Genetic Analyses of the FRNK Motif Function of *Turnip mosaic virus* Uncover Multiple and Potentially Interactive Pathways of Cross-Protection

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Cross-protection triggered by a mild strain of virus acts as a prophylaxis to prevent subsequent infections by related viruses in plants; however, the underlying mechanisms are not fully understood. Through mutagenesis, we isolated a mutant strain of *Turnip mosaic virus* (TuMV), named Tu-GK, that contains an Arg182Lys substitution in helper component-proteinase (HC-Pro^K) that confers complete cross-protection against infection by a severe strain of TuMV in *Nicotiana benthamiana*, *Arabidopsis thaliana* Col-0, and the *Arabidopsis dcl2-4/dcl4-1* double mutant defective in DICER-like ribonuclease (DCL)2/DCL4-mediated silencing. Our analyses showed that HC-Pro^K loses the ability to interfere with microRNA pathways, although it retains a partial capability for RNA silencing suppression triggered by DCL. We further showed that Tu-GK infection triggers strong salicylic acid (SA)-dependent and SA-independent innate immunity responses. Our data suggest that DCL2/4-dependent and -independent RNA silencing pathways are involved, and may crosstalk with basal innate immunity pathways, in host defense and in cross-protection.

Cross-protection results from a primary infection with a mild virus (termed the protective virus) that confers resistance against secondary infections by severe viruses (termed challenge viruses) that share sequence homology with the

protective virus (Gal-On and Shibolet 2006; Lin et al. 2007a and b; McKinney 1929). The features of cross-protection resemble the feature of current defense models for plants. First, cross-protection shares biochemical similarities to post-transcriptional gene silencing (PTGS), which has been regarded as a mechanism of RNA-mediated resistance that is produced via a transgenic plant approach (English et al. 1996; Prins and Goldbach 1996). However, the protective virus must persist in the host to trigger cross-protection, implying that cross-protection is not identical to RNA-mediated resistance, which can completely eliminate an invading virus by PTGS (Ratcliff et al. 1997; Simón-Mateo and García 2011). Second, the protective virus in the host plant provides protein-mediated resistance through interference with the uncoating of the challenge virus (Lu et al. 1988; Osbourn et al. 1990; Register and Beachy 1988), functional interference by a dominant negative effect, such as truncated replicase (Hellwald and Palukaitis 1995; Nguyen et al. 1996), or sequestration of key host factors that are essential for the replication and spreading of the challenge virus (Hofius et al. 2007; Lellis et al. 2002; Ziebell and Carr 2010). Third, the innate immune mechanisms of a host plant, such as the salicylic-acid (SA)-associated defense response that is triggered during viral infections, provide a basal defensive reaction against pathogens that do not have viral sequence- or species-specific resistance (Jovel et al. 2011; Kim et al. 2008; Love et al. 2012; Zhu et al. 2011). Although various defense models have been proposed, none of the models fully explain cross-protection; therefore, the mechanism of cross-protection remains unclear.

RNA-mediated resistance is based on the sense-PTGS mechanism of protecting the host against RNA-virus infection (Bouché et al. 2006; Ruiz et al. 1998; Simón-Mateo and García 2011; Wang et al. 2011b). The replicating viral RNA has a double-stranded structure and is regarded as a pathogen-associated molecular pattern that can initiate antiviral silencing and be cleaved by DICER-like ribonuclease 2 (DCL2) and DCL4 into fragments of 22 and 21 nucleotides (nt) of primary viral-derived short-interfering RNAs (vsiRNAs), respectively (Nakahara et al. 2012). García-Ruiz and associates (2010) demonstrated that DCL4 is important for antiviral silencing in

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inoculated leaves, whereas DCL2 and DCL4 are involved in silencing in systemic leaves or inflorescences. In addition, the plant loss-of-function mutants *dcl2* and *dcl4* are more susceptible to virus infection (Bouché et al. 2006; Deleris et al. 2006; Wang et al. 2011b). These primary vsiRNAs induce the host RNA-dependent RNA polymerases and other host components to generate secondary vsiRNA to amplify and maintain the antiviral silencing response (Bouché et al. 2006; Deleris et al. 2006; Eamens et al. 2008; Garcia-Ruiz et al. 2010; Wang et al. 2011b). Specifically, the vsiRNAs are loaded into ARGONAUTE1 (AGO1), which is a core protein of the RNA-induced silencing complex (RISC), to form a vsiRISC that targets viral RNAs. Due to the sequence identity of vsiRNAs, vsiRISC has a strain specificity that targets and cleaves the RNA genome of the second invading virus that shares sequence homology (Morel et al. 2002).

