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Double-Stranded RNA-Binding Protein 4 Is Required for Resistance Signaling against Viral and Bacterial Pathogens

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SUMMARY

Plant viruses often encode suppressors of host RNA silencing machinery, which occasionally function as avirulence factors that are recognized by host resistance (R) proteins. For example, the *Arabidopsis* R protein, hypersensitive response to TCV (HRT), recognizes the turnip crinkle virus (TCV) coat protein (CP). HRT-mediated resistance requires the RNA-silencing component double-stranded RNA-binding protein 4 (DRB4) even though it neither is associated with the accumulation of TCV-specific small RNA nor requires the RNA silencing suppressor function of CP. HRT interacts with the cytosolic fraction of DRB4. Interestingly, TCV infection both increases the cytosolic DRB4 pool and inhibits the HRT-DRB4 interaction. The virulent R8A CP derivative, which induces a subset of HRT-derived responses, also disrupts this interaction. The differential localization of DRB4 in the presence of wild-type and R8A CP implies the importance of subcellular compartmentalization of DRB4. The requirement of DRB4 in resistance to bacterial infection suggests a universal role in R-mediated defense signaling.

INTRODUCTION

The various modes of defense induced upon the recognition of pathogen-derived molecules provide species-level resistance to nonhost pathogens, local and systemic resistance to race-specific pathogens, and basal resistance to virulent pathogens. Resistance (*R*) gene-mediated or species-specific immunity is induced when a strain-specific avirulence (avr) effector from the pathogen associates directly/indirectly with a cognate plant R protein (Kachroo et al., 2006). R proteins mediating defense against bacteria, fungi, viruses, oomycetes, nematodes, and insects have been identified from a variety of plants. A majority of the known R proteins belong either to the coiled coil (CC)-nucleotide binding site (NBS)-leucine rich repeat (LRR) or toll-interleukin 1 receptor (TIR)-NBS-LRR class.

Plants lacking cognate R proteins can activate the less robust basal defense response. In case of viruses, this form of defense often involves RNA silencing (reviewed in Carr et al., 2010; Ding, 2010). RNA silencing is induced upon the formation of double-stranded RNA (dsRNA), which is processed to small (s) 20–30 nucleotide (nt) dsRNA with staggered ends. The ribonuclease-type III enzymes called Dicers mediate the processing of dsRNA into sRNA. *Arabidopsis* plants encode four Dicer-like proteins (DCL1–DCL4), and of these DCL2, DCL3, and DCL4 process long dsRNA molecules of various cellular origins into sRNA that are 22, 24, or 21 nt in length, respectively. One strand of the dsRNA is then incorporated into a large ribonucleoprotein complex called the RNA-induced silencing complex (RISC), which then cleaves the target viral RNA. RISC complexes are formed of sRNA strand and a member of the Argonaute (AGO) protein family, which are also called slicer proteins because they cleave target single-stranded RNA at the duplex formed with the guide-strand sRNA. Emerging results show a role for some AGO proteins and small RNAs in R protein-mediated resistance (Navarro et al., 2006; Katiyar-Agarwal et al., 2007; Bhattacharjee et al., 2009), although the relationship between R protein-mediated signaling and RNA silencing pathway remains largely unclear.

Viruses, in turn, have evolved to express suppressors that target host RNA silencing components and thereby ensure replication in the host (Carr et al., 2010; Ding, 2010). Interestingly, in many cases these suppressors of silencing also acts as avr factors, and their interaction with the host R proteins leads to activation of defense responses. Many R-avr protein interactions are thought to occur indirectly and involve other host proteins that are targeted by the pathogen-encoded effector proteins. The interaction between R-avr proteins is thought to follow the

"guard model" or modified version thereof, where R protein guards other host protein(s) referred to as "guardee" (Van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008). Any alteration in the "guardee" protein, brought about by avr factor, is thought to activate the R protein, resulting in initiation of defense responses against the pathogen.

HRT, a CC-NBS-LRR protein, confers resistance to turnip crinkle virus (TCV), a single-stranded, positive-sense RNA virus. Most *Arabidopsis* ecotypes are susceptible to TCV; however, a resistant line, designated Di-17, was isolated from the Dijon (Di) ecotype (Dempsey et al., 1997). Following TCV infection, Di-17 plants develop hypersensitive response (HR), express several defense genes, including pathogenesis-related 1 (*PR-1*), and accumulate salicylic acid (SA) (Dempsey et al., 1997; Kachroo et al., 2000; Chandra-Shekara et al., 2004). In contrast, plants lacking the dominant gene *HRT* fail to develop these phenotypes and allow systemic spread of the virus resulting in death of the plant (Kachroo et al., 2000; Chandra-Shekara et al., 2004). However, *HRT* alone is not sufficient to confer TCV resistance, because all F_1 plants and ~75% of HR-developing F_2 plants derived from a cross between resistant (Di-17) and susceptible (Col-0) ecotypes succumb to disease. Furthermore, ~90% of transgenic Col-0 plants expressing the *HRT* transgene are susceptible to TCV even though these plants develop HR upon TCV inoculation (Cooley et al., 2000). The recessive allele of a second, as-yet-unidentified locus designated *rrt* (*r*egulates *r*esistance to *T*CV) is also required for resistance (Kachroo et al., 2000; Chandra-Shekara et al., 2004).

Even though SA acts downstream of HRT, exogenous treatment with SA is unable to confer TCV resistance in *hrt* plants. Thus, SA likely operates via a feedback loop with HRT and this SA-induced expression of *HRT* is dependent on *PAD4* (*p*hyto*a*lexin *d*eficient) (Chandra-Shekara et al., 2004). Consistent with these observations, *NPR1* (nonexpressor of PR-1), which acts downstream of SA, is not required for TCV resistance (Kachroo et al., 2000). Interestingly, HR and resistance are two distinct phenotypes in the *Arabidopsis*-TCV interaction that do not necessarily share all downstream signaling components (Chandra-Shekara et al., 2004; Venugopal et al., 2009; Jeong et al., 2010). These observations suggest that HRT triggers at least two distinct sets of events that culminate in HR and resistance responses.

HRT-mediated signaling is activated in response to coat protein (CP) of the virus (Cooley et al., 2000; Zhao et al., 2000; Jeong et al., 2008). However, direct interaction between HRT and TCV CP has not been detectable (Zhu et al., 2011). Resistance to TCV is dependent upon SA pathway and blue-light photoreceptors, of which *CRY2* (cryptochrome) and *PHOT2* (phototropin) are required for the stability of HRT (Chandra-Shekara et al., 2006; Jeong et al., 2010). HRT interacts with CRT1 (*c*ompromised for *r*ecognition of *T*CV), and a mutation in *CRT1* compromises resistance to TCV (Kang et al., 2008). More recently, we showed that HRT also associates with EDS1 (enhanced *d*isease *s*usceptibility), which facilitates CP-triggered HR (Zhu et al., 2011). Although EDS1 forms a ternary complex with two other related proteins, SAG101 (*s*enescence-*a*ssociated *g*ene) and PAD4, neither of these facilitate HRT-CP-triggered HR (Zhu et al., 2011).

Here, we evaluated the role of components of RNA silencing pathway in HRT-mediated resistance to TCV. We show that RDR6, DCL4, and DRB4 are required for the HRT-mediated resistance to TCV. Biochemical analysis showed that HRT forms a complex containing DRB4 and DRB4 is required for the stability of HRT. DRB4 is also required for resistance signaling mediated by RPS2 and RPM1 proteins. These results, together with our findings that the silencing suppressor function of CP is not required for the activation of resistance response against TCV and that accumulation of viral-specific small RNAs is inversely related to activation of HRT, suggest a specific role for DRB4 in R-mediated defense.

RESULTS

TCV CP Mutants Deficient in Silencing Suppressor Function Can Activate HRT-Mediated Signaling

The TCV CP, which activates resistance signaling via HRT, also serves as a suppressor of RNA silencing (Choi et al., 2004; Cao et al., 2010). To determine if the RNA silencing suppressor function of CP was required for the activation of HRT-mediated resistance, we analyzed the response of Di-17 (resistant ecotype) plants inoculated with TCV mutants carrying the R130T (CPB) or R137H (CPC) mutations in CP. These mutant proteins are impaired in RNA silencing suppressor activity (Cao et al., 2010). Typical phenotypes associated with the activation of HRT include HR development on the inoculated leaf, which is associated with induction of *PR-1* expression. Inoculation of wild-type (WT) and CPB and CPC TCV mutants induced discrete visible necrotic lesions with trypan blue staining showing dead cells characteristic of an HR similar to that seen on Di-17 leaves (Figures 1A and 1B). Furthermore, the HR correlated with the levels of *PR-1* expression in the inoculated leaves of Di-17 plants (Figure 1C), suggesting that WT and mutant viruses elicit comparable response on Di-17 plants. Neither WT nor mutant viral strains were able to induce a high level of *PR-1* expression in plants lacking HRT (Col-0; susceptible ecotype) (Figure 1C). Like WT TCV, the CPB and CPC mutants were unable to cause disease on Di-17 plants (Figure 1D). Consistent with their impaired RNA silencing suppressor activity, CPB and CPC mutants showed reduced virulence on Col-0 plants, which correlated with their reduced replication (Figure 1C, left panels). In comparison, replication of CPB and CPC viruses in Di-17 leaves was either slightly lower or comparable to plants inoculated with WT TCV, respectively (Figure 1C, right panels). Together, these results suggest that the silencing suppressor activity of TCV CP is not required for its ability to activate HRT-mediated resistance.

