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The NB-LRR proteins RGA4 and RGA5 interact functionally and physically to confer disease resistance

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Abstract

Plant resistance proteins of the class of nucleotide-binding and leucine-rich repeat domain proteins (NB-LRRs) are immune sensors which recognize pathogen-derived molecules termed avirulence (AVR) proteins. We show that RGA4 and RGA5, two NB-LRRs from rice, interact functionally and physically to mediate resistance to the fungal pathogen *Magnaporthe oryzae* and accomplish different functions in AVR recognition. RGA4 triggers an AVR-independent cell death that is repressed in the presence of RGA5 in both rice protoplasts and *Nicotiana benthamiana*. Upon recognition of the pathogen effector AVR-Pia by direct binding to RGA5, repression is relieved and cell death occurs. RGA4 and RGA5 form homo- and hetero-complexes and interact through their coiled-coil domains. Localization studies in rice protoplast suggest that RGA4 and RGA5 localize to the cytosol. Upon recognition of AVR-Pia, neither RGA4 nor RGA5 is re-localized to the nucleus. These results establish a model for the interaction of hetero-pairs of NB-LRRs in plants: RGA4 mediates cell death activation, while RGA5 acts as a repressor of RGA4 and as an AVR receptor.

Introduction

Plants possess a highly efficient, two-layered innate immune system that renders them resistant against most microbial pathogens (Jones & Dangl, 2006; Dodds & Rathjen, 2010; Dangl *et al*, 2013). The first layer of defense relies on the recognition of conserved microbe-associated molecular patterns (MAMPs) by the so-called pattern recognition receptors (PRRs). PRRs are generally plasma membrane receptors which are often coupled to intracellular kinase domains (Monaghan & Zipfel, 2012). Adapted pathogens are able to suppress or to bypass this first layer of resistance through the secretion of effector proteins that interfere with plant immunity. The second layer of plant immunity relies on the specific recognition of pathogen effectors by disease resistance proteins (R). This recognition leads to a strong immune response which is often associated with a localized programmed cell death termed the hypersensitive response (HR). Since effectors are generally species or isolate specific, this second layer of immunity is only efficient against isolates that carry the recognized effector which is then called an avirulence protein (AVR).

Most R-proteins belong to the nucleotide-binding and leucine-rich repeat (NB-LRR) family and comprise a multidomain architecture with a C-terminal leucine-rich repeat domain (LRR) and a central nucleotide-binding domain (NB-ARC). In most cases, they also carry at their N-terminus a coiled-coil (CC) domain or a domain with homologies to the TOLL/interleukin-1 receptor (TIR) (Takken & Goverse, 2012).

The molecular mechanisms and the structural bases governing NB-LRR function are still insufficiently understood, but the detailed investigation of a limited number of NB-LRR proteins generated a conceptual framework for the role of the different domains (Bernoux *et al*, 2011a). The LRR domain often confers recognition specificity to NB-LRR proteins and is supposed to act in certain cases as a receptor domain (Ellis *et al*, 1999, 2007; Jia *et al*, 2000; Wang *et al*, 2007; Krasileva *et al*, 2010). In addition, it is involved in complex intramolecular interactions that contribute to proper regulation of NB-LRR proteins (Leister, 2005; Ade *et al*, 2007; Van

Ooijen *et al*, 2008a; Slootweg *et al*, 2013). N-termini of NB-LRR proteins, including the CC, NB-ARC, or TIR domains, are believed to be important for the activation of resistance signaling and HR induction (Frost *et al*, 2004; Leister, 2005; Ade *et al*, 2007; Van Ooijen *et al*, 2008a; Swiderski *et al*, 2009; Krasileva *et al*, 2010). Recently, the CC domain of MLA10 from barley and the TIR domain of L6 from flax were demonstrated to be necessary and sufficient for cell death induction and to mediate immune receptor homodimerization (Bernoux *et al*, 2011b; Maekawa *et al*, 2011). The central NB-ARC domain shows homologies to the metazoan apoptosis regulators Apaf-1 and CED-4 and consists of the subdomains NB, ARC1 (for Apaf-1, R-protein and CED-4), and ARC2. These proteins form the NB-ARC family within the class of signal transduction ATPases with numerous domains (STAND) proteins. The NB-ARC domain was shown to bind and hydrolyze nucleotides (Tameling, 2002) and, in analogy to other STAND proteins, nucleotide binding and exchange is supposed to control the activity of NB-LRR proteins. ADP binding is believed to stabilize the inactive state, while ATP binding stabilizes the signaling competent state (Bernoux *et al*, 2011a; Williams *et al*, 2011).

AVR recognition by NB-LRR proteins can occur in a direct manner by physical binding of the AVR effector to the NB-LRR protein or in an indirect manner (Dangl & Jones, 2001; Van der Hoorn & Kamoun, 2008; Collier & Moffett, 2009). In the latter case, a plant protein modified by the AVR effector or the complex between the AVR effector, and the plant protein binds to the NB-LRR protein and triggers resistance. This plant protein may be either the operational target of the effector and in this case it is called a guardee since it is guarded by the NB-LRR, or a mimic of the operational target in which case it is called a decoy (Dangl & Jones, 2001; Van der Hoorn & Kamoun, 2008).

In most cases, individual NB-LRR proteins seem sufficient to mediate AVR effector recognition and to confer resistance. However, in recent years, an increasing number of cases have been reported where two NB-LRR proteins are required for resistance in both mono- and dicotyledonous plants (Sinapidou *et al*, 2004; Ashikawa *et al*, 2008; Lee *et al*, 2009; Loutre *et al*, 2009; Narusaka *et al*, 2009; Eitas & Dangl, 2010; Okuyama *et al*, 2011; Yuan *et al*, 2011; Brotman *et al*, 2012; Cesari *et al*, 2013; Wang *et al*, 2013). Why pairs of NB-LRR proteins are required for resistance in these cases, and how these paired proteins interact functionally and mechanistically, is largely unknown.

Blast disease caused by the fungus *Magnaporthe oryzae* is the most important disease in rice (Pennisi, 2010; Dean *et al*, 2012). It causes frequent and devastating epidemics in all rice-producing areas of the world and is therefore a severe threat for the cultivation of the most important crop for human nutrition. More than 20 rice blast resistance genes have been cloned, and with one exception, they all code for CC-NB-LRR proteins (Liu *et al*, 2010). A particular feature of rice blast NB-LRR proteins is that in a relatively high proportion (6 cases: Pik, Pikp, Pikm, Pi-a, Pi-CO39, and Pi-5), resistance is conferred by pairs of CC-NB-LRRs (Ashikawa *et al*, 2008; Lee *et al*, 2009; Okuyama *et al*, 2011; Yuan *et al*, 2011; Cesari *et al*, 2013; Wang *et al*, 2013). Examples of this are Pik-1 and Pik-2 which confer resistance to AVR-Pik-expressing *M. oryzae* strains (Zhai *et al*, 2011; Kanzaki *et al*, 2012) and RGA4 and RGA5 which recognize two unrelated effectors AVR-Pia and AVR1-CO39 (Okuyama *et al*, 2011; Cesari *et al*, 2013). The genes coding these matching

NB-LRR pairs show a conserved type of organization in the rice genome consisting of extremely tight physical linkage and arrangement in inverted orientation.

To date, seven *M. oryzae* AVR proteins have been cloned and six of them code for small secreted proteins translocated into host cells suggesting that the majority of AVRs of the rice blast fungus are cytoplasmic effectors. For 4 of these AVRs, AVR-Pia, AVR1-CO39, AVR-Pita, and AVR-Pik, recognition by a matching CC-NB-LRR protein or a CC-NB-LRR protein pair has been described and was found in all cases to be direct (Jia *et al*, 2000; Kanzaki *et al*, 2012; Cesari *et al*, 2013). AVR-Pita interacts with the LRR domain of its R-protein Pi-ta (Jia *et al*, 2000), while AVR-Pik, AVR-Pia, and AVR1-CO39 bind physically to only one member of their respective CC-NB-LRR pair, Pik-1 and RGA5, respectively (Kanzaki *et al*, 2012; Cesari *et al*, 2013). Interestingly, the AVR interaction domain is, in both cases, a conserved domain with homologies to ATX1, a copper chaperone present in most eukaryotic organisms and containing a heavy metal-associated (HMA) domain (Cesari *et al*, 2013). This RATX1 domain (Related to ATX1), which is uncommon in NB-LRR proteins, is located between the CC and NB-ARC domains in Pik-1 and after the LRR domain in RGA5. In the cases of Pik-1/Pik-2 and RGA4/RGA5, it therefore seems that one of the members of the CC-NB-LRR pair acts as an AVR receptor, while the function of the other partner as well as the functional and molecular interactions between the two partners remain unknown.

To better understand the molecular mechanism by which RGA4 and RGA5 interact and trigger pathogen recognition, we undertook a functional analysis of this pair of R-proteins using both the homologous rice system and the heterologous *Nicotiana benthamiana* system. This study reveals a novel mechanism involving physical and functional interactions between the two CC-NB-LRR proteins. We show that RGA4 and RGA5 interact through their CC domains and form homo- and hetero-complexes. Furthermore, constitutive RGA4 expression triggers an effector-independent cell death response that is repressed by the presence of RGA5. Upon recognition of AVR-Pia, repression is relieved and HR-like cell death occurs.

Therefore, it seems that evolution has led each CC-NB-LRR partner in this and perhaps other hetero-pairs to undertake distinct functions (receptor or signaling inducer) in an immune receptor complex that mediates pathogen recognition and defense signaling.

Results

RGA4 acts as a constitutively active cell death inducer and is regulated by RGA5

To investigate functional and molecular interactions between RGA4 and RGA5, these proteins were transiently expressed in *N. benthamiana* leaves by *Agrobacterium tumefaciens*-mediated transformation. Several constructs driven by the *35S* promoter and allowing expression of RGA4 or RGA5 with or without fusion to the HA epitope tag or fluorescent proteins (CFP or YFP) were tested. Western blot analysis showed that all fusion proteins were properly expressed (Fig 1A and Supplementary Fig S1A). Expression of RGA4:HA triggered strong and rapid HR-like cell death which became visible 2 days after inoculation (Fig 1B and Supplementary

Fig S2A). RGA4 and RGA4:CFP induced the same response (Supplementary Fig S2A). These results suggest that RGA4 acts as a constitutively active cell death inducer.

Unlike RGA4, expression of RGA5 alone did not induce any response in *N. benthamiana* leaves, suggesting that it has no cell death-inducing activity (Fig 1B). However, co-expression of RGA4 with RGA5, HA:RGA5, or YFP:RGA5 attenuated or completely abolished RGA4-triggered cell death (Fig 1B and Supplementary Fig S2B). The extent of cell death suppression was dependent on the ratio between *35S::RGA4-* and *35S::RGA5*-carrying agrobacteria in the infiltration inoculum. Using a ratio of 1:2, no symptom or only slight chlorosis was observed. These findings suggest that RGA5 negatively regulates RGA4 and acts as an inhibitor of RGA4-triggered cell death.

To assess the specificity of RGA5 repression activity, RGA5 was transiently co-expressed with the auto-active rice CC-NB-LRR Orin1 (Kawano *et al*, 2010) and an auto-active mutant of the flax TIR-NB-LRR L6 (L6$_{MHV}$, Howles *et al*, 2005) in *N. benthamiana* and *N. tabacum.* Neither Orin1- nor L6$_{MHV}$-mediated cell death was suppressed by RGA5 showing that its inhibition activity is specific to RGA4 (Supplementary Fig S3).

