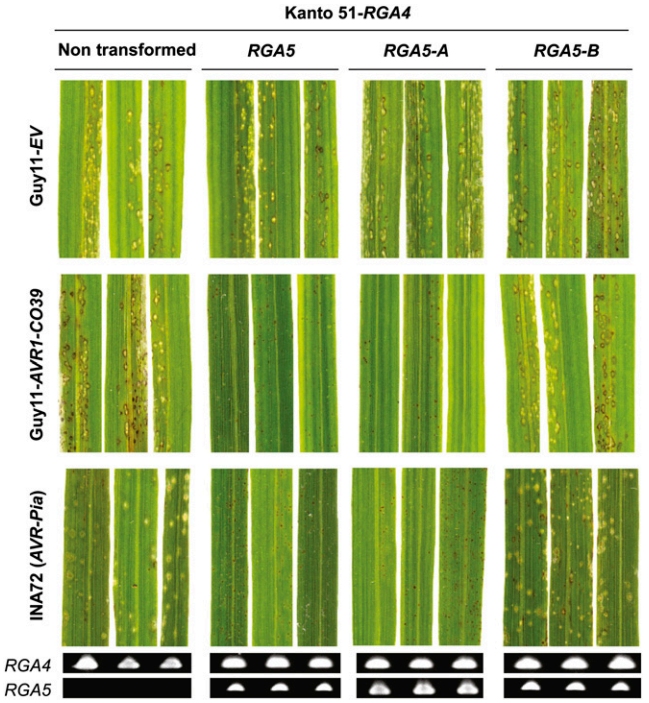

Figure 4. RGA5-A Is the Functional Isoform Required for *Pi-CO39* and *Pia* Resistance.

A transgenic line of rice cultivar Kanto51 carrying *RGA4* was transformed with a genomic construct for wild-type *RGA5* (*RGA5*) or engineered genomic constructs for *RGA5-A* or *RGA5-B* (details in Supplemental Figure 2 online). Plants of the transgenic lines were spray inoculated with the transgenic strains Guy11-*AVR1-CO39* or Guy11-*EV* or the field isolate INA72 carrying *AVR-Pia* (Okuyama et al., 2011), and symptoms were recorded until 7 d after inoculation. Only *RGA4 RGA5-A* and *RGA4 RGA5* rice lines were resistant to *M. oryzae* strains expressing *AVR1-CO39* or *AVR-Pia*. Presence of the transgenes was determined by direct PCR with *RGA4*- and *RGA5*-specific primers (bottom). Rice transformation was performed twice, and identical results were obtained in two independent inoculation experiments using at least 15 independent transgenic lines for each construct (see Supplemental Figure 3 online for the replicate experiment). Pictures show typical symptoms at 7 d after inoculation.

AVR-Pia and AVR1-CO39 Physically Interact with a C-Terminal, Non-LRR Domain of RGA5-A

Since RGA5-A, but not RGA5-B, is responsible for *Pia*- and *Pi-CO39*–driven resistance, the interaction of AVR1-CO39 and AVR-Pia with RGA5-A was examined using the yeast two-hybrid system. In addition, interaction of AVR-Pia with RGA5-B was also analyzed. Constructs carrying the C-terminal non-LRR domain of RGA5-A or RGA5-B in fusion with the Gal4 activation domain were generated and named *RGA5-A_L* (*AD:RGA5-A*$_{883-1116}$) and *RGA5-B_L* (*AD:RGA5-B*$_{883-1069}$), respectively (Figure 5A). *RGA5-B_L* was analogous to the largest clone *ACI1-L* identified in the two-hybrid screen. For AVR-Pia, a construct coding a fusion protein between the Gal4 DNA binding domain and the mature AVR-Pia protein, deleted for its secretion peptide (AVR-Pia$_{20-85}$), was generated and named *BD:AVR-Pia*. Yeast clones carrying *RGA5-A_L* in combination with *BD:AVR1-CO39* or *BD:AVR-Pia* grew on selective medium, suggesting that both Avr proteins interact physically with the C-terminal non-LRR domain of RGA5-A (Figure 5B). Yeast clones carrying *RGA5-B_L* grew only in the presence of *BD:AVR1-CO39* on selective medium but not in combination with *BD:AVR-Pia* (Figure 5B), indicating that AVR-Pia does not interact with RGA5-B.

To narrow down the interaction domain in the RGA5-A C terminus, N- and C-terminal deletions of *RGA5-A_L* were generated (Figure 5A). The N-terminal deletion construct *RGA5-A_S* (*AD:RGA5-A*$_{982-1116}$) contained the RATX1 domain and 48 additional C-terminal amino acids. The C-terminal deletion construct *RGA5_ΔC* (*AD:RGA5*$_{883-1022}$) contained the first part of the non-LRR sequence and missed the second half of the RATX1 sequence encoded by exon 4 and specific to RGA5-A. Yeast clones carrying *RGA5-A_S* in combination with *BD:AVR1-CO39* or *BD:AVR-Pia* grew on selective medium, indicating interaction between both Avr proteins and the C-terminal RGA5-A fragment containing the RATX1 domain (Figure 5B). Conversely, yeast isolates expressing *RGA5_ΔC* and *BD:AVR1-CO39* or *BD:AVR-Pia* did not grow on selective medium. Proper expression of the fusion proteins in yeast was verified by immunoblotting (see Supplemental Figure 4 online). These results suggest that the RATX1 domain and/or downstream amino acids are sufficient for RGA5-A–Avr interaction.

However, an open question remains as to how RGA5-B interacts with AVR1-CO39 because the RGA5_ΔC fragment, which is common to RGA5-A and RGA5-B, does not mediate interaction with AVR1-CO39 in the two-hybrid system and the

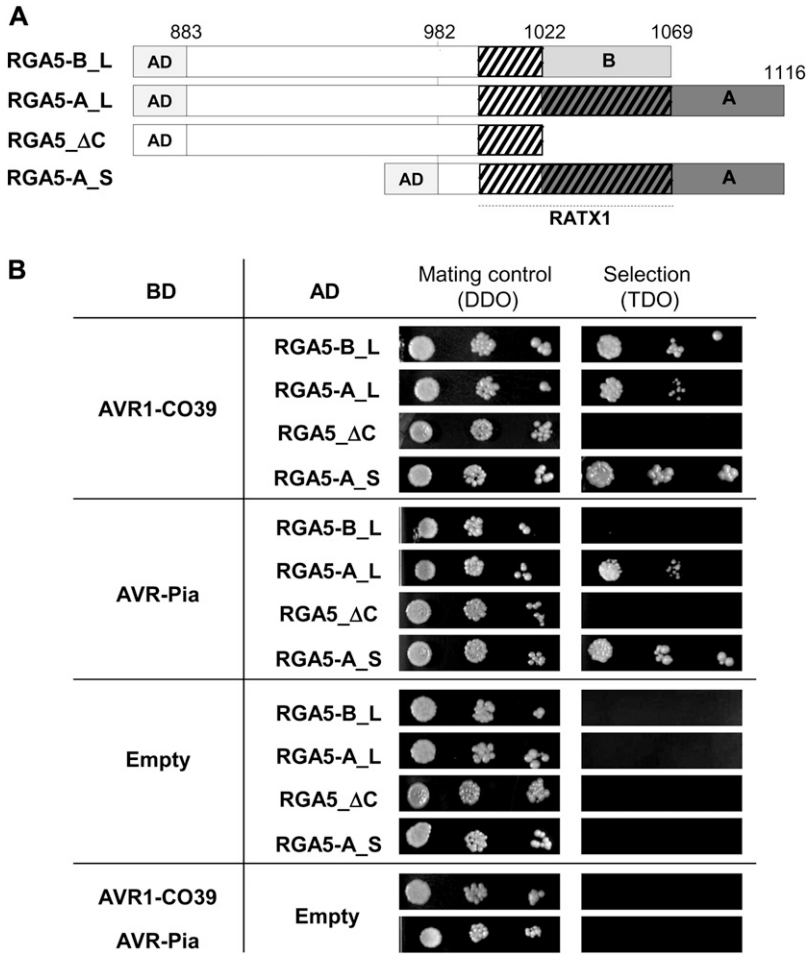

Figure 5. AVR1-CO39 and AVR-Pia Interact with the C-Terminal Non-LRR Domain of RGA5-A in Yeast Two-Hybrid Assays.

(A) RGA5-A_L and RGA5-B-L constructs carry the C-terminal non-LRR domain of RGA5-A or RGA5-B, respectively, in fusion with the Gal4 activation domain (AD:RGA5-$A_{883-1116}$ and AD:RGA5-$B_{883-1069}$). RGA5_ΔC and RGA5-A_S are C- and N-terminal deletions, respectively, of RGA5-A_L (AD: $RGA5_{883-1022}$ and AD:RGA5-$A_{982-1116}$).