To counteract the host defensive response, plant viruses encode various viral suppressors to repress host RNA silencing (Gal-On and Shibolet 2006; Simón-Mateo and García 2011; Ziebell and Carr 2010). For example, the helper component-proteinase (HC-Pro) of the genus *Potyvirus* and 2b of *Cucumber mosaic virus* (CMV) inhibit DCL2- and DCL4-mediated PTGS (Lin et al. 2007b; Ziebell et al. 2007). Specific mutations in these viral suppressors lead to mild viral symptoms with lower viral titers in the infected plants, implying that the capability of viral suppressors to antagonize the DCL2- and DCL4-mediated PTGS may be affected (Lin et al. 2007b; Ziebell et al. 2007).

Previous studies demonstrated that the highly conserved Arg180 of the FRNK motif of HC-Pro of the *Zucchini yellow mosaic virus* (ZYMV) is essential for viral pathogenicity (Gal-On 2000; Lin et al. 2007b). The ZYMV mild strain of Taiwan isolate (GAC) and Israel isolate (AG), which has an Arg180Ile mutation of the FRNK motif, causes mild symptoms with moderate viral titer in infected zucchini squash plants and confers complete cross-protection (Gal-On 2000; Lin et al. 2007b). In addition, the Arg of the FRNK motif of HC-Pro is also essential for the suppression of the microRNA (miRNA)-mediated host response that regulates several genes during plant development (Shibolet et al. 2007; Wu et al. 2010). These results indicated that the Arg of the FRNK motif of HC-Pro plays an important role in mild strain-mediated cross-protection.

In this study, we used the *Turnip mosaic virus* (TuMV) severe strain YC5 carrying a *green fluorescent protein* (GFP) gene as a model virus (denoted as Tu-GR) (Chen et al. 2003; Niu et al. 2006) to generate attenuated mutants to further investigate mechanisms of cross-protection. Nineteen mutants with different amino acid substitutions of Arg182 of the FRNK motif were screened. Mutant Tu-GK, which contains an Arg182Lys substitution in HC-Pro (HC-Pro^K) and causes symptomless infection in *Arabidopsis thaliana* Col-0 plants and moderate infection in the *Arabidopsis dcl2/4* mutant plants, was selected for detailed functional analyses in cross-protection. Our results indicated that Tu-GK provided complete cross-protection against Tu-GR infection in both Col-0 and *dcl2/4* mutant plants. HC-Pro^K lost the ability to interfere with the miRNA pathway, resulting in infection without conspicuous symptoms, although it retained partial capability for RNA silencing suppression triggered by DCL. We found that DCL2/DCL4-independent vsiRNAs existed in *dcl2/4* plants. In addition, the DCL2/DCL4-mediated gene silencing pathway crosstalk with basal innate immunity pathways mediated by SA-dependent pathway and SA-independent pathway. Our results raise the intriguing possibility that multiple and likely cooperative mechanisms of resistance are involved in viral cross-protection.

RESULTS

Mutagenesis identified a TuMV strain that conferred effective cross-protection in *Nicotiana benthamiana*.

A useful and effective viral strain for mechanistic studies of cross-protection should sustain a moderate titer for a long period of time in an infected plant and does not cause severe disease symptoms. To identify such a TuMV strain, we used mutagenesis targeting the highly conserved Arg in the FRNK motif of HC-Pro that is essential for *Potyvirus* pathogenicity (Fig. 1A, upper panel) (Gal-On 2000; Lin et al. 2007a and b). In TuMV, the conserved Arg is at position 182 (Fig. 1A, upper panel). Nineteen amino acid substitutions were generated for Arg182 in the severe strain YC5 background, which carries a GFP gene (denoted as Tu-GR) (Chen et al. 2003; Niu et al. 2006) (Fig. 1A, lower panel). These mutants were named Tu-GX, where X is the one-letter abbreviation of the introduced amino acid (Fig. 1A, lower panel). All mutants were evaluated for their infectivity and pathogenicity in *Nicotiana benthamiana*, which is relatively easy to inoculate, to select for a mild TuMV strain that would confer cross-protection for further mechanistic studies in *Arabidopsis*. Upon inoculation onto *N. benthamiana*, only nine of the mutants (Tu-GH, Tu-GK, Tu-GS, Tu-GT, Tu-GP, Tu-GA, Tu-GI, Tu-GF, and Tu-GY) showed infectivity (Fig. 1A, lower panel).

Based on their titers and severity of the symptoms they caused in the infected *N. benthamiana* plants, the nine infectious TuMV mutants as well as the wild-type virus could be classified into four types. Type I included Tu-GR, the wild type, which caused mosaic and leaf distortion symptoms with strong GFP fluorescence under UV illumination (Fig. 1A and B). Furthermore, it accumulated to high titers (indirect enzyme-linked immunosorbent assay [ELISA] reading at an optical density at 405 nm [OD₄₀₅] of 1.2 to 1.6) from 5 to 15 days postinoculation (dpi) (Fig. 1D). Type II included Tu-GF, Tu-GP, and Tu-GY. They caused necrotic symptoms in the inoculated leaves and curling of systemic leaves with detectable GFP fluorescence (Fig. 1A and C). Their titers varied during infection, suggesting unstable viral accumulations (Fig. 1D). Type III included Tu-GH, Tu-GK, and Tu-GT. They induced mild mottling in the inoculated leaves and no symptoms in systemic leaves, with low GFP fluorescence in systemic leaves (Fig. 1A and C). Although Tu-GH had a low titer (OD₄₀₅ < 0.5), Tu-GK and Tu-GT showed a stable middle range of titers compared with that of Tu-GR (Fig. 1D). However, the Tu-GK titer had a shorter recovery time (<5 dpi) than Tu-GH (>10 dpi) during infection (Fig. 1D). Type IV (Tu-GI, Tu-GS, and Tu-GA) did not cause conspicuous symptoms and exhibited very low GFP fluorescence (Fig. 1A and C). These strains had low titers (OD₄₀₅ < 0.5) (Fig. 1D).

The type II mutants caused severe symptoms in inoculated and systemic leaves and type IV mutants showed relatively constant low titers that were difficult to sustain in the host plant. Moreover, none of the type IV mutants provided effective cross-protection in *N. benthamiana* against Tu-GR (*data not shown*). Therefore, they were not suitable as protective viruses for cross-protection studies. For the type III mutants, Tu-GH had a very low titer and Tu-GT required an excessively long recovery time. They were not suitable for cross-protection studies either. In contrast, Tu-GK caused mild mottling symptoms in the inoculated leaves and no symptoms in systemic leaves after 10 dpi (Fig. 1C). It was stably sustained in the host with a moderate viral titer compared with Tu-GR (Fig. 1D). Moreover, Tu-GK provided effective cross-protection in *N. benthamiana* plants against Tu-GR (Supplementary Fig. S1). Therefore, the Tu-GK mutant was selected for further studies on cross-protection in *Arabidopsis*.

Fig. 1. Construction of Turnip mosaic virus (TuMV) mild strain. **A**, The upper schematic represents the conserved Arg position in the helper component-proteinase (HC-Pro) of Zucchini yellow mosaic virus (ZYMV) and TuMV. Another conserved position, E396, is indicated in the HC-Pro of ZYMV to explain the mild strain (GAC) mutation position. The lower schematic represents the wild-type TuMV HC-Pro protein (Tu-GR) and the Arg182 mutants in the Tu-GX series for the selection of the TuMV mild strains. The 19 single-substitution mutations at position 182 in HC-Pro and the corresponding names of the mutant viruses are indicated to the left of the diagram. Tu-GR contains the green fluorescent protein (GFP) expression cassette. R refers to Arg at position 182. In the mutant viruses, the X refers to the substituted amino acid at position 182. The “+” and “-” symbols indicate the presence and absence, respectively, of viral infection in *Nicotiana benthamiana* plants. Types of viral symptoms in the infected *N. benthamiana* plants for each mutant virus are indicated to the right side of the mutants. **B**, Type I symptoms were caused by Tu-GR in *N. benthamiana* plants. Photographs were taken at 14 days postinoculation (dpi) under white light (left panel) and UV light (right panel). **C**, Symptoms that developed in *N. benthamiana* plants were classified into type II, type III, and type IV. A representative plant from each category and the names of the mutants are shown. “Mock” indicates buffer-inoculated control. Photographs were taken at 14 dpi under white light (left panels) and UV light (right panels). Bar = 2 cm. **D**, Time courses of Tu-GR and Tu-GX virus titers in the infected *N. benthamiana* plants. Each absorbance value represents the average reading from three plants. The virus titer was determined using an antiserum against TuMV CP (Lin et al. 2013) at a 1/20,000 dilution.

Tu-GK caused mild symptoms and retained partial silencing suppression function in *Arabidopsis*.

To establish the utility of Tu-GK for cross-protection studies in *Arabidopsis*, we first analyzed its infectivity, in comparison with Tu-GR as a control, in wild-type *Arabidopsis* Col-0 and mutant *dcl2/4* plants. This mutant line was chosen because DCL2 and DCL4 are primarily responsible for dicing viral RNAs to generate vsRNAs to initiate anti-viral silencing (Bouché et al. 2006). The *dcl2/4* plants flowered earlier than Col-0 (Fig. 2A). Tu-GR caused curling inflorescence, stunted growth, and yellowing mosaic leaf in both Col-0 and *dcl2/4* plants (Fig. 2A and B). In contrast, Tu-GK-infected Col-0 plants were symptomless, phenotypically similar to mock-

inoculated Col-0 plants (Fig. 2A and B). Tu-GK induced slight yellowing, leaf distortion, and shortened inflorescence symptoms in the *dcl2/4* plants, which were less severe than Tu-GR-infected *dcl2/4* plants (Fig. 2A and B).

The accumulation levels of Tu-GR and Tu-GK viral RNAs at different time points of infection in the Col-0 and *dcl2/4* plants were measured by real-time reverse-transcription polymerase chain reaction (RT-PCR) using *GFP-coat protein* (CP)-specific primers (Fig. 2C). The relative viral RNA levels at 5 and 10 dpi were compared with the RNA level at 0 dpi. Pairwise *t* tests were used to determine if there were significant differences among samples at 5 and 10 dpi, respectively (Fig. 2C). At 5 dpi, the expression level of Tu-GK in *Arabi-*

Fig. 2. Infectivity of Tu-GK in the *Arabidopsis* Col-0 and *dcl2/4* plants. **A**, Inflorescence symptoms in the Col-0 (upper panel) and the *dcl2/4* (lower panel) *Arabidopsis* plants infected with Tu-GR or Tu-GK. Tu-GR was used as a positive control. Photographs were taken at 14 days postinoculation (dpi). Bar = 2.5 cm. **B**, Yellowing symptoms on the leaves of Col-0 and *dcl2/4* plants after inoculation with Tu-GR or Tu-GK. Photographs were taken at 14 dpi. Bar = 1 cm. **C**, Time courses of Tu-GR and Tu-GK viral RNA levels in the infected Col-0 and *dcl2/4* plants as evaluated by real-time reverse-transcription polymerase chain reaction with green fluorescent protein coat protein (GFP-CP)-specific primers. The value at each time point represents the average relative viral RNA levels (log₁₀) from three plants. Relative viral RNA levels were compared with those of the 0-dpi plants. Bars represent standard deviations (*n* = 3). Letters represent significant differences between the groups by *t* test. **D**, GFP expression levels of Tu-GR or Tu-GK in the infected Col-0 or *dcl2/4* plants at 10 dpi were observed by confocal microscopy. Mock-inoculated Col-0 and *dcl2/4* plants were used as negative controls. Bar = 300 μ m. **E**, Relative average GFP expression (log₂) of Tu-GR- or Tu-GK-infected leaves compared with that of Tu-GR-infected Col-0 plants as determined by confocal microscopy (*n* = 3). Relative expressions levels were significantly different from those of the Tu-GR/Col-0 plants in each RNA sample, based on a Student *t* test; ** indicates *P* values < 0.01.

dopsis Col-0 was significantly lower than that of Tu-GR ($P < 0.05$); Tu-GK in the *Arabidopsis dcl2/4* mutant had a lower expression level than Tu-GR but the difference did not reach the $P < 0.05$ significance level. A similar scenario was also observed at 10 dpi; Tu-GR had a higher expression level than Tu-GK in *Arabidopsis* Col-0, although the difference was not significant at the $P < 0.05$ level.

The different accumulation levels of Tu-GK and Tu-GR in these plants revealed by real-time RT-PCR were confirmed by confocal microscopic observation (Fig. 2D) and quantification (Fig. 2E) of GFP fluorescence derived from the tagged viruses.

Altogether, our results indicated that Tu-GK has compromised but still functional activity in suppressing host anti-viral defense. The role of Tu-GK in cross-protection was directly tested by the following experiments.

Tu-GK conferred complete cross-protection in both Col-0 and *dcl2/4* plants.

The cross-protection function of the Tu-GK mutant was evaluated in the Col-0 and *dcl2/4* plants. *Arabidopsis* plants were first inoculated with Tu-GK as the protective virus (Fig. 3A). At 7 dpi, the plants were inoculated with YC5 as the challenge virus. ELISA analyses with anti-TuMV CP antibodies showed a significant reduction in the accumulation level of viral CP in Tu-GK-protected plants (Fig. 3B). To distinguish between Tu-GK and YC5 viruses in the protected plants, specific primers for each virus were used to amplify the *GFP-CP* region of Tu-GK and the *Nib-CP* region of YC5 (Fig. 3C). The real-time RT-PCR results showed that YC5 was present abundantly in unprotected plants but barely detectable in the protected plants (Fig. 3D). Our data indicated that Tu-GK infection of *Arabidopsis* conferred complete cross-protection against YC5 infection in both Col-0 and *dcl2/4* plants, consistent with earlier observations in *N. benthamiana*. Therefore, Tu-GK is a suitable mild strain for cross-protection in different plants. To gain insights into the potential mechanisms of Tu-GK function in cross-protection, or to understand the functional features of a protective, mild virus in cross-protection for future design of

such a virus, we conducted the following experiments to examine various aspects of Tu-GK function in comparison with the Tu-GR control.

DCL2/4-dependent and -independent accumulation of vsRNAs.

DCL2 and DCL4 targeted the double-stranded (ds), replicative forms of viral RNAs to generate 22- and 21-nt vsRNAs that guide sequence-specific viral RNA degradation (Bouché et al. 2006). To determine whether Tu-GK would affect vsRNA accumulation in comparison with the control Tu-GR, we used deep sequencing to analyze the vsRNA profiles in Col-0 and *dcl2/4* plants infected by the two viral strains. In Tu-GR-infected Col-0 plants, the 21-nt sense and antisense vsRNA reads were higher than those in the Tu-GR-infected *dcl2/4* plants (Fig. 4A). The scatter plot and the regression analysis of the vsRNA positions also showed the read counts moving toward the Col-0 side (the red dashed line below the 45° red solid line) (Fig. 4B, left panel). It is possible that redundant function of DCL1 (Bouché et al. 2006; Deleris et al. 2006) or other ribonucleases compensated for the defective *dcl4* function to generate the 21-nt vsRNAs (Bouché et al. 2006). In contrast, there were no significant differences in the 21-nt vsRNA reads in the Tu-GK-infected Col-0 and *dcl2/4* plants (Fig. 4A). This was confirmed by scatter plots (Fig. 4B, right panel; the red dashed line was close to the 45° red solid line). We suggest that the inefficient suppression of DCL2 and DCL4 by HC-Pro^K in the Col-0 plants resulted in a low titer of Tu-GK RNA as substrate for vsRNA production.

The 22-nt vsRNA levels in Tu-GR-infected *dcl2/4* plants showed a twofold reduction compared with those of Tu-GR-infected Col-0 plants, likely as a result of loss of *dcl2* function (Fig. 4A). In addition, the 22-nt vsRNA levels were considerably lower than the 21-nt vsRNA levels in the Tu-GR-infected Col-0 plants, suggesting that DCL2 plays a less efficient role in TuMV resistance (Fig. 4A). In contrast to the 21- and 22-nt vsRNAs, the 24-nt vsRNA levels increased approximately 95-fold in the Tu-GR- or Tu-GK-infected *dcl2/4* plants com-

Fig. 3. Cross-protection in *Arabidopsis* Col-0 and *dcl2/4* plants caused by infection with mild-strain viruses. **A**, The procedure for cross-protection (upper panel). Arrows indicate the time points of inoculation and detection for the protective virus (Tu-GK) and the challenge virus (YC5). **B**, Tu-GK confers cross-protection against the YC5 in the Col-0 (black column) and *dcl2/4* mutant (gray column) strains of *Arabidopsis*. The enzyme-linked immunosorbent assay (ELISA) analysis was performed at 14 day postchallenge using the TuMV coat protein (CP) antibody at a 1/20,000 dilution. Bars represent standard deviations ($n = 3$). **C**, The schematic represents the primer sets for the detection of Tu-GK or YC5 by real-time reverse-transcription polymerase chain reaction (RT-PCR). Arrowheads indicate the primer positions. **D**, Distinguishing between YC5 and Tu-GK from co-infected plants by real-time RT-PCR using specific primer sets. Relative expressions levels were normalized to the *ubiquitin* level. Bars represent standard deviations ($n = 3$).

pared with those in the Col-0 plants (Fig. 4A). These data suggested that DCL3 compensatorily cleaves the viral dsRNAs in the absence of *dcl2* and *dcl4* functions (Bouché et al. 2006).

P1/HC-Pro^K sustained a low level of sense-PTGS suppression activity.

The above viral infection experiments suggested that Tu-GK retained partial RNA silencing function. To test directly

the suppression ability of HC-Pro^K on sense-PTGS, the *P1/HC-Pro^K* and *P1/HC-Pro^R* (control) genes were expressed under the control of the 35S promoter in the β -glucuronidase (*GUS*) sense-PTGS transgenic *Arabidopsis* L1 line, which normally shows low or no *GUS* expression (Fig. 4C, lower panel). Two independent lines having similar expression levels of HC-Pro for each construct were selected for further anal

Fig. 4. Effects of TuMV wild-type P1/HC-Pro^R and mutant (P1/HC-Pro^K) on the morphological and molecular phenotypes of transgenic *Arabidopsis* plants. **A**, Accumulation of virus-derived siRNAs of Tu-GR and Tu-GK in the Col-0 and *dcl2/4* plants. The different sizes of the sense and antisense of TuMV-derived short-interfering (si)RNAs accumulated in the Col-0 and *dcl2/4* plants. The dark blue (21 nucleotides [nt]), red (22 nt), and green (24 nt) boxes indicate the sense-strand siRNAs, whereas the light blue (21 nt), pink (22 nt), and light green (24 nt) boxes indicate the antisense-strand siRNAs. The scale of the reads per kilobase of exon model per million mapped reads (RPKM) was divided by 10⁷. The read counts were significantly different from those of the Col-0 plants in each small RNA sample based on the Poisson test; ** indicates *P* values < 10⁻¹⁰⁰. **B**, Scatter plots of the read counts of the *dcl2/4* plants against the read counts of the Col-0 plants. The x- and y-axes are indicated in the log₂ read counts. The red solid line is the 45° straight line; the red dashed line is based on the results of a simple regression using the read counts of the Col-0 plants as the predictor variables and the read counts of the *dcl2/4* plants as the response variables. **C**, Representative phenotypes of transgenic *Arabidopsis* plants expressing *P1/HC-Pro^R* and *P1/HC-Pro^K* genes in the L1 line background. Photographs were taken using 2-week-old seedlings. The L1 line is a post-transcriptional gene silencing transgenic *Arabidopsis* expressing the β -glucuronidase (*GUS*) gene and was used as a control. Bar = 0.5 cm. **D**, HC-Pro expression levels in transgenic *Arabidopsis* lines. At least two independent lines for each construct were analyzed. The numbers on the panel represent the signal strengths of the HC-Pro levels after normalization against ribulose-1,5-bisphosphate carboxylase (*) using the large subunit levels as loading controls. A polyclonal antibody against HC-Pro was used at a 1/10,000 dilution for Western blot detection. **E**, Evaluation of the *P1/HC-Pro^R* and *P1/HC-Pro^K* suppression efficiency in *GUS* expression in the L1 line background by real-time reverse-transcription polymerase chain reaction. Relative expression levels were normalized with the *ubiquitin* level. Bars represent standard deviations (*n* = 3). **F**, Effects of *P1/HC-Pro^R* and *P1/HC-Pro^K* on microRNA (miRNA) and miRNA* accumulation. Oligonucleotides complementary to miRNA and miRNA* were used as probes. 5S ribosomal RNA (rRNA) and transfer RNA (tRNA) were used as loading controls. Numbers on the panels represent the average signal strengths of the miRNAs relative to those in the non-transformed wild-type plants (Col-0) after normalization against the loading control. Levels in the Col-0 plants were set at 1.0. Two independent lines for each construct were analyzed.

Both *P1/HC^R* and *P1/HC^K* plants stained positively for GUS, with indistinguishable levels (Fig. 4C). However, quantification of *GUS* mRNA levels with real-time RT-PCR revealed a significant difference between plants expressing the two *P1/HC-Pro* constructs. Although *P1/HC^R* plants showed approximately 59-fold increase in *GUS* mRNA level compared with that of the L1 line, *P1/HC^K* plants showed a 19-fold increase (Fig. 4E). Thus, *P1/HC^K* retained the function of suppressing sense-PTGS but at a significantly lower level than *P1/HC^R*. This was consistent with the results from the above viral infection analyses.

***P1/HC-Pro^K* did not cause abnormal phenotypes or interfere with miRNA pathways in *Arabidopsis*.**

The *P1/HC^R* plants showed a severe serrated leaf phenotype that is typical of a defective miRNA pathway, as reported earlier (Kasschau et al. 2003; Lin et al. 2007b). However, the *P1/HC^K* plants did not exhibit any abnormal phenotypes (Fig. 4C). This

suggested that *P1/HC^K*, while still retaining partial silencing-suppression function, might not negatively affect miRNA metabolism. To test this directly, we analyzed several miRNAs that have altered expression levels in correlation with the altered plant phenotype in transgenic *Arabidopsis* expressing the TuMV HC-Pro (Kasschau et al. 2003). Our results showed that miR165/miR165* and miR168/miR168* exhibited increased accumulations in the *P1/HC^R* plants compared with that in the Col-0 plant (Fig. 4F), as previously reported (Kasschau et al. 2003). In contrast, the *P1/HC^K* plants had expression levels of these miRNAs similar to the Col-0 plants (Fig. 4F). Therefore, *P1/HC-Pro^K* retained partial sense-PTGS suppression but did not appear to interfere with the general miRNA pathways.

HC-Pro^K had stability similar to the wild-type HC-Pro^R in planta.

The partial loss of suppression activity of *P1/HC^K* could be attributed to faster turnover of the protein caused by the mutation or to other factors. To address this, we next analyzed the stability of HC-Pro^K in comparison with HC-Pro^R in *P1/HC^K* and *P1/HC^R* plants treated with 200 μ M cyclohexamide (CHX). Both HC-Pro^R and HC-Pro^K exhibited similar stability during the 4-h period of CHX treatment (Fig. 4A). As a control, RING1, an E3 ubiquitin ligase with a rapid turnover rate (Lin et al. 2008), was degraded 1 h after CHX treatment (Fig. 5A). Thus, the partial loss of suppression activity of *P1/HC^K* was due to factors other than protein stability.

We further investigated whether *P1/HC^K* would have a dominant negative effect in protein-mediated resistance. We challenged the *P1/HC^K*-transgenic plants with Tu-GR virus. At 14 dpi, the plants showed severe symptoms (Fig. 5B). Thus, HC-Pro^K did not provide protein-mediated resistance to Tu-GR infection.

Evidence for crosstalks between RNA silencing and plant innate immunity.

In addition to RNA silencing-based adaptive immunity, various innate immunity mechanisms have also evolved in plants. To test whether RNA silencing would crosstalk with plant innate immunity pathways, we analyzed the expression of several genes that play critical roles in innate immunity in Col-0 and *dcl2/4* plants infected by Tu-GR and Tu-GK, respectively. At 10 dpi, Tu-GK had lower viral titers than Tu-GR in Col-0 and *dcl2/4* plant (Fig. 6A). First, we analyzed the expression of the SA-dependent defense marker gene *PATHOGENESIS-RELATED GENE 1 (PR1)*. Col-0 and *dcl2/4* plants infected by Tu-GR or Tu-GK all showed elevated expression of *PR1* (approximately 42- to 64-fold increase compared with expression in the mock-inoculated plants) (Fig. 6B).

Next, we measured the expression of defense genes involved in SA biosynthesis (*SID2* and *EDS5*) (Fig. 6C), SA accumulation and signaling (*WIN3*, *FMO1*, and *PAD4*) (Fig. 6D), and SA downstream signaling (*WRKY51*) (Fig. 6E) pathways (Ho et al. 2013; Mishina and Zeier 2006; Nawrath et al. 2002; Wang et al. 2011a; Wildermuth et al. 2001; Zhou et al. 1998). We also measured the expression of SA-independent defense genes *AIG1* and *DOX1* (Fig. 6F). These measurements revealed two major patterns. First, their expression levels were generally lower in *dcl2/4* plants than in Col-0 plants infected by both TuMV strains. This indicates that expression of DCL2/4 is necessary for the proper expression of these genes under viral infection conditions. Second, the expression levels of these genes were generally higher in both types of plants infected by Tu-GK than those infected by Tu-GR. Thus, dampened silencing suppressor activities, and perhaps also low viral titers, appear to trigger stronger innate immune responses. Taken together, the requirement for the RNA silencing machinery for

Fig. 5. Evaluation of HC-Pro^K protein-mediated resistance. **A**, Stability of HC-Pro^R and HC-Pro^K in vivo. Two-week-old *Arabidopsis* seedlings over-expressing *P1/HC-Pro^R* and *P1/HC-Pro^K* were used. Samples were treated with or without 200 μ M cyclohexamide (CHX). HC-Pro protein levels were analyzed after various treatment durations. Migration positions of protein markers (kDa) are indicated on the left. The ubiquitin E3 ligase RING1-HA was used as a positive control. **B**, Examination of protein-mediated resistance in *P1/HC^K* plant by challenge with Tu-GR. Photographs were taken at 14 days postinoculation (dpi).

the proper expression and the downregulated expression by a full-functional viral silencing suppressor of these defense genes suggests crosstalks between adaptive immunity based on RNA silencing and innate immunity based on other mechanism. How these two immunity pathways interact for general plant defense for cross-protection is an outstanding question for future investigations.

DISCUSSION

Knowledge of the mechanisms of cross-protection has significant implications in understanding the evolution and pathogenesis of viruses and in developing practical methods to control viral diseases. Using mutagenesis, we generated Tu-GK as a mild strain of TuMV that provided complete cross-protection in *N. benthamiana* and *Arabidopsis* plants. Characterization of this strain allowed us to define several important criteria for a useful protective virus that may help future development of new protective strains for other viruses. Furthermore, studies of this strain shed new light on the potential interplay between RNA silencing and innate immunity pathways in cross-protec-

tion, which may enable further in-depth studies to better understand plant defense mechanisms.

Criteria for a suitable mild strain for cross-protection.

Based on the findings from this study and from the literature, we proposed four criteria for the identification of a useful mild strain for effective cross-protection. First, a mild strain should have an inefficient suppressor that does not interfere with miRNA pathways and does not induce severe symptoms in protected plants, as exemplified by P1/HC-Pro^K characterized in this study. As further support of this criterion, the 2b deletion mutant of CMV (Fny-CMVΔ2b) also caused no symptoms and conferred cross-protection against the Fny-CMV severe strain in Col-0 plant (Ziebell and Carr 2009).

Second, for survival and maintenance of replication, the silencing suppressor of a mild strain should retain partial suppression of the DCL2/DCL4-mediated viral defense. P1/HC-Pro^K retains 33% of the wild-type efficiency in sense-PTGS suppression, enabling the virus to sustain a moderate titer in the Tu-GK-protected Col-0 plants (Fig. 2C). Similar results

Fig. 6. Differential expression of defense genes assessed by real-time reverse-transcription polymerase chain reaction in Tu-GK- or Tu-GR-infected *Arabidopsis* Col-0 and *dcl2/4* plants. **A**, Detection of TuMV accumulation at 10 days postinoculation by monitoring the green fluorescent protein coat protein (GFP-CP) region. **B**, Expression of defense marker gene (*PR1*). **C**, Expression of salicylic acid (SA) biosynthesis genes (*SID2* and *EDS5*). **D**, Expression of *WIN3*, *FMO1*, and *PAD4*, which are regulated in SA accumulation and signaling pathways. **E**, Expression of *WRKY51*, which is involved in SA downstream signaling. **F**, Expression of *AIG1* and *DOX1*, which are involved in other plant defense response-associated pathways. Bars represent standard deviations ($n = 3$). Letters represent significant differences between the Tu-GK/Tu-GR and Col-0/*dcl2/4* using the *t* test.

were obtained with infection of Fny-CMVΔ2b in *dcl2/3/4* or *dcl2/4* plants (Ziebell and