RDR6 and DCL4 Are Required for HRT-Mediated Resistance but Not HR

Next, we evaluated whether RNA silencing components are required for HRT-mediated signaling. The *rdr1*, *rdr2*, *rdr6*, *dcl1*, *dcl2*, *dcl3*, and *dcl4* alleles (Col-0 ecotype) were crossed into the Di-17 background. F_2 progeny derived from these crosses were genotyped and inoculated with TCV. All of the *HRT*-containing F_2 progeny from these crosses developed visible and microscopic HR following TCV infection (Figures 2A and 2B)

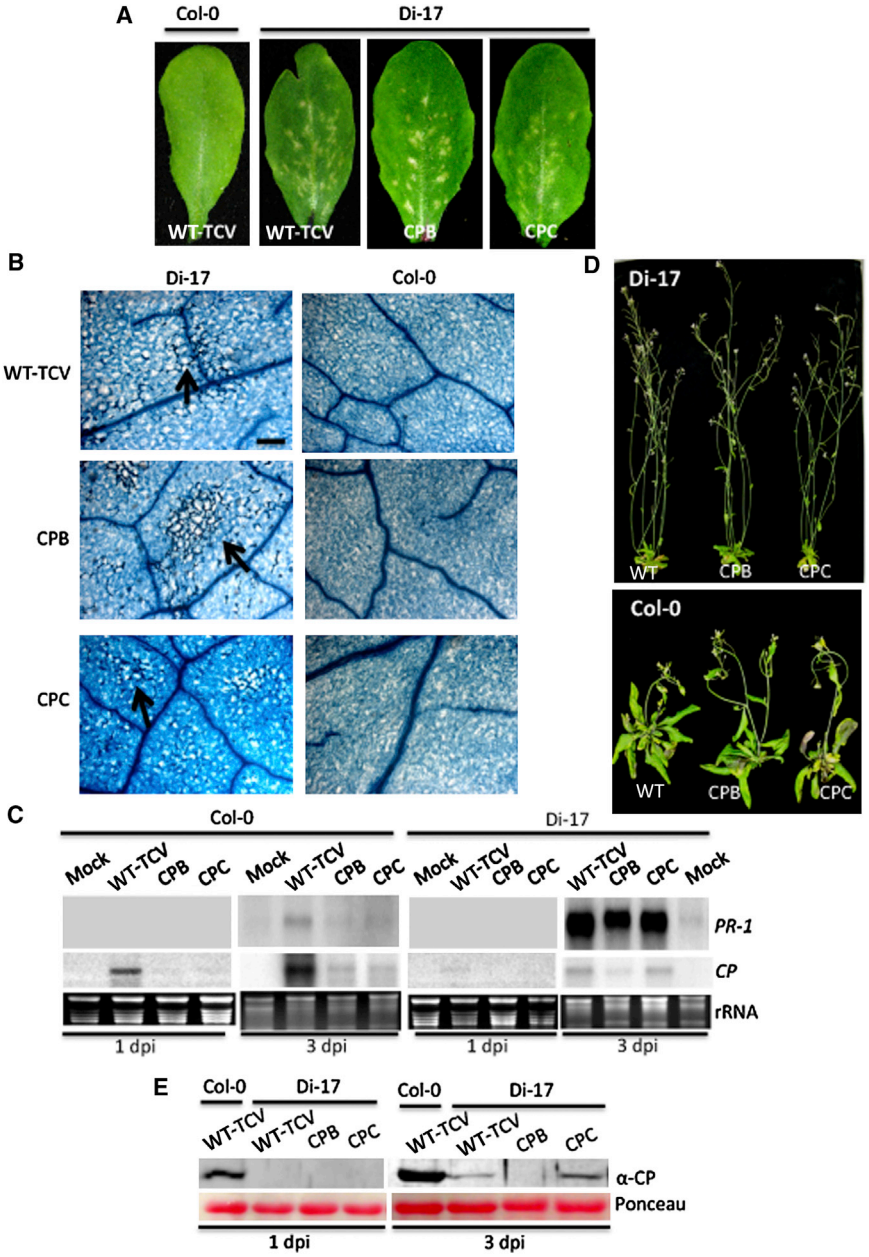

Figure 1. RNA Silencing Suppressor Function Is Not Required for the Activation of HRT-Mediated Resistance

(A) Visual phenotypes in Col-0 and Di-17 leaf at 3 days postinoculation (dpi) with WT-TCV, CPB, or CPC strains of TCV. The HR phenotype was evaluated in at least 35 plants in four separate experiments.

(B) Trypan-blue-stained leaves from Di-17 and Col-0 plants inoculated with WT-TCV, or CPB and CPC mutants. Leaves were sampled at 3 dpi, and the arrows indicate isolated cell death zones corresponding to visible HR lesions. This experiment was repeated three times with similar results.

(C) RNA gel blot analysis showing expression of *PR-1* and TCV *CP* in indicated genotypes after inoculation with buffer (mock), WT-TCV, or CPB and CPC mutants. Total RNA was extracted from inoculated leaves at 1 or 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. The experiment was repeated twice with similar results.

(D) Typical morphological phenotypes of Di-17 and Col-0 plants inoculated with WT-TCV, CPB, or CPC viruses. Plants were photographed at 18 dpi. Approximately 30–40 plants were inoculated in three separate experiments and analyzed for disease phenotypes.

(E) Western blot showing relative CP levels in Col-0 and Di-17 plants at 1 or 3 dpi with WT-TCV, CPB, or CPC viruses. Ponceau-S staining of the western blot was used as the loading control. This experiment was repeated three times with similar results.

and exhibited induced *PR-1* gene expression (Figure 2C). These results suggested that these RNA silencing components are not required for HR development or the associated *PR-1* expression. As expected, all *hrt*/*hrt* and ~75% of *HRT*/- (homo/heterozygous for *HRT*) of F_2 progeny from a Di-17 × Col-0 control cross showed typical phenotypes associated with susceptible plants. Furthermore, only 25% (homo/heterozygous for *HRT,* but homozygous for *rrt*) of these HR-developing progeny were able to resist TCV infection (Table S1). The resistance phenotype in F2 progeny obtained from Di-17 × *rdr1*/*rdr2*/*dcl1*/*dcl2*/*dcl3* crosses also showed expected Mendelian segregation (Table S1), suggesting that mutations in *RDR1*, *RDR2, DCL1*, *DCL2*, or *DCL3* do not affect HRT-mediated resistance (Figure 2D). In contrast, mutations in *RDR6* or *DCL4* abrogated *HRT*-mediated resistance; all plants containing *HRT* and *rdr6* or *dcl4* alleles showed typical disease phenotypes associated with susceptible plants (Figure 2D; Table S1). The appearance of disease symptoms also correlated with the presence of TCV transcript in the uinoculated tissues (Figure S1A). Together, these data suggest that RDR6 and DCL4 are required for HRT-mediated resistance.

Next, we determined the effect of *rdr6* and *dcl4* mutations on viral replication as assessed by CP levels in the inoculated leaves. CP levels in the inoculated leaves of *HRT rdr6* and *HRT dcl4* plants were similar to that in resistant Di-17 even though the mutant plants showed typical systemic viral symptoms (Figure 2E). Likewise, CP levels in the inoculated leaves of *rdr6* and *dcl4* plants were similar to that in Col-0 plants (Figure 2E). Together, these results suggest that the *rdr6* and *dcl4* mutations do not affect viral replication in the inoculated leaves of Col-0 or Di-17 plants but do enable escape of the virus to systemic tissues in the *HRT* background.

SA accumulation is a critical signaling event required for resistance to TCV. Both free and conjugated (SAG) forms of SA

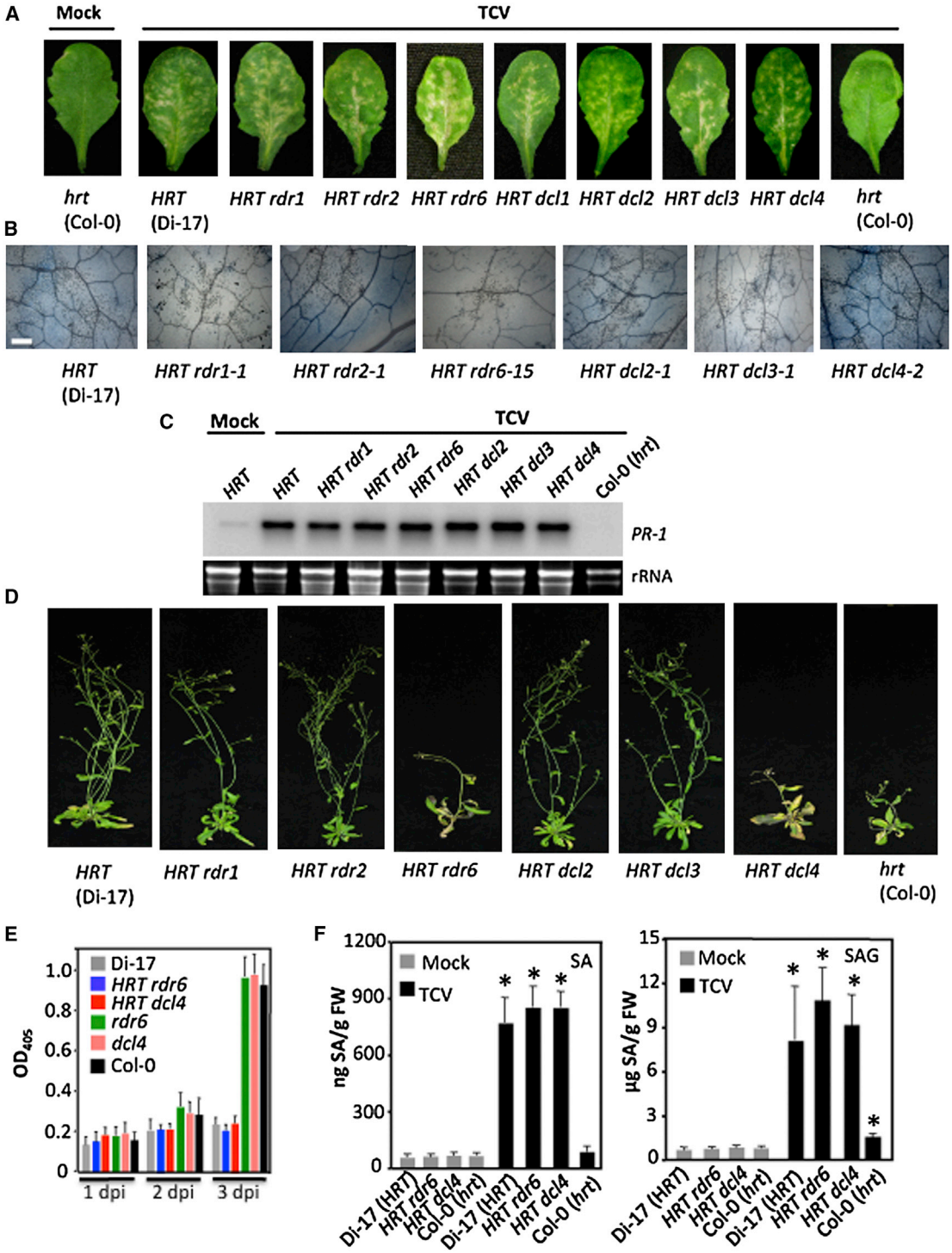

Figure 2. HRT-Mediated Resistance to TCV Is Dependent on RDR6 and DCL4

(A) HR formation in mock- or TCV-inoculated WT plants (Col-0 and Di-17) or F2 progeny derived from various crosses at 3 dpi. The HR phenotype was evaluated in at least 100 F2 progeny.

(B) Trypan-blue-stained leaves showing microscopic cell death phenotype after TCV inoculation. Scale bars, 270 μm. The cell death phenotype in TCV-inoculated *HRT dcl1* plants was similar to Di-17. At least five independent leaves were analyzed with similar results.