(B) Interaction of AVR1-CO39 and AVR-Pia (BD:AVR1-CO39 and BD:AVR-Pia) with RGA5 constructs was assayed by a yeast two-hybrid experiment. Empty-AD and empty-BD vectors were used as controls. Cultures of diploid yeast clones were adjusted to an OD of 0.2, and three dilutions (1/10, 1/100, and 1/1000) were spotted on synthetic TDO medium (-Trp/-Leu/-His supplemented with 3-amino-1,2,4-triazole) to assay for interactions and on synthetic double drop out (DDO) medium (-Trp/-Leu) to monitor proper growth. Photos were taken after 4 d of growth.

major part of the interacting RGA5-A_S fragment is absent from RGA5-B. Also, RGA5-B–specific sequences interact with AVR1-CO39 or sequences present in RGA5_ΔC (i.e., the N-terminal half of the RATX1 domain interacts with AVR1-CO39) but are not correctly folded in the RGA5_ΔC fusion protein in yeast.

Gal4-AD fusion constructs for full-length RGA5-A, RGA5-B, and RGA4, or C-terminal fragments of RGA5-A and RGA5-B, including the LRR domain (RGA5-$A_{577-1116}$ and RGA5-$B_{577-1069}$), were also generated. However, they did not result in protein production in yeast, according to immunoblot analysis, and did not support yeast growth in the presence of *BD:AVR1-CO39* or *BD:AVR-Pia* (data not shown).

Taken together, these results suggest that AVR1-CO39 and AVR-Pia interact physically with the RGA5-A resistance protein through a small and well-defined, C-terminal, non-LRR domain consisting essentially of the RATX1 domain.

In Planta Validation of Avr–R Interaction by Coimmunoprecipitation

To investigate whether RGA5-A interacts with AVR1-CO39 or AVR-Pia in planta and to test for possible interactions between the Avr proteins and RGA4, coimmunoprecipitation experiments were performed. RGA4 and RGA5-A were fused to a C-terminal triple HA tag (RGA5-A:HA and RGA4:HA) and expressed together with cyan fluorescent protein (CFP)–tagged AVR-Pia (AVR-Pia:CFP) or AVR1-CO39 (AVR1-CO39:CFP) under the control of the 35S promoter in *Nicotiana benthamiana* leaf cells by *Agrobacterium tumefaciens*–mediated transient transformation.

As a negative control, RGA4:HA and RGA5:HA were coexpressed with green fluorescent protein (GFP). All constructs were highly expressed as shown by immunoblotting (Figure 6A), and anti-GFP antibodies immune precipitated CFP-tagged Avr effectors and GFP. RGA5 was coprecipitated with AVR-Pia and AVR1-CO39 but not with GFP (Figure 6A), while RGA4 did not coprecipitate with either the Avr proteins or GFP. In this assay, RGA5-A seems to interact better with AVR-Pia than AVR1-CO39 since stronger signals are obtained for coprecipitation of RGA5-A:HA with AVR-Pia:CFP compared with AVR1-CO39 (Figure 6A).

To validate the role of the non-LRR C-terminal domain of RGA5-A in Avr protein binding, the C-terminal RGA5-A domain used in yeast two-hybrid experiments (RGA5-$A_{883-1116}$) was fused to an N-terminal triple HA tag (HA:RGA5-A_L) and expressed together with CFP-tagged AVR-Pia (AVR-Pia:CFP) or AVR1-CO39 (AVR1-CO39:CFP) in *N. benthamiana*. To test the specificity of interactions, the *M. oryzae* Avr effector PWL2 (Sweigard et al., 1995) was used (PWL2:CFP). Immunoblotting showed high expression for all constructs (Figure 6B). Immunoprecipitation with anti-GFP antibodies resulted in enrichment of CFP-tagged Avr effectors. RGA5-A_L coprecipitated with AVR-Pia and AVR1-CO39 but not with PWL2 (Figure 6B), indicating that the RGA5-A C terminus binds AVR-Pia and AVR1-CO39 in planta in a specific manner (Figure 6B). Taken together, these results provide strong evidence for direct and specific interaction of RGA5-A with AVR-Pia and AVR1-CO39 in plant cells and for a central role of the RGA5-A C terminus in Avr binding.

FRET-FLIM Measurements Validate in Planta Interaction between AVR-Pia and RGA5-A

To further validate in planta interactions, in planta fluorescence resonance energy transfer–fluorescence lifetime imaging (FRET-FLIM) analysis was performed. AVR1-CO39 and AVR-Pia fused to CFP and RGA5-A and RGA5-B fused to yellow fluorescent protein (YFP) were expressed in *N. benthamiana* leaf cells. Immunoblot analysis with anti-GFP antibodies confirmed proper expression of the recombinant proteins (see Supplemental Figure 5 online). Confocal laser scanning microscopy of AVR1-CO39:CFP– and AVR-Pia:CFP–expressing cells detected blue fluorescent signals in both the cytoplasm and the nucleus of *N. benthamiana* epidermal cells, indicating that both fusion proteins diffuse freely between the nucleus and the cytoplasm, which is in accordance with their low molecular weight (see Supplemental Figure 6 online). Expression of YFP:RGA5-A or YFP:RGA5-B resulted in yellow fluorescent staining of the nuclei and the cytoplasm, and FRET-FLIM experiments were performed on nuclei of cotransformed cells (see Supplemental Figure 6 online). The lifetime of CFP fluorescence was significantly reduced in cells coexpressing *AVR-Pia*:*CFP* and *YFP:RGA5-A* compared with cells expressing *AVR-Pia*:*CFP* alone (Table 1). This is indicative of in planta interaction between AVR-Pia:CFP and YFP:RGA5-A. Conversely, coexpression of *AVR-Pia*:*CFP* and *YFP:RGA5-B* resulted only in a slight and nonsignificant reduction of CFP fluorescence lifetime (Table 1).

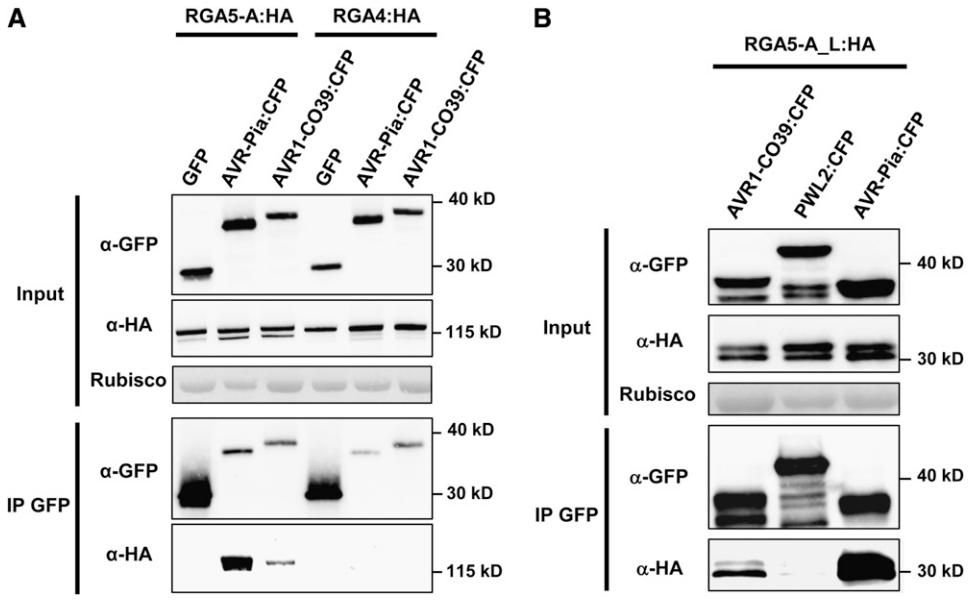

Figure 6. AVR1-CO39 and AVR-Pia Interact with the C-Terminal Non-LRR Domain of RGA5-A in Planta.

(A) RGA4:HA, RGA5:HA, AVR-Pia:CFP, AVR1-CO39:CFP, and GFP were transiently expressed in *N. benthamiana* leaves by *Agrobacterium* infiltration. Protein extracts were analyzed by immunoblotting with anti-GFP (α-GFP) and anti-HA antibodies (α-HA) (Input). In addition, immunoprecipitation was performed with anti-GFP beads (IP GFP) and analyzed by immunoblotting with anti-GFP antibodies for the detection of immunoprecipitated Avr proteins and with anti-HA antibodies for the detection of coprecipitated RGA4-A or RGA5.