To test whether the RGA4-RGA5 pair is responsive to AVR-Pia and AVR1-CO39 in the heterologous *N. benthamiana* system, both NB-LRRs were co-expressed with AVR1-CO39, AVR-Pia, or the inactive H3 allele of AVR-Pia which does not interact physically with RGA5 and does not confer avirulence to *M. oryzae* (Cesari *et al*, 2013). Co-expression of AVR-Pia, RGA4, and RGA5 induced rapid and strong cell death indistinguishable in timing and appearance from the response induced by RGA4 alone (Fig 1B). On the contrary, expression of AVR-Pia and RGA5 alone induced no symptoms, supporting the idea that RGA5 has no cell death-inducing activity (Fig 1B). Expression of AVR-Pia_H3, RGA4, and RGA5 induced no response or only slight chlorosis, resembling co-expression of RGA4 and RGA5 (Supplementary Fig S2C). Taken together, these results suggest that AVR-Pia removes RGA5-mediated repression of RGA4-triggered cell death. The lack of activity of AVR-Pia_H3 indicates that binding of AVR-Pia to RGA5 is crucial to relieve RGA5 repressor activity. N- or C-terminally tagged versions of AVR-Pia did not activate cell death in the presence of RGA4 and RGA5, although they accumulated to high levels according to Western blot experiments (Supplementary Fig S1B) and interacted with RGA5 co-immunoprecipitation experiments (Cesari *et al*, 2013). The same was true for AVR1-CO39 or tagged variants of AVR1-CO39, which were detected in Western blot experiments but did not trigger cell death when co-expressed with RGA4 and RGA5 (Supplementary Figs S1B and S2C).

To validate the functional interactions observed in *N. benthamiana* in rice, combinations of RGA4, RGA5, and AVR-Pia were co-expressed with the luciferase reporter protein in leaf protoplasts of the susceptible rice variety Himenomochi or protoplasts from Oc rice suspension culture (Baba *et al*, 1986). Compared to the empty vector control, expression of RGA4 alone or co-expression of RGA4, RGA5, and AVR-Pia resulted in significant reduction of luciferase reporter activity, indicating induction of HR-like cell death (Fig 1C and Supplementary Fig S4A, B and D). Expression of RGA5 alone had no effect as luciferase activity was not significantly reduced compared to the empty vector control (Fig 1C and Supplementary Fig S4A and C). However, when RGA5 was co-expressed with

RGA4, RGA4-triggered reduction of luciferase activity was abolished, indicating that RGA5 also suppresses RGA4-induced cell death in rice (Fig 1C and Supplementary Fig S4A and D). RGA4 and RGA5 seem therefore to interact in the same manner in rice and in the heterologous *N. benthamiana* system.

To exclude that cell death induction by RGA4 is a consequence of overexpression and does not reflect its activity at normal physiological levels, RGA5 was silenced in rice protoplasts of the resistant Sasanishiki variety by transfection with RNAi constructs (Fig 1D and Supplementary Fig S5). Depletion of RGA5 by two different RNAi constructs induced cell death, while depletion of RGA4 had no significant effect. In the *rga4* loss-of-function-mutant line Sas1493 (Okuyama *et al*, 2011), RGA5 depletion had no effect indicating that the cell death induced by RGA5 depletion relies entirely on RGA4.

Taken together, these results suggest that RGA4 acts as an HR executer which is regulated in its activity by RGA5. RGA5 can be regarded as an AVR receptor since it physically binds AVR1-CO39 and AVR-Pia (Cesari *et al*, 2013) and transduces AVR signal perception into a relief of RGA4 repression, resulting in resistance induction and HR.

A functional nucleotide-binding pocket is required for RGA4 but not for RGA5 activity

To further test this model, mutant alleles of RGA4 and RGA5 in motifs required for the nucleotide-binding and exchange functions of NB-LRR proteins were generated and tested, respectively, for cell death-inducing or repressing activity. The central lysine (K) in the P-loop, a highly conserved motif in all nucleotide-binding proteins, was replaced by an arginine (R), giving rise to rga4$_{K209R}$ (rga4$_{K/R}$) and rga5$_{K210R}$ (rga5$_{K/R}$). Such P-loop mutations strongly impair nucleotide binding and abolish resistance and cell death induction in many NB-LRR proteins (Dinesh-Kumar *et al*, 2000; Tao *et al*, 2000; Tameling, 2002; Howles *et al*, 2005; Ade *et al*, 2007; Williams *et al*, 2011; Bai *et al*, 2012).

Tagged versions of rga4$_{K/R}$ and rga5$_{K/R}$, with a C-terminal HA tag (rga4$_{K/R}$:HA) or an N-terminal YFP tag (YFP:rga5$_{K/R}$), were transiently expressed in *N. benthamiana,* while untagged rga4$_{K/R}$ and rga5$_{K/R}$ proteins were tested in rice protoplasts. Western blotting confirmed proper expression of the fusion proteins in *N. benthamiana* (Fig 2A and B). Expression of rga4$_{K/R}$ alone or in combination with RGA5 and AVR-Pia did not trigger cell death in *N. benthamiana* nor in rice (Fig 2C, D and E). On the contrary, rga5$_{K/R}$ still repressed RGA4-triggered cell death (Fig 2D, E and F) and co-expression of RGA4, rga5$_{K/R,}$ and AVR-Pia triggered cell death to the same extent as co-expression of RGA4, RGA5, and AVR-Pia (Fig 2E and F). Thus, an intact RGA4 nucleotide-binding pocket is required for cell death induction by RGA4, either on its own or in the presence of RGA5 and AVR-Pia. However, an intact RGA5 P-loop motif is not necessary for repression of RGA4-induced cell death or for removal of this repression after AVR-Pia recognition. This supports the notion that cell death is exclusively activated by RGA4 while RGA5 acts as an AVR receptor and negative regulator of RGA4. Binding of AVR-Pia to RGA5 may either not lead to nucleotide exchange and RGA5 activation or such nucleotide exchange may not be required for AVR-induced de-repression of RGA4.
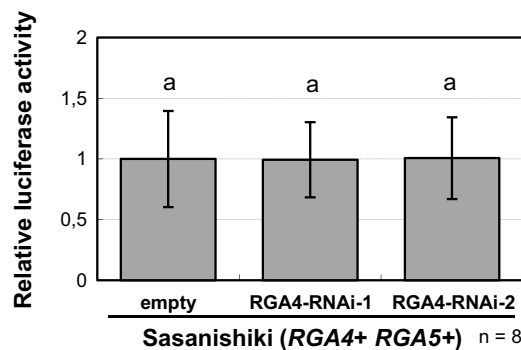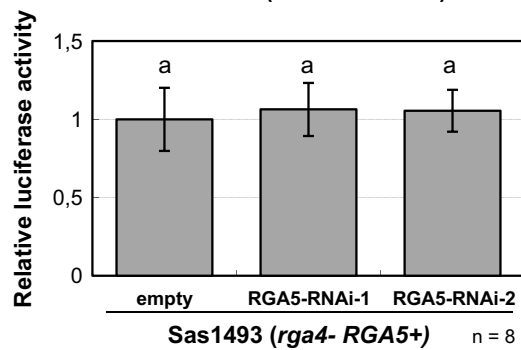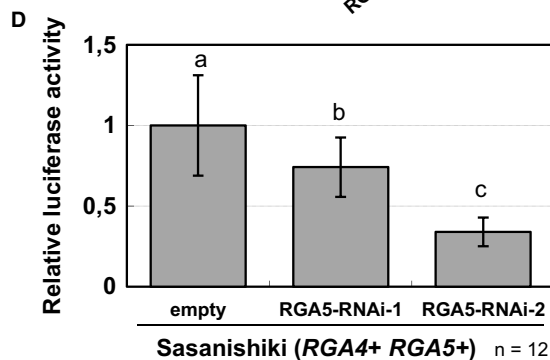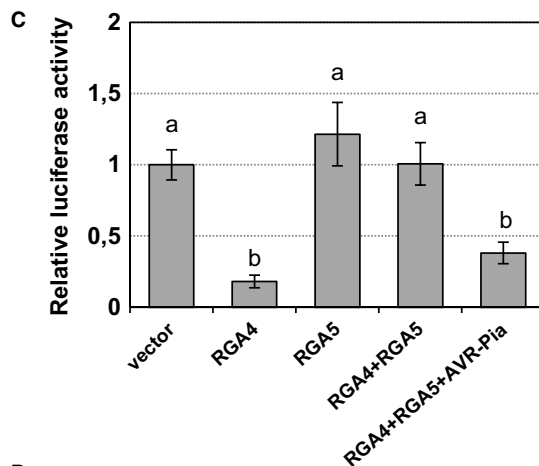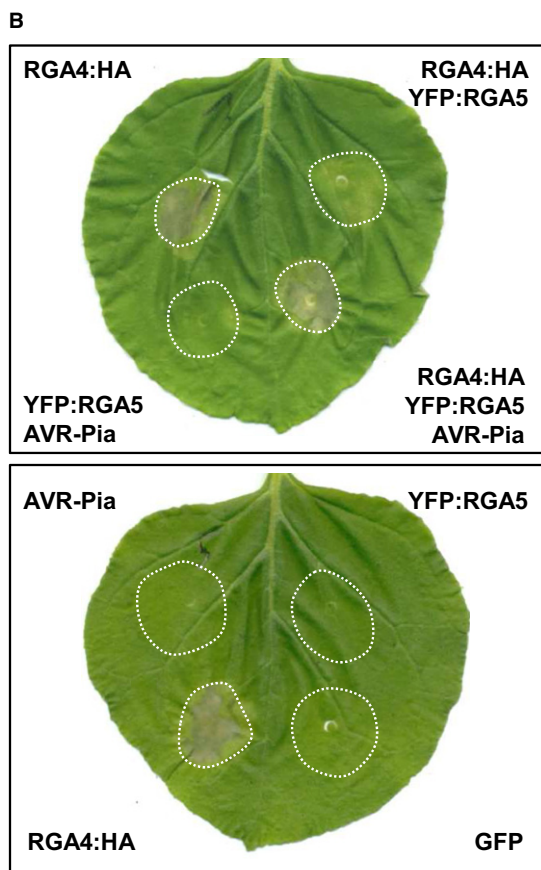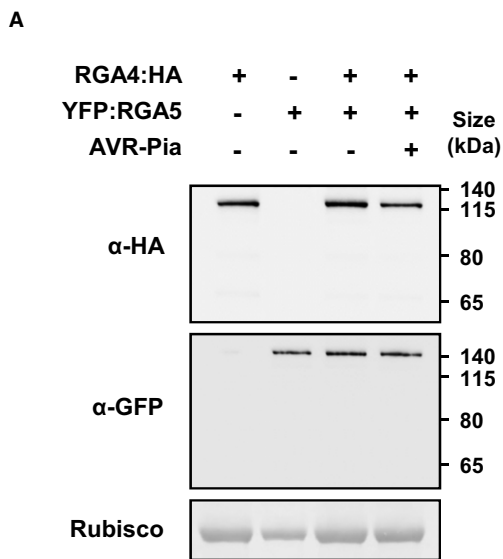
A non-canonical MHD motif is crucial for RGA4 but not for RGA5 activity

Within the ARC2 domain, the conserved MHD motif is particularly important for the regulation of NB-LRR proteins. Replacement of the conserved histidine or aspartate in the MHD core sequence by other amino acids leads in most cases to auto-active NB-LRR proteins, which constitutively activate immune responses and cell death (Bendahmane *et al*, 2002; De La Fuente van Bentem *et al*, 2005; Howles *et al*, 2005; Van Ooijen *et al*, 2008b; Kawano *et al*, 2010; Gao *et al*, 2011; Maekawa *et al*, 2011; Bai *et al*, 2012; Zhang *et al*, 2012; Roberts *et al*, 2013). Sequence analysis of RGA4 and RGA5

Figure 1. RGA4 auto-activates HR-like cell death that is repressed by RGA5.