(C) RNA gel blot analysis showing expression of *PR-1* transcript in indicated genotypes after mock or TCV inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. This experiment was repeated twice with similar results. Ethidium bromide staining of rRNA was used as the loading control. The *PR-1* levels in TCV-inoculated *HRT dcl1* plants were similar to Di-17.

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increase by ~10- and ~15-fold, respectively, in TCV-inoculated Di-17 plants (Kachroo et al., 2000). Therefore, SA/SAG levels in TCV-inoculated *HRT rdr6* and *HRT dcl4* plants were tested to determine if the loss of resistance in these mutants was due to defects in TCV-induced SA accumulation. No defects in SA or SAG accumulation were evident (Figure 2F), which correlated with normal expression of the SA responsive *PR-1* expression seen in TCV-inoculated *HRT rdr6* and *HRT dcl4* (Figure 2C). The effects of mutations in the *RDR6* or *DCL4* genes on HRT levels were analyzed using Di-17 *HRT-FLAG* plants containing the *rdr6* or *dcl4* mutations. Neither mutation significantly altered basal levels of HRT-FLAG (Figure S1B). In addition, as in Di-17 plants, HRT-FLAG in *HRT rdr6* and *HRT dcl4* backgrounds was only detected in the membranous fraction (Figure S1C), and the HRT levels did not change after TCV inoculation (Figure S1D).

The DCL4-Interacting DRB4 Is Required for Normal HR and Resistance to TCV

We next assessed whether the interacting partner of DCL4, the double-stranded RNA-binding protein 4 (DRB4) (Hiraguri et al., 2005; Fukudome et al., 2011), contributed to HRT-mediated resistance. The *drb4* mutant in Col-0 background was crossed with Di-17, and the F2 progeny were evaluated for HR and resistance. All of the *HRT/- drb4/drb4* F2 progeny developed a HR (Figures 3A and 3B) and exhibited induced *PR-1* gene expression following TCV infection (Figures 3C and S2A). However, unlike Di-17, *HRT rdr6*, and *HRT dcl4* plants, *HRT drb4* developed a spreading HR, characterized by more extensive cell death with eventual collapse of inoculated leaves by 10 days postinoculation (dpi) (Figures 3A and 3B). Absence of necrotic lesions on Col-0 leaves suggested that spreading lesion seen on *HRT drb4* leaves were associated with HR phenotype (Figure S2B). The spreading HR phenotype was similar to that of Di-17 *crt1* mutant (Kang et al., 2008). Because *HRT drb4* plants were derived from a cross between Di-17 and Col-0 plants, whereas *crt1* was isolated in the Di-17 background, it was important to determine if genetic background difference(s) contributed to the spreading HR phenotype. To this end, we generated a homozygous T-DNA KO line for *CRT1* in the Col-0 background and crossed it to Di-17. Several F3 pools of *HRT crt1* plants were created, two of which were evaluated for their HR phenotypes (Figure 3A). Both of the F3 pools consistently showed spreading HR in response to TCV inoculation, similar to the Di-17 *crt1* mutant (Kang et al., 2008). Comparison of HR phenotypes showed that *HRT drb4* plants displayed a more pronounced spreading HR (Figure 3A), which correlated with increased replication of the virus in their inoculated leaves (Figures 3C, 3D, and S2C); the *HRT drb4* plants showed higher *CP* transcript and CP protein levels compared to *HRT crt1* plants. Additionally, compared to Di-17 plants, the avirulent strains CPB and CPC also replicated to higher levels in *HRT drb4* plants (Figure 3E). Consistent with the avirulence function of CP in HRT plants, mutant virus carrying a null mutation in CP (designated TCV-stop) neither produced visible symptoms on the inoculated leaves of *HRT drb4*, *HRT crt1*, Col-0, or Di-17, nor did it induce *PR-1* expression (Figures 3F and 3G, also see Figures 3A and 3C). As expected, no CP protein was detected in TCV-stop-inoculated plants, even though all genotypes accumulated low levels of *CP* transcript (Figure 3G). We next evaluated replication of mutant R8A virus, which contains an arginine (R) to alanine (A) substitution at amino acid 8 of CP and is virulent on Di-17 plants (Choi et al., 2004). Like WT-TCV, CPB, and CPC, the R8A derivative also replicated to higher levels in the inoculated leaves of *HRT drb4* plants than in the Di-17 plants (Figures 3E and S2C). This correlated with increased CP levels in the inoculated leaves of *drb4* as compared to Col-0 plants (Figures S2D and S2E). Notably, in spite of the increased accumulation of R8A in the inoculated tissues, the *drb4* plants showed Col-0-like susceptibility and accumulated Col-0-like levels of CP in the systemic bolt tissues (Figure S2F). Together, these data suggest that *DRB4* regulates HR to TCV and a mutation in *DRB4* increases TCV levels in the inoculated leaves of *HRT* plants.

Unlike *HRT drb4* and *HRT crt1*, a number of TCV-susceptible genotypes tested here or in our earlier studies (including *HRT pad4*, *HRT sid2*, *HRT eds1*, *HRT sag101*, *HRT rdr6*, *HRT dcl4*) accumulate similar levels of CP in their inoculated leaves as the resistant Di-17 plants (Kachroo et al., 2000; Chandra-Shekara et al., 2007; Venugopal et al., 2009; Zhu et al., 2011; Figure 2E). This, and the fact that both *DRB4* and *CRT1* are involved in host RNA silencing pathways (Hiraguri et al., 2005; Fukudome et al., 2011; Moissiard et al., 2012), prompted us to check the effect of *drb4* and *crt1* mutations on viral-specific small RNA (VsRNA) levels in the *HRT* background. Notably, VsRNA only accumulated in the TCV-inoculated Col-0 plants but not in the *HRT* backgrounds, regardless of TCV levels in the inoculated leaves or their resistant (Di-17) or susceptible (*HRT drb4*, *HRT crt1*, *HRT dcl4*, *HRT sag101*, *HRT eds1*) response to TCV (Figures 3H, left panel, S2G, also see Figures 3C–3E and S2C). This suggested that the lack of accumulation of VsRNA was likely associated with the presence/activation of HRT. To test this further, we assayed VsRNA levels in TCV-stop-inoculated Col-0, Di-17, *HRT drb4*, and *HRT crt1* plants. All these hosts accumulated VsRNA when infected with TCV-stop (Figure 3H, middle panel). Next, we assayed VsRNA in CPB and R8A-inoculated Di-17 and *HRT drb4* plants. Like TCV-stop, R8A-infected Di-17, *HRT drb4*, or *HRT crt1* plants accumulated significant amounts of VsRNA (Figure 3H, right panel, S2G), but CPB behaved like WT-TCV and did not induce accumulation of VsRNA. Together these results suggested that accumulation of VsRNA was inversely associated with the presence/activation of HRT and was independent of disease phenotype.

(D) Typical morphological phenotypes of TCV-inoculated *HRT* (Di-17), *HRT rdr*, *HRT dcl*, and *hrt* (Col-0) genotypes. Plants were photographed at 18 dpi.
(E) Time-course analysis of TCV CP levels in the inoculated leaves of indicated genotypes at 1, 2, and 3 dpi. Enzyme-linked immunosorbent assay was used to quantify the CP levels. This experiment was repeated three times with similar results. The error bars indicate SD.
(F) SA and SAG levels in mock- or TCV-inoculated plants at 3 dpi. Asterisks indicate data statistically significant from mock-inoculated plants ($p < 0.05$, $n = 6$). The error bars indicate SD. The experiment was repeated twice with similar results.
See also Figure S1.

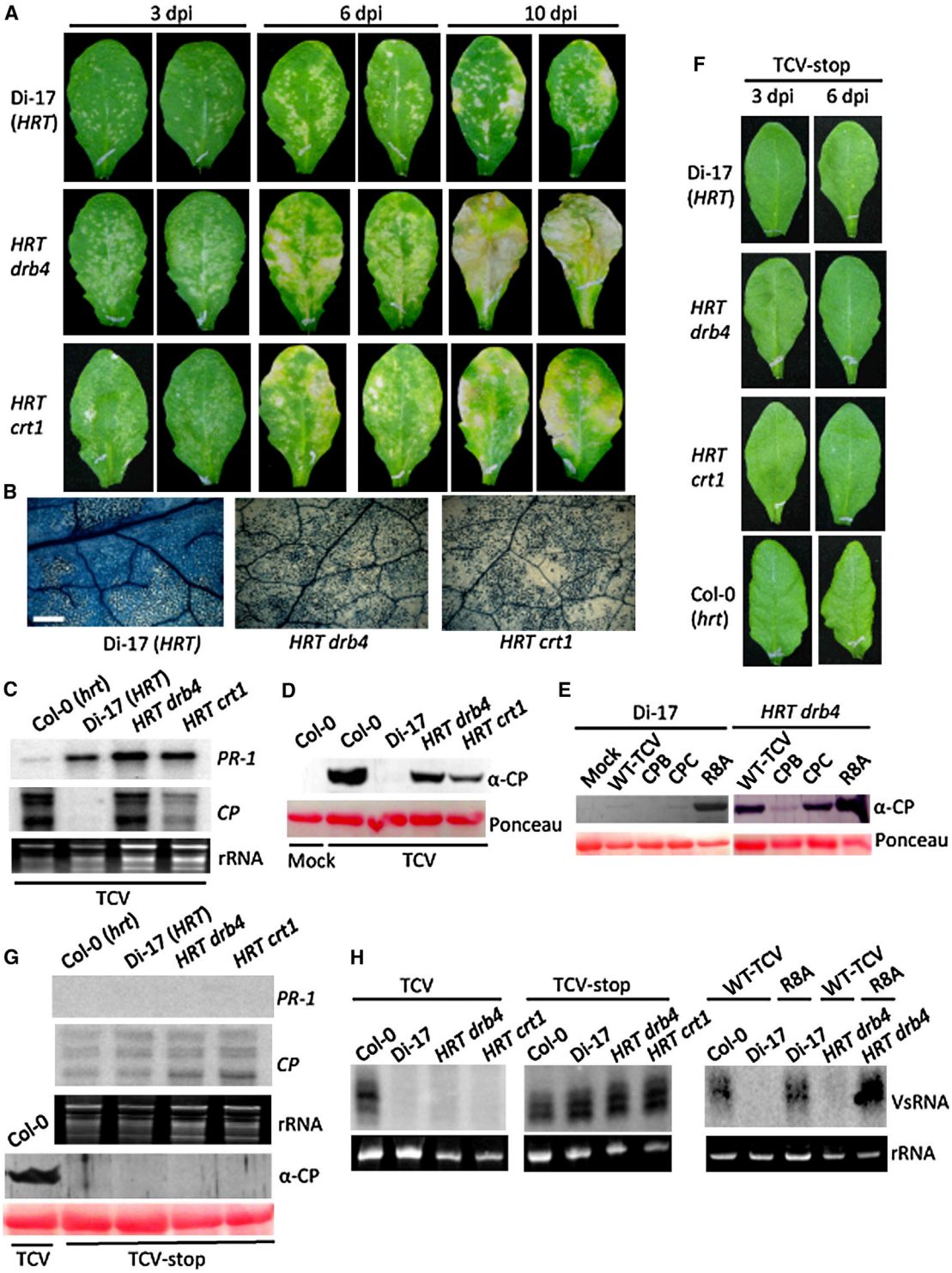

Figure 3. The *HRT drb4* Plants Show Spreading HR and Support Increased Replication of Virus

(A) HR formation in TCV-inoculated Di-17, *HRT drb4*, and *HRT crt1* plants at 3, 6, and 10 dpi. The HR phenotype was evaluated in ~40 plants that were analyzed in three separate experiments.