(B) 3HA:RGA5-A_L (RGA5-$A_{883-1116}$), AVR-Pia:CFP, AVR1-CO39:CFP, and PWL2:CFP were transiently expressed in *N. benthamiana*, and samples were analyzed as described in **(A)**.

When the lifetime of CFP fluorescence in cells coexpressing *AVR1-CO39*:*CFP* and *YFP*:*RGA5-A* was compared with cells expressing *AVR1-CO39*:*CFP* alone, no significant reduction was detected (Table 1). However, coexpression of *AVR1-CO39*:*CFP* and *YFP*:*RGA5-B* led to a strong and significant reduction in CFP fluorescence lifetime. Taken together, these results support the in planta interaction between AVR-Pia and RGA5-A and between AVR1-CO39 and RGA5-B. Interaction between RGA5-A and AVR1-CO39 was not detected by FRET-FLIM experiments, which may be due to weaker interaction of RGA5-A with AVR1-CO39 than with AVR-Pia in the *N. benthamiana* system, which was already concluded from coimmunoprecipitation experiments.

Natural Polymorphisms in AVR-Pia Impair Interaction with RGA5-A and Recognition by *Pia*

We next exploited the natural genetic diversity of *AVR-Pia* to investigate whether physical Avr–RGA5-A interaction is required for Avr recognition and activation of ETI. A core collection of 50 rice-infecting *M. oryzae* isolates, representative of the worldwide genetic diversity of the rice blast fungus (Tharreau et al., 2009), was inoculated on the rice variety Aichi Asahi, diagnostic for *Pia* resistance, to determine if they possess a functional *AVR-Pia* allele (see Supplemental Table 2 online). Only four strains were avirulent on Aichi Asahi, indicating that AVR-Pia–mediated avirulence is rare. In addition, all 50 strains were analyzed for the presence of the *AVR-Pia* gene by PCR analysis with *AVR-Pia*–specific primers and sequencing of PCR products in order to detect new *AVR-Pia* alleles. In all four avirulent strains, *AVR-Pia*–specific primers amplified a specific band of the expected size, and sequencing of this DNA fragment showed 100% identity with the previously published *AVR-Pia* sequence (see Supplemental Table 2 online). Forty-three strains were virulent on Aichi Asahi and did not amplify the *AVR-Pia* gene, suggesting that *AVR-Pia* is deleted in these strains. Three strains, IN17, IN58, and IN73, were virulent on Aichi Asahi but gave a fragment of the expected size in PCR analysis (see Supplemental Table 2 online). Sequencing of the fragments revealed that in all three strains, *AVR-Pia* contains the same two nonsynonymous polymorphisms leading to changes of amino acids 24 and 46 (F24S and T46N) (Figure 7A). This apparently inactive *AVR-Pia* allele was named *AVR-Pia-H3*, while the active wild-type allele was named *AVR-Pia-H0*.

To rule out the possibility that loss of recognition of *AVR-Pia-H3* by *Pia* is due to a lack of expression, *AVR-Pia-H3* expression during rice infection was analyzed by quantitative RT-PCR in the three *AVR-Pia-H3* isolates, IN17, IN58, and IN73, and compared with expression in the strain FR13 carrying the active *AVR-Pia-H0* allele. As a negative control, the strain Guy11, which does not contain *AVR-Pia*, was used. *AVR-Pia-H0* and *AVR-Pia-H3* alleles proved to be expressed during the early biotrophic phase of infection, which lasts 3 d, but in the Guy11 strain, no *AVR-Pia* transcripts were detected (see Supplemental Figure 7 online). This indicates that lack of activity of *AVR-Pia-H3* is due to a lack of recognition by *Pia*.

To investigate whether the polymorphisms in AVR-Pia-H3 affect the direct interaction with RGA5-A, yeast two-hybrid experiments were performed. A *BD*:*AVR-Pia-H3* fusion construct (*BD*:*AVR-Pia-H3*$_{20-85}$) carrying the F24S and T46N substitutions and a construct carrying only the F24S substitution (*BD*:*AVR-Pia-H3.1*) were generated and compared with *BD*:*AVR-Pia*. Immunoblotting showed that all AVR-Pia fusion proteins are expressed properly and to comparable levels (see Supplemental Figure 4 online). In contrast with *BD*:*AVR-Pia*, the *BD*:*AVR-Pia-H3* and *BD*:*AVR-Pia-H3.1* constructs did not confer growth on selective medium to yeast strains carrying *RGA5-A_L*, indicating that AVR-Pia-H3 does not interact with the C terminus of RGA5-A (Figure 7B) and that the F24S substitution alone is sufficient to abolish AVR-Pia/RGA5-A interaction. Taken together, these results support that physical binding of AVR-Pia to RGA5-A is required for AVR-Pia recognition and *Pia*-mediated resistance.

The C-Terminal RATX1 Domain of RGA5-A Is Shared with the Pik-1 Resistance Protein and Is Involved in Specific Avr Protein Binding

BLAST searches with the minimal AVR-Pia- and AVR1-CO39 binding domain of RGA5-A (RGA5-A$_{994-1116}$) identified homologies with the rice blast R protein Pik-1 (Zhai et al., 2011) and revealed that Pik-1 contains a previously undescribed RATX1

Table 1. FRET-FLIM Measurements in *N. benthamiana* Show That AVR-Pia and AVR1-CO39 Interact with RGA5-A and RGA5-B, Respectively

Donor	Acceptor	τ[a]	SE	Δ[b]	n[c]	FRET %[d]	P Value[e]
AVR1-CO39:CFP	no acceptor	2.837	0.039	–	72	–	–
AVR1-CO39:CFP	YFP:RGA5-A	2.707	0.051	0.130	20	3	0.21
AVR1-CO39:CFP	YFP:RGA5-B	2.223	0.053	0.614	33	21.7	3×10^{-14}
AVR-Pia:CFP	no acceptor	2.523	0.041	–	63	–	–
AVR-Pia:CFP	YFP:RGA5-A	2.129	0.042	0.394	29	15.5	1.2×10^{-9}
AVR-Pia:CFP	YFP:RGA5-B	2.381	0.040	0.142	20	6	0.015

[a]Mean lifetime (in nanoseconds). For each nucleus, average fluorescence decay profiles were plotted and fitted with exponential function using a nonlinear square estimation procedure, and the mean lifetime was calculated according to $\tau = \sum \alpha_i\tau_i^2/\sum \alpha_i\tau_i$ with $I(t) = \sum\alpha_i e^{-t/\tau i}$.

[b]$\Delta t = \tau_D - \tau_{DA}$ (in nanoseconds).

[c]Total number of measured nuclei.

[d]Percentage of FRET efficiency: $E = 1 - (\tau_{DA}/\tau_D)$.

[e]P value of the difference between the donor lifetimes in the presence and absence of acceptor (Student's *t* test). –, treated.

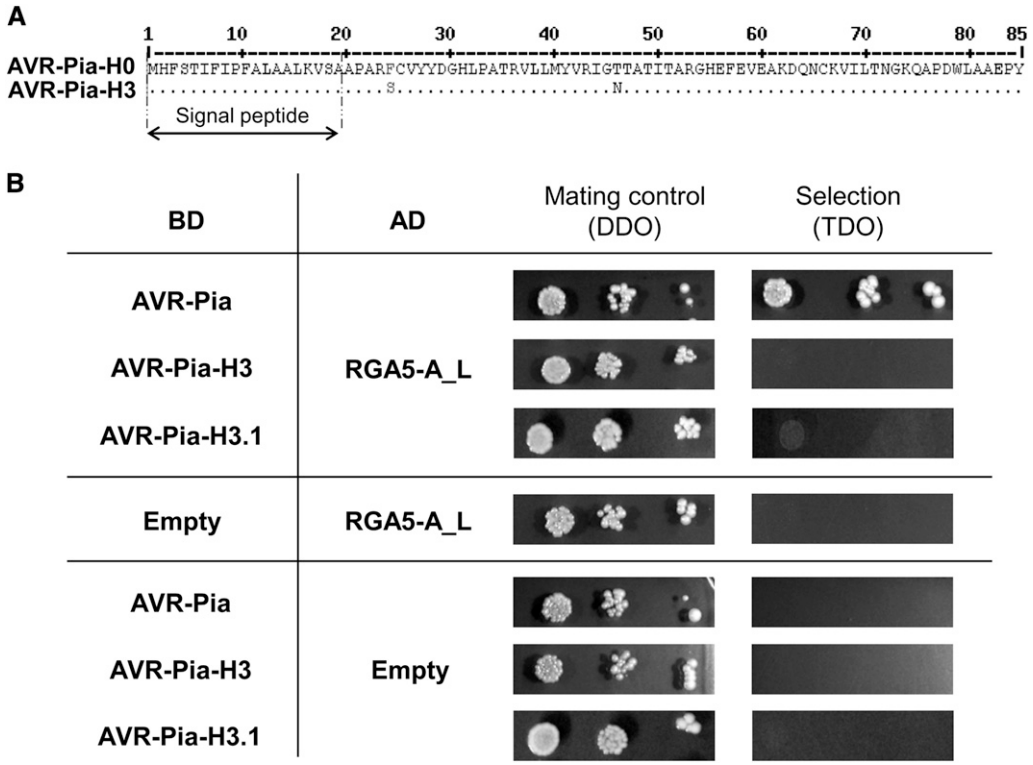

Figure 7. AVR-Pia-H3 Does Not Interact with RGA5-A.

