RCY1*-Mediated Resistance to *Cucumber mosaic virus* Is Regulated by LRR Domain-Mediated Interaction with CMV(Y) Following Degradation of *RCY1

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RCY1, which encodes a coiled coil nucleotide-binding site leucine-rich repeat (LRR) class R protein, confers the hypersensitive response (HR) to a yellow strain of *Cucumber mosaic virus* (CMV[Y]) in *Arabidopsis thaliana*. *Nicotiana benthamiana* transformed with hemagglutinin (HA) epitope-tagged *RCY1* (*RCY1-HA*) also exhibited a defense response accompanied by HR cell death and induction of defense-related gene expression in response to CMV(Y). Following transient expression of *RCY1-HA* by agroinfiltration, the defense reaction was induced in *N. benthamiana* leaves infected with CMV(Y) but not in virulent CMV(B2)-infected *N. benthamiana* leaves transiently expressing *RCY1-HA* or CMV(Y)-infected *N. benthamiana* leaves transiently expressing HA-tagged *RPP8* (*RPP8-HA*), which is allelic to *RCY1*. This result suggests that *Arabidopsis RCY1*-conferred resistance to CMV(Y) could be reproduced in *N. benthamiana* leaves in a gene-for-gene manner. Expression of a series of chimeric constructs between *RCY1-HA* and *RPP8-HA* in CMV(Y)-infected *N. benthamiana* indicated that induction of defense responses to CMV(Y) is regulated by the LRR domain of *RCY1*. Interestingly, in CMV(Y)-infected *N. benthamiana* manifesting the defense response, the levels of both *RCY1* and chimeric proteins harboring the *RCY1* LRR domain were significantly reduced. Taken together, these data indicate that the *RCY1*-conferred resistance response to CMV(Y) is regulated by an LRR domain-mediated interaction with CMV(Y) and seems to be tightly associated with the degradation of *RCY1* in response to CMV(Y).

Our current knowledge of the plant immune system indicates that plants use pattern recognition receptors to initially recognize conserved pathogen-associated molecular patterns (PAMPs) of fungal and bacterial pathogens, and thereby induce active defense responses that reduce disease severity (PAMP-triggered immunity [PTI]) (Hammond-Kosack and Parker 2003; Jones and Dangl 2006; McDowell and Simon 2008). Potential fungal and bacterial pathogens enhance their virulence

by secreting pathogen effectors that either reduce or completely suppress PTI (Chisholm et al. 2006). When some effector molecules are specifically detected by a resistance (R)-protein-mediated complex, a defense reaction (also known as effector-triggered immunity [ETI]) that includes the hypersensitive response (HR) is then activated.

This two-tiered immune system may also be manipulated to respond to viral pathogens. In virus-infected plants, an abundance of foreign and double-stranded viral RNAs seem to be recognized as conserved molecule patterns that induce antiviral RNA silencing, which is considered a virus-specific version of PTI (Ding 2010; Ding and Voinnet 2007). Because RNA silencing is inhibited by virus-encoded suppressors of silencing, viruses are able to overcome PTI and establish infection (Diaz-Pendon and Ding 2008). In such an instance, R proteins recognize a broad array of viral proteins, including coat proteins, movement proteins, replicases, and silencing suppressors, as a second line of defense that corresponds to ETI (Cournoyer and Dinesh-Kumar 2011).

R genes confer resistance to many evolutionarily diverse pathogens; however, many of them encode a protein possessing conserved nucleotide binding site (NB) and leucine-rich repeat (LRR) domains that interact similarly with this array of pathogens (Dangl and Jones 2001; Hammond-Kosack and Jones 1997). Thus far, nine virus R genes encoding NB-LRR class R proteins have been cloned from *Arabidopsis thaliana* and from species within the Solanaceae (Cournoyer and Dinesh-Kumar 2011; Kachroo et al. 2006; Tomita et al. 2011). Most of these cloned viral R genes, including *Rx*, *Rx-2*, *Sw-5*, *Tm2*, *Tm2²*, *HRT*, *RCY1*, and *L*, encode a coiled-coil (CC)-NB-LRR protein, whereas only *Tobacco mosaic virus* (TMV) resistance protein N contained an anti-viral Toll interleukin receptor (TIR)-NB-LRR (Whitham et al. 1994).

NB-LRR proteins can be activated upon recognition of fungal and bacterial effectors and viral proteins either directly or indirectly (Dodds and Rathjen 2010; Jones and Dangl 2006). In several cases, this recognition seems to be mediated through a cellular cofactor interacting with both the NB-LRR protein and the pathogen effector (Collier and Moffett 2009; Mukhtar et al. 2011), which then activates NB-LRR proteins. The primary interaction between NB-LRR proteins and pathogen effectors or recognition cofactors has been shown to be effected by the LRR domain in domain-swapping experiments between closely related NB-LRR proteins with differing rec-

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ognition specificities (Ellis et al. 2007; Hwang and Williamson 2003; Raidan and Moffett 2006; Shen et al. 2003). In addition, recent studies have suggested that the N-terminal CC or TIR domains of NB-LRR proteins also function as cofactors for TMV and *Potato virus X* recognition (Burch-Smith et al. 2007; Caplan et al. 2008; Collier and Moffett 2009; Raidan et al. 2008).

Cucumber mosaic virus (CMV) is one of the best-characterized tripartite positive-sense single-stranded RNA viruses (Palukaitis and García-Arenal 2003). Viral silencing suppressor 2b, which is translated from subgenomic RNA4A transcribed from the second open reading frame of RNA2, inhibits antiviral RNA silencing via PTI in CMV-infected host plants (Palukaitis and García-Arenal 2003). Thus, several strains of CMV can overcome antiviral RNA silencing systems by synthesizing the 2b protein to systemically infect host plants. *A. thaliana* ecotypes host various CMV strains. However, *A. thaliana* ecotype C24 is resistant to a yellow strain of CMV (CMV[Y]), to which it exhibits the HR, but is susceptible to an Indonesian strain, CMV(B2). In contrast, *A. thaliana* ecotype Col-0 can be systemically infected by either strain (Takahashi et al. 1994, 2001). Resistance to CMV(Y) in C24 is conferred by the combination of the viral coat protein and a CC-NB-LRR class R protein, RCY1 (resistance to CMV[Y]) (Sekine et al. 2006; Takahashi et

al. 2002). This *RCY1*-conferred resistance to CMV(Y) comprises an ETI-based two-tiered immune system response. *RCY1*-mediated resistance to CMV(Y) is accompanied by the development of necrotic local lesions at the primary infection sites, elevated expression of defense-related genes (e.g., pathogenesis-related [*PR*]-1a), and accumulation of salicylic acid (Ishihara et al. 2008; Takahashi et al. 2004). Increased expression of *RCY1* results in conversion of the HR resistance to an extreme resistance to CMV(Y) (Sekine et al. 2008), suggesting that the level of RCY1 protein regulates the strength of resistance to CMV(Y) in *Arabidopsis*. However, a need for further functional analysis of the RCY1 protein remains.