A   *RGA4:HA*, *YFP:RGA5*, and *AVR-Pia* constructs were transiently expressed in *Nicotiana benthamiana* leaves by *A. tumefaciens* infiltration. Proteins were extracted 24 h after infiltration, and protein extracts were analyzed by immunoblotting with anti-GFP (α-GFP) and anti-HA (α-HA) antibodies. Ponceau staining of Rubisco small subunit was used to verify equal protein loading.

B   The indicated combinations of fusion proteins were transiently expressed in *N. benthamiana*. The *RGA4:HA* strain was infiltrated at OD_{600} of 0.2, while strains carrying all other constructs were infiltrated at OD_{600} of 0.4. A bacterial strain carrying the *GFP* construct was used to equilibrate the total OD_{600} to 1 before infiltration. Cell death induced by the different combinations was visualized 3 days after infiltration. Equivalent results were obtained in at least three independent experiments.

C   Leaf protoplasts of the susceptible rice cultivar Himenomochi (*pia*) were transfected with a plasmid for constitutive LUC expression in combination with constructs allowing expression of RGA4, RGA5, RGA4+RGA5, or RGA4+RGA5+AVR-Pia. LUC activity was determined in protein extracts of protoplast samples harvested 40 h after transfection. Average values and standard deviations were calculated from three replicate samples, and values were normalized with respect to the average values of the empty vector samples. The experiment was repeated three times with equivalent results. Different letters indicate groups that are significantly different ($P < 0.05$ in pairwise *t*-test).

D   Rice protoplast of the resistant rice variety Sasanishiki or of the *rga4* mutant line Sas1493 was co-transfected with RNAi constructs directed against *RGA4* or *RGA5* and the luciferase reporter gene. Luc activity was determined and analyzed as in (C). Average values and standard deviations were calculated from the indicated number of replicate samples (*n*). The experiment was repeated three times with equivalent results.

Source data are available online for this figure.

ARC2 domains revealed that both NB-LRR proteins contain non-canonical MHD motifs (Fig 3A and Supplementary Fig S6). In RGA4, the motif is highly degenerated, and all three amino acids differ from the consensus MHD sequence ($T_{500}Y_{501}G_{502}$). The hydrophobic methionine is substituted by a hydrophilic threonine, while the hydrophilic positively charged histidine and the negatively charged aspartate are replaced by a hydrophobic tyrosine and an uncharged glycine, respectively. In RGA5, the motif is less disturbed ($L_{504}H_{505}H_{506}$) since the methionine is replaced by a leucine, which has similar biophysical properties, and the central histidine is conserved. However, the acidic, negatively charged aspartate is substituted by a basic, positively charged histidine.

To determine whether RGA4 auto-activity could be due to its non-canonical TYG motif, point mutations were introduced in the *RGA4* sequence to generate a MHD consensus sequence. This construct was fused to a C-terminal HA tag (rga4$_{MHD}$:HA) and transiently expressed in *N. benthamiana*, alone or in combination with YFP:RGA5 or YFP:RGA5 and AVR-Pia (Fig 3B, C and D). None of these combinations were effective in triggering cell death, whereas RGA4:HA, used as a positive control, induced a strong cell death response (Fig 3C). To test whether single amino acids in the non-canonical core sequence TYG are required for auto-activity, they were individually replaced by M, H, or D, respectively (Fig 3B). In addition, an AAA mutant was generated. The mutant forms rga4$_{MYG}$ and rga4$_{THG}$ retained auto-activity, while rga4$_{TYD}$ lost auto-activity indicating that the threonine and the tyrosine are neither required nor sufficient for auto-activity (Fig 3D) and that the glycine is crucial. The rga4$_{AAA}$ mutant still induced cell death, however, to a much lower rate than wild-type RGA4. Similar strong reduction of cell death after replacement of multiple amino acids in the MHD sequence was also observed for the tomato I-2 NB-LRR protein and was attributed to a potential disturbance of the ATP-binding pocket (Van Ooijen *et al*, 2008b).

These results suggest that the disturbed MHD motif of RGA4 is crucial for its activity as an auto-active cell death inducer. In addition, they indicate that recognition of AVR-Pia by RGA5 does not lead to activation of rga4$_{MHD}$ supporting the hypothesis that auto-activity of RGA4 is necessary for AVR-Pia-induced cell death.

To determine whether RGA5 can function as an activator of cell death signaling, point mutations leading generally to NB-LRR auto-activity were introduced in its MHD motif. The RGA5 LHH core

motif was replaced by an LHV or an LAH sequence, and the resulting constructs, N-terminally fused to YFP (YFP:rga5$_{LHV}$ and YFP:rga5$_{LAH}$), were transiently expressed in *N. benthamiana*. Both rga5$_{LHV}$ and rga5$_{LAH}$ were expressed at similar levels as the RGA5 wild-type protein (Fig 3B), but they did not trigger any cell death when expressed alone and are therefore not auto-active (Fig 3E). Nevertheless, they were fully functional in repressing RGA4-mediated cell death and in recognizing AVR-Pia (Supplementary Fig S7A and B). The RGA5 MHD motif contains like several other NB-LRR proteins (Van Ooijen *et al*, 2008b) a second MHD-like sequence, VHD, separated by one amino acid from the MHD core sequence (Fig 3A). To test whether mutation of this sequence leads to auto-activity, the aspartate in the VHD sequence was replaced by valine. The corresponding rga5$_{D510V}$ mutant behaved like the wild-type RGA5. It did not activate cell death but repressed RGA4 and recognized AVR-Pia (Supplementary Fig S8).

These results support the conclusion that cell death signaling is activated exclusively by RGA4 and that AVR-Pia binding does not lead to RGA5 activation but solely to RGA4 de-repression.

RGA4 and RGA5 form hetero- and homo-complexes

To investigate whether the functional interaction between RGA4 and RGA5 may rely on the formation of RGA4-RGA5 hetero-complexes, co-immunoprecipitation experiments with *in planta*-expressed tagged variants of RGA4 and RGA5 were performed. In addition, the formation of RGA4 and RGA5 homo-complexes was investigated since NB-LRR proteins have been reported to form homo-dimers (Mestre & Baulcombe, 2006; Ade *et al*, 2007; Gutierrez *et al*, 2010; Bernoux *et al*, 2011b; Maekawa *et al*, 2011).

In rice protoplasts, full-length RGA4 fused to a C-terminal T7 tag (RGA4:T7) was co-expressed with full-length RGA5 fused to an N-terminal 10×Myc tag (10×Myc:RGA5). These fusion proteins were fully functional as shown by cell death assays in rice protoplasts (Supplementary Fig S9). As a negative control, RGA4:T7 was expressed with 10×Myc-tagged GFP (10×Myc:GFP) and un-tagged RGA5 which served to prevent cell death. All fusion proteins were detected in protoplast extracts by immunoblotting with anti-Myc or anti-T7 antibodies (Fig 4A). 10×Myc:RGA5 and 10×Myc:GFP were expressed at similar levels and were precipitated by anti-Myc antibodies. RGA4 co-precipitated specifically with RGA5 but not with GFP (Fig 4A).
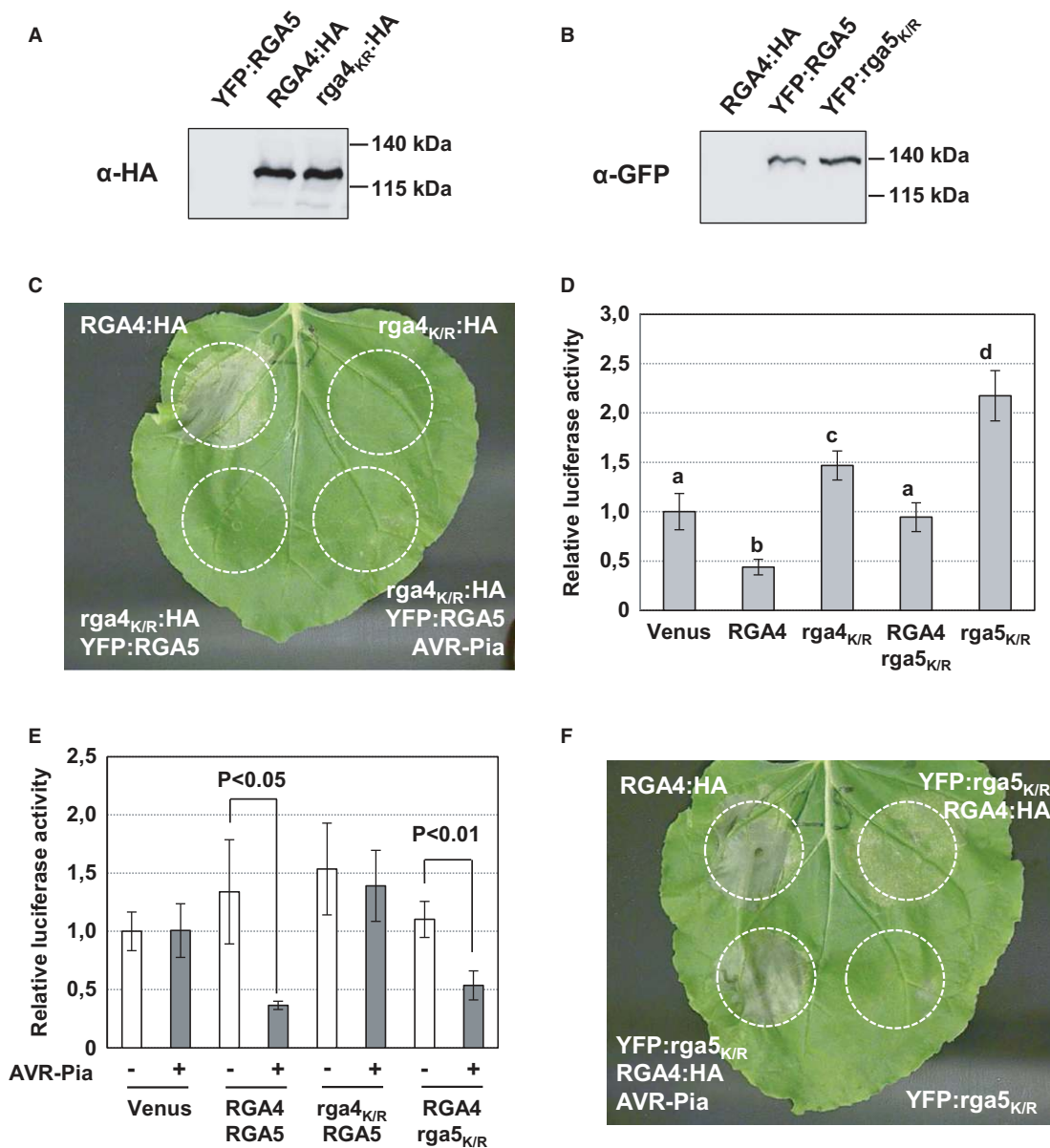
Figure 2. An intact nucleotide-binding pocket is required for RGA4 but not for RGA5 activity.

A, B   Constructs were transiently expressed in *N. benthamiana*, and protein extracts were analyzed by immunoblotting with anti-HA (α-HA) and anti-GFP (α-GFP) antibodies.

C   The *rga4_{K/R}:HA* strain carrying a construct for the rga4_{K209R} P-loop mutant was infiltrated alone and in combination with *YFP:RGA5* or *YFP:RGA5* and *AVR-Pia* strains in *N. benthamiana* leaves. The *RGA4:HA* strain was infiltrated alone as a positive control. Cell death was visualized 3 days after infiltration. Equivalent results were obtained in at least three independent experiments.

D, E   Rice protoplasts from Oc suspension culture were transfected with plasmids for constitutive LUC expression and different combinations of constructs for expression of: Venus, RGA4, rga4_{K209R}, RGA5, rga5_{K210R}, and AVR-Pia. LUC activity was determined in protoplast protein extract samples 40 h after transfection. Average values and standard deviations were calculated from three replicate samples, and values were normalized with respect to the average values of the corresponding Venus samples. The experiments were repeated three times with equivalent results. Average values significantly different from the average value of the corresponding control (Venus) samples are indicated ($P < 0.05$ in Student's t-test).