(A) The H3 allele of *AVR-Pia* harbors two nonsynonymous polymorphisms generating the amino acid changes F24S and T46N.
(B) Interaction of RGA5-A_L (AD:RGA5-$A_{883-1116}$) with AVR-Pia (BD:AVR-Pia), AVR-Pia-H3 (F24S, T46N) (BD:AVR-Pia-H3), and AVR-Pia-H3.1 (F24S) (BD:AVR-Pia-H3.1) was assayed by a yeast two-hybrid experiment. Empty-AD and empty-BD vectors were used as controls. All interactions were assayed on TDO medium (-Trp/-Leu/-His) in three independent experiments, which gave identical results.

domain with homology to RGA5-A (68% similarity and 54% identity) (Figure 8A). In contrast with RGA5-A, this domain is located in the Pik-1 N terminus (Pik-$1_{191-264}$) between the CC and NB domains. As in RGA5-A, the Pik-1 RATX1 domain seems to mediate Avr recognition (Kanzaki et al., 2012) since it constitutes, together with the CC-domain, the minimal Pik-1 fragment necessary and sufficient for AVR-Pik binding. In addition, specificity of the *Pik* alleles *Pik*, *Pikp*, and *Pikm* for the *AVR-Pik* alleles *A*, *C*, *D*, and *E* and specificity in binding of Pik-1, Pikp-1, and Pikm-1 to the products of the different AVR-Pik alleles can be correlated to individual polymorphic amino acids within the RATX1 domain (Kanzaki et al., 2012). It therefore appears that RGA5-A and Pik-1 interact with their cognate Avr proteins through homologous RATX1 domains.

To assess the binding specificity of these homologous RATX1 domains, the pairwise interactions between, on the one side, the RGA5-A C terminus and the Pik-1 N terminus and, on the other side, AVR-Pik, AVR-Pia, and AVR1-CO39 were tested in the yeast two-hybrid system (Figure 8B). BD fusions of AVR-Pia, AVR-Pik, or AVR1-CO39 were coexpressed with AD fusions of RGA5-A_L or the Pik-1 N terminus (Pik-1 N-ter), including the CC and RATX1 domains (Okuyama et al., 2011), and AD fusions of AVR-Pia or AVR-Pik were coexpressed with BD fusions of RGA5-A_L and Pik-1 N-ter. Yeast carrying RGA5-A_L in combination with AVR-Pik and yeast carrying Pik-1 N-ter in combination with AVR-Pia or AVR1-CO39 showed no growth on selective medium (Figure 8B). Only in the case of BD-AVR-Pik combined with AD-RGA5-A_L was weak growth observed. By contrast, combinations of RGA5-A_L with AVR-Pia or AVR1-CO39 and expression of Pik-1 N-ter with AVR-Pik conferred strong growth (Figure 8B). This indicates high specificity in the binding of RGA5-A and Pik-1 RATX1 domains to their corresponding Avr proteins. In the future, detailed structure-function analysis should elucidate the structural and mechanistic basis of RATX1 domain binding and recognition specificities.

Phylogenetic Analysis Indicates That RGA5-A and Pik-1 Acquired the RATX1 Domain Independently

Whole-genome analysis indicates that the RATX1 domain occurs only in one additional rice NB-LRR protein, present in the rice varieties 93-11 and Nipponbare (accession number NP_001062892.2). This NB-LRR protein has no known recognition specificity and was previously named *pi5-3* because it is located at the *Pi5* locus of varieties without *Pi5* resistance (Lee et al., 2009). In this protein, the RATX1 domain is located in the C terminus as in RGA5-A. In addition, a RATX1 domain is present in the product of the recessive quantitative rice blast

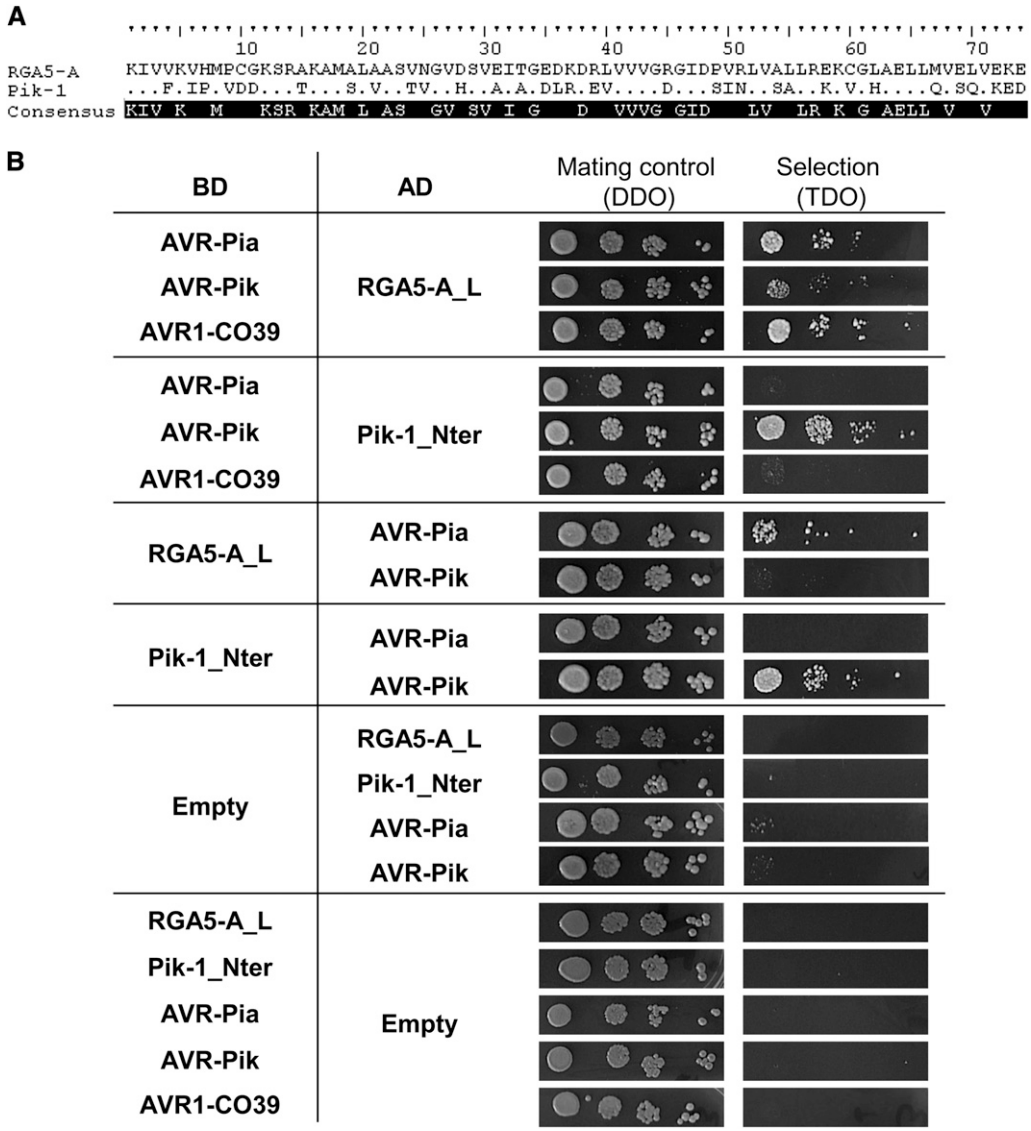

Figure 8. RGA5 and the Unrelated Pik-1 Resistance Protein Possess Homologous RATX1 Domains Involved in Specific Avr Protein Binding.

(A) Alignment of RGA5-A and Pik-1 RATX1 domain sequences.