NB-LRR proteins exhibit narrow recognition specificity against pathogens. To recognize a wide range of pathogens, several NB-LRR genes are often arranged in clusters that originate by tandem duplications due to unequal crossing over and gene conversion, while some NB-LRR genes are unique in some plant genomes (Holub 2001). In the *Arabidopsis* genome, approximately 150 genes encode NB-LRR proteins (Bakker et al. 2006). Interestingly, *RCY1* is allelic to *HRT*, a gene for resistance to *Turnip crinkle virus* in ecotype Di-17, and to *RPP8*, a gene for resistance to *Hyaloperonospora arabidopsidis* in ecotype Landsberg (Cooley et al. 2000; McDowell et al. 1998; Takahashi et al. 2002). Thus, the *RCY1/HRT/RPP8* locus may

Fig. 1. Phenotypes of *RCY1-HA*-transformed *Nicotiana benthamiana* lines accumulating *RCY1-HA* protein at differential levels. **A**, Phenotype of *N. benthamiana* lines (numbers 10-5, 10-7, and 10-2) transformed with genomic *RCY1* DNA fused to a sequence encoding a hemagglutinin (HA) epitope at the 3' end (*g.RCY1-HA*). **B**, Spontaneous cell death in transgenic line 10-21 showing ectopic over-accumulation of *RCY1-HA*. **C**, Visualization of spontaneous cell death by trypan blue staining in fully expanded leaves of transgenic lines 10-7 and 10-21. **D**, Immunological detection of *RCY1-HA* protein in four transgenic lines (*Nb::g.RCY1-HA* numbers 10-5, 10-7, 10-2, and 10-21) using an anti-HA antibody (α -HA). Rubisco stained with Coomassie Brilliant Blue R-250 was shown as an internal control.

have evolved from an ancient NB-LRR gene and, subsequently, diversified among these three different ecotypes to specifically recognize three different pathogens (Kuang et al. 2008).

Nicotiana benthamiana is the most widely used experimental host in plant virology (Goodin et al. 2008). In this study, we characterized the defense responses to CMV(Y) of *RCY1*-transformed *N. benthamiana*, and then analyzed the major determinant in the *RCY1* protein for CMV(Y) recognition specificity by transient expression of a series of chimeric *RCY1/RPP8* constructs in *N. benthamiana*. Our results demonstrate that the induction of HR in *N. benthamiana* was likely to be tightly associated with the degradation of the *RCY1* protein, which seems to be regulated by recognition of CMV(Y) by the LRR domain of *RCY1*.

RESULTS

Phenotype of *RCY1*-HA-transformed *N. benthamiana*.

N. benthamiana was transformed with a genomic clone of the *RCY1* gene fused to a sequence encoding a hemagglutinin (HA) epitope at its 3' end (*g.RCY1*-HA), under the control of the approximately 1.5-kb promoter region of the genomic *RCY1* sequence. Among the 20 independent transgenic lines initially produced, five lines (numbers 10-2, 10-5, 10-7, 10-15, and 10-21) displaying distinct phenotypes were selected (Fig. 1A and B; Supplementary Fig. S1A). The level of *g.RCY1*-HA expression varied among these transgenic lines (Figs. 1D and 2A). A high level of *RCY1*-HA protein accumulated in *g.RCY1*-HA-

transgenic line 10-21, which showed a severe dwarf phenotype, whereas line 10-7, which contained a relatively lower level of *RCY1*-HA protein, appeared normal, as did line 10-5, in which levels of *RCY1*-HA protein were below detectable levels (Fig. 1A, B, and D). The accumulated level of *RCY1*-HA protein in line 10-2 showing a dwarf phenotype seems to be intermediate between those of lines 10-21 and 10-7 (Fig. 1A and D). Furthermore, spontaneous cell death was likely to develop in fully expanded leaves of the *RCY1*-overexpressing line 10-21 (Fig. 1B), whereas fully expanded leaves of number 10-2 showed little cell death (Fig. 1A). This cell death in line 10-21 could be visualized by trypan blue staining but not in line 10-7 (Fig. 1C).

To clarify the relation of the degree of dwarfing and the accumulated level of *RCY1*-HA protein, the progenies of transgenic line 10-15 were used. The progenies of transgenic line 10-15, which were designated numbers 10-15-1, 10-15-2, and 10-15-4, segregated for varying degrees of dwarfing and accumulated various levels of *RCY1*-HA protein (Fig. 2A). The differential phenotype in the progeny lines was probably caused by segregation of several transgenic inserts with varying levels of expression in parent line 10-5 plants into the progenies. When *PR-1a* gene expression was analyzed in these lines, the *PR-1a* transcript was abundant in *g.RCY1*-HA-overexpressing transgenic line 10-15-4, which exhibited a severe dwarf phenotype with spontaneous cell death visualized by trypan blue staining (Fig. 2A to C). On the other hand, the cell death and *PR-1a* transcript were not detected in non-dwarf transgenic

Fig. 2. Induction of pathogenesis-related (*PR*)-1a in *Nicotiana benthamiana* lines differentially accumulating the *RCY1*-HA protein. Accumulation of *RCY1*-HA protein, induction of spontaneous cell death, and *PR-1a* expression were analyzed in three progenies (Nb::g.*RCY1*-HA numbers 10-15-1, 10-15-2, and 10-15-4) of *g.RCY1*-HA-transformed *N. benthamiana* lines 10-15 and nontransgenic control (wild type [wt]). **A**, Immunological detection of *RCY1*-HA protein in three progenies (Nb::g.*RCY1*-HA numbers 10-15-1, 10-15-2, and 10-15-4) of transgenic line 10-15 using an anti-hemagglutinin antibody (α -HA). Rubisco was detected as an internal protein loading control. **B**, Induction of *PR-1a* expression in three progenies (Nb::g.*RCY1*-HA 10-15-1, 10-15-2, and 10-15-4) of transgenic line 10-15. *rRNA* was detected as an internal RNA control. **C**, Amount of *RCY1*-HA protein in Nb::g.*RCY1*-HA 10-15-1, 10-15-2, and 10-15-4. The relative amount of *RCY1*-HA protein in three individual plants derived from each transgenic line (10-15-1, 10-15-2, and 10-15-4), which was segregated from parent transgenic line 10-15 and nontransgenic control (wt), was quantified by measurement of the band intensity visualized with the chemiluminescent substrate ECL-Prime in immunoblotting. The average and standard deviation (SD) of the band intensity in triplicate immunoblotting of each progeny were shown.

line 10-15-1, which accumulated the least RCY1-HA protein (Fig. 2A to C; Supplementary Fig. S2A). In transgenic line 10-15-2, which accumulated to intermediate levels of RCY1-HA protein (Fig. 2A and C), *PR-1a* expression was induced at a level intermediate between that seen in lines 10-15-1 and 10-15-4 (Fig. 2B). Thus, the accumulated level of RCY1-HA protein appeared to be correlated with the degree of dwarfing in each transgenic line, with auto-activation of cell death, and with induction of *PR-1a* gene expression. These results indicate that the overaccumulation of the RCY1-HA protein seems to auto-activate the defense response independently on infection with CMV(Y) in transgenic *N. benthamiana*.

Response of *RCY1-HA*-transformed *N. benthamiana* to CMV(Y).

When *g.RCY1-HA*-transformed *N. benthamiana* line 10-15-1 was inoculated with CMV(Y), HR-like cell death developed in virus-inoculated leaves at 4 days after inoculation (Fig. 3A). However, cell death was not observed in leaves inoculated with the virulent CMV(B2) strain, although the virus coat protein accumulated to the same level in both CMV(Y)- and

CMV(B2)-inoculated leaves (Fig. 3A and B). HR-like cell death also developed in inoculated leaves four days after CMV(Y) inoculation in *g.RCY1-HA*-transformed *N. benthamiana* lines 10-7 and 10-2, which show no or little spontaneous cell death but do accumulate RCY1-HA protein, and line 10-5, neither showing spontaneous cell death nor accumulating detectable levels of RCY1-HA (Fig. 1A and D). Interestingly, in line 10-7, HR-like cell death also appeared at noninoculated upper leaves at 10 days after CMV(Y) inoculation, suggesting that virus may partially escape from virus-inoculated leaves and, thereby, spread and induce HR-like cell death in noninoculated upper leaves.