F   The *YFP:rga5_{K/R}* strain carrying a construct for the rga5_{K210R} P-loop mutant was infiltrated alone and in combination with *RGA4:HA* or *RGA4:HA* and *AVR-Pia* strains. The *RGA4:HA* strain was infiltrated alone as a positive control. Cell death was visualized 3 days after infiltration. Equivalent results were obtained in at least 3 independent experiments.

Source data are available online for this figure.

Similar results were obtained using the *N. benthamiana* system where different combinations of RGA4:CFP, RGA4:HA, YFP:RGA5, HA:RGA5, and GFP (used as a negative control) were expressed.

Immunoblotting using anti-GFP and anti-HA antibodies showed proper expression of all proteins (Fig 4B). Immunoprecipitation with anti-GFP antibodies resulted in enrichment of RGA4:CFP, YFP:RGA5
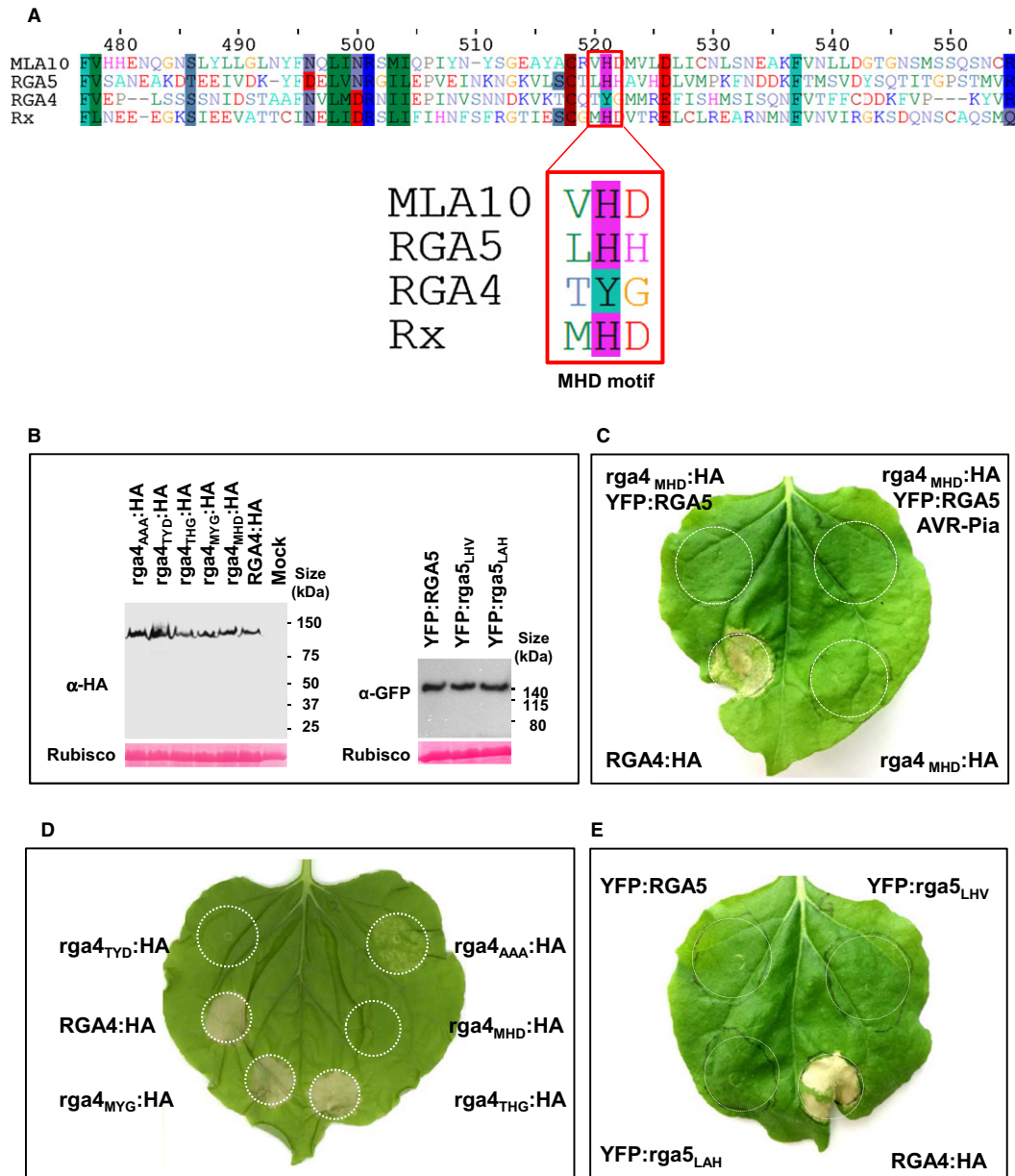
Figure 3. A disturbed MHD motif in RGA4 is crucial for its function.

A   Alignment of a portion of the ARC2 domain sequences of RGA4, RGA5, Rx, and MLA10 containing the MHD motif (red box).

B   Proteins were extracted 24 h after infiltration of the indicated *RGA4:HA* or *YFP:RGA5* constructs in *N. benthamiana* and analyzed by immunoblotting with anti-HA or anti-GFP antibodies (α-HA and α-GFP). Ponceau staining of Rubisco small subunit was used to verify equal protein loading.

C   The *rga4$_{MHD}$:HA* strain harboring a construct for expression of an RGA4 variant with the MHD consensus motif was infiltrated alone and in combination with *YFP:RGA5* or *YFP:RGA5* and *AVR-Pia* strains in *N. benthamiana*. Cell death was visualized 3 days after infiltration. The same results were obtained in at least three independent experiments.

D   The RGA4 MHD mutant constructs *rga4$_{MHD}$:HA*, *rga4$_{MYG}$:HA*, *rga4$_{THG}$:HA*, *rga4$_{TYD}$:HA* and *rga4$_{AAA}$:HA* were expressed in *N. benthamiana* and analyzed for cell death induction.

E   *YFP:RGA5*, *YFP:rga5$_{LHV}$*, *YFP:rga5$_{LAH}$*, and *RGA4:HA* were expressed in *N. benthamiana*, and cell death was visualized 3 days after infiltration. Similar results were obtained in at least three independent experiments.

Source data are available online for this figure.

(Fig 4B), and GFP (Supplementary Fig S10). RGA4:HA specifically co-precipitated with YFP:RGA5 but not with GFP (Fig 4B), and HA:RGA5 was efficiently co-precipitated by RGA4:CFP but not by GFP. Interestingly, HA:RGA5 was also co-precipitated by YFP:RGA5, and RGA4:HA co-precipitated with RGA4-CFP. Taken together, these results indicate that *in planta* RGA4-RGA5 hetero-complexes exist and suggest that RGA4 binds RGA5 in a specific manner. In addition, RGA4 and RGA5 may interact with themselves and form homo-complexes.
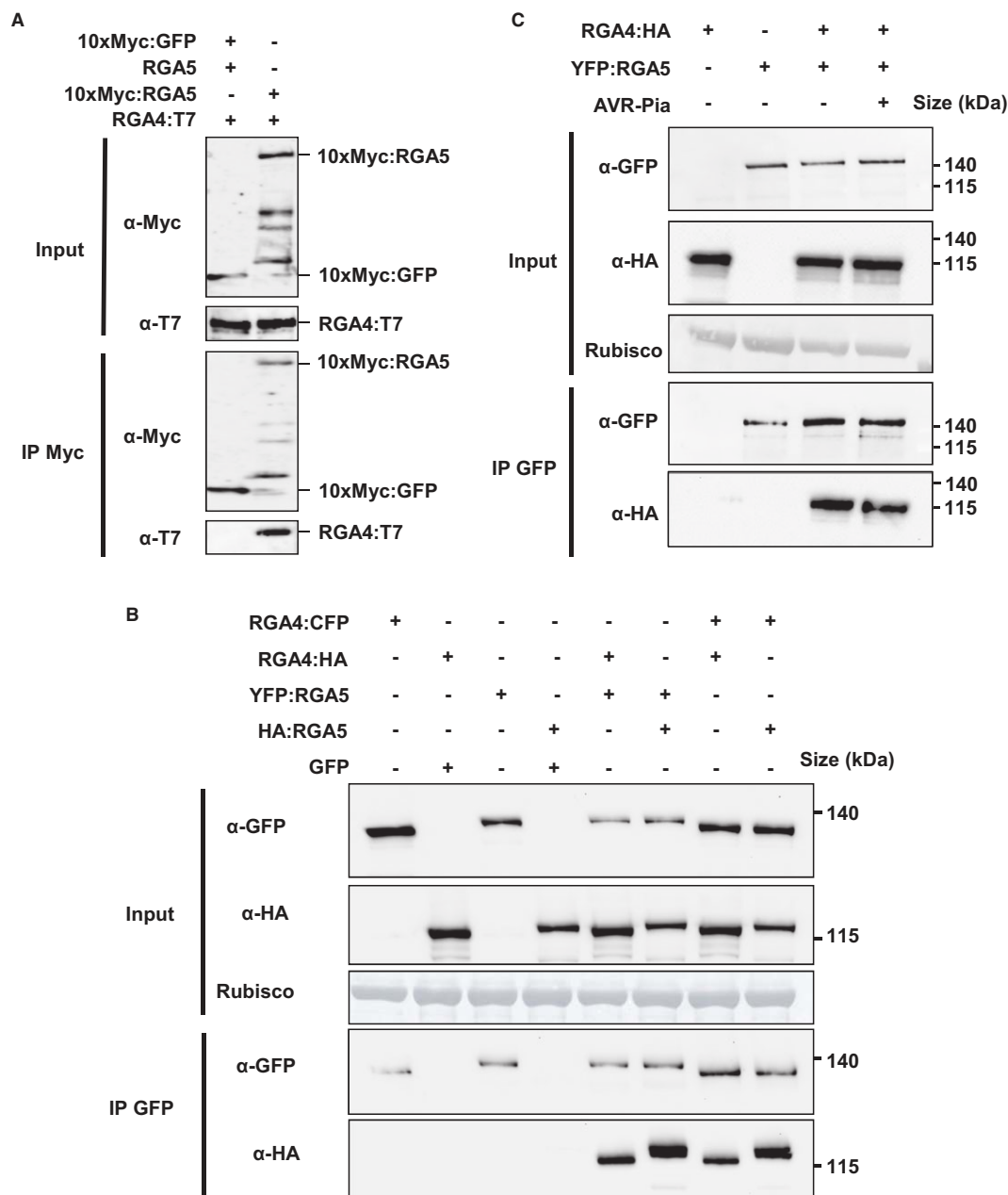
Figure 4. RGA4 and RGA5 form hetero- and homo-complexes *in planta*.

A   RGA4:T7, 10×Myc:RGA5, RGA5, and 10×Myc:GFP were transiently expressed in rice protoplasts from Oc cells. Proteins were extracted 16 h after transfection and analyzed by immunoblotting with anti-c-Myc (α-c-Myc) and anti-T7 antibodies (α-T7) (Input). In addition, immunoprecipitations were performed with anti-c-Myc beads (IP c-Myc) and analyzed by immunoblotting with anti-c-Myc antibodies for the detection of immunoprecipitated 10×Myc:RGA5 and 10×Myc:GFP and with anti-T7 antibodies for the detection of co-precipitated RGA4:T7.

B, C   Different combinations of *RGA4:CFP*, *RGA4:HA*, *YFP:RGA5*, *HA:RGA5*, *GFP*, and *AVR-Pia* constructs were transiently expressed in *N. benthamiana*. Protein extracts were analyzed by immunoblotting with anti-HA (α-HA) and anti-GFP antibodies (α-GFP) (Input). In addition, immunoprecipitations were performed with anti-GFP beads (IP GFP) and analyzed by immunoblotting with anti-GFP antibodies for the detection of immunoprecipitated RGA4:CFP, YFP:RGA5, and GFP proteins and with anti-HA antibodies for the detection of co-precipitated RGA4:HA and HA:RGA5 proteins.

Source data are available online for this figure.

To investigate whether AVR-Pia disrupts RGA4–RGA5 hetero-complexes, additional co-immunoprecipitation experiments were performed where RGA4:HA and YFP:RGA5 were co-expressed in *N. benthamiana* in the presence or in the absence of AVR-Pia. As observed previously, AVR-Pia expression had no influence on the abundance of

RGA4 or RGA5 (Fig 4C). In addition, AVR-Pia had no influence on the co-precipitation of RGA4:HA by YFP:RGA5 (Fig 4C). This indicates that RGA4–RGA5 hetero-complexes still exist in the presence of AVR-Pia.

Taken together, these results suggest that RGA5 regulates RGA4 through direct interaction. However, complete disruption of

RGA4–RGA5 hetero-complexes is not induced by AVR-Pia recognition and seems not necessary for HR.

The CC domains of RGA4 and RGA5 form homo- and hetero-complexes and the RATX1 domain of RGA5 homo-complexes

To investigate whether formation of RGA4–RGA5 hetero-complexes and RGA4 and RGA5 homo-complexes involves direct physical binding and to identify the protein domains involved, pairwise interactions of isolated CC, NB-ARC, and LRR domains of RGA4 and RGA5 and of the C-terminal RATX1 domain of RGA5 (Supplementary Fig S11A and B) were analyzed in the yeast two-hybrid system. NB-ARC domains and LRR domains showed no interactions with the exception of the RGA5 NB-ARC domain which interacted weakly with the RGA5 RATX1 domain (Fig 5A and Supplementary Fig S12A and B). Expression of the different domains was confirmed by Western blot analysis (Supplementary Fig S12C). RGA4 and RGA5 CC domains ($RGA4_{1-176}$ and $CC-RGA5_{1-177}$) interacted with themselves but did not interact with one another (Fig 5A and Supplementary Fig S12A and B). The RATX1 domain showed also homo-dimerization.

To further investigate possible hetero-interactions of CC-RGA4 and CC-RGA5, three additional fragments were analyzed for each protein (Supplementary Fig S13C). $CC-RGA4_{1-130}$ and $CC-RGA5_{1-133}$ correspond to a fragment of the MLA10 CC domain that homo-dimerizes *in vitro* and has been structurally characterized by crystallography (Maekawa *et al*, 2011). $CC-RGA4_{1-130}$ and $CC-RGA5_{1-133}$ showed homo- and hetero-interactions (Supplementary Fig S13). $CC-RGA4_{1-164}$ and $CC-RGA5_{1-167}$ correspond to a minimal MLA10 fragment sufficient for cell death induction (Maekawa *et al*, 2011; Bai *et al*, 2012). $CC-RGA4_{1-164}$ interacted with itself but $CC-RGA5_{1-167}$ interacted neither with itself nor with $CC-RGA4_{1-164}$ (Supplementary Fig S13). $CC-RGA4_{1-229}$ and $CC-RGA5_{1-228}$ contain, in addition to the CC domain, a part of the NB domain including the P-loop motif, and correspond to an MLA10 fragment that homo-dimerizes in the yeast two-hybrid system (Maekawa *et al*, 2011). $CC-RGA5_{1-228}$ interacted with itself and with $CC-RGA4_{1-229}$, and $CC-RGA4_{1-229}$ interacted with itself although interactions seemed much weaker than $CC-RGA4_{1-130}$ and $CC-RGA4_{1-164}$ homo-interactions (Fig 5B and Supplementary Fig S13). CC-homo- and hetero-interactions were specific since no interaction with the CC domain of the sequence-unrelated wheat rust resistance NB-LRR protein Sr33 (Periyannan *et al*, 2013) was detected (Fig 5B). Proper expression of RGA4, RGA5, and Sr33 CC domains was verified by immunoblotting (Fig 5C).

Taken together, the yeast two-hybrid analyses suggest that CC domain interactions are involved in RGA4-RGA5 hetero-complex and RGA4 and RGA5 homo-complex formation. However, it becomes evident that CC domain interactions depend strongly on the length of the CC fragments. In addition, RATX1 self-interaction may contribute to RGA5 homo-complex formation.

To validate the interactions of the RGA4 and RGA5 CC domains, co-immunoprecipitation experiments were performed. $CC-RGA4_{1-229}$:HA, $CC-RGA4_{1-229}$:CFP, $HA:CC-RGA5_{1-228}$, $CC-RGA5_{1-228}$:CFP, and GFP (used as negative control) were co-expressed in *N. benthamiana*. Immunoblotting using anti-GFP and anti-HA antibodies showed proper expression of all constructs in the input (Fig 6). Immunoprecipitation with anti-GFP antibodies resulted in enrichment of $CC-RGA4_{1-229}$:CFP, $CC-RGA5_{1-228}$:CFP, and GFP (Fig 6). $CC-RGA4_{1-229}$:HA and $HA:CC-RGA5_{1-228}$ specifically co-precipitated with $CC-RGA4_{1-229}$:CFP and $CC-RGA5_{1-228}$:CFP but not with GFP (Fig 6). Taken together, these results indicate that *in planta* RGA4 and RGA5 CC domains can form hetero- and homo-complexes.

The RGA5 CC domain is necessary but not sufficient for repression of RGA4-mediated cell death, while its RATX1 domain is dispensable

To elucidate the role of individual domains of RGA5- in RGA4-mediated cell death repression and to identify eventually a minimal domain for this activity, a deletion series of RGA5 was analyzed. Individual CC, NB, ARC, LRR, and RATX1 domains (the latter with and without flanking sequences) as well as combinations of two, three, or four of these five domains were expressed together with RGA4 in *N. benthamiana* (Fig 7 and Supplementary Fig S14). None of the individual domains or combinations of two or three domains was sufficient to repress RGA4-mediated cell death. In addition, deletion of the CC domain alone abolished repressor activity indicating that the CC domain is important for RGA4 repression but not sufficient (Fig 7). Only the RGA5 variant where the RATX1 domain but not the sequence between the LRR domain and RATX1 domain is deleted retained RGA4 repressor activity indicating that the RATX1 domain is not required for RGA4 repression. These results further highlight a role of the RATX1 domain in AVR recognition but not in RGA4 repression and indicate that the CC domain is crucial for functional interactions between RGA5 and RGA4.

RGA4 and RGA5 are mainly localized in the cytosol of rice protoplasts

To determine whether RGA4 and RGA5 act in the same cellular compartments, translational fusions of RGA4 or RGA5 with fluorescent proteins (RGA4:GFP and Venus:RGA5) were expressed in rice protoplasts (Supplementary Fig S15) and visualized by confocal laser scanning microscopy (Fig 8). Protoplast cell death assays demonstrated that the fusion proteins are functional (Supplementary Fig S9). Green fluorescence of RGA4:GFP was observed mainly in the cytosol labeled by mCherry, whereas no or only weak signal could be detected in nuclei labeled by mOrange fused to a nuclear localization signal (NLS:2xmOrange; Fig 8A). Similar results were obtained for Venus:RGA5 which co-localized with Cerulean in the cytosol but not with NLS:Cerulean in the nuclei (Fig 8B). These results suggest that RGA4 and RGA5 localize to the cytosol and are excluded from the nucleus. Co-expression of RGA4 and RGA5 or RGA4, RGA5, and AVR-Pia did not change the localization patterns of the NB-LRR proteins and did not induce their re-localization to the nucleus (Fig 9).

Discussion

Plant resistance is often conferred by single NB-LRR proteins that are polymorphic between different accessions/genotypes of a plant species. It is therefore generally supposed that these NB-LRRs do not need to interact with other NB-LRR proteins. However, in recent years, an increasing number of plant resistances conferred by pairs
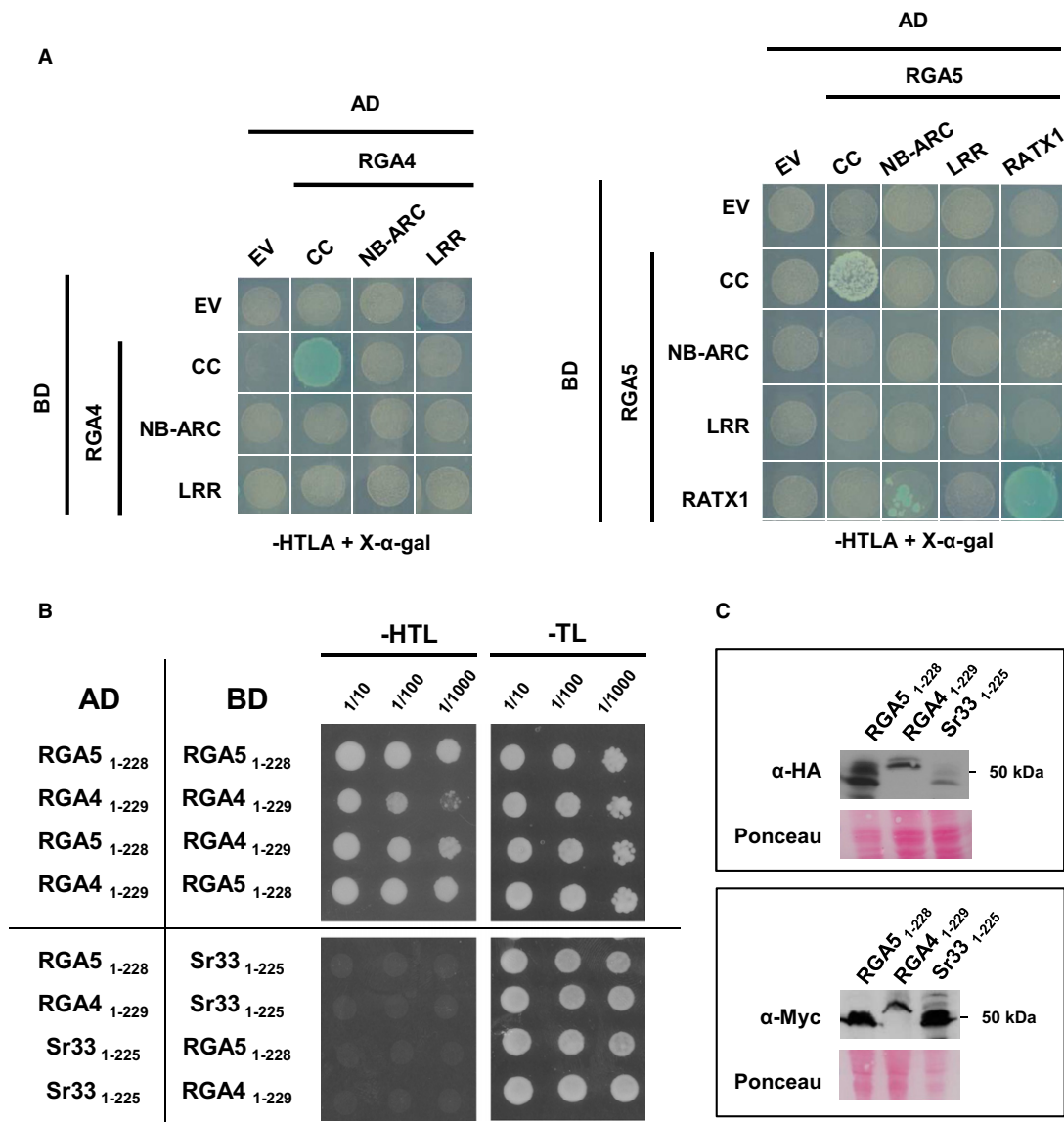
Figure 5. The CC domains of RGA4 and RGA5 form homo- and hetero-complexes and the RATX1 domain of RGA5 self-interacts in yeast.

A Pairwise interaction of CC, NB-ARC, and LRR domains from RGA4 and RGA5, as well as the RGA5 RATX1 domain (see Supplementary Fig S9A for domain borders) was evaluated by blue coloration and growth of yeast cells on basal media lacking Trp, Leu, Ade, and His and supplemented with 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-a-gal). Pictures were taken after 3 days of growth.
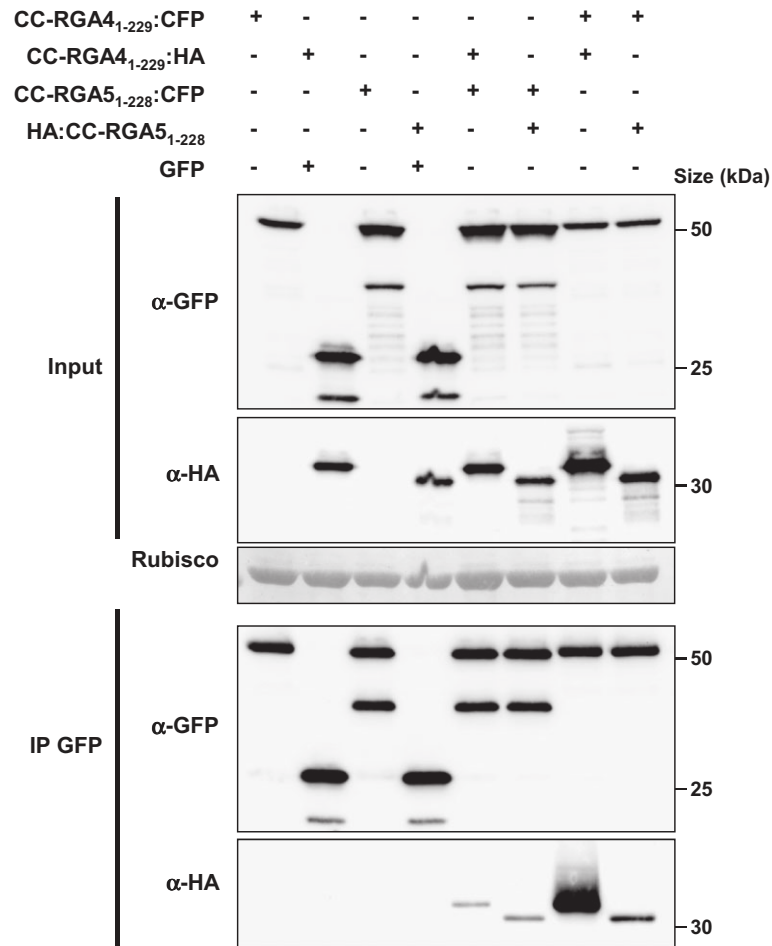
B RGA4 and RGA5 CC domain homo- and hetero-complexes. Dilution series of yeast cells expressing GAL4-AD and GAL4-BD fusions of RGA5 1–228 and/or RGA4 1–229 CC domains on non-selective synthetic media lacking Trp and Leu (-TL) and selective media additionally lacking His (-HTL). The wheat Sr33 (1–225) CC domain was used as a negative control. Pictures were taken after 3 days of growth. Growth on -HTL media is indicative of interaction.

C GAL4-AD and -BD fusions of CC domains of RGA4, RGA5, and Sr33 were detected by immunoblotting of yeast protein extracts with, respectively, anti-HA and anti-Myc antibodies. Equal loading was verified by red Ponceau staining.

Source data are available online for this figure.

of two distinct NB-LRR proteins, and not individual NB-LRRs, have been described. The first example was the TIR-NB-LRRs RPP2A and RPP2B which are both required for *A. thaliana* resistance against the *Hyaloperonospora parasitica* isolate Cala2 (Sinapidou *et al*, 2004). More recently, *A. thaliana* TIR-NB-LRRs RPS4 and RRS1 were found to be both required for resistance to multiple pathogens, by recognizing the unrelated effectors AvrRps4 from *Pseudomonas syringae,* PopP2 from *Ralstonia solanacearum,* and an uncharacterized

factor produced by *Colletotrichum higginsianum* (Gassmann *et al*, 1999; Deslandes *et al*, 2002; Narusaka *et al*, 2009). Fom1 and Prv, from melon, were recently described to provide resistance against *Fusarium oxysporum* and papaya ring-spot virus (Brotman *et al*, 2012). Interestingly, several *R* gene pairs, encoding CC-NB-LRRs, have also been characterized in monocotyledonous plants. For instance, *Lr10* and *RGA2* are both required for rust resistance in wheat (Loutre *et al*, 2009), and *Rpg5* and *HvRGA1* confer, together

Figure 6.   RGA4 and RGA5 CC domains form homo- and hetero-complexes *in planta*.
RGA4$_{1-229}$:CFP, RGA4$_{1-229}$:HA, YFP:RGA5$_{1-228}$, HA:RGA5$_{1-228}$, and *GFP* constructs were transiently expressed in *N. benthamiana*. Protein extracts were analyzed by immunoblotting with anti-HA (α-HA) and anti-GFP antibodies (α-GFP) (Input). Immunoprecipitations were performed with anti-GFP beads (IP GFP) and analyzed by immunoblotting with anti-GFP antibodies for the detection of immunoprecipitated RGA4$_{1-229}$:CFP, YFP:RGA5$_{1-228}$, and GFP proteins and with anti-HA antibodies for the detection of co-precipitated RGA4$_{1-229}$:HA and HA:RGA5$_{1-228}$ proteins.

Source data are available online for this figure.

with *HvAdf3*, rust resistance to barley (Wang *et al*, 2013). In the rice blast pathosystem, resistance protein pairs seem to be particularly frequent (Sinapidou *et al*, 2004; Ashikawa *et al*, 2008; Lee *et al*, 2009; Okuyama *et al*, 2011; Yuan *et al*, 2011; Cesari *et al*, 2013; Wang *et al*, 2013).

Why, in certain cases, two distinct plant NB-LRR proteins are required for the functions often executed by individual R-proteins and how those paired NB-LRR proteins interact functionally are important questions. Our previous investigation of RGA4/RGA5 and Pik-1/Pik-2 indicated that RGA5 and Pik-1 mediate AVR protein recognition through physical binding to their RATX1 domains and thus act as AVR receptors, while RGA4 and Pik-2 have no AVR-binding property (Kanzaki *et al*, 2012; Cesari *et al*, 2013). This suggested that each pair partner accomplishes on its own a subset of the functions frequently executed by one individual NB-LRR protein rather than cooperating with its partner to accomplish together R-protein tasks such as AVR recognition or resistance activation.

RGA4 is an activator of cell death and HR

This hypothesis is strengthened in the present study, which shows that cell death activation is mediated exclusively by RGA4. RGA4 but not RGA5 acts as a constitutively active inducer of HR-like cell death as demonstrated by transient overexpression experiments in rice and *N. benthamiana*. Constitutive activity is an intrinsic property of RGA4 and not a consequence of overexpression since silencing of RGA5 leads to RGA4 de-repression and cell death when driven by its own promotor. Interestingly, previously generated transgenic rice lines expressing *RGA4* in the Kanto51 variety lacking *Pi-a* and *Pi-CO39* resistance do not show spontaneous cell death (Okuyama *et al*, 2011; Cesari *et al*, 2013). The lack of cell death induction may be due to the fact that rice varieties lacking *Pi-a* and *Pi-CO39* resistance carry at the *Pi-a/Pi-CO39* locus, a highly homologous allele of *RGA5* and a pseudogene homologous to *RGA4* (respectively, *Os11g11810* and *Os11g11790* in the Nipponbare reference variety). This RGA5 allele *Os11g11810* lacks the RATX1
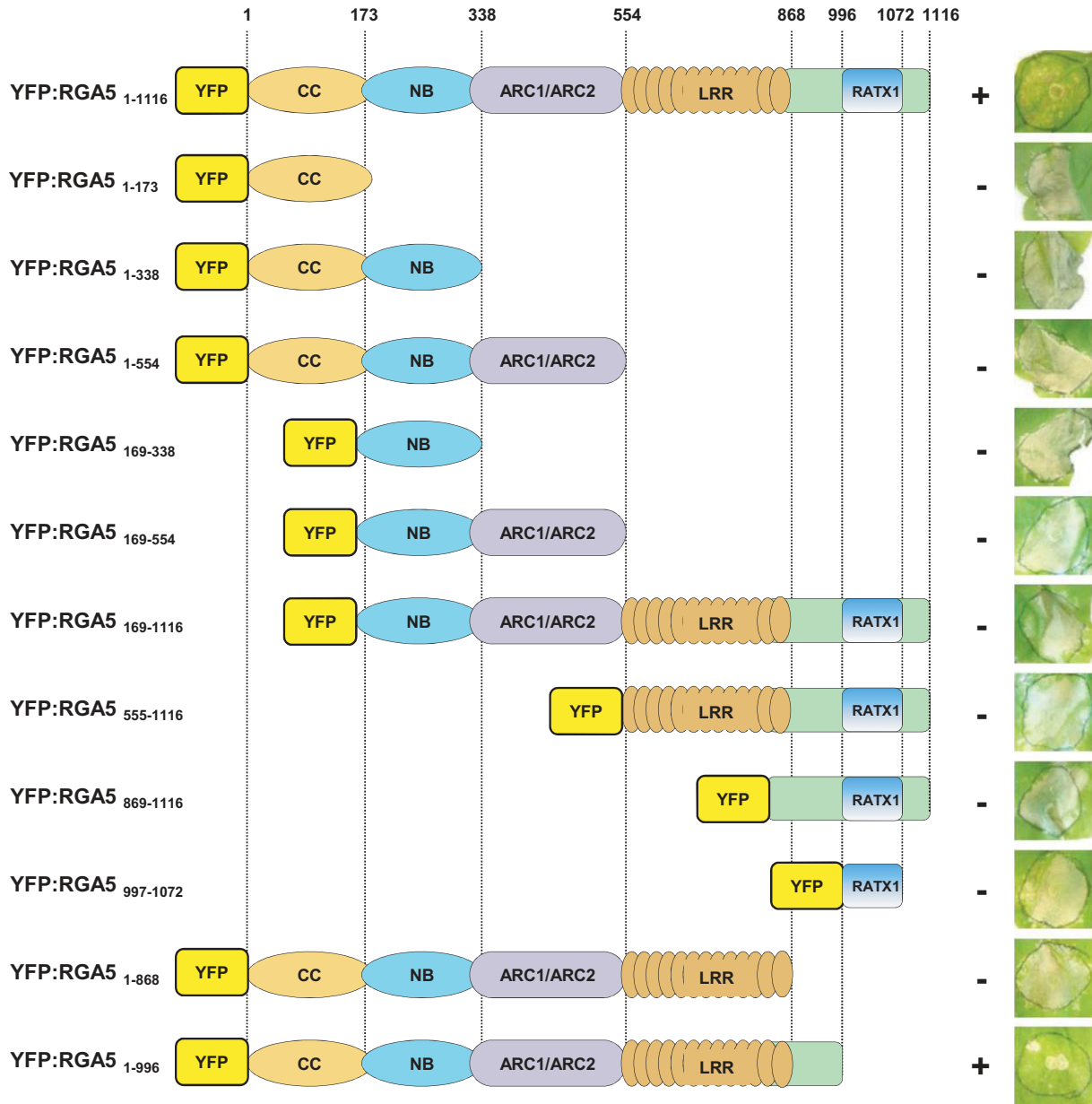
Figure 7. The RGA5 CC domain is required but not sufficient for repression of RGA4-mediated cell death, while the RATX1 domain is dispensable.
The indicated RGA5 deletion constructs were co-expressed with RGA4 in *N. benthamiana*, and development of cell death was visualized 4 days after infiltration.

sequence and is non-functional in recognizing AVR-Pia and AVR1-CO39 but may be active in repressing the product of the *RGA4* transgene since it shows high overall similarity to RGA5 (> 70% identity).