(B) Interaction of AVR-Pia, AVR-Pik, and AVR1-CO39 (BD:AVR-Pia, BD:AVR-Pik, BD:AVR1-CO39, AD:AVR-Pia, and AD:AVR-Pik) with RGA5 C-terminal non-LRR sequences (BD:RGA5-A_L and AD:RGA5-A_L) or Pik-1 N-terminal sequences (BD:Pik-1_Nter and AD:Pik-1_Nter) was assayed by a yeast two-hybrid experiment. Empty-AD and empty-BD vectors were used as controls. Cultures of diploid yeast clones were adjusted to an OD of 2, and four dilutions (1/10, 1/100, 1/1000, and 1/10,000) were spotted on synthetic TDO medium (-Trp/-Leu/-His supplemented with 3-amino-1,2,4-triazole) to assay for interactions and on synthetic double dropout (DDO) medium (-Trp/-Leu) to monitor proper growth. Photos were taken after 4 d of growth.

resistance gene *pi21*, which is not an NB-LRR protein (Fukuoka et al., 2009). The pi21 RATX1 domain is only distantly related to the one of RGA5-A and Pik-1 (see Supplemental Figure 8 online).

To assess whether the RATX1 domains in rice CC-NB-LRR proteins have a common origin or were acquired independently, these domains were aligned with the 11 closest RATX1 proteins from the rice cultivar Nipponbare (Panther domain family PTHR22814, subfamily 32) and one rice RATX1 protein that belongs to a distinct subfamily and serves as an outgroup (Panther domain family PTHR22814, subfamily 15) (see Supplemental Figure 9 online). The neighbor-joining distance phylogeny inferred from this alignment (Figure 9) showed that the RGA5-A RATX1 domain clusters in a well-supported monophyletic clade (node support = 80) with the RATX1 proteins encoded by *Os04g39010* and a gene cluster on chromosome 4 comprising four genes (*Os04g39350*, *Os04g39360*, *Os04g39370*, and *Os04g39380*). The same result was obtained when the phylogenies were reconstructed by parsimony or maximum likelihood (data not shown). In the neighbor-joining tree (Figure 9),

the Pik-1 RATX1 domain branched with the same clade, although with less strong support (node support = 59). This branching was also found with the maximum likelihood method but not with the parsimony method (data not shown). This suggests that the Pik-1 RATX1 domain originated from the same common ancestor as the RGA5-A domain and the chromosome 4 cluster genes. It appears that the common ancestral RATX1 protein has been acquired first by the Pik-1 protein in its N terminus and later by the RGA5-A protein in its C terminus to serve as an Avr recognition domain. In parallel, it underwent duplications and diversification leading to the formation of the four clustered RATX1 genes on chromosome 4. However, it cannot be ruled out that the Pik-1 RATX1 domain has been acquired from a close homolog of the ancestor of the RGA5-A RATX1 domain that has since been lost. Further phylogenetic analyses with sequences from additional grasses and wild rice species are necessary to test these hypotheses. The pi5-3 RATX1 domain displays homology to a more distantly related RATX1 protein and clearly has another ancestral origin (Figure 9; see Supplemental Figures 8 and 9 online).

DISCUSSION

Cloning of *Pi-CO39*, Conferring Resistance to *AVR1-CO39* and *AVR-Pia*

The rice *Pi-CO39* locus, conferring resistance to *M. oryzae* strains expressing the avirulence gene *AVR1-CO39*, had previously been mapped to the short arm of rice chromosome 11 in the resistant variety CO39 (Chauhan et al., 2002). The locus was restricted to one BAC clone containing a cluster of NB-LRR protein-coding candidate genes (Leong et al., 2004). In this study, we show that the two NB-LRR–coding genes, *RGA4* and *RGA5*, located within the gene cluster, are necessary and sufficient to confer *Pi-CO39* resistance. *RGA5* was identified in a yeast two-hybrid screen for physical interactors of AVR1-CO39, and the role of both *RGA4* and *RGA5* was confirmed genetically. The identification of a plant resistance gene by yeast two-hybrid screening using an avirulence protein as bait seems therefore, in certain cases, to be a good alternative to map-based cloning.

Interestingly, *RGA4* and *RGA5* also confer resistance against *M. oryzae* isolates expressing the avirulence gene *AVR-Pia*, which shows no sequence similarity to *AVR1-CO39* (Yoshida et al., 2009; Okuyama et al., 2011). Therefore, *RGA4* and *RGA5* together constitute the genetically defined *Pia* and *Pi-CO39* resistance genes. Accordingly, perfect association between *Pia* and *Pi-CO39* resistance was observed when a collection of rice cultivars was analyzed for resistance to *M. oryzae* strains carrying either *AVR-Pia* or *AVR1-CO39*. Hence, our study demonstrates that the pair of CC-NB-LRR proteins RGA4 and RGA5 possesses a dual Avr recognition specificity. Such dual specificity for a pair of NB-LRR proteins had previously been demonstrated for RPS4 and RRS1, a TIR-NB-LRR pair that is required to recognize the *P. syringae* effector AvrRps4, the *Ralstonia solanacearum* effector PopP2, and a still uncharacterized factor produced by *Colletotrichum higginsianum* (Gassmann et al., 1999; Deslandes et al., 2002; Birker et al., 2009; Narusaka et al., 2009). The present work provides therefore an example of dual recognition mediated by a pair of distinct CC-NB-LRR proteins.

The Relevance of Alternative Splicing of CC-NB-LRR Coding Genes Is Unknown

Alternative splicing is a frequent phenomenon in plants; however, its functional relevance is largely unknown (Filichkin et al., 2010; Lu et al., 2010, Severing et al., 2011). Different studies have highlighted the importance of alternative splicing in TIR-NB-LRR R protein-coding genes. For instance, analyses of the tobacco *N* and the *Arabidopsis RPS4* genes showed that intron-deprived genes (genomic construct with all introns removed) have no or reduced activity (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003). For genes encoding proteins of the CC-NB-LRR class, alternative splicing has also been documented, but its functional relevance has not been investigated.

In this study, we identified two *RGA5* transcript variants: *RGA5*-A and *RGA5-B*. Intron retention in *RGA5-B* leads to a frame shift and results in different C-terminal amino acid sequences. *RGA5-A* confers resistance to AVR1-CO39 and AVR-Pia, while *RGA5-B* is neither necessary nor sufficient for resistance. This is striking and not understood, since RGA5-B interacts both in planta and in yeast with AVR1-CO39. It is possible that RGA5-A–specific C-terminal sequences are involved in additional intra- and intermolecular interactions and that they are necessary for the overall activity of the RGA5 protein or involved in the activation of downstream responses. Hence, the potential function of alternative splicing in CC-NB-LRR proteins remains to be elucidated.

Recognition of AVR1-CO39 and AVR-Pia through Direct Binding to RGA5-A

In recent years, an increasing number of mutually matching R and Avr proteins from agronomically relevant or naturally occurring pathosystems have been cloned. These studies revealed that, particularly, in fungal and oomycete pathosystems, recognition by direct interaction is much more frequent than previously postulated. In all cases where the recognition of flax-rust and rice blast Avr proteins has been studied in detail, strong evidence indicates recognition by direct binding. Indeed, the fungal Avr proteins AvrM and AvrL567 from *Melampsora lini* have been shown to be directly recognized by the flax TIR-NB-LRR R proteins M and L5 or L6 (Dodds et al., 2006; Ellis et al., 2007; Catanzariti et al., 2010). Likewise, the *M. oryzae* effectors AVR-Pita and AVR-Pik/km/mp are perceived through direct interaction with their specific CC-NB-LRR R proteins (Jia et al., 2000; Kanzaki et al., 2012). Conversely, indirect recognition of fungal Avr proteins by NB-LRR proteins has not been described yet.