To confirm whether the resistance response was induced in CMV(Y)-inoculated leaves in *g.RCY1-HA*-transformed plants, defense-related gene expression was analyzed in line 10-15-1. The expression of *PR-1a* and *PR-1b* was clearly induced at 48 h after CMV(Y) inoculation but not after inoculation with virulent CMV(B2) (Fig. 3C and D). Thus, in *N. benthamiana*, the HR resistance reaction seems essentially to be activated by the combination of RCY1-HA protein and CMV infection in a gene-for-gene manner.

Fig. 3. Induction of hypersensitive response (HR) cell death and defense-related gene expression in *g.RCY1-HA*-transformed *Nicotiana benthamiana* lines infected with CMV(Y). **A**, Response of *g.RCY1-HA*-transformed *N. benthamiana* line number 10-15-1 to CMV(Y) and CMV(B2). A single leaf from line 10-15-1 was partially inoculated with CMV(Y), CMV(B2), and 0.05 mM phosphate buffer (pH 7.0) as a control (Mock). At 4 days after inoculation, the virus-inoculated leaf was photographed under bright field (left panel) and then stained with trypan blue for visualization of cell death (right panel). **B**, Immunological detection of the virus coat protein in leaf (Nb::g.RCY1-HA 10-15-1) inoculated with CMV(Y), CMV(B2), or mock control. The virus coat protein in the inoculated *N. benthamiana* leaf was detected immunologically with an anti-coat protein antibody (α-CP). Rubisco was detected as an internal protein loading control. **C**, Detection of pathogenesis-related (*PR*)-1*a* and *PR*-1*b* transcripts in a leaf (Nb::g.RCY1-HA 10-15-1) inoculated with CMV(Y), CMV(B2), or mock control at 48 h after inoculation by Northern hybridization. rRNA was detected as an internal RNA control. **D**, Time course of induction of *PR*-1*a* and *PR*-1*b* expression in a CMV(Y)-inoculated leaf (Nb::g.RCY1-HA 10-15-1) of line 10-15-1. Transcripts of *PR*-1*a* and *PR*-1*b* were analyzed at 0, 24, 48, and 72 h after CMV(Y) inoculation (hpi) by Northern hybridization. rRNA was detected as an internal RNA control.

Auto-activation of cell death by transient expression of *RCY1*-HA, but not by *RPP8*-HA.

When *RCY1* cDNA fused to a sequence encoding an HA epitope at the 3' end (*RCY1*-HA; Fig. 4) was transiently expressed under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter in *N. benthamiana* by the agroinfiltration method, cell death appeared in the infiltrated region where *RCY1*-HA protein accumulated (Fig. 5A and B). However, in a *N. benthamiana* leaf transiently expressing the cDNA for *RPP8* fused to a sequence encoding an HA epitope at the 3' end (*RPP8*-HA; Fig. 4), although the accumulated level of the *RPP8*-HA protein was similar to that of the *RCY1*-HA protein (Fig. 5B), cell death did not develop at the region infiltrated with *RPP8*-HA (Fig. 5A).

To identify the domain or domains controlling auto-activation of cell death in *N. benthamiana* transiently expressing *RCY1*-HA, four kinds of chimeric cDNAs were constructed by exchanging CC- and LRR-encoding domains between cDNAs for *RCY1*-HA and *RPP8*-HA. Four pRI201-AN binary vectors, in which each chimeric cDNA was cloned under the CaMV 35S promoter, were designated *C/C/P*-HA, *C/P/P*-HA, *P/C/C*-HA, and *P/P/C*-HA (Fig. 4). When *RCY1*-HA, *RPP8*-HA, and these four chimeric constructs were transiently expressed by agroinfiltration in a single leaf of *N. benthamiana*, cell death was only induced in the region agroinfiltrated with *RCY1*-HA (Fig. 5C) but not in the corresponding regions agroinfiltrated with these four chimeric constructs and *RPP8*-HA, even though those proteins accumulated to a level similar to that of *RCY1*-HA (Fig. 5D). These results indicate that each and every one of the CC, NB, and LRR domains of the *RCY1* protein seem to be indispensable for induction of CMV(Y)-independent auto-activation of cell death in *N. benthamiana* leaves.

Response of CMV(Y)-infected *N. benthamiana* leaves transiently expressing *RCY1*-HA or *RPP8*-HA by agroinfiltration.

To confirm whether cell death was induced in CMV(Y)-inoculated leaves of *N. benthamiana* transiently expressing *RCY1*-

HA or *RPP8*-HA, one side of a single, fully expanded leaf of *N. benthamiana* was infiltrated with *Agrobacterium* harboring *RCY1*-HA and the other side was done with *RPP8*-HA at 48 h after CMV(Y) inoculation. HR-like cell death appeared in the CMV(Y)-inoculated region transiently expressing *RCY1*-HA (Fig. 6A). However, interestingly, in the CMV(Y)-inoculated region agroinfiltrated with *RCY1*-HA, *RCY1*-HA protein was not immunologically detected (Fig. 6B). On the other hand, in the CMV(Y)-inoculated region accumulating *RPP8*-HA protein, cell death was not induced, although the virus coat protein accumulated equally in the CMV(Y)-inoculated region transiently expressing either *RCY1*-HA or *RPP8*-HA (Fig. 6B). These results suggest that induction of HR-like cell death in the leaves agroinfiltrated with *RCY1*-HA may be associated with disappearance of *RCY1*-HA protein in response to CMV(Y).

In CMV(Y)-inoculated half leaf sides infiltrated with *Agrobacterium* harboring either *g.RCY1*-HA or the genomic *RCY1* sequence fused to a sequence encoding an HA epitope at the 5' end (*HA-g.RCY1*), HR-like cell death was induced in both *g.RCY1*-HA- and *HA-g.RCY1*-agroinfiltrated regions (Fig. 7A). However, *RCY1* protein was not immunologically detected in either agroinfiltrated region (Fig. 7B). On the other hand, *RCY1* protein was detected at the corresponding regions of the mock-inoculated side in the same leaf (Fig. 7B), although cell death was induced in these infiltrated tissues (Fig. 7A). The disappearance of *RCY1*-HA and *HA-g.RCY1* in response to CMV(Y) suggests that the actual *RCY1* protein tagged with an HA epitope, not just the HA-tagged epitope, seems to be degraded in CMV(Y)-inoculated leaves that have developed HR-like cell death upon agroinfiltration with *g.RCY1*-HA and *HA-g.RCY1*.