Mutation of the conserved P-loop motif abolishes RGA4 cell death and HR signaling activation, while mutation of the RGA5 P-loop motif has no such effect. This finding suggests that RGA4 activity is likely to be dependent on nucleotide binding and exchange while RGA5 activity is not.

Structurally, the constitutive activity of RGA4 could be caused by its degenerated MHD motif. Modeling studies and functional analysis suggest that the MHD motif located at the C-terminus of the

ARC2 subdomain is important for maintaining the NB-ARC domain in a compact inactive state by stabilizing ADP-binding and physical interactions with the NB-subdomain (Riedl *et al*, 2005; Van Ooijen *et al*, 2008b). In RGA4, the highly conserved histidine and aspartate are changed to uncharged amino acids, and the normally hydrophobic amino acid preceding the histidine is replaced by a hydrophilic threonine. This degeneration of the MHD motif may trap RGA4 in an active state. This is supported by the finding that introduction of a consensus MHD motif abolishes constitutive activity of RGA4.

RGA5 also carries a modified MHD domain since the conserved aspartate is replaced by a histidine. This altered MHD motif or the introduction of additional mutations usually rendering NB-LRR
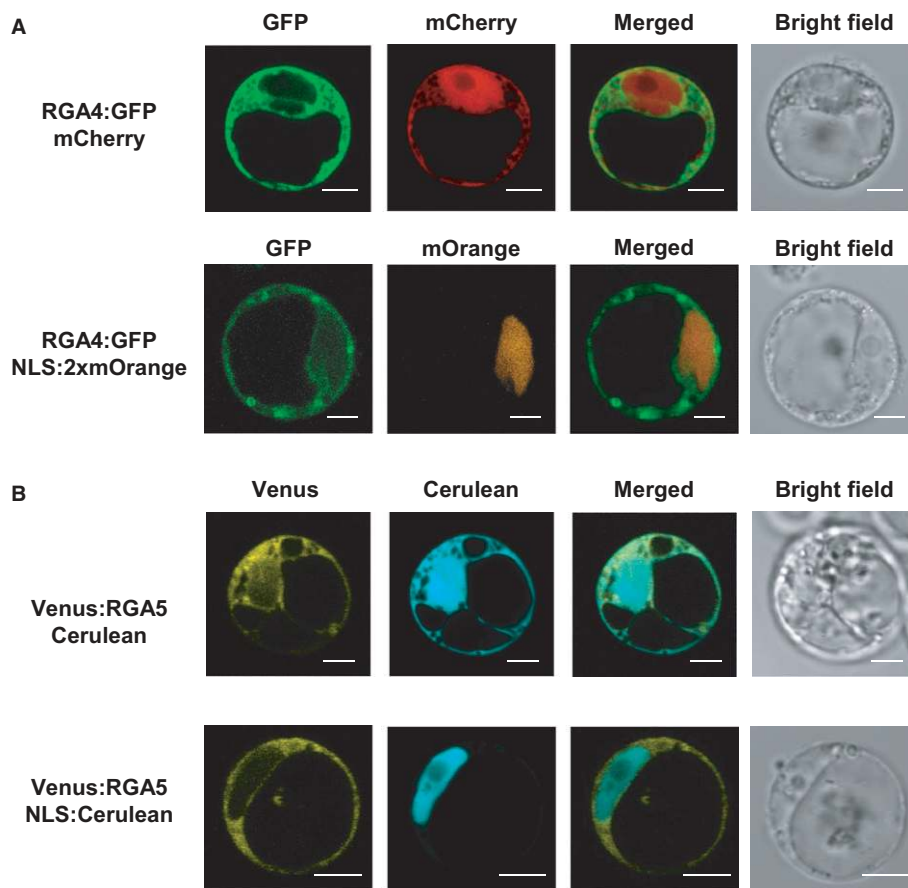
Figure 8. RGA4 and RGA5 are mainly localized in the cytosol of rice protoplasts.

A, B Rice protoplasts derived from Oc cells (Baba *et al*, 1986) were transiently transformed with combinations of constructs allowing the expression of RGA4:GFP, Venus:RGA5, NLS:2×mOrange, NLS:Cerulean, mCherry, and Cerulean. Confocal images of protoplasts were taken 16–22 h after transfection. Scale bars, 5 μm.

proteins auto-active does not lead to constitutive activity of RGA5. It may be that a closed inactive conformation of RGA5 is stabilized by additional intramolecular interactions or that RGA5 is unable to interact with downstream signaling partners and is therefore intrinsically inactive in resistance activation. This further supports the view that cell death and HR activation is mediated exclusively by RGA4.

The inspection of other characterized NB-LRR protein pairs from rice did not identify other NB-LRRs with MHD motifs degenerated as in RGA4. However, in several cases, the conserved aspartate is replaced either by an asparagine or a proline, but it remains to be determined if these changes lead to constitutive activity. It may also be that auto-activity is caused by other structural features. It remains to be investigated whether other NB-LRR pairs also rely on auto-active NB-LRRs or interact in another manner.

RGA5 regulates RGA4 in an AVR-Pia-dependent manner

Co-expression of *RGA4* and *RGA5* in rice and *N. benthamiana* shows that RGA5 inhibits RGA4-mediated cell death. This suggests that RGA5 regulates RGA4 signaling activity by acting as a repressor in the absence of recognized effectors. This regulation is specific since cell death triggered by the Orin1 CC-NB-LRR auto-active protein (Kawano *et al*, 2010) or an auto-active allele of L6 (Howles

et al, 2005) is not repressed by RGA5. Upon AVR recognition by direct binding, RGA5-mediated repression of RGA4 is relieved and cell death occurs. This is reminiscent of the interaction of other well-studied NB-LRR immune receptors with their corresponding guardees or decoys (Jones & Dangl, 2006; Van der Hoorn & Kamoun, 2008). These host proteins are operational targets of pathogen effectors or their mimic, and binding to effectors or modification by them is recognized by the corresponding NB-LRR immune receptor, generally by direct physical binding. Since there is no indication that RGA5 has other functions in disease or immunity than Avr recognition, RGA5 can be interpreted formally as a decoy surveyed by RGA4. However, AVR-Pia and AVR1-CO39 do not bind the CC, NB, or LRR domains but the uncommon RATX1 domain, and this event is sensed by the immune receptor hetero-pair. It is therefore more appropriate to consider the RATX1 domain as the true decoy integrated into one partner of the NB-LRR hetero-pair and allowing to monitor modification of other RATX1 proteins (Cesari *et al*, 2013). It will be interesting to test in the future if other hetero-pairs, such as Pik-1/Pik-2 where one of the partners contains a RATX1 domain and RRS1/RPS4 where one of the partners contains a WRKY domain, could also function by such an integrated decoy mechanism (Deslandes *et al*, 2003; Narusaka *et al*, 2009; Okuyama *et al*, 2011; Cesari *et al*, 2013).
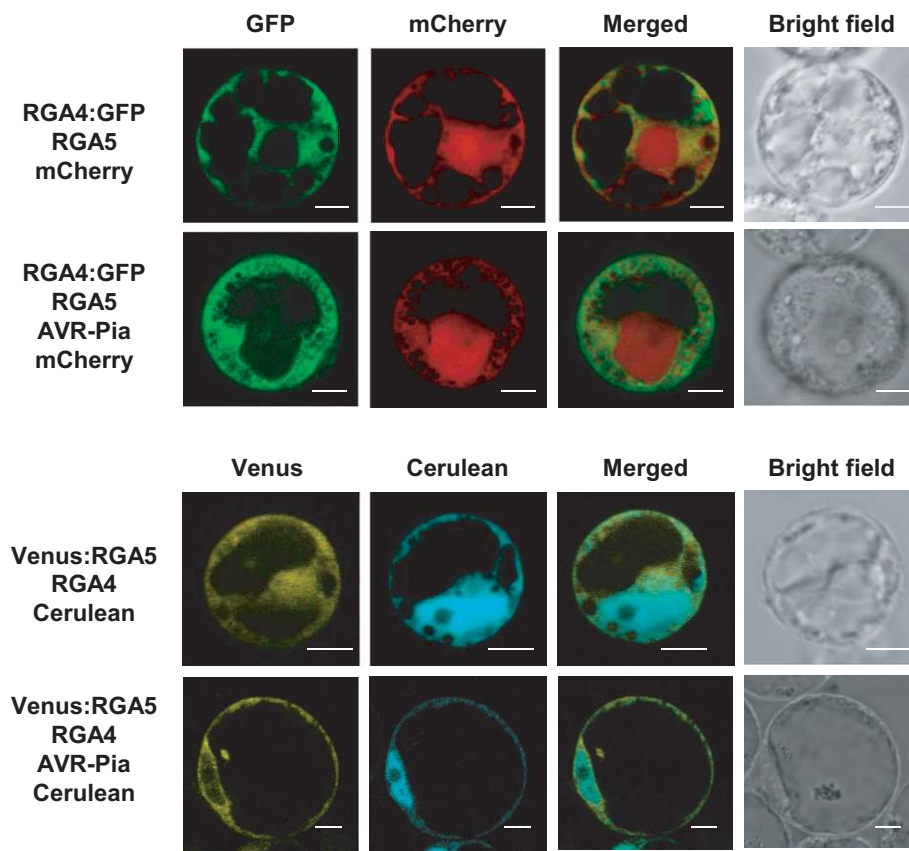
Figure 9. Localization patterns of RGA4 and RGA5 are not affected by the presence of AVR-Pia or RGA4–RGA5 co-expression.
Rice protoplasts derived from Oc cells (Baba *et al*, 1986) were transfected with combinations of constructs for the expression of RGA4:GFP, Venus:RGA5, AVR-Pia, mCherry and Cerulean, and un-labeled RGA4 and RGA5. Confocal images of protoplasts were taken 16–22 h after transfection. Scale bars, 5 μm.

RGA4 and RGA5 form hetero-complexes *in planta* which appears to be mediated, at least in part, by interaction between their CC domains. Likewise, both proteins individually form homo-complexes *in planta* which are also apparently mediated via CC domain interactions. Thus, a simple hypothesis is that the RGA4–RGA5 hetero-complex competes with and prevents formation of a signaling active RGA4 homo-complex. Binding of AVR-Pia to RGA5 could disrupt RGA4–RGA5 hetero-complexes resulting in RGA4 homo-complex formation and de-repression. Such homo-complexes are important for activation of the MLA and L6 NB-LRR proteins (Maekawa *et al*, 2011; Bernoux *et al*, 2011b). However, co-immuno-precipitation of RGA4 by RGA5 is still observed in the presence of AVR-Pia. Therefore, under this hypothesis, it must be assumed that disruption of a portion of the complexes frees sufficient RGA4 to signal cell death. An alternative model is that AVR-Pia binding to the RATX1 domain of RGA5 induces conformational changes within the intact RGA4–RGA5 hetero-complexes leading to their transition from a repressed to an activated state.

The dissection of RGA4 and RGA5 homo- and hetero-complex formation indicates that they are mainly mediated through interactions between the CC domains. This is consistent with the finding that removal of the RGA5 CC domain leads to a loss of RGA4 repression and the previous finding that MLA10 homo-complex formation is mediated by its CC domain (Maekawa *et al*, 2011). It will be interesting to analyze in the future the impact of mutations in the RGA4

and RGA5 CC domains on homo- and hetero-complex formation and on cell death activation and repression.

Striking similarities in the interaction of the RPS4–RRS1 and RGA4–RGA5 hetero-pairs

The RGA4–RGA5 interaction shows striking similarities to the interaction of RPS4 and RRS1 described in a recent publication (Williams *et al*, 2014). RPS4 was shown to act as RGA4 as an activator of resistance and cell death, while RRS1 represses RPS4 and binds the Avr proteins PopP2 and AvrRPS4 and resembles in this sense RGA5. RPS4 and RRS1 interact physically, and RPS4–RRS1 hetero-complexes are not or not completely disrupted by Avr protein recognition. The N-terminal TIR domains of RPS4 and RRS1 are important for resistance signaling and repression and form hetero- and homo-dimers like the RGA4–RGA5 CC domains. A P-loop mutation in RPS4 leads to loss of resistance, while an RRS1 P-loop mutant is fully functional (Williams *et al*, 2014). It therefore looks as if different NB-LRR hetero-pairs possessing different types of N-terminal domains interact molecularly and functionally in a similar manner.

Differences between RPS4 and RGA4 can, however, also be observed. RPS4 has a canonical MHD motif and causes cell death in a temperature-sensitive manner when overexpressed in *A. thaliana* but not when expressed from its own promoter (Wirthmueller *et al*,

2007). It will therefore be interesting to investigate in different hetero-pairs in detail the link between effector recognition and activation of signaling.

Similarities exist also with the *A. thaliana* NB-LRR proteins ADR1 and ADR1-like 1 and 2 (ADR1-L1 and -L2) that interact functionally with NB-LRR immune receptors and are required for their activity (Bonardi *et al*, 2011). However, on the contrary to RGA5 or RRS1, ADR1 proteins are not involved in Avr recognition, and an MHD mutant of ADR1-L2 shows auto-immune responses. On the contrary to RGA4 and RPS4, ADR1 proteins interact not just with one but with several different NB-LRR immune receptors that are physically not linked to them, they are required for certain but not all downstream responses, and a p-loop mutation in ADR1-L2 does not impair its activity in resistance signaling (Bonardi *et al*, 2011). ADR1 proteins are therefore considered as helper NB-LRRs and define another type of NB-LRR interactions, different from NB-LRR hetero-pairs such as RAG4–RGA5 and RPS4–RRS1.

Genomic organization: a key feature for proper control and co-evolution of NB-LRR pairs?

The RGA4-RGA5 system which contains a constitutively active cell death activator is potentially dangerous for the plant and requires tight control since loss or modification of RGA5 may lead to inappropriate activation of immune responses and cell death. Other NB-LRR proteins, that are harmless in their native genetic context, were demonstrated to cause constitutive activation of the plant immune system and trigger hybrid necrosis after inter- or intraspecific crosses (Bomblies *et al*, 2007). The clustered genomic organization of *RGA4-RGA5* in an inverted head-to-head orientation in a 7-kb interval where they share the same intergenic region reduces the risk of misregulation and modification or loss of one partner by recombination or genomic rearrangements. However, it is intriguing that the same genomic organization as in *RGA4-RGA5* is found in almost all *R* gene pairs identified, although they are not phylogenetically related. Investigation of other NB-LRR pairs is needed to determine whether clustered organization is a universal feature of *NB-LRR* gene pairs to favor efficient transmission of resistance after sexual reproduction or whether it is restricted to those that code constitutively active cell death inducers and related to the control of inappropriate activation of immune responses.

Convergent evolution generated NB-LRR pairs in animals and plants

Interestingly, cooperation of two distinct NB-LRR proteins in the recognition of pathogens is not restricted to plants but also occurs in mammals. Activation of immune responses in mice by the inner rod protein of the type III secretion systems (T3SS) of diverse bacterial species (for instance PrgJ of *Salmonella typhimurium*) requires the cytoplasmic NB-LRR proteins NLRC4 and NAIP2 (NLR family, apoptosis inhibitory protein 2), whereas NLRC4 and NAIP5 are required for recognition of bacterial flagellin in the cytoplasm of mouse macrophages and activation of downstream immune responses (Kofoed & Vance, 2011; Zhao *et al*, 2011). Biochemical dissection of the NLRC4/NAIP system showed that NAIP2 physically and specifically associates with T3SS inner rod proteins from different bacteria, whereas NAIP5 specifically associates with

C-terminal sequences of flagellin. In both cases, ligand binding induces NAIP–NLRC4 association followed by NLRC4 oligomerization and activation of downstream responses such as caspase activation, interleukin maturation and release, and pyroptosis in an NLRC4-dependent manner (Kofoed & Vance, 2011; Zhao *et al*, 2011; Von Moltke *et al*, 2013).

The RGA4/RGA5 pair resembles in many instances to the mammalian NLRC4/NAIP system and adds thus one more piece to the striking similarities between plant and animal NB-LRR proteins in domain architectures and molecular and biological functions. These similarities appear even more appealing if one considers that evolutionary analysis strongly suggests that mammalian and plant NB-LRR proteins appeared independently by convergent evolution in plants and animals (Ausubel, 2005; Yue *et al*, 2012). The frequent observation of NB-LRR pairs in mammals and plants may thus indicate that interaction of two different NB-LRR proteins is a fundamental element of NB-LRR protein function.

Materials and Methods

Growth of plants

For leaf protoplast preparation, etiolated rice plants (*Oryza sativa* L.) were grown as described (Chen *et al*, 2006). Faivre-Rampant *et al* (2008) *Nicotiana benthamiana* plants were grown in a growth chamber at 22°C with a 16-h light period.

Constructs

All PCR products used for cloning were generated using Phusion High-Fidelity DNA Polymerase (Finnzymes) or PrimeSTAR GLX DNA polymerase (Takara Bio) using primers listed in Supplementary Table S1. Molecular cloning was performed by restriction-ligation, Gateway recombination (Invitrogen) or Quickchange site-directed Mutagenesis (Stratagene) as detailed in Supplementary Table S2. For yeast two-hybrid analysis, pGBKT7-BD and pGADT7-AD (Clontech) or Gateway-compatible vectors derived from pGADT7 and pGBKT7 were used (Bernoux *et al*, 2011b). For the creation of Gateway entry clones, pDONR207 or pENTR D-TOPO (Invitrogen) were used. For agro infiltration experiments, pBIN19-35S::GTW:3HA, pBIN19-35S:: YFP:GTW, pBIN19-35S::GTW:CFP, pBIN19-35S::HA:GTW (Cesari *et al*, 2013), and pMCD83-35S::GTW:GFP (Curtis & Grossniklaus, 2003) were used. Constructs used for protoplast transfection were generated with pAHC17-pUbi (Yoshida *et al*, 2009; Okuyama *et al*, 2011), p35s:GTW (Takahashi *et al*, 2009), p35s::GTW:GFP (Akamatsu *et al*, 2013), p35s::Venus:GTW (Kawano *et al*, 2010), pGII pUbi::GW-T7, and pGII pUbi::10xMyc-GW (Taoka *et al*, 2011). Constructs used for RNAi in rice protoplasts were generated in pENTR D-TOPO (Invitrogen) and inserted in pPANDA (Miki & Shimamoto, 2014) by LR gateway reaction. Details of constructs and primers are given in Supplementary Tables S1 and S2.

Yeast two-hybrid assays

BD or AD constructs used in Fig 6A and Supplementary Fig S10 were transformed into the yeast strain AH109. Co-transformants were plated on synthetic medium lacking leucine, tryptophan,

adenine, and histidine (supplemented with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and incubated at 28°C for 3 days.

AD or BD fusions of RGA5$_{1-133}$, RGA5$_{1-167}$, RGA5$_{1-228}$, RGA4$_{1-130}$, RGA4$_{1-164}$, RGA4$_{1-229}$, and Sr33$_{1-225}$ constructs used in Fig 6B and Supplementary Fig S11 were transformed into HF7c yeast strain as described in the Yeast Protocols Handbook (Clontech).

Transient protein expression and cell death assays 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 rifampicin, 15 mg/ml gentamycin, and 25 mg/ml kanamycin at 28°C for 24 h. Bacteria were harvested by centrifugation, resuspended in infiltration medium (10 mM MES pH 5.6, 10 mM MgCl$_2$, 150 µM acetosyringone) to an OD$_{600}$ of 1, and incubated for 2 h at room temperature before leaf infiltration. The infiltrated plants were incubated in growth chambers under controlled conditions for co-immunoprecipitation experiments and cell death assays, respectively. For documentation of cell death, leaves were photographed or scanned with a Typhoon FLA9000 fluorescence scanner (GE Healthcare) with excitation at 635 nm and use of a long-pass red filter (LPR-665 nm) 3–5 days after infiltration.

Confocal microscopy

Protoplast isolation from rice Oc suspension culture and protoplast transformation using polyethylene glycol (PEG) method were performed as described (Chen *et al*, 2006). 2.5 µg of RGA4 or 5 µg of RGA5 expression vector with 2 µg of subcellular localization markers was mixed with 0.1 ml of aliquots of 2 × 10^6 cells. After incubation from 16 to 22 h at 30°C, the protoplasts were observed under a confocal microscope (FV1000; Olympus). Spectral settings for detection of each fluorescence proteins were as follows: CFP; excitation, 458 nm, detection, 475–500 nm, GFP; excitation, 488 nm, detection, 500–525 nm, Venus; excitation, 515 nm, detection, 530–560 nm, mOrange; excitation, 543 nm, detection, 555–600 nm, mCherry; excitation, 543 nm, detection, 610–650 nm.

Protein extraction, Western blot and co-immunoprecipitation

Yeast protein extraction for immunoblotting analysis was performed following a post-alkaline extraction method (Kushnirov, 2000). Protein fusion detection was performed using anti-HA-HRP (Roche, clone 3F10) and anti-Myc (Roche, clone 9E10) antibodies.

Protein extracts of *N. benthamiana* leaves and related immunoblotting and co-IP experiments were performed as described (Cesari *et al*, 2013).

For rice protoplast co-immunoprecipitation assays, a large-scale protoplast transformation procedure was used. DNA plasmids (50 µg of pUbi::RGA4-T7 and 250 µg of pUbi::10xMyc-RGA5 or 10 µg of pUbi::10xMyc-GFP) were mixed with 1 ml of aliquots of 5 × 10^6 cells in each sample (Chen *et al*, 2006). Protein extract of rice protoplast was prepared in protein extraction buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 5 mM MgCl$_2$, 10% glycerol, 1% (w/v) Triton X-100, phosphatase inhibitor cocktail 2, phosphatase inhibitor cocktail 3 and 1× cOmplete, EDTA free or

1× cOmplete ULTRA, EDTA free). Cell debris was removed by centrifugation, and extracts were immunoprecipitated by using the µMacs c-Myc isolation kits and µMacs GFP isolation kits (Miltenyi Biotec). Samples were separated by SDS-PAGE and subjected to immunoblotting using anti-c-Myc antibody (Nacalai Tesque, Inc.), anti-T7 antibody (MBL) or anti-GFP antibody (Abcam), and secondary antibody conjugated to horseradish peroxidase (GE Healthcare).

Cell viability assay in rice protoplasts

Rice protoplasts were prepared as described previously from rice-etiolated seedlings of cultivars Himenomochi (Yoshida *et al*, 2009) or rice Oc cells (Chen *et al*, 2006). The firefly luciferase gene (*LUC*) expressed under the control of the maize ubiquitin promoter was used as a reporter to monitor protoplasts viability. Specified combinations of constructs were co-transfected together with the LUC plasmid into rice protoplasts by electroporation or polyethylene glycol (PEG) method (Chen *et al*, 2006; Yoshida *et al*, 2009). Luciferase activity was measured 40 h after transfection using a luciferase assay system (Promega), and the reduction in luminescence was compared with a control comprising empty or Venus expression vector-transfected protoplasts.

Author contributions

SC, HK, TF, RT, and TK designed the research. SC, HK, TF, MB, VC, and TK performed the research. SC, HK, TF, MB, RT, PD, and TK analyzed the data. SC and TK wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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