In our study, we add an example to this emerging picture. The ETI elicitors AVR1-CO39 and AVR-Pia bind to a defined small, C-terminal, non-LRR domain of the CC-NB-LRR resistance protein RGA5-A in the yeast two-hybrid system and in planta, as demonstrated by coimmunoprecipitation experiments. Binding to the entire RGA5-A protein was shown *in* planta by

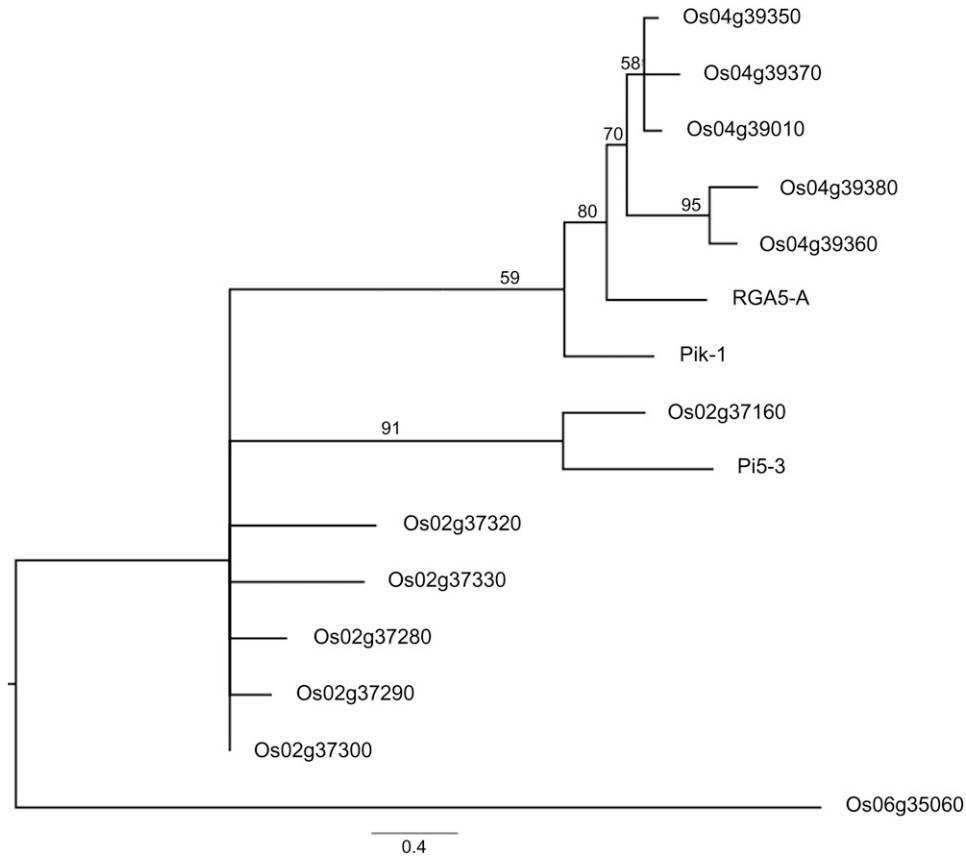

Figure 9. Phylogenetic Analysis Indicates That Independent Events Lead to the Integration of the RATX1 Domain into RGA5-A and Pik-1.

Phylogenetic relationship of the RATX1 domains of RGA5-A, Pik-1, Pikp-1, pi5-3, and closely related homologous RATX1 proteins from rice, reconstructed using the neighbor-joining distance method based on the alignment shown in Supplemental Figure 9 and Supplemental Data Set 1 online. Node supports are given in percentage of 1000 bootstrap replicates. The topology shows the condense consensus tree of the 1000 bootstrap replicates, with nodes with a bootstrap support <50% being collapsed. Branch lengths are proportional to phylogenetic distances estimated from the JTT + G amino acid substitution model.

coimmunoprecipitation and FRET-FLIM analysis. It is worth noting that several techniques were used to provide a convincing demonstration, as each method is associated with specific limitations. In the case of AVR-Pia, the inactive allele AVR-Pia-H3 does not confer avirulence and does not interact with the RGA5-A interaction domain, supporting the view that direct binding is necessary for ETI activation.

RGA5-A Possesses a Novel Avr Recognition Domain Also Present in Pik-1

The structural details underlying the direct binding of Avr proteins to R proteins are largely unknown because data on direct Avr–R interactions are often incomplete and indirect, and only in rare cases could the interaction domain be identified. The LRR domain is in many cases the domain conferring specificity to NB-LRR proteins, and for the R proteins L5, L6, Pi-ta, and RPP1, it was demonstrated to be the key receptor domain mediating direct Avr binding (Ellis et al., 1999, 2007; Jia et al., 2000; Wang et al., 2007; Krasileva et al., 2010). Recently, two studies described examples where N-terminal non-LRR domains of NB-LRR proteins confer Avr recognition specificity and may confer direct binding, indicating that the LRR domain is not necessarily the Avr receptor domain (Chen et al., 2012; Kanzaki et al., 2012). The *Phytophthora infestans* IPI-O1 effector associates in planta with the CC domain of the RB R protein of *Solanum bulbocastanum*, also known as Rpi-blb1, and elicits resistance (Chen et al., 2012). The effector IPI-O4 blocks recognition of IPI-O1 by binding to the same RB CC domain, leading to inactivation of RB-mediated programmed cell death (Chen et al., 2012). In rice, AVR-Pik interacts specifically with N-terminal sequences of Pik-1, comprising the CC domain and additional sequences located before the NB domain. Remarkably, this direct binding, observed both in yeast two-hybrid and coimmunoprecipitation assays, determines recognition specificities for the multiples alleles of *AVR-Pik* and *Pik-1* (Kanzaki et al., 2012).

In this study, we identified a non-LRR sequence in RGA5-A, interacting physically with AVR1-CO39 and AVR-Pia and acting as an Avr receptor domain. In contrast with the previously cited examples, the RGA5-A interaction domain is located in the very

C terminus of the protein, downstream of the LRR domain. This receptor domain is related to the copper binding proteins ATX1 from *S. cerevisiae* (Lin and Culotta, 1995) and ATOX1 in humans (Klomp et al., 1997). Most eukaryotes have only one ATX1-related protein that is involved in copper homeostasis. In higher plants, however, RATX1 proteins have proliferated (69 in *Arabidopsis thaliana* and 61 in rice according to Panther database (www.pantherdb.org/panther/family.do?clsAccession=PTHR22814) and the corresponding genes are organized in clusters in plant genomes. Like ATX1, they generally do not contain additional domains and are small proteins of 100 to 130 amino acids. Two RATX1 proteins of *Arabidopsis* have been investigated for their role in copper homeostasis (Shin et al., 2012), but for the majority of plant RATX1 proteins no function is known. In many cases, one of the two Cys residues mediating copper binding in ATX1 is not conserved, suggesting they may be involved in other functions than metal homeostasis. Some of them could have a role in immunity, such as the quantitative rice blast resistance protein pi21, which contains a RATX1 domain (Fukuoka et al., 2009).

Phylogenetic analysis suggests that a common RATX1 protein has been independently recruited in the N terminus of Pik-1 and in the C terminus of RGA5-A, allowing the recognition of at least three sequence-unrelated Avr proteins. Interestingly, the insertion of the RATX1 domain in NB-LRR proteins seems to be rare and restricted to monocots. In the future, it will be interesting to further characterize this domain to better understand the specificity of Avr recognition and its link to resistance activation. In addition, it will be interesting to investigate if other uncommon domains integrated into NB-LRR resistance proteins are involved in Avr recognition.

RGA4 and RGA5 Interact Functionally to Recognize Two Sequence-Unrelated Effectors

The gene-for-gene hypothesis states that a single plant *R* gene product recognizes a unique avirulence protein (Flor, 1971). However, numerous examples illustrate a higher level of complexity in pathogen recognition by R proteins. For instance, an increasing number of resistances mediated by pairs of distinct NB-LRR proteins have been described. Pairs of resistance proteins can be formed by two TIR-NB-LRR proteins, as in the case of RPS4 and RRS1 mediating multiple resistances in *Arabidopsis* (Gassmann et al., 1999; Deslandes et al., 2002; Birker et al., 2009; Narusaka et al., 2009) or by two CC-NB-LRR proteins, such as Lr10 and RGA2, mediating resistance against wheat leaf rust caused by *Puccinia triticina* (Loutre et al., 2009). The functional interaction of one CC-NB-LRR and one TIR-NB-LRR protein has also been described in the case of RPM1 and TAO1 (Eitas et al., 2008). However, RPM1 and TAO1 are genetically not linked, and RPM1 can still recognize AvrB in the absence of TAO1, suggesting that the interaction of these two NB-LRR proteins is mechanistically different from the previously described cases.

Functional couples of R proteins frequently have been described in the rice–*M. oryzae* pathosystem where the *Pik/km/kp* (Ashikawa et al., 2008; Yuan et al., 2011; Zhai et al., 2011), *Pi-5* (Lee et al., 2009), and *Pia* (Okuyama et al., 2011) resistance loci all comprise two complementary CC-NB-LRR coding genes. Remarkably, in all cases, both R genes display physical linkage and are located next to each other in opposite directions. Such conserved features strongly suggest a common evolutionary mechanism for these resistance genes.