(B) Trypan-blue-stained leaves showing microscopic cell death phenotype at 10 dpi with TCV. Scale bars, 270 μm. At least five independent leaves were analyzed with similar results.

(C) RNA gel blot analysis showing expression of *PR-1* and *CP* transcripts in indicated genotypes at 3 dpi after TCV inoculation. This experiment was repeated three times with similar results. Ethidium bromide staining of rRNA was used as the loading control.

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To determine if *DRB4* was required for resistance to TCV (preventing spread of the virus to uninoculated tissues), we evaluated F2 progeny derived from Di-17 × *drb4* cross. All *HRT/-drb4* plants showed typical disease symptoms, suggesting that DRB4 was required for HRT-mediated resistance (Table S1; Figure S2H). We next estimated SA/SAG levels in TCV-inoculated *HRT drb4* plants to determine if the loss of resistance in these mutant backgrounds was due to a defect in SA accumulation. No significant differences in levels of SA or SAG accumulation were evident (Figure S2I), which correlated well with the observation that *HRT drb4* plants developed HR and had WT-like expression of *PR-1* (Figures 3C and S2A).

DRB4 Associates with HRT and Regulates Its Stability

Analysis of HRT levels in *HRT-FLAG drb4* compared to Di-17 *HRT-FLAG* revealed significantly reduced HRT protein (Figure 4A), but not transcript levels (Figure S3A). This result provides a possible explanation for the spreading HR phenotype seen in *HRT drb4* plants and suggested that a certain threshold level of HRT might be required to contain the spread of the necrotic lesions. To test this possibility, we evaluated HRT levels in *HRT crt1* plants, because these also showed spreading lesions in response to TCV. As expected, *HRT crt1* plants also showed reduced levels of HRT-FLAG (Figure 4B). As an additional test, we evaluated HR and viral levels in *HRT cry2* plants, which, like *HRT drb4* and *HRT crt1* plants, accumulated reduced levels of HRT (Jeong et al., 2010). Similar to *HRT drb4* and *HRT crt1* plants, the *HRT cry2* plants showed spreading lesions (Figures S3B and S3C) and also elevated levels of CP in the inoculated leaf (Figure S3D). Together, these results suggested that, like CRY2, CRT1 and DRB4 are also required for the stability of HRT and that reduction in HRT levels results in inability of the plant to fully suppress viral replication and spread, ultimately leading to a spreading HR.

Previous work has shown that the T-DNA insertion mutant in *drb4-1* is also defective in the expression of the DRB4-neighboring gene, At3g62810 (Nakazawa et al., 2007). To confirm that degradation of HRT in *drb4* background was specific to the mutation in *DRB4*, we generated transgenic *drb4* plants expressing the *DRB4-MYC* transgene under its own promoter and crossed these to *HRT-FLAG drb4* plants. The F2 plants were scored for *HRT-FLAG* and *DRB4-MYC* transgenes and evaluated for their HRT and DRB4 levels. The *drb4*::*DRB4-MYC* plants contained normal levels of HRT-FLAG (Figure 4C), suggesting that reduced stability of HRT in *drb4* plants was specifically due to the *drb4* mutation. Notably, immunoblot analysis with α-MYC at times showed a doublet of DRB4, suggesting that this protein might undergo posttranslational modifications (Figure 4C).

To determine if DRB4 stabilizes the HRT R-protein through physical interaction, we assessed interaction between HRT and DRB4 using bimolecular fluorescence complementation (BiFC) assays. As shown previously (Jeong et al., 2010), HRT interacted with CRT1, and this interaction was detected in the endosomes as well as the cell periphery (Figures 4D and S3E). Also, DRB4 was found to be associated with DCL4 in the nucleus (Figures 4D and S3E), as previously reported (Hiraguri et al., 2005). In contrast, no interaction was detected between HRT and DCL4 using BiFC (Figure 4D). However, HRT interacted with DRB4, and this interaction was primarily seen in the cell periphery (Figures 4D and S3E). The interaction between HRT and DRB4 was verified by coimmunoprecipitation (IP) assays when these proteins were coexpressed under their native promoters in *Arabidopsis* (Figure 4E) or in *Nicotiana benthamiana* (Figure 4F). Coimmunoprecipitation (coIP) assays in *N. benthamiana* also confirmed the lack of interactions between HRT and DCL4 (Figure 4F) and between DRB4 and GST (Figure S3F) when these proteins were expressed using the 35S promoters.

Notably, only a small fraction of the total DRB4 protein was coimmunoprecipitated with HRT (Figures 4E and 4F), which was consistent with the fact that most of the cellular pool of DRB4 is present in the nucleus and might not be available to complex with HRT (Figure 4G; Hiraguri et al., 2005). To test this, we fused DRB4 with nuclear export signal (NES) or nuclear localization signal (NLS) and assayed localization of these proteins in *N. benthamiana*. As expected, DRB4-NLS was exclusively detected in nucleus, whereas DRB4-NES remained in the extranuclear compartment. In comparison, DRB4 fused to the mutant NES/NLS sequences (DRB4-nes/nls-GFP) behaved like WT protein, localizing to both nuclear and extranuclear compartments (Figure 4G). BiFC and coIP interaction assays carried out between HRT and DRB4-NES/NLS proteins showed that HRT interacted only with the DRB4-NES protein, but not with DRB4-NLS (Figures 4H, S3G, and S3H).

We next tested interaction between the CC, NBS, and LRR domains of HRT with DRB4 in *N. benthamiana*. Interestingly, DRB4 bound to all three HRT domains (CC, NBS, and LRR) of HRT (Figures S4A and S4B). This raised the possibility that the

(D) Western blot showing relative CP levels in TCV-inoculated genotypes shown in (C). Ponceau-S staining of the western blot was used as the loading control. This experiment was repeated three times with similar results.
(E) Western blot showing relative CP levels in Di-17 and *HRT drb4* plants inoculated with CPB, CPC, R8A, and WT TCV. Leaves were sampled at 3 dpi. Ponceau-S staining of the western blot was used as the loading control. This experiment was repeated three times with similar results.
(F) TCV-stop-inoculated Col-0, Di-17, *HRT drb4*, and *HRT crt1* leaves with absence of visual HR. Plants were photographed at 4 dpi. This experiment was repeated three times with similar results.
(G) RNA (upper three panels; *PR-1*, *CP*, and rRNA) and protein (lower two panels; α-CP and Ponceau-S) gel blot analyses showing expression of *PR-1* and *CP* transcripts and CP protein levels in TCV-stop-inoculated plants. TCV-inoculated Col-0 plants were included as a positive control in protein gel blot. Total RNA and protein were extracted from inoculated leaves at 3 dpi. This experiment was repeated two times with similar results. Ethidium bromide staining of rRNA and Ponceau-S staining of protein were used as the loading controls.
(H) Levels of TCV-CP-specific small RNA in Col-0, Di-17, *HRT drb4*, and *HRT crt1* plants inoculated with TCV, TCV-stop, or R8A. Leaves were sampled at 3 dpi. This experiment was repeated four times with similar results.
See also Figures S2 and S3.

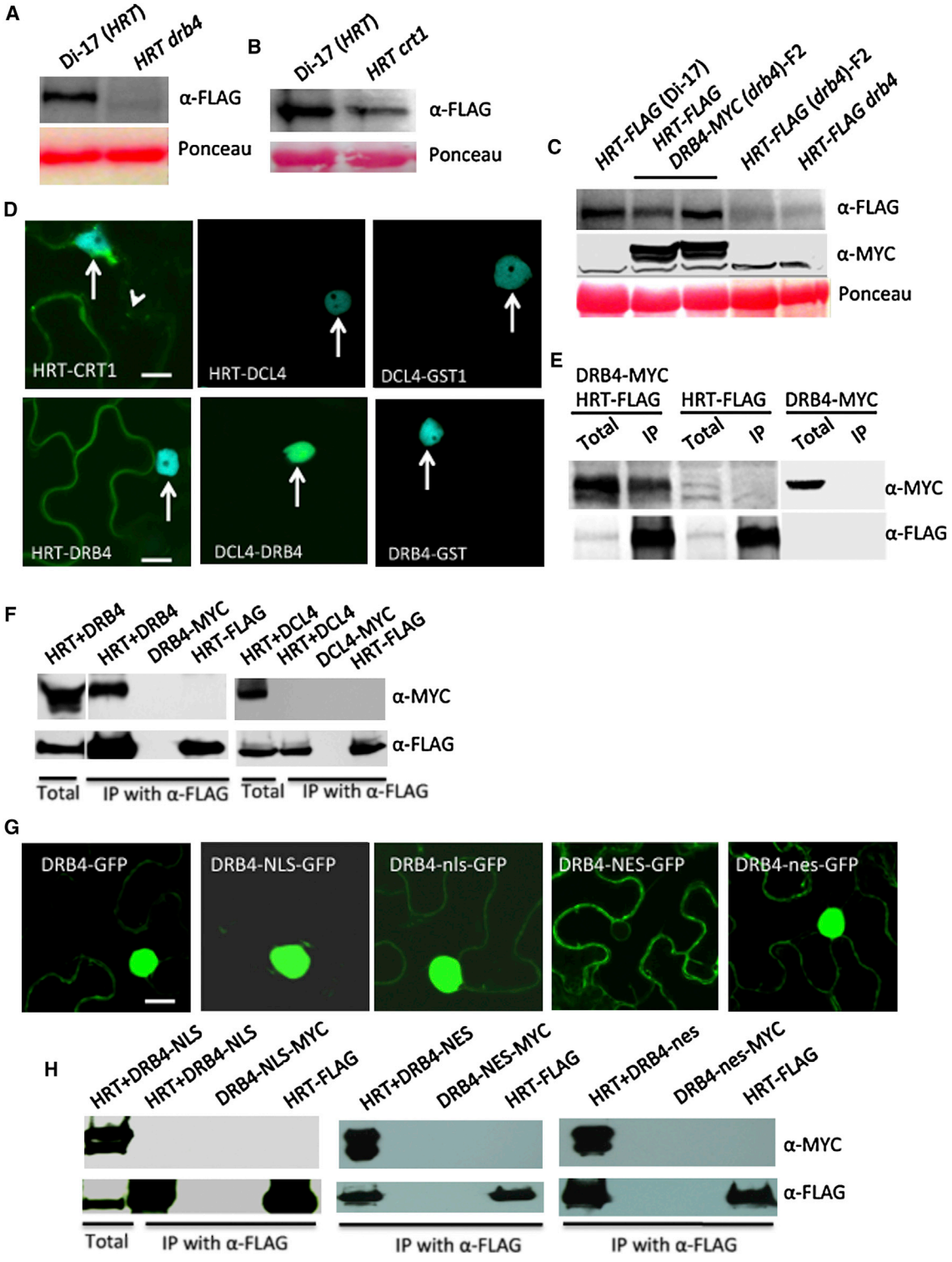

Figure 4. DRB4 Interacts with and Is Required for the Stability of HRT

(A and B) Western blots showing relative levels of HRT-FLAG in Di-17 (designated *HRT*) and *HRT drb4* (A) or *HRT crt1* (B) plants. Ponceau-S staining of the western blots was used as the loading control. This experiment was repeated four times with similar results.