To directly compare the timing and degree of *RCY1*-HA-induced HR cell death and concurrent disappearance of *RCY1*-HA protein in CMV(Y)-inoculated leaves or mock-inoculated leaves, one side of a single *N. benthamiana* leaf was inoculated with CMV(Y) and the other side was mock inoculated as a control. Both sides were then infiltrated with *Agrobacterium* harboring *RCY1*-HA at 48 h after inoculation. At 24, 48, 72, and 96 h after agroinfiltration, the induction of cell death was visualized

Fig. 4. Schematic structure of *RCY1*-HA, *RPP8*-HA, and their chimeric proteins. CC_{RCY1}, NB_{RCY1}, and LRR_{RCY1} domains of *RCY1* are shown as black rectangles and those of *RPP8* (CC_{RPP8}, NB_{RPP8}, and LRR_{RPP8}) are shown as gray rectangles. CC_{RCY1}, NB_{RCY1}, or LRR_{RCY1} correspond to the positions 1 to 163, 164 to 508, or 509 to 906 amino acid residues of *RCY1*; and CC_{RPP8}, NB_{RPP8}, or LRR_{RPP8} to the positions 1 to 166, 167 to 511, or 512 to 906 amino acid residues of *RPP8*. A hatched rectangle indicates a hemagglutinin (HA)-epitope tag sequence. *RCY1* was tagged with the HA-epitope at the N-terminus (*HA-RCY1*) and C-terminus (*RCY1*-HA), respectively. The C-terminal end of *RPP8* was tagged with the HA sequence (*RPP8*-HA). A chimeric protein, *C/C/P*-HA, comprised the CC-NB domains of *RCY1* (CC_{RCY1}-NB_{RCY1}) and the LRR domain of *RPP8* (LRR_{RPP8}) with the HA tag at its C terminus; chimeric protein *C/P/P*-HA comprised *RCY1* CC (CC_{RCY1}) and *RPP8* NB-LRR (NB_{RPP8}-LRR_{RPP8}) domains with the HA tag; chimeric protein *P/C/C*-HA comprised *RPP8* CC (CC_{RPP8}) and *RCY1* NB-LRR (NB_{RCY1}-LRR_{RCY1}) domains with the HA tag; and chimeric protein *P/P/C*-HA comprised *RPP8* CC (CC_{RPP8}) and *RCY1* LRR (LRR_{RCY1}) domains with the HA tag.

with trypan blue staining, and the accumulation of RCY1-HA protein was detected immunologically. Cell death began to appear at 48 h and continued to develop until 96 h after agroinfiltration in both CMV(Y) and mock-inoculated leaves whereas RCY1-HA protein was detected at significant levels in the mock-inoculated region but not in the CMV(Y)-inoculated region (Fig. 8). There was no significant difference in the timing or degree of development of cell death between the CMV(Y)-inoculated and mock-inoculated regions (Fig. 8A), whereas RCY1-HA protein disappeared only on the side inoculated with CMV(Y) (Fig. 8B). This suggests that degradation of RCY1-HA protein in CMV(Y)-inoculated leaves agroinfiltrated with *RCY1-HA* seems to be caused by the combined presence of RCY1-HA and CMV(Y), rather than from CMV(Y)-independent auto-activation of cell death induced by overaccumulation of RCY1-HA protein in *N. benthamiana*.

Response of CMV(Y)-infected *N. benthamiana* leaves transiently expressing *RCY1-HA* and *RPP8-HA* chimeras.

To investigate the response of CMV(Y)-infected leaves to the accumulation of chimeric proteins encoded by *C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA* (Fig. 4), a single leaf of

N. benthamiana was agroinfiltrated with the four chimeric construct, at 48 h after CMV(Y) inoculation to express *C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, or *P/P/C-HA* protein, respectively. HR-like cell death was induced in the regions agroinfiltrated not only with *RCY1-HA* but also with two of the chimeric constructs (*P/C/C-HA* and *P/P/C-HA*), which encode chimeric proteins that share the LRR domain from the RCY1 protein (Fig. 9A). Both *P/C/C-HA* and *P/P/C-HA* proteins in CMV(Y)-inoculated tissues disappeared, as did RCY1-HA, in response to CMV(Y) inoculation (Fig. 9B). On the other hand, HR-like cell death did not appear in the regions agroinfiltrated with *C/C/P-HA* and *C/P/P-HA*, which encode chimeric proteins that share the LRR domain from RPP8, although the *C/C/P-HA* and *C/P/P-HA* proteins and the virus coat protein did significantly accumulate in the agroinfiltrated regions (Fig. 9B). Furthermore, *PR-1a* expression was induced in CMV(Y)-inoculated leaves transiently expressing *P/P/C-HA* by agroinfiltration (Fig. 9C). In addition, individual expression of HA-tagged CC-NB domains of RPP8 (*HA-CC_{RPP8}-NB_{RPP8}*) and HA-tagged LRR domain of RCY1 (*LRR_{RCY1}-HA*) did not activate HR-like cell death at the agroinfiltrated regions in CMV(Y)-inoculated leaves, whereas significant amounts of each protein accumu-

Fig. 5. Induction of cell death in mock-inoculated *Nicotiana benthamiana* leaves agroinfiltrated with *RCY1-HA*, *RPP8-HA*, and their chimeric constructs. **A**, Development of cell death in a mock-inoculated leaf transiently expressing *RCY1* and *RPP8* cDNA fused to a sequence encoding a hemagglutinin (HA) epitope at the 3' end (*RCY1-HA* or *RPP8-HA*). The left side of an individual leaf was agroinfiltrated with *RCY1-HA* and the right side of the same leaf was done with *RPP8-HA*. The inoculated leaf was photographed under bright field (upper panel) and then stained with trypan blue for visualization of cell death (lower panel). **B**, Immunological detection of *RCY1-HA* and *RPP8-HA* proteins in the mock-inoculated leaf transiently expressing *RCY1-HA* and *RPP8-HA* with an anti-HA monoclonal antibody (α -HA). Rubisco was detected as an internal protein loading control. **C**, Development of cell death in a mock-inoculated leaf transiently expressing *RCY1-HA*, *RPP8-HA*, and four chimeric constructs between them (*C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA*) under the control of the *Cauliflower mosaic virus* 35S promoter. The mock-inoculated leaf was stained with trypan blue for visualization of cell death. **D**, Immunological detection of *RCY1-HA*, *RPP8-HA*, and chimeric proteins *C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA* in the mock-inoculated leaf with α -HA. Rubisco was detected as an internal protein loading control.

lated in them (Supplementary Fig. S3). These results indicate that the LRR domain of RCY1 protein seems to determine the activation of *RCY1*-mediated defense reactions accompanied by the development of HR cell death and disappearance of the RCY1 protein in CMV(Y)-infected *N. benthamiana*.

To further confirm whether the combination of CMV(Y) and the LRR domain from RCY1 determines the induction of HR cell death, a single leaf inoculated with the CMV2-A1 vector expressing enhanced green fluorescent protein (*EGFP*) (CMV::GFP) or mock-inoculated on each side was agroinfiltrated with *P/P/C-HA*. The infectivity of CMV::GFP was adjusted by appropriate dilution of the inoculum to result in a single infection site on each inoculated leaf. The virus infection site was visualized by GFP signal (Fig. 10A). HR cell death was only induced at the virus-infected site in the region agro-

infiltrated with *P/P/C-HA* but not at the mock-inoculated site in the region agroinfiltrated with *P/P/C-HA* (Fig. 10A). These results clearly indicate that the combination of CMV(Y) and the LRR domain from RCY1 is necessary to induce HR cell death in *N. benthamiana* leaves agroinfiltrated with *RCY1-HA*.

In addition, when CMV::GFP-inoculated leaves were agroinfiltrated with either *P/P/C-HA* or *RCY1-HA*, the induction of HR cell death was only detected in the virus-infected site in the region agroinfiltrated with *P/P/C-HA*, whereas cell death appeared in all regions agroinfiltrated with *RCY1-HA* independent of virus infection (Fig. 10B). These results suggest that two events occur at once in *N. benthamiana* transiently expressing *RCY1-HA*: induction of spontaneous cell death by CMV(Y)-independent auto-activation of RCY1-HA, and induction of HR cell death by specific activation of RCY1-HA by LRR domain-mediated recognition of CMV(Y).

To analyze CMV strain-specific induction of HR cell death, one side of a single *N. benthamiana* leaf previously inoculated

Fig. 6. Induction of hypersensitive response (HR)-like cell death in CMV(Y)-inoculated *Nicotiana benthamiana* leaf agroinfiltrated with *RCY1-HA* and *RPP8-HA*. At 48 h after CMV(Y) inoculation, the left side of the inoculated individual leaf was agroinfiltrated with *RCY1* cDNA fused to a sequence encoding a hemagglutinin (HA) epitope at the 3' end (*RCY1-HA*), and the right side of the same leaf was done with *RPP8* cDNA fused to a sequence encoding an HA epitope at the 3' end (*RPP8-HA*). **A**, Development of HR-like cell death in a CMV(Y)-inoculated leaf transiently expressing *RCY1-HA* and *RPP8-HA*. The CMV(Y)-inoculated leaf was photographed under bright field (upper panel) and then stained with trypan blue for visualization of cell death (lower panel). **B**, Immunological detection of *RCY1-HA* and *RPP8-HA* proteins in the CMV(Y)-inoculated leaf transiently expressing *RCY1-HA* and *RPP8-HA* with an anti-HA monoclonal antibody (α -HA). The virus coat protein in the CMV(Y)-inoculated leaf transiently expressing *RCY1-HA* and *RPP8-HA* was detected immunologically with an anti-coat protein antibody (α -CP). As a control, the homogenate of noninfiltrated leaf (Control) was used. Rubisco was detected as an internal protein loading control.

Fig. 7. Induction of hypersensitive response (HR)-like cell death and accumulation of RCY1 protein tagged with the hemagglutinin (HA) epitope at either its N- or C-terminus in CMV(Y)- or mock-inoculated *Nicotiana benthamiana* leaf. The right side of an individual leaf was inoculated with CMV(Y) and the left side of the same leaf was mock inoculated. **A**, Development of HR cell death in a CMV(Y)- and mock-inoculated leaf transiently expressing genomic *RCY1* that encodes the RCY1 protein tagged with HA at its N-terminus (*HA-g.RCY1*) and C-terminus (*g.RCY1-HA*). Cell death was visualized by trypan blue staining. **B**, Immunological detection of RCY1 protein (α -HA) and virus coat protein (α -CP) in the CMV(Y)- and mock-inoculated leaf transiently expressing *HA-g.RCY1* and *g.RCY1-HA*, respectively. As a control, the homogenate of noninfiltrated leaf (Control) was used. Rubisco was detected as an internal protein loading control.

with CMV(Y) and the other side previously inoculated with virulent CMV(B2) were agroinfiltrated with *P/P/C-HA* at 48 h after CMV inoculation. HR cell death was induced only in the agroinfiltrated region in CMV(Y)-infected tissues but not in the agroinfiltrated region in CMV(B2)-infected tissues (Fig. 11A), whereas there was no significant difference in virus coat protein accumulation between CMV(Y)- and CMV(B2)-infected leaf tissues (Fig. 11B). This result indicates that the induction of HR cell death by agroinfiltration with *P/P/C-HA* in CMV(Y)-infected *N. benthamiana* leaves is controlled in a gene-for-gene manner.

Comparative analysis of differential decreases in the levels of RCY1 protein with severity of HR cell death.

The role of CMV(Y)-dependent disappearance of the RCY1 protein in activation of the *RCY1*-mediated defense reaction in *N. benthamiana* remains unclear. To clarify this process, the severity of CMV(Y)-dependent cell death was compared with the level of RCY1-HA protein in an *RCY1-HA*-agroinfiltrated *N. benthamiana* leaf. The severity of cell death in both CMV(Y)-inoculated and mock-inoculated leaf tissues agroinfiltrated with *RCY1-HA* was comparable with the concentration of *Agrobacterium* harboring *RCY1-HA* (Fig. 12A). The level of RCY1-HA

protein decreased in CMV(Y)-inoculated regions infiltrated with various concentrations of *Agrobacterium* harboring *RCY1-HA*, whereas RCY1-HA protein accumulated significantly and remained at a constant level in mock-inoculated regions agroinfiltrated with a corresponding concentration of bacteria (Fig. 12B). The comparison of the level of RCY1-HA protein in CMV(Y)-inoculated regions to that measured in mock-inoculated regions indicated an inverse relationship in which decreased levels of RCY1-HA correlated with increased severity of HR cell death (Fig. 12A and C).

DISCUSSION

Phenotype of transgenic *N. benthamiana* constitutively expressing *RCY1-HA*.

Transgenic plants carrying NB-LRR-class *R* genes derived from closely related species exhibit resistance to the avirulent pathogens corresponding to those *R* genes (Liu et al. 2002a). In the current study, transfer of *RCY1* from *A. thaliana* (Brassicaceae) into *N. benthamiana* (Solanaceae) induced HR cell death and *PR* gene expression in response to avirulent CMV(Y) but not in response to virulent CMV(B2). Furthermore, *RCY1-HA* expression led to a dose-dependent auto-activation of this de-

Fig. 8. Time course of induction of hypersensitive response (HR)-like cell death in CMV(Y)- and mock-inoculated *Nicotiana benthamiana* leaf transiently expressing *RCY1-HA*. The lower side of an individual leaf was inoculated with CMV(Y) and the upper side of the same leaf was mock inoculated. **A**, Detection of HR cell death in a CMV(Y)- and mock-inoculated leaf transiently expressing *RCY1-HA* at 24, 48, 72, and 96 h after agroinfiltration (hpi). Cell death was visualized by trypan blue staining. **B**, Immunological detection of RCY1-HA (α -HA) and virus coat proteins (α -CP) in a CMV(Y)- and mock-inoculated leaf transiently expressing *RCY1-HA* at 24, 48, 72, and 96 hpi. As a control, the homogenate of noninfiltrated leaf (Control) was used. Rubisco was detected as an internal protein loading control.

fense reaction in *N. benthamiana*. Thus, *Arabidopsis* *RCY1* is functional in *Nicotiana* spp. and ectopic overaccumulation of *RCY1* protein can activate the signaling cascade to induce this defense system, independent of pathogen recognition. On the other hand, transgenic *N. benthamiana* lines expressing a lower level of *RCY1-HA* gave a trailing necrosis phenotype in response to CMV(Y). This is probably because *RCY1* still may not function optimally in *N. benthamiana* and, thus, it only works effectively if highly expressed. In those cases, there may still be some factors involved in the recognition of CMV(Y) by *RCY1* that differ between *Arabidopsis* and *N. benthamiana*.

Auto-activation of defense reaction by transient expression of *RCY1* protein.

Transient expression of truncated but not intact NB-LRR-class R proteins often induces auto-HR signaling in *N. benthamiana*.

iana and *N. tabacum*, thereby triggering spontaneous cell death (Bendamane et al. 2002; Collier et al. 2011; Gabriëls et al. 2007; Rairdan et al. 2008). Auto-activation of HR signaling is also observed in chimeric NB-LRR proteins in which amino acid sequences have been swapped between closely related paralogs or alleles (Howles et al. 2006; Hwang et al. 2000; Rairdan et al. 2008; Sun et al. 2001). On the other hand, transient overexpression of intact NB-LRR-class R genes in *N. benthamiana* is generally not sufficient to induce HR cell death. As an exception, avirulence (*avr*) gene-independent HR is induced in tobacco transiently expressing *Arabidopsis* *RPS4*, which encodes a TIR-NB-LRR-class R protein required for HR to *Pseudomonas syringae* carrying *avrRps4* (Zhang et al. 2004). Interestingly, in the current study, when HA-tagged *RCY1* or *RPP8* was transiently expressed in *N. benthamiana* leaves, auto-activation of cell death was induced in the leaves expressing *RCY1-HA* but

Fig. 9. Induction of hypersensitive response (HR)-like cell death and pathogenesis-related (*PR*)-*1a* expression in a CMV(Y)-inoculated *Nicotiana benthamiana* leaf agroinfiltrated with *RCY1-HA*, *RPP8-HA*, and their chimeric constructs. **A**, Development of HR-like cell death in a CMV(Y)-inoculated leaf transiently expressing cDNA for *RCY1-HA*, *RPP8-HA*, and four chimeric constructs between them (*C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA*) under the control of the *Cauliflower mosaic virus* 35S promoter. As a control, the leaf was infiltrated with *Agrobacterium* harboring the empty binary vector pRI201-AN (Vector). The CMV(Y)-inoculated leaf was stained with trypan blue for visualization of cell death. **B**, Immunological detection of *RCY1-HA*, *RPP8-HA*, and four chimeric proteins (*C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA*) in the CMV(Y)-inoculated leaf (α -HA). The virus coat protein in the CMV(Y)-inoculated leaf transiently expressing *RCY1-HA*, *RPP8-HA*, and four chimeric constructs was also detected immunologically (α -CP). As a control, the homogenate of either CMV(Y)-inoculated leaf infiltrated with *Agrobacterium* harboring empty binary vector pRI201-AN (Vector) or noninfiltrated leaves (Control) was used. Rubisco was detected as an internal protein loading control. **C**, Detection of *PR-1a* transcript in a CMV(Y)- or mock-inoculated *N. benthamiana* leaf, which was agroinfiltrated with a chimeric construct *P/P/C-HA*, by Northern hybridization. *rRNA* was detected as an internal RNA control.

not in those expressing *RPP8-HA*. This auto-activation of the defense reaction is consistent with the phenotype of stable transgenic *N. benthamiana* overexpressing *RCY1-HA*. On the other hand, transient expression of some chimeric constructs harboring the *RCY1* LRR domain did not give auto-activation of cell death, suggesting that we may have come up with a way to make functional chimeric *R* genes that can have high-enough expression to give us full resistance but that would not be dwarfed due to the auto-activation of defense reaction.

Function of LRR domain in *RCY1*-conferred resistance to CMV(Y).

Experiments on the potential signaling functions of domains in NB-LRR-class R proteins indicate that the LRR domain or the combined action of the LRR domain and N-terminal domain has been implicated in pathogen recognition (Banerjee et al. 2001; Burch-Smith et al. 2007; Ellis et al. 1999, 2007; Gao et al. 2007; Luck et al. 2000; Rairdan and Moffett 2006; Rairdan et al. 2008; Tomita et al. 2011). The LRR domain is a structural motif characterized by a conserved pattern of hydrophobic leucine residues and displays a broad surface for protein–protein interactions (Kobe and Kajara 2001; Padmanabhan et al. 2009). Variation in amino acid sequences between *RCY1* and *RPP8* proteins indicates that their LRR domains undergo strong positive selection and possibly interact with pathogens (McDowell et al. 1998; Takahashi et al. 2002). As expected, the LRR domain of *RCY1* was found to be the primary determinant of the specificity of CMV(Y) recognition, whereas the CC-NB portions of *RCY1* and *RPP8* were interchangeable in this respect.

Degradation of the *RCY1* protein.

Many NB-LRR-class R proteins require molecular chaperones, such as HEAT SHOCK PROTEIN 90 (HSP90), SUPPRESSOR OF G-2 ALLELE OF SKIP1 (SGT1), and RE-

QUIRED FOR Mla12 RESISTANCE1 (*RAR1*) (Belkadir et al. 2004; Shirasu and Schulze-Lefert 2003). The HSP90-SGT1-*RAR1* complex, which associates with the 26S proteasome via the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complex, targets specific substrate proteins for ubiquitination. Thus, the coordination of the folding and stability of NB-LRR class R proteins with the HSP90-SGT1-*RAR1* complex should be necessary for efficient activation of defenses against pathogen attack (Cheng et al. 2011; Liu et al. 2002b). Although the dependence of *RCY1* on *HSP90*, *SGT1*, and *RAR1* remains to be investigated, a significant level of *RCY1* protein must accumulate under multiple levels of tight positive and negative control to induce resistance to CMV(Y) in *Arabidopsis* (Sekine et al. 2008). In the current study, the overexpression of *RCY1-HA* protein that leads to auto-activation of defense reactions implicates the importance of precise control of *RCY1-HA* protein levels. Furthermore, interestingly, *RCY1-HA* disappeared at time points coincident with the onset of defense reactions (HR cell death and *PR-1* expression) against CMV(Y) in *N. benthamiana* leaves. The loss of *RCY1-HA* seems to be caused by rapid degradation of *RCY1* proteins, which was coincident with the induction of the HR through either direct or indirect interaction between the LRR domain of *RCY1* and CMV(Y), rather than by nonspecific global protein degradation resulting from HR. Such coordinated degradation of *RCY1* protein may be associated with elaborate regulation of efficient activation of defenses to CMV(Y).

Possible role of *RCY1* degradation for inducing the resistance to CMV(Y).

Recent studies have suggested that an interplay between the LRR domain and the ARC2 motif in the NB domain seems to be critical for controlling the activity of the NB-LRR protein, which is often described as a “bait and switch” model (Collier

Fig. 10. CMV(Y)-dependent hypersensitive response (HR) cell death of *Nicotiana benthamiana* leaf tissues expressing *P/P/C*-hemagglutinin (HA). **A**, Development of HR cell death in a leaf infected with CMV(Y) carrying enhanced green fluorescent protein (EGFP) (CMV[Y]::GFP) and transiently expressing *P/P/C*-HA which encodes CC_{RPP8}-NB_{RPP8}-LRR_{RCY1}-HA. The right side of an individual leaf was inoculated with CMV(Y)::GFP and the left side of the same leaf was mock inoculated. At 48 h after inoculation with CMV(Y)::GFP or mock inoculation (Mock), the leaf was partially infiltrated with *Agrobacterium* harboring *P/P/C*-HA. The track of the plastic syringe for agroinfiltration was indicated by a black arrow and the primary virus infection site was indicated by a blue arrow. The *Agrobacterium*-infiltrated region in the leaf was shown by a dotted line. At 24 h after agroinfiltration, no induction of HR cell death was confirmed under bright field microscopy, and a GFP signal due to virus infection was observed under 417-nm-excitation and 525-nm-emission filter conditions. At 48 h after agroinfiltration, induction of HR cell death was confirmed under bright field and by trypan blue staining for visualization of cell death. **B**, Detection of HR cell death and GFP signal derived from infection with CMV(Y)::GFP in leaf tissue infiltrated with *Agrobacterium* harboring *RCY1-HA* and *P/P/C*-HA, respectively. The *Agrobacterium*-infiltrated region in the leaf was shown by dotted line. A GFP signal derived from virus infection was detected at 48 h after agroinfiltration (upper panel). Induction of HR cell death was confirmed by trypan blue staining at 48 h after agroinfiltration (lower panel).

and Moffett 2009). According to this model, activation of NB-LRR-class R proteins likely leads to a conformational change in the R protein (Leister et al. 2005; Mestre and Baulcombe 2006; Moffett et al. 2002; Rairdan and Moffett 2006). Therefore, the recognition of CMV(Y) by RCY1 protein should induce a conformational change in the RCY1, possibly leading to its interaction with unknown host factors to activate downstream signaling for resistance to CMV(Y) (Fig. 13). Furthermore, the interaction of RCY1 with unknown host factors may be promoted dependently on the level of RCY1 accumulation, because *RCY1-HA* expression led to a dose-dependent auto-activation of defense reaction in *N. benthamiana*. At the same time, the alteration in the conformation of RCY1 makes it more susceptible to degradation (Fig. 13). This, in turn, could be a way of having a negative feedback mechanism to precisely control HR resistance to CMV(Y).

Previously, it has been shown that the *Arabidopsis* CC-NB-LRR protein RPM1, which confers resistance to *P. syringae* expressing either *avrRpm1* or *avrB*, is rapidly degraded at the onset of the HR (Boyce et al. 1998; Gao et al. 2011; Grant et al. 1995). The possible role of RPM1 degradation has been proposed to be suppression of HR lesion size and response amplitude at the site of infection. Recently, loss-of-function mutations in *Arabidopsis* F-box protein CPR30/CPR1 have revealed that the NB-LRR-class R protein SNC1 is regulated through SCF complex-mediated protein degradation to prevent auto-activation of defense reactions (Cheng et al. 2011; Gou et al. 2012). Furthermore, very recently, SUPPRESSOR OF *rps4-RLD* (SRFR1) has been shown to negatively regulate NB-LRR-class R protein accumulation and prevent auto-activation of the plant immune system (Kim et al. 2010; Li et al. 2012). Therefore, the degradation of the RCY1 protein may serve as a negative feedback loop, limiting the extent of cell death and the amplitude of the overall disease resistance response (Fig. 13). Taken together, the degradation of RCY1 seems to play an important part in appropriate and effective induction of HR resistance to CMV(Y). Identification of the specific ubiquitin ligase that targets RCY1 will give a new insight into the *RCY1*-mediated system of immunity to CMV(Y).

MATERIALS AND METHODS

Plant and viruses.

N. benthamiana were grown on soilless mix (PRO-MIX B; Premier Horticulture, Ltd., Quakertown, PA, U.S.A.) in a growth chamber (KG-50HLA; Koito Manufacturing Co. Ltd., Tokyo) at day and night temperatures of 25 and 22°C, respectively, under a 14-h photoperiod at 171.43 µE/m²/s. CMV was propagated on tobacco (*N. tabacum* 'Xanthi nc'), and the virus was purified as previously described (Takahashi and Ehara 1993). CMV(Y) (Tomaru and Hidaka 1960) and an Indonesian strain, CMV(B2) (Suastika et al. 1995), were used as an inoculum. CMV(Y) vector CMV2-A1 expressing EGFP was used according to the procedure of Takeshita and associates (2012).

Virus inoculation and detection.

Fully expanded leaves of 6-week-old *N. benthamiana* plants were inoculated with CMV(Y) or CMV(B2) at 100 µg/ml as previously described (Takahashi et al. 1994). Virus was detected immunologically by Western blot according to the method described previously (Takahashi et al. 1994). The amount of total protein for Western blot was determined using the Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.).

Plant transformation.

Binary vector pGA482/RCY1-HA, containing an HA epitope encoding sequence-fused genomic *RCY1* sequence with

an approximately 1.5-kbp promoter region (Sekine et al. 2008), designated *g.RCY1-HA* in this article, was used to transform *N. benthamiana*. The leaf discs of *N. benthamiana* were co-cultivated with *Agrobacterium tumefaciens* LBA4404 containing *g.RCY1-HA* according to the standard protocol (Takahashi et al. 1997). Transgenic plants were screened on Murashige and Skoog medium (Murashige and Skoog 1962) with 0.8% agar and kanamycin at 50 µg/ml. Transformation of *g.RCY1-HA* into *N. benthamiana* plants was confirmed in the second and third generations by the accumulation of RCY1-HA protein, and independent transgenic lines showing distinct levels of accumulated RCY1-HA protein were selected for further study. Detection of HA-tagged RCY1 protein was performed by immunoblotting using a rat anti-HA monoclonal antibody (clone 3F10, dilution 1:10,000; Roche, Indianapolis, IN, U.S.A.) according to the method described previously (Sekine et al. 2008). The protein concentration in the leaf homogenate for immunoblot analysis was determined using the Bradford reagent (Bio-Rad). As an internal control,

Fig. 11. Response of a *Nicotiana benthamiana* leaf transiently expressing *P/P/C-HA* to virulent CMV(B2). The right side of an individual leaf was inoculated with virulent CMV(Y) and the left side of the same leaf was virulent CMV(B2) inoculated. At 48 h after CMV inoculation, the leaf was agroinfiltrated with *P/P/C-HA*, which encodes CC_{RPP5}-NB_{RPP5}-LRR_{RCY1}-HA. **A**, Induction of hypersensitive response (HR) cell death in a leaf agroinfiltrated with *P/P/C-HA* in response to infection with avirulent CMV(Y) or virulent CMV(B2) strain. Development of HR cell death was detected by bright field (upper panel) and trypan blue staining (lower panel). **B**, Detection of the virus coat protein of CMV(Y) and CMV(B2) in the leaf agroinfiltrated with *P/P/C-HA* in response to infection with avirulent CMV(Y) or virulent CMV(B2) strain. A mock-inoculated leaf agroinfiltrated with *P/P/C-HA* was used as a control (Mock). Rubisco was detected as an internal protein loading control.

ribulose-1,5-bisphosphate carboxylase (Rubisco) was detected by staining with Coomassie Brilliant Blue R-250 (CBB-R250).

Detection of cell death.

Cell death in CMV(Y)-inoculated leaves was photographed under bright field, and then visualized by staining with trypan blue according to a standard protocol (Bowling et al. 1994).

Analysis of gene expression by northern hybridization.

Expression of *PR* genes *PR-1a* and *PR-1b* in *g.RCY1-HA*-transformed *N. benthamiana* lines and nontransgenic control plants was analyzed by Northern hybridization as described previously (Takahashi et al. 2004). To detect the expression of *PR-1a* and *PR-1b*, approximately 400-bp fragments of *PR-1a* and *PR-1b* were amplified from genomic DNA of *N. benthamiana* by polymerase chain reaction (PCR) with a set of primers: *PR-1aF* (5'-CGGGATCCCGATGGTCGCCGTGAAATCTT-3', *Bam*HI site underlined) and *PR-1aR* (5'-CGGAATTCGCGAGTTATGCCAAACCACCT-3', *Eco*RI site un-

derlined) for *PR-1a*, and *PR-1bF* (5'-CGGGATCCCGCCAAACCCCAAATGGGATA-3', *Bam*HI site underlined) and *PR-1bR* (5'-CGGAATTCGCGTAGGGACGTTGTCCTCTC-3', *Eco*RI site underlined) for *PR-1b*, respectively. The template genomic DNA for PCR was isolated from *N. benthamiana* leaves by the cetyltrimethylammonium bromide method (Murray and Thompson 1980). The PCR product was purified and cloned into the *Bam*HI and *Eco*RI sites of the pBluescript SKII+ vector (Stratagene, La Jolla, CA, U.S.A.) according to the procedure of Takahashi and Ehara (1993). To confirm that the expected DNA was cloned, the nucleotide sequence of each insert was determined by the Sanger method using a CEQ 8000 automated DNA sequencer (Beckman Coulter, Brea, CA, U.S.A.). PCR probes for *PR-1a* and *PR-1b* were labeled with digoxigenin (DIG)-11-dUTP using a DIG PCR labeling kit (Roche). The DIG-labeled probe was detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and visualized with the chemiluminescent substrate CDP-Star according to the manufacturer's instructions (New England Biolabs, Beverly, MA, U.S.A.).

Fig. 12. Relationship between the strength of *RCY1-HA*-induced cell death and decreased *RCY1-HA* protein level in *Nicotiana benthamiana*. The right side of an individual leaf was inoculated with CMV(Y) and the left side of the same leaf was mock inoculated. At 48 h after CMV(Y) and mock inoculation, the inoculated leaf was agroinfiltrated with different concentrations of *Agrobacterium* harboring *RCY1-HA*. The concentration of bacteria was adjusted to 0.02, 0.1, 0.2, 0.4, or 0.6 of the value for absorbance at 600 nm (*A*₆₀₀). Cell death was detected by trypan blue staining. **B**, Immunological detection of *RCY1-HA* (α-*HA*) and virus coat protein (α-*CP*) in a CMV(Y)- and mock-inoculated leaf infiltrated with different concentrations of *Agrobacterium* harboring *RCY1-HA*. Rubisco was detected as an internal protein loading control. **C**, Decreased level of *RCY1-HA* protein in a CMV(Y)-inoculated leaf. The amount of *RCY1-HA* protein was measured as the average of the band intensity visualized with the chemiluminescent substrate ECL-Prime in triplicate immunoblotting. The decreased protein level is shown as a percentage of the decreased amount of *RCY1-HA* protein in CMV(Y)-inoculated leaf tissue relative to that in mock-inoculated leaf tissue infiltrated with *Agrobacterium* whose concentration was adjusted to 0.02, 0.2, or 0.6 of the *A*₆₀₀ value.

Vector construction.

The cDNAs for the CMV(Y) resistance gene *RCY1*, the *Hyloperonospora arabidopsidis* resistance gene *RPP8*, and four cDNA clones chimeric between *RCY1* and *RPP8*, which were fused to a sequence encoding an HA epitope at the 3' ends, were chemically synthesized using nucleoside phosphoramidites, and then cloned between the CaMV 35S promoter and NOS terminator of pRI201-AN (Takara-BIO, Mie, Japan). To confirm that desired constructs were obtained, independent clones of each plasmid were sequenced using a CEQ 8000 automated DNA sequencer (Beckman Coulter). The resulting plasmids pRI201::RCY1-HA, pRI201-AN::RPP8-HA, pRI201-AN::CC_{RCY1}-NB_{RCY1}-LRR_{RPP8}-HA.1-1 encoding the CC-NB domains of RCY1 and the LRR domain of RPP8, pRI201-AN::CC_{RCY1}-NB_{RPP8}-LRR_{RPP8}-HA.2-3 encoding RCY1 CC domain and RPP8 NB-LRR domains, pRI201-AN::CC_{RPP8}-NB_{RCY1}-LRR_{RCY1}-HA.3-6 encoding RPP8 CC domain and RCY1 NB-LRR domains, and pRI201-AN::CC_{RPP8}-NB_{RPP8}-LRR_{RCY1}-HA.4-3 encoding RPP8 CC-NB domains and RCY1 LRR domain were designated as *RCY1*-HA, *RPP8*-HA, *C/C/P*-HA, *C/P/P*-HA, *P/C/C*-HA, and *P/P/C*-HA, respectively (Fig. 4).

A binary vector containing an *RCY1* genomic DNA fused to a sequence encoding an HA epitope at the 5' end was constructed by PCR amplification of the genomic *Arabidopsis*

RCY1 fragment using the primer set RCY1-8D-AscI-2 (5'-AGAGGCGCGCCAATTTTGATTCCCTGCTTGCATCA-3') and RCY1-Ter-SmaR (5'-CCCGGGTTCCATGCACAACATCGAAAA-3'), to which *AscI* and *SmaI* sites (underlined) were added, respectively. The *RCY1* fragment obtained was then sub-cloned into the *AscI* and *SmaI* sites of binary vector pSMHd-N632L-M2GUS after deletion of β -glucuronidase (GUS) gene, and this construct was designated as pSMHdN632L/RCY1. To obtain an *RCY1* genomic DNA fused to a sequence encoding an HA epitope at the 5' end, an approximately 3.5-kbp partial fragment including the promoter and N-terminal region of *RCY1* was amplified by PCR with the primer set rpp8D (5'-CAATTTTGATTCCCTGCTTGCATCATCAA-3') and RCY-aR3 (5'-AACAAGCCTCCCAACGCTTTA-3'), and was then cloned into the *EcoRV* site of the pBluescript SKII+ vector (Stratagene). The sequence encoding the HA epitope was introduced into the plasmid by site-directed mutagenesis using the primer set RCY1-N-HA-F (5'-ATGGCTTACCCATACGACGTTCCAGACTACGCTA-TGGCTGAAGGATTTGTGTCGTTTGGGCTTCAGAA-3', epitope underlined and start codon in bold) and RCY1-N-HA-R (5'-AGCGTAGTCTGGAACGTCGTATGGGTAGCCATAACAACATAGACGTCGAGTATCACCTACGATGAA-3', antisense epitope underlined and start codon in bold). The insertion of the sequence encoding the HA

Fig. 13. Model of RCY1 activation and degradation. Folding of the RCY1 protein is mediated by the interaction of coiled-coil (CC), nucleotide binding site (NB), and leucine-rich repeat (LRR) domains. Direct or indirect interaction of CMV(Y) with the LRR domain of RCY1 induces a conformational change. This change then results in disruption of an interaction between the CC and NB domains, thereby permitting interaction with an unknown host factor X, which then activates downstream signaling and confers resistance to CMV(Y). In the subsequent step, degradation of the CC, NB, and LRR domains of RCY1 releases the host factor X and attenuates downstream signaling to limit the overall disease resistance response.

epitope was confirmed by sequencing and the plasmid was then digested at the *NcoI* sites in the *RCY1* gene. The *NcoI*-digested fragment containing the N-terminal region of HA-tagged *RCY1* with an approximately 1.5-kbp promoter was introduced into pSMAHdN632L/*RCY1* by exchanging the corresponding fragment between *NcoI* sites. The resulting plasmid, pSMAHdN632L/*HA-RCY1*, was designated as *HA-g.RCY1* (Fig. 4).

A diagram of vector constructs is shown in Figure 4. Each plasmid (1 µg) was introduced into *A. tumefaciens* LBA4404 (Takara-BIO) by the electroporation method according to the standard protocol (Sambrook and Russell 2001).

Agrobacterium-mediated transient expression (agroinfiltration).

A. tumefaciens LBA4404 cultures harboring *g.RCY1-HA*, *HA-g.RCY1*, *RCY1-HA*, *RPP8-HA*, *C/C/P-HA*, *C/P/P-HA*, *P/C/C-HA*, and *P/P/C-HA* were grown in YEB medium containing acetosyringone at 150 ng/ml and 30°C for 48 h. Bacteria were collected by centrifugation at 7,000 rpm for 10 min at room temperature, and suspended in 10 mM MES (pH 7.5) containing 10 mM MgCl₂. After centrifugation at 7,000 rpm for 10 min at room temperature, the pellet was again suspended in 10 mM MES (pH 7.5) containing 10 mM MgCl₂ and acetosyringone at 500 ng/ml, and incubated at 25°C for 2 h. Fully expanded leaves of *N. benthamiana* were infiltrated with *Agrobacterium* containing *RCY1-HA* adjusted to absorbance at 600 nm (A_{600}) = 0.2, or with *Agrobacterium* carrying other constructs adjusted to A_{600} = 0.75, using a plastic syringe. As a control, the same leaves of *N. benthamiana* were agroinfiltrated with LBA4404 harboring empty vectors pGA482 or pRI201-AN. At 48 h after agroinfiltration, accumulation of HA-tagged proteins was detected by immunoblotting with rat anti-HA monoclonal antibody (clone 3F10, dilution 1:10,000; Roche) according to the method described previously (Sekine et al. 2008). To quantitatively analyze the level of HA-tagged protein, the intensity of the band visualized with chemiluminescent substrate ECL-Prime (GE Healthcare, Piscataway, NJ, U.S.A.) was measured using a VersaDoc MP 4000 system (Bio-Rad).

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