An unanswered key question is why in certain cases two distinct NB-LRR proteins are required for Avr-triggered resistance. As each NB-LRR protein fails to confer resistance on its own, it can be assumed that the functions normally executed by individual R proteins are performed by the cooperative action of R protein pairs. Alternatively, it may be that each protein of the pair accomplishes by its own a subset of the functions normally executed by one individual NB-LRR protein. Our study suggests that RGA5-A recognizes AVR-Pia and AVR1-CO39 by direct binding and independently of RGA4. It could therefore be that RGA5-A acts as an Avr receptor with dual recognition specificity and that RGA4 is involved in downstream activities, such as the activation of resistance signaling. How this interaction between RGA4 and RGA5-A takes place at the molecular level is unknown, but the previously described dimerization of NB-LRR proteins via their N-terminal TIR or CC domains could play an important role (Bernoux et al., 2011; Maekawa et al., 2011). Binding of Avrs to RGA5-A could lead to the formation of signaling competent NB-LRR complexes formed of RGA4 and/or RGA5-A. Therefore, the investigation of RGA4 and RGA5-A protein–protein interactions promises to give in the future new and important insights into NB-LRR protein function.

METHODS

Growth of Plants and Fungi and Infection Assays

Rice plants (*Oryza sativa*) were grown as described (Faivre-Rampant et al., 2008). *Nicotiana benthamiana* plants were grown in a growth chamber at 22°C with a 16-h light period. The *rga4* mutant lines Sas1493 and Sas2127 as well as transgenic rice lines of cultivar Kanto51 carrying *pRGA5:RGA5*, *pRGA4:RGA4*, or *pRGA4:SasRGA4* + *pRGA5:RGA5* were described elsewhere (Okuyama et al., 2011).

Magnaporthe oryzae isolates and transgenic strains Guy11-*AVR1-CO39* and Guy11-*EV* (Ribot et al., 2013) were grown on rice flour agar for spore production (Berruyer et al., 2003). For the determination of interaction phenotypes, a suspension of fungal conidiospores (5×10^4 spores·mL^{-1}) was spray-inoculated on the leaves of 3-week-old plants (Berruyer et al., 2003). For gene expression analysis, an inoculum of 2×10^6 spores·mL^{-1} was used.

Constructs

All PCR products used for cloning were generated using Phusion High-Fidelity DNA polymerase (Finnzymes). Plasmids, PCR primers, and PCR experiments used in this study are listed in Supplemental Tables 3 to 5 online.

For yeast two-hybrid assays, the AVR1-CO3922-89 PCR product (see Supplemental Table 3 online) was cloned into pGBKT7-BD (Clontech) using *Bam*HI and *Eco*RI restriction sites. The AVR-Pia20-85 PCR product was cloned in pGBKT7-BD using *Nde*I and *Sal*I restriction sites to generate the BD:AVR-Pia bait construct. The BD:AVR-Pia-H3 and BD:AVR-Pia-H3.1 constructs were obtained from BD:AVR-Pia20-85 by site-directed mutagenesis using the Quikchange Lightening site-directed mutagenesis kit (Stratagene). RGA5-A883-1116 and RGA5-B883-1069 PCR products were cloned in pGADT7-AD using *Nde*I and *Xho*I restriction

sites to create AD:RGA5-B_L and AD:RGA5-A_L prey constructs. N-terminal and C-terminal deletions were performed in the AD:RGA5-A_L construct using the Quikchange Lightning site-directed mutagenesis kit to create AD:RGA5-A_S and AD:RGA5-A_ΔC constructs.

For stable rice transformation or transient expression in *N. benthamiana*, genomic and cDNA constructs were created for RGA5-A and RGA5-B. To generate pRGA5:RGA5-A and pRGA5:RGA5-B genomic constructs, site-directed mutagenesis was performed from the pAHC17.pRGA5:RGA5 genomic construct (Okuyama et al., 2011). To create pAHC17.pRGA5:RGA5-A, the Δ4288-4366 nucleotide deletion, corresponding to RGA5 third intron, was performed. For pAHC17.pRGA5:RGA5-B, the T4289C point mutation in the third exon donor splice site was introduced to prevent splicing of the third intron and create the RGA5-B genomic construct. To create Kanto51 transgenic rice lines expressing pRGA5:RGA5-A, pRGA5:RGA5-A, or pRGA5:RGA5-B, the previously described pAHC17 vectors carrying either pRGA5:RGA5 (Okuyama et al., 2011), pRGA5:RGA5-A, or pRGA5:RGA5-B were digested using *Hin*dIII and *Bam*HI restriction enzymes to release pRGA5:RGA5, pRGA5:RGA5-A, and pRGA5:RGA5-B, respectively. The resulting fragments were cloned in pCambia2300 (Cambia) by ligation using the same restriction sites, creating the three binary vectors pCambia.pRGA5:RGA5, pCambia.pRGA5:RGA5-A, and pCambia.pRGA5:RGA5-B.

To create the RGA5-B full-length cDNA PCR product, a two-step PCR approach was used. The pAHC17.pUBI:RGA5 construct carrying RGA5 cDNA sequence (Okuyama et al., 2011) and the pAHC17.pRGA5:RGA5-B construct were used as PCR templates. Briefly, RGA5 5′ cDNA sequence was amplified from pAHC17.pUBI:RGA5, and specific RGA5-B 3′ sequence (harboring the T4289C point mutation) was amplified from pAHC17.pRGA5:RGA5-B. The two PCR products contain overlapping sequences. Both PCR products were mixed in a 1:1 ratio and incubated for 5 min with DNA polymerase before PCR was performed to generate the full-length RGA5-B artificial cDNA PCR product subsequently used for gateway cloning.

For FRET-FLIM and coimmunoprecipitation experiments, all plasmids were generated by Gateway technology (Invitrogen) following the instructions of the manufacturer. AVR1-CO3922-89, AVR-Pia20-85, PWL222-145, RGA5-A (cDNA), RGA5-B (cDNA), RGA5-A883-1116, and RGA5-B883-1069 PCR products flanked by attB sites were recombined into the pDONR207 vector (Invitrogen) via a BP reaction to create corresponding entry clones. Appropriate LR reactions were performed to generate AVR1-CO39:CFP, AVR-Pia:CFP, PWL2:CFP, YFP:RGA5-A, YFP:RGA5-B, 3HA:RGA5-A_L, and 3HA:RGA5-B_L constructs using pBIN19-35S:GTW:CFP, pBIN19-35S:YFP:GTW, or pBIN19-35S:3HA:GTW binary vectors.

Yeast Two-Hybrid Library Screening and Interaction Analysis

Total RNA from leaves of 3-week-old plants of the rice cultivar CO39 was extracted, and mRNAs were purified using the NucleoTrap mRNA purification kit (Macherey-Nagel). The CO39 yeast two-hybrid library was built in the pGADT7-Rec vector (Clontech) using the Make Your Own "mate and plate" library system (Clontech) protocol and transformed in the yeast strain Y187 (Clontech). Library screening was performed according to the Matchmaker Gold yeast two-hybrid system protocol (Clontech). Briefly, after mating between the Gold strain transformed with *BD:AVR1-CO39* and the Y187 CO39 library, diploid yeasts were plated on triple dropout synthetic selective medium lacking Trp, Leu, and His and supplemented with 3-amino-1,2,4-triazole. Colonies that grew on this medium were transferred to selection medium to confirm growth. Plasmids were extracted, amplified in bacteria, and transformed back into Y187 to validate the interaction with BD:AVR1-CO39 and exclude interaction with the BD domain alone. Validated plasmids were sequenced, and BLAST was used to compare the inserts nucleotide sequences to the rice genome of both Nipponbare and 93-11 to identify corresponding genes.

For directed yeast two-hybrid tests, all BD or AD constructs were transformed into Gold or Y187 yeast strain, respectively (Clontech). Interaction tests were performed according to the Matchmaker Gold yeast two-hybrid system protocol (Clontech). Briefly, after mating, diploid yeasts were plated on synthetic DDO (mating control) and TDO stringent medium (supplemented with various concentrations of 3-amino-1,2,4-triazole) and incubated at 28°C for 5 d. Growth of all diploid yeasts carrying both pGADT7-AD and pGBKT7-BD transformed vectors on DDO was examined. Interaction was considered relevant when diploid yeasts were able to grow on both DDO and stringent TDO medium, while corresponding controls (AD or BD empty vectors) could not.