(C) Western blot showing WT-like levels of HRT-FLAG in *drb4* plants expressing *HRT-FLAG* and *DRB4-MYC*. The F2 plants were derived from a cross between *drb4*::*HRT-FLAG* and *drb4*::DRB4-*MYC* transgenic plants. Ponceau-S staining of the western blot was used as the loading control. This experiment was repeated twice with similar results.

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N-terminal CC and C-terminal LRR domains of HRT protein might fold over the NBS domain, such that DRB4 was able to interact with all three domains of HRT (Figure S4C). Furthermore, both CC and NBS domains self-interacted (Figure S4A), suggesting intramolecular interactions within HRT. To assess this further, we studied the self-interaction of the full-length HRT protein. Using both *Arabidopsis* and *N. benthamiana*, HRT was found to interact with itself (Figure S4D). These results suggested that HRT exist as a dimer or multimer. Interestingly, DRB4 also self-interacted, as shown using the yeast two-hybrid assays as well as by coIP in *N. benthamiana* (Figures S4E–S4G). Together, these results suggest that the HRT complex likely contains multiple units of DRB4 proteins (Figure S4B).

TCV Inoculation Increases the Cytosolic Pool of DRB4

We next considered the possibility that TCV inoculation might modulate HRT-DRB4 complex formation. To test this, we generated transgenic Di-17 plants expressing *DRB4-GFP* under its own promoter and assayed levels and localization of DRB4-GFP in mock- and TCV-inoculated plants. Approximately 65% of leaves inoculated with WT-TCV or R8A mutant showed an increase in DRB4-GFP levels (Figure 5A), which correlated with an ~2.8-fold increase in *DRB4* transcript levels in TCV-inoculated Di-17 plants (Figures S5A and S5B). Exogenous application of SA did not increase DRB4-GFP levels (Figure 5A), suggesting that a factor other than SA was responsible for the WT-TCV- and R8A-triggered increase in DRB4-GFP (Figure 5A; Table S2).

In the absence of virus inoculations, the majority of DRB4-GFP was present in the nucleus with only a small fraction in the extranuclear compartment, just as seen in *N. benthamiana* (Figures 4G and 5B). Interestingly, TCV, CPB, or CPC inoculations significantly increased cytosolic levels of DRB4-GFP at 3 dpi; all TCV-inoculated leaves showed increased DRB4-GFP fluorescence outside the nucleus, and a majority of these showed fluorescence only in the extranuclear compartment (Figures 5B and S5C). A time-course analysis of TCV-inoculated *DRB4-GFP* leaves showed that cytosolic levels of DRB4-GFP increased within 24 hr of infection (Figure 5C) and prior to visible (Figure S5D) or microscopic HR formation (Figure 5C, bottom panel). Like WT-TCV, the virulent R8A mutant also increased cytosolic levels of DRB4-GFP, but a majority of these plants retained some DRB4-GFP in the nucleus (indicated by an arrow, Figure 5B). Moreover, DRB4-GFP also formed punctate structures in the R8A-inoculated leaves, not seen with WT-TCV (indicated by arrowheads, Figure 5B).

The partial or complete exclusion (monitored as visual absence of fluorescence) of DRB4 from the nucleus of Di-17 plants inoculated with avirulent (WT-TCV, CPB, CPC) or virulent (R8A) TCV, respectively, correlated with increased extranuclear levels of DRB4 in virus-infected plants. This, together with the fact that TCV-dependent nuclear exclusion of DRB4 occurred in response to both virulent and avirulent pathogens, raised two possibilities: (1) the R8A CP derivative is able to induce some HRT-mediated responses; (2) virulent and avirulent TCV derivatives possibly induce differential subcellular compartmentalization of cytosolic DRB4 that correlates with susceptible and resistance phenotypes, respectively. To evaluate the first possibility, we assayed HRT-triggered HR and *PR-1* expression in R8A-inoculated Di-17 plants. Although R8A-inoculated Di-17 plants did not show any visible HR (Figure S6A), they did show microscopic cell death (Figure S6B). However, the lesion size in R8A-inoculated plants was significantly smaller than that seen in WT-TCV-inoculated Di-17 plants. Consistent with microscopic HR formation, the R8A and WT-TCV-inoculated Di-17 plants accumulated similar levels of *PR-1* transcript (Figure S6C). In comparison, WT-TCV or R8A-inoculated Col-0 plants showed basal expression of *PR-1* (Figure S6C). Together, these results suggested that R8A was capable of eliciting at least a subset of HRT-mediated defense responses (also see below).

Next, we tested the subcellular compartmentalization by assaying colocalization of DRB4-GFP and CP-RFP or R8A-RFP in *N. benthamiana*. When expressed alone DRB4 localized primarily to the nucleus, and CP or its mutant derivative R8A were seen in inclusion-like structures (Figure 5D). Interestingly, coexpression of CP-RFP with DRB4-GFP not only increased the peripheral localization of DRB4, but also directed DRB4 and CP to punctate foci along the periphery (shown by arrowheads, Figure 5E, left upper panel). Notably, although R8A-RFP also colocalized with DRB4-GFP, a majority of R8A remained in the inclusion bodies (shown by arrowheads, Figure 5E, left middle panel). Colocalization of DRB4 and CP-/R8A-RFP was also tested in leaves coexpressing DRB4-NES/NLS-GFP proteins with CP-RFP or R8A-RFP. Leaves coexpressing DRB4-NES-GFP and CP-RFP showed more prominent punctate GFP fluorescence along the periphery (shown by arrowheads, Figure 5E, left bottom panel). In contrast, when coexpressed with R8A-RFP, the majority of the DRB4-NES-GFP

(D) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (scale bar, 10 μM). The micrographs shown are YFP and CFP overlay images. Individual YFP and CFP micrographs are shown in Figure S3E. Arrow and arrowhead indicates nucleus and endosomes, respectively. All interactions were confirmed in three separate experiments.
(E) Coimmunoprecipitation (IP) of DRB4-MYC with HRT-FLAG. Total protein extracted from the transgenic *Arabidopsis* plants expressing HRT-FLAG or DRB4-MYC or both was immunoprecipitated using anti-FLAG affinity beads, and the immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. The experiment was repeated three times with similar results.
(F) CoIP of DRB4-MYC with HRT-FLAG. *N. benthamiana* plants were agroinfiltrated, and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. Right panel shows IP assay of DCL4-MYC with HRT-FLAG. HRT and DRB4 were expressed using their respective native promoters, and DCL4 was expressed under 35S promoter. This experiment was repeated twice with similar results.
(G) Confocal micrographs showing localization of DRB4-GFP, DRB4-NLS-GFP, DRB4-nls-GFP (mutant NLS), DRB4-NES-GFP, and DRB4-nes-GFP (mutant NES) in *N. benthamiana* (scale bar, 10 μM). The experiment was repeated four times with similar results.
(H) CoIP of HRT-FLAG with DRB4-NLS or DRB4-NES/nes proteins. *N. benthamiana* plants were agroinfiltrated and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. *HRT* and *DRB4* were expressed under the 35S promoter. This experiment was repeated twice with similar results.
See also Figures S3 and S4.

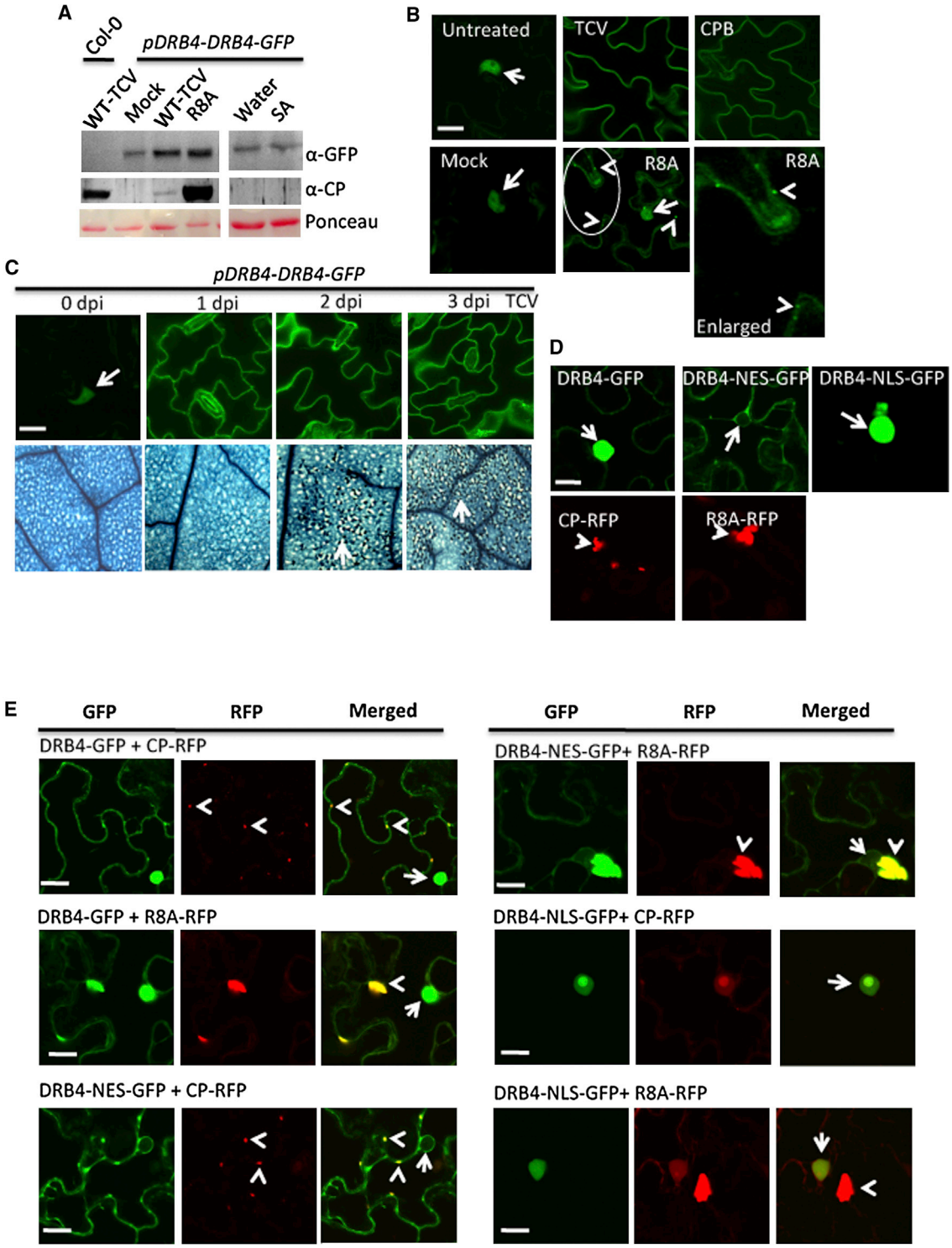

Figure 5. TCV Inoculation Increases the Cytosolic Pool of DRB4

(A) Western blot showing DRB4-GFP levels in mock- and WT-TCV-/R8A-inoculated and water- or SA- (500 μM) treated transgenic Di-17 plants expressing *DRB4-GFP* under its native promoter. Pathogen- and SA-treated leaves were sampled at 3 dpi and 2 days posttreatment, respectively. This experiment was repeated three times with similar results.

(B) Confocal micrographs showing localization of DRB4-GFP in untreated, mock-, or TCV-/CPB-/R8A-inoculated *Arabidopsis* plants. The leaves were analyzed at 3 dpi. The experiment was repeated three times with similar results. Scale bars, 10 μM. Bottom-right panel is an enlarged view of micrograph (shown by circle, R8A panel).

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colocalized to the inclusion bodies (shown by arrowheads, Figure 5E, right upper panel). Furthermore, coexpression of DRB4-NLS-GFP with CP-RFP or R8A-RFP directed all visually detectable CP-RFP to the nucleus, whereas a majority of R8A-RFP remained in the inclusion bodies (Figure 5E, right middle and bottom panels). Complete and partial nuclear localization of CP and R8A in the presence of DRB4-NLS-GFP, respectively, was consistent with the presence of both mono- and bipartite-NLS at the N terminus of CP (Figure S6D) and the fact that the R8A mutation mapped within the mono/bipartite NLS. The localization pattern of DRB4 and CP/R8A in leaves coexpressing DRB4-nes/nls-GFP and CP-/R8A-RFP was similar to that seen in control leaves (Figures 5E and S6E). These results, together with the fact that only DRB4-NES interacts with HRT (Figures 4H and S3G), suggest that CP-mediated increase in cytosolic levels of DRB4 and colocalization of CP and DRB4 may be important for the induced resistance response against TCV.

TCV CP Prevents HRT-DRB4 Complex Formation

Colocalization of DRB4 and CP prompted us to assay the interaction between CP and DRB4. The transgenic *Arabidopsis* Col-0 plants expressing *DRB4-MYC* under the native promoter were inoculated with buffer or TCV, and at 3 dpi the inoculated leaves were processed for IP using MYC antibodies. The TCV-inoculated WT Col-0 plants were used as an additional control in this experiment. Immunoblot analysis showed that CP coimmunoprecipitated with DRB4 only in TCV-inoculated *DRB4-MYC* transgenic plants (Figure 6A). This result, together with the observations that HRT interacts with DRB4 and DRB4 colocalizes with CP, suggested that DRB4 might act as a link between R protein and their corresponding avr protein, reminiscent of "guardee" proteins. However, unlike the R protein RPS2, which is activated by the absence of its guardee RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003), the absence of functional DRB4 protein did not autoactivate HRT; no significant difference in *PR-1* transcript was noticed in mock- and TCV-inoculated Di-17 and *HRT drb4* plants (Figure S2A).

A larger cytosolic pool of DRB4 in TCV-infected plants and the fact that HRT interacted only with the DRB4-NES protein (Figures 4H, S3G, 5B, and 5C) suggested that activation of HRT might be associated with increased association between HRT and DRB4. To test this, we assayed HRT-DRB4 complex formation in TCV-inoculated *Arabidopsis* plants. The *Arabidopsis* plants expressing *HRT-FLAG* and *DRB4-MYC* under their respective native promoters were mock- or TCV-inoculated, and at 3 dpi the infected leaves were processed for IP using FLAG antibodies. Interestingly, in three independent experiments we noticed that the TCV-inoculated plants showed a significant reduction in the levels of HRT-DRB4 complex, which we were only able to detect using a large amount of protein extract for coIP (Figure 6B). To determine if the reduced interaction was specifically the effect of the avr factor CP (as against other viral-encoded proteins), we carried out coIP assays in *N. benthamiana*, where HRT and DRB4 were coexpressed with and without CP. Consistent with results obtained with *Arabidopsis*, HRT did not interact with DRB4 in the presence of CP (Figure 6C). In contrast, the presence of CP did not alter the interaction between DRB4 and DCL4 or between HRT and CRT1 (Figures 6D and 6E), suggesting that CP specifically inhibited HRT-DRB4 interaction. An alternate possibility is that HRT-CP-mediated HR, rather than the presence of CP itself, abolished HRT-DRB4 interaction. To test this, we first monitored the HR phenotype of *N. benthamiana* plants expressing HRT, DRB4, and CP. As shown earlier, coexpression of HRT and CP did not trigger HR, it but did so in the presence of EDS1 (Zhu et al., 2011). Interestingly, coexpression of DRB4 with HRT and CP also potentiated HR, albeit less effectively than EDS1; HRT-CP-DRB4-expressing plants showed significantly less ion leakage than plants expressing HRT-CP-EDS1 (Figures S7A and S7B). Coexpression of CP did not alter interaction between HRT and EDS1 (Figure 6F), indicating that neither HR nor the presence of CP affects the association between HRT and EDS1. To assess further the possible effect of HR induction on HRT-DRB4 interaction, HRT and DRB4 were coexpressed in *N. benthamiana* in the presence or absence of AvrB, which induces cell death in *N. benthamiana* (Figure 6G, Selote et al., 2013). The presence of AvrB-induced cell death did not alter interaction between HRT and DRB4 (Figure 6H). Together these results suggested that cell death is not responsible for abolishing interaction between HRT and DRB4. To determine if the CP-mediated dissociation and/or obstruction of the HRT-DRB4 complex formation was required for HRT-mediated resistance, we evaluated HRT-DRB4 interaction in the presence of the coat protein from virulent strain R8A. Notably, R8A also resulted in the dissociation of the HRT-DRB4 complex (Figure 6I), which correlated with the fact that R8A partially activated HRT-mediated HR (Figures S6A–S6C) and triggered partial increase in the cytosolic pool of DRB4-GFP. Thus, dissociation of the HRT-DRB4 complex alone is not sufficient for complete activation of HRT and thereby full induction of resistance against TCV.

DRB4 Contributes to Bacterial Resistance

To determine if DRB4 serves as a general regulatory component in basal and/or R protein-mediated signaling, we evaluated the requirement for DRB4 in bacterial resistance. The *drb4* plants accumulated ~7- to 10-fold higher levels of virulent *Pseudomonas syringae pv tomato* (*Pst*), suggesting compromised basal resistance to *Pst* (Figure S8A). Interestingly, a mutation in *DRB4*

(C) Confocal micrographs showing localization of DRB4-GFP in TCV-inoculated *Arabidopsis* plants at 1–3 dpi (upper panel). The bottom panel shows corresponding trypan-blue-stained leaves. The experiment was repeated two times with similar results. Scale bars, 10 μM. Arrows indicate nucleus (upper panel) and dead cells (bottom panel).

(D) Confocal micrographs showing localization of DRB4-GFP, DRB4-NLS-GFP, DRB4-NES-GFP, CP-RFP, and R8A-RFP in *N. benthamiana* (Scale bar, 10 μM). Arrows and arrowheads indicate nucleus and inclusion structures, respectively.

(E) Confocal micrographs showing localization of the indicated proteins when coexpressed in *N. benthamiana* (scale bars, 10 μM). Experiments shown in (D) and (E) were carried out together. Arrows and arrowheads indicate nucleus and inclusion structures, respectively. This experiment was repeated three times with similar results.

See also Figure S5.

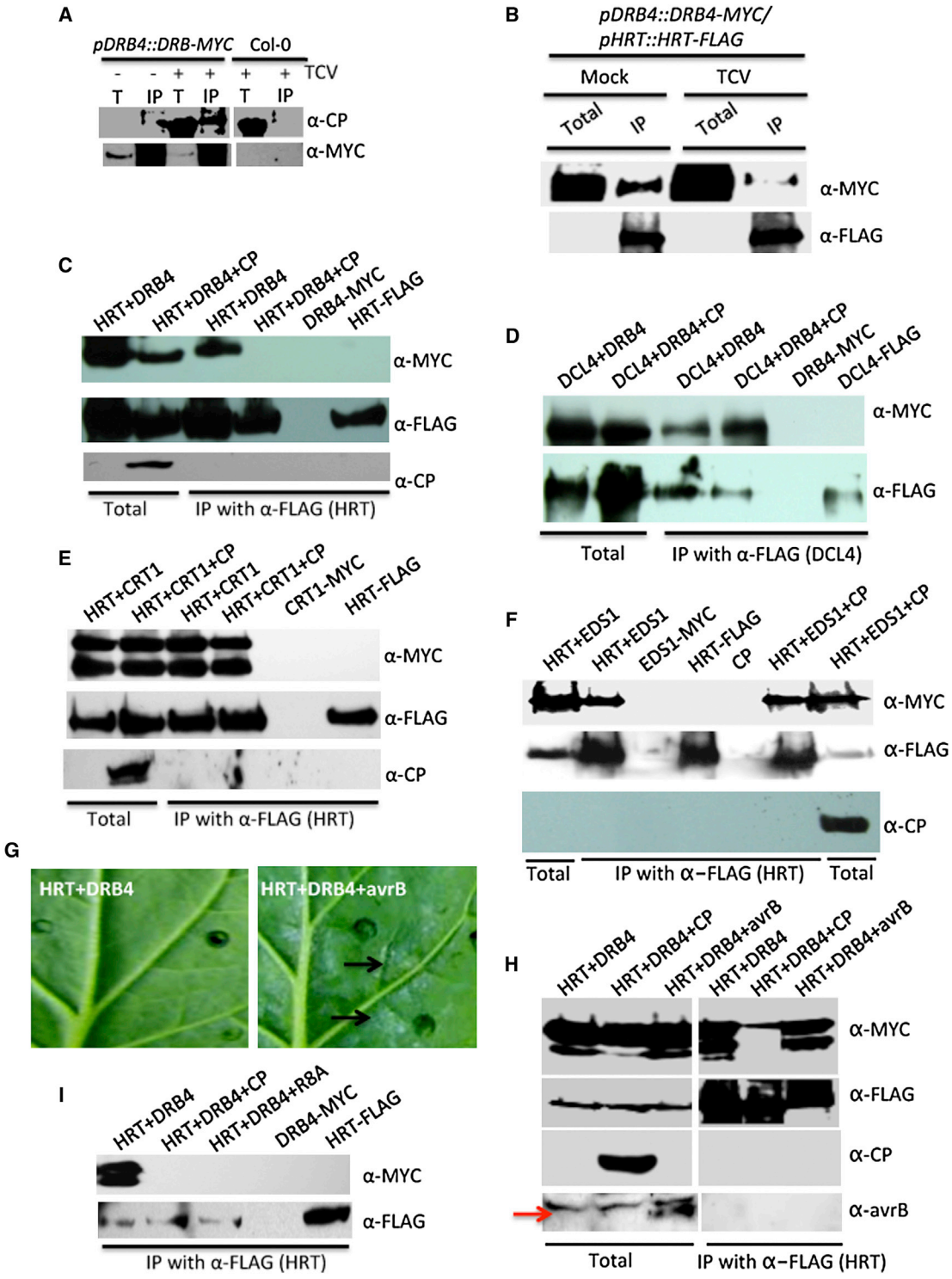

Figure 6. TCV Coat Protein Prevents HRT-DRB4 Complex Formation

(A) CoIP of TCV-CP with DRB4-MYC. The transgenic *Arabidopsis* plants expressing DRB4-MYC were inoculated with buffer (indicate by −) or TCV (indicated by +), and the total protein extracted at 3 dpi was immunoprecipitated using anti-MYC affinity beads and analyzed with α-MYC and α-CP. TCV-inoculated Col-0 plants were used as an additional control. This experiment was repeated three times with similar results.

(B) IP of DRB4-MYC with HRT-FLAG in mock- and TCV-inoculated plants. Total protein extracted from the transgenic *Arabidopsis* plants expressing DRB4-MYC and HRT-FLAG was immunoprecipitated using anti-FLAG affinity beads and analyzed with α-MYC and α-FLAG. Double the amount of total protein (2 mg/ml) extracted from TCV-inoculated plants was used for IP. The experiment was repeated three times with similar results.

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had a more severe effect on RPS2- and RPM1-mediated resistance to *Pst* expressing *avrRpt2* or *avrRpm1*, respectively; the *drb4* plants supported ~10- to 20-fold higher growth of these bacteria (Figures 7A and 7B). We next compared response of *drb4, sid2*, and *rps2* plants to *avrRpt2*. Consistent with an earlier report (Tsuda et al., 2009), *rps2* plants supported maximum bacterial growth followed by the *sid2* mutant (Figure 7A). Notably, *drb4* plants were as susceptible as *sid2* plants (Figure 7A). As observed in *Arabidopsis*-TCV interactions, inoculation of *Pst avrRpt2* or *Pst avrRpm1* resulted in increased cell death on *drb4* plants, which correlated with increased ion leakage (Figures 7C, 7D, S8B, and S8C). A time-course analysis of *PR-1* expression in *avrRpt2*-inoculated plants showed that *drb4* plants were compromised in pathogen induced *PR-1* levels (Figure 7E). Likewise, the *avrRpm1*-inoculated *drb4* plants also showed reduced *PR-1* expression (Figure S8D).

To determine if the compromised R-mediated resistance to bacterial pathogens in *drb4* plants was due to the instability of RPS2 and/or RPM1 proteins, we first evaluated interaction between DRB4 and RPS2/RPM1 proteins. DRB4 interacted with both RPS2 and RPM1 proteins in the coIP and BiFC assays (Figures 7F and 7G). Consistent with the plasma-membrane-specific localization of RPS2 and RPM1, the interaction between DRB4 and RPS2 or RPM1 was detected in the periphery of the cell. We next evaluated levels of RPM1-MYC and RPS2-HA in the *drb4* background. These plants were generated by crossing *drb4* with transgenic plants expressing RPM1-MYC and RPS2-HA under their respective native promoters. At least five independent F2 plants were tested, and all showed dramatic reduction in RPM1-MYC and RPS2-HA levels (Figures 7H and 7I). Normal *RPS2* and *RPM1* transcript levels in *drb4* plants (Figure S8E) suggested that degradation of RPS2/RPM1 proteins in *drb4* plants is a posttranscriptional response. The fact that *drb4* plants showed better resistance compared to *rps2* plants suggested that these plants were markedly reduced but not depleted in their RPS2 levels (Figure 7A). Because RPM1/RPS2 stability is dependent on RAR1 (Tornero et al., 2002), it was possible that a mutation in *drb4* affected stability of these R proteins by destablizing RAR1. However, normal levels of RAR1 in *drb4* plants suggested that DRB4 dependent RPM1/RPS2 stability was likely RAR1 independent (Figure 7J).

Because avr protein-induced systemic acquired resistance (SAR) is dependent on the presence of cognate R protein, we predicted that SAR would be compromised in *drb4* plants. To test this, we inoculated the WT (Col-0) and *drb4* plants with $MgCl_2$ or *avrRpt2/avrRpm1*-expressing *Pst*, and 48 hr later the distal leaves of all plants were challenged with a virulent strain of *Pst*. The WT plants previously inoculated with an avirulent *Pst* strain, showed an ~10-fold reduced growth of virulent bacteria compared to plants previously infiltrated with $MgCl_2$ (Figures S8F and S8G). In contrast, the *drb4* plants showed no reduction in the growth of virulent bacteria at 3 dpi, when pre-exposed to avirulent bacteria. Thus, *drb4* mutant plants were defective in their ability to induce SAR. Together, these results suggest that DRB4 is required for the stability of RPS2 and RPM1 proteins and thereby resistance mediated by these R proteins.

DISCUSSION

RNA silencing is a conserved pathway in both plants and animals. However, its role in defense has primarily been studied with relation to basal resistance against viral pathogens. In plants, the RNA silencing pathway targets viral RNA for degradation. Viruses, in turn, encode suppressors that target one or more components of the host RNA silencing machinery. Compromised RNA silencing in the host leads to increased accumulation of viral RNA, thereby enhancing susceptibility to the viral pathogens (Carr et al., 2010; Ding, 2010). However, in several cases the requirements for host RNA silencing components in basal resistance are not evident when the viral pathogen encodes an RNA silencing suppressor. In such cases, basal resistance has been evaluated using viral mutants that lack silencing suppressor activity and/or hosts that lack multiple components of the RNA silencing pathway (Deleris et al., 2006; Qu et al., 2008; Cao et al., 2010). Such analyses have shown that DCL2 and DCL4 are redundant, such that mutations in both DCL2 and DCL4 are required to compromise basal resistance against TCV (Deleris et al., 2006; Qu et al., 2008). In contrast, we demonstrated that this is not the case for R protein-mediated resistance because the *dcl4* mutation alone is sufficient to compromise HRT-mediated resistance, whereas a mutation in *DCL2* has no effect on HRT-mediated signaling. Moreover, mutations in *RDR6* and *DCL4* compromise HRT-mediated resistance but not basal resistance to WT-TCV (Qu et al., 2008). A role for RDRs in resistance signaling is further highlighted by the recent discovery that *R* genes against *Tomato yellow leaf curl virus* code for DFDGD-class RDRP (Verlaan et al., 2013).

Our results show that the RNA silencing suppressor function of CP is not essential for HRT-mediated resistance because the mutant CPs (CPB and CPC) lacking this function can activate HRT-mediated signaling. Conversely, mutant R8A virus, which retains suppressor activity, is virulent on resistant Di-17 plants.

(C–F) CoIP of DRB4-MYC with HRT-FLAG (C), DRB4-MYC with DCL4-FLAG (D), CRT1-MYC with HRT-FLAG (E), and EDS1-MYC with HRT-FLAG (F), in the presence or absence of CP. *N. benthamiana* plants were agroinfiltrated, and total extracts and immunoprecipitated proteins were analyzed with α-MYC, α-FLAG, and α-CP. The experiments shown in (C)–(F) were repeated three times with similar results.

(G) Visual phenotype of *N. benthamiana* leaves expressing HRT+DRB4 or HRT+DRB4+avrB. Arrows indicate cell death lesions. Agroinfiltration was used to express HRT and DRB4. For avrB, the *N. benthamiana* plants were inoculated with *Pseudomonas syringae* expressing avrB. The leaves were photographed at 3 days posttreatment.

(H) CoIP of DRB4-MYC with HRT-FLAG in the presence or absence of CP or avrB. Total and immunoprecipitated proteins were analyzed with α-MYC, α-FLAG, α-CP, and α-avrB. Arrow indicates band corresponding to avrB. The experiment was repeated two times with similar results.

(I) CoIP of DRB4-MYC with HRT-FLAG in the presence or absence of CP or R8A. *N. benthamiana* plants were agroinfiltrated and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. This experiment was repeated two times with similar results.

See also Figures S6 and S7.

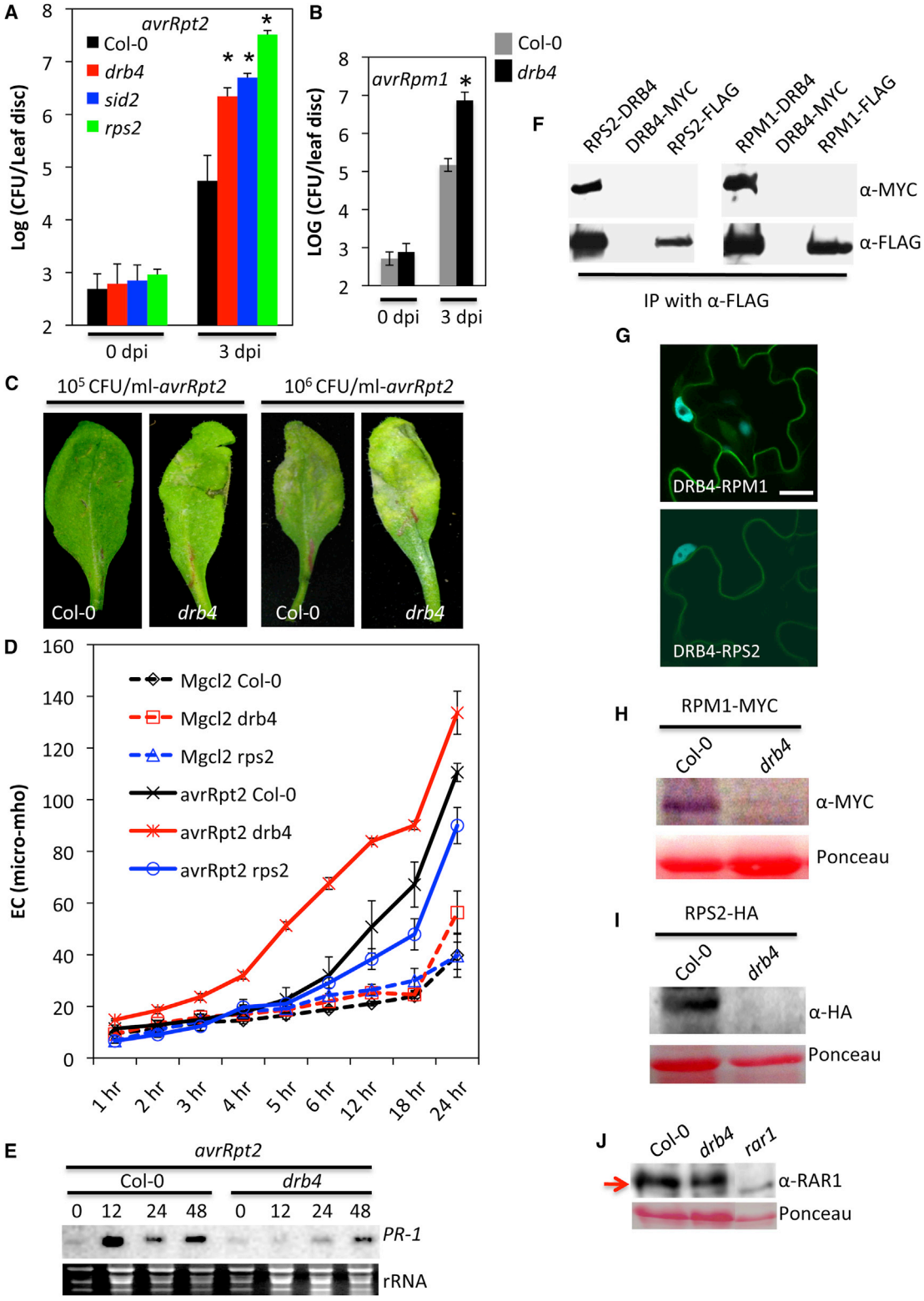

Figure 7. DRB4 Is Required for RPS2- and RPM1-Mediated Resistance to Bacterial Pathogens

(A and B) Growth of avirulent (*avrRpt2*, A; *avrRpm1*, B) *P. syringae* (*Pst*) strains on indicated genotypes. Error bars indicate SD. Asterisks indicate data statistically significant from that of control (Col-0) ($p < 0.05$, $n = 4$).

(C) Morphological phenotype of Col-0 and *drb4* plants inoculated with 10^5 or 10^6 CFU/ml *avrRpt2 Pst*. The leaves were photographed at 3 dpi.

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These results are also consistent with our observation that CP-mediated activation of HRT inversely correlates with accumulation of viral sRNA. This, in turn, suggests that processes leading to the synthesis of viral sRNA either are not active or are negatively regulated during a resistance response. Increased viral sRNA accumulation and high expression of the prototypic SA-responsive gene *PR-1* in R8A-inoculated plants suggest that increased levels of SA may not be associated with inhibition of viral sRNA synthesis.

We further show that DRB4 interacts with HRT and is required for its stability; a mutation in *DRB4* leads to spreading HR necrotic lesion, which correlates with degradation of HRT and increased replication of TCV. Similarly, the *cry2* and *crt1* mutants have reduced levels of HRT, which allows more replication and spread of the virus, leading to spreading HR lesions. These results are consistent with the observations that transgenic plants overexpressing HRT or plants with elevated HRT (induced by SA treatment) exhibit more resistance to TCV, with no visible HR development (Cooley et al., 2000; Chandra-Shekara et al., 2004; Jeong et al., 2010). The fact that overexpression of HRT or exogenous SA can overcome negative effects of RRT suggests that perhaps RRT might perform ADR1-like helper function (Bonardi et al., 2011). A requirement for RRT for resistance was reestablished by performing backcrosses between F1s (*HRT*/*hrt RRT*/*rrt*) derived from a Di-17 (*HRT*/*HRT rrt*/*rrt*) × Col-0 (*hrt*/*hrt RRT*/*RRT*) cross with resistant (Di-17) and susceptible (Col-0) parent, respectively. As expected, resistant plants were only observed in a test cross with Di-17 parent and segregated in a Mendelian fashion (50% resistant:50% susceptible). Whether rrt participates in the RNA silencing pathway awaits its cloning.

Interestingly, DRB4 was exclusively present in the extranuclear compartment when the resistant Di-17 plants were inoculated with avirulent (WT-TCV, CPB, and CPC) TCV but not virulent R8A virus. The fact that DRB4 and WT-CP colocalize to different subcompartments from DRB4 and R8A suggests that the relative localization of these and/or their interacting partners might play an important role in resistance signaling. A recent report showing relocalization of DRB4 from the nucleus to the cytoplasm in response to *Turnip yellow mosaic virus* suggests that the subcellular redistribution of DRB4 also occurs in other pathosystems (Jakubiec et al., 2012). This and the observations that DRB4 interacts with CP and P6 proteins of TCV and *Cauliflower mosaic virus* (Haas et al., 2008), respectively, suggest that DRB4 might be a common target of many viral pathogens. Increased extranuclear levels of DRB4 in TCV-inoculated plants, and the fact that only extranuclear DRB4 interacts with HRT, correlate with plasma-membrane-specific localization of HRT (Jeong et al., 2010). Our attempts to confirm HRT-DRB4-NES interaction using the native system were unsuccessful because DRB4-NES transgenic lines did not show any detectable expression of DRB4-NES. A possible explanation could be that constitutive extranuclear localization of DRB4 can potentially alter localization of other cellular protein that complex with DRB4, thereby affecting plant growth and/or viability. Our result that EDS1 localization can influence the subcellular localization of its interacting partners, SAG101 and PAD4, supports this possibility (Zhu et al., 2011).

In addition to DRB4, HRT also interacts with EDS1 (Zhu et al., 2011), but a mutation in EDS1 does not result in reduced levels of HRT and the resulting spreading HR (Chandra-Shekara et al., 2004). Notably, both HRT and DRB4 are degraded in a 26S-proteasome-dependent manner (Jeong et al., 2010), likely via their interaction with the E3 ubiquitin ligases, COP1 (Jeong et al., 2010) and APC/C (Marrocco et al., 2012), respectively. Whether APC/C participates in HRT degradation and if DRB4 regulates HRT stability by affecting COP1 or APC/C E3 ligase activities remains to be determined.

The fact that HRT interacts with EDS1, CRT1, and DRB4 raises the possibility that these proteins exist as multiprotein complex(s), as shown for other R proteins (Qi and Katagiri, 2009; Rivas et al., 2002). Interestingly, the pathogen-encoded avr effector, CP, specifically prevents the HRT-DRB4 interaction, but not the DRB4-DCL4, HRT-EDS1, or HRT-CRT1 interactions. The dissociation of HRT-DRB4 complex alone might not be sufficient for resistance signaling because CP from either avirulent (WT-TCV) or virulent (R8A) strains were able to disrupt the HRT-DRB4 interaction. However, both WT-TCV and R8A viruses were able to induce HRT-mediated SA pathway, which correlates with the dissociation of HRT-DRB4 complex in the presence of WT or R8A CPs. Thus, it is possible that HRT-DRB4 interaction and dissociation governs a subset of HRT-induced responses, which eventually culminate into a resistance response. Perhaps additional proteins in the HRT complex also regulate its activation. Consistent with this notion EDS1 is required for HRT-CP-mediated induction of HR. Furthermore, HR induced by coexpression of HRT, CP, and EDS1 in *N. benthamiana* is much stronger than when HRT and CP are coexpressed together with DRB4 (Zhu et al., 2011 and this study). Whether DRB4 and EDS1 regulate HR to TCV in a cooperative manner requires further investigation.

(D) Electrolyte leakage in Col-0, *drb4*, and *rps2* plants infiltrated with $MgCl_2$ or *avrRpt2 Pst*. Error bars represent SD (n = 6).
(E) RNA gel blot analysis showing expression of *PR-1* in Col-0 and *drb4* plants after inoculation with $MgCl_2$ (mock) or *avrRpt2 Pst*. Total RNA was extracted at indicated hours postinoculation. Ethidium bromide staining of rRNA was used as the loading control. The experiment was repeated twice with similar results.
(F) CoIP of DRB4-MYC with RPS2-FLAG (left panel) or RPM1-FLAG (right panel). *N. benthamiana* plants were agroinfiltrated and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. This experiment was repeated twice with similar results.
(G) Confocal micrographs showing BiFC for DRB4-RPM1 and DRB4-RPS2. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (scale bar, 10 μM). The micrographs shown are YFP and CFP overlay images. These interactions were confirmed in three separate experiments.
(H–J) Western blots showing relative levels of RPM1-MYC (H), RPS2-HA (I), or RAR1 (J) in Col-0 and *drb4* plants. The *rar1-21* mutant was used as a negative control for Figure 7J. Ponceau-S staining of the western blot was used as the loading control. This experiment was repeated four (H and I) or two (J) times with similar results. The arrow in Figure 7J indicates band corresponding to RAR1.
See also Figure S8.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions, Genetic Analysis, and Generation of Transgenic Plants

Plants were grown in MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and 14 hr photoperiod. The WT and mutant alleles were identified by PCR, CAPS, or dCAPS analysis (Table S3).

RNA Extraction, Conductivity Assays, Pathogen Infections, and Protein Work

RNA extraction, conductivity assays, and pathogens infections were carried out as described earlier (Kachroo et al., 2000; Yu et al., 2013). Protein analysis and coimmunoprecipitations were carried out as described earlier (Jeong et al., 2010; Zhu et al., 2011).

Detailed experimental procedures are included in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, eight figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.018.

AUTHOR CONTRIBUTIONS

S.Z., R.-D.J., and G.-H.L. carried out most of the biochemical, molecular, and genetic analysis with contributions from K.Y. and C.W. D.N. carried out SA estimations. All the authors analyzed data. P.K. and A.K. wrote the manuscript with contributions from all the authors.

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