Transgenic Rice Lines

pRGA5:RGA5, *pRGA5:RGA5-A*, *pRGA5:RGA5-B*, and *pUBI:GFP* were used for *Agrobacterium tumefaciens*–mediated transformation (strain EH1) (Toki et al., 2006) of transgenic Kanto51 carrying *RGA4* (Okuyama et al., 2011) or wild-type Kanto51 rice lines. Infected calli were selected on medium containing 50 mg·L^{-1} geneticin. Geneticin-resistant calli were transferred to regeneration medium. At least 20 independent transgenic lines were obtained for each construct in each transformation experiment. T0 generation plants were used for the evaluation of rice blast resistance 3 weeks after transfer to soil. For this, regenerated plants with at least three tillers were split in three plantlets, replanted in soil in independent pots, and grown in soil for 3 weeks before inoculation by *M. oryzae*. The presence of the transgenes in the T0 generation plants was checked by PCR with primer pairs listed in Supplemental Table 4 online.

Transient Protein Expression in *N. benthamiana*

For *Agrobacterium*-mediated *N. benthamiana* leaf transformations, transformed *GV3101 pMP90* strains were grown in Luria-Bertani liquid medium containing 50 mg mL^{-1} rifampicin, 15 mg mL^{-1} gentamycin, and 25 mg mL^{-1} kanamycin at 28°C for 24 h before use. Bacteria were harvested by centrifugation, resuspended in infiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 mM acetosyringone) to an OD_{600} of 1, and incubated for 2 h at room temperature before leaf infiltration. The infiltrated plants were incubated for 36 or 48 h in growth chambers under controlled conditions for FRET-FLIM or coimmunoprecipitation experiments, respectively.

Confocal Microscopy and Fluorescence Lifetime Microscopy

Fluorescence confocal laser scanning microscopy was performed as described (Tasset et al., 2010). Fluorescence lifetime of the donor was measured in the presence and absence of the acceptor. FRET efficiency (E) was calculated by comparing the lifetime of the donor in the presence (τ_{DA}) or absence (τ_D) of the acceptor: $E = 1 - (\tau_{DA})/(\tau_D)$. Statistical comparisons between control (donor) and assay (donor + acceptor) lifetime values were performed by Student's *t* test. FRET-FLIM measurements were performed using a FLIM system coupled to a streak camera (Krishnan et al., 2003). The light source (l = 439 nm) was a pulsed diode laser working at 2 MHz (Hamamatsu Photonics). All images were acquired with a ×60 oil immersion lens (Plan Apo 1.4–numerical aperture, IR) mounted on an inverted microscope (Eclipse TE2000E; Nikon) coupled to the FLIM system. The fluorescence emission was directed back out into the detection unit through a band-pass filter. The FLIM unit was composed of a streak camera (Streakscope C4334; Hamamatsu Photonics) coupled to a fast and high-sensitivity charge-coupled device camera (model C8800-53C; Hamamatsu). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with triexponential function using a nonlinear least-squares estimation procedure.

Protein Extraction Immunoblot and Coimmunoprecipitation

Protein extracts of yeast cells were prepared according to the trichloroacetic acid protein extraction method described in the Yeast Protocols Handbook (Clontech). Protein extracts of *N. benthamiana* leaves were prepared in protein extraction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 0.1% Nonidet P-40, supplemented with a Complete protease inhibitor cocktail [Roche] and polyvinylpolypyrrolidone 0.5%). For anti-GFP immunoprecipitations, 20 μL of magnetic GFP-trap_M beads (Chromotek), prewashed three times in protein extraction buffer, were added to 1 mL of protein extract and incubated with gentle rotation for 2 h at 4°C. Beads were magnetically separated and washed three times with 500 μL of protein extraction buffer (without polyvinylpolypyrrolidone). Bound proteins were eluted by boiling for 10 min in 50 μL of Laemmli buffer and separated on 10% Tris-Gly SDS-PAGE gels, transferred to nitrocellulose membrane (Millipore), and analyzed by immunoblotting. Other protein samples were mixed with 2× Laemmli buffer and boiled for 5 min. For immunodetection of proteins, anti-c-Myc-peroxidase antibodies (Roche), mouse anti-GFP (Roche), goat anti-mouse-horseradish peroxidase (Sigma-Aldrich), or rat anti-HA-horseradish peroxidase (clone 3F10; Roche) were used in combination with the Immobilon western kit (Millipore).

RNA Extraction and qRT-PCR Analysis

RNA was extracted from infected rice leaves with Trizol reagent (Invitrogen). Reverse transcription was performed with oligo$(dT)_{18}$ primers, and quantitative PCR was performed using LC 480 SYBR Green I Master mix (Roche) and a Lightcycler 480 instrument (Roche). For normalization, a fragment of the *M. oryzae* gene *MGG_03641* coding for elongation factor 1α was amplified (see Supplemental Tables 4 and 5 online).

Phylogenetic Analysis

To identify homologous protein sequences in the Nipponbare rice reference genome, BLASTp searches (Altschul et al., 1997) against the OrygenesDB database were performed (http://orygenesdb.cirad.fr/index.html) (Droc et al., 2006). The protein alignment generated with ClustalX (Larkin et al., 2007) was manually edited and curated, and gaps were removed for further analyses. We used MEGA 5.05 (Tamura et al., 2011) to reconstruct maximum parsimony, maximum likelihood, and distance trees. For the maximum parsimony analysis, we used the heuristic search algorithm to explore the possible topologies. For the maximum likelihood analysis, we used the JTT + G amino acid substitution model. According to the smallest Akaike information criterion (AIC), this model was determined to be the best-fit model using ProtTest 3 (Darriba et al., 2011), which estimates the likelihood and the parameter values of 112 different protein evolution models using a maximum likelihood framework. For the distance analysis, we used neighbor joining with the JTT + G amino acid substitution model. For the three analyses, we performed 1000 bootstrap replicates to assess the support for the nodes and displayed the bootstrap consensus tree.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *AVR-Pia* (AB498873), AVR-Pia-H3 (KC777366), *AVR1-CO39* (AF463528), *PWL2* (U26313), *RGA4* (AB604622), Sasanishiki *RGA5-A* (AB604627), Sasanishiki *RGA5-B* (KC777365), CO39 *RGA5-B* (KC777364), *Pik-1* (ADZ48537), pi5-3 (BAF24806), Os02g37160 (BAF09168), Os04g39380 (BAH92703), Os04g39360 (CAE06008), Os04g39350 (BAF14955), Os04g39370 (CAE06009), Os04g39010 (BAF14929), Os02g37330 (BAF09179), Os02g37320 (BAF09178), Os02g37290 (BAH91774), Os02g37280 (BAD17465), Os02g37300 (BAF09176), Os06g35060 (BAF19722), and Pi21 (BAF14599).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Validation of Alternative Splicing of *RGA5* by RT-PCR.

Supplemental Figure 2. Genomic *RGA5-A* and *RGA5-B* Constructs for Rice Transformation.

Supplemental Figure 3. A Replicate Experiment Confirms That *RGA5-A* Is the Functional Isoform Required for *Pi-CO39* and *Pia* Resistance.

Supplemental Figure 4. Immunoblotting Analyses Indicate Proper Expression of Bait and Prey Constructs in Yeast.

Supplemental Figure 5. Immunoblotting Shows Expression of AVR-Pia:CFP, AVR1-CO39:CFP, YFP:RGA5-A, and YFP:RGA5-B in *N. benthamiana*.

Supplemental Figure 6. Colocalization of AVR-Pia:CFP, AVR1-CO39:CFP, YFP:RGA5-A, and YFP:RGA5-B in *N. benthamiana* Leaf Epidermal Cells.

Supplemental Figure 7. *AVR-Pia-H3* Is Specifically Expressed during Plant Infection.

Supplemental Figure 8. Multiple Sequence Alignment of RGA5-A, Pik-1, and pi5-3 RATX1 Domains with 12 RATX1 Proteins from Rice.

Supplemental Figure 9. Multiple Sequence Alignment of RATX1 Proteins and Domains for Phylogenetic Analysis.

Supplemental Table 1. *Pi-CO39* and *Pia* Resistance Is Linked in Rice Cultivars.

Supplemental Table 2. Occurrence of *AVR-Pia* in *M. oryzae* Isolates.

Supplemental Table 3. Constructs.

Supplemental Table 4. Primers.

Supplemental Table 5. PCR, RT-PCR, and qRT-PCR.

Supplemental Data Set 1. Alignment Used for Phylogenetic Analysis in Figure 9.

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AUTHOR CONTRIBUTIONS

S.C. and T.K. designed the research. S.C., G.T., C.R., V.C., C.M., A.J., L.A., S.R., E.F., D.T., and T.K. performed the research. H.K., Y.O., and R.T. contributed important tools and biological materials. S.C., S.R., A.J., E.F., D.T., and T.K. analyzed data. S.C., S.R., J.-B.M., R.T., and T.K. wrote the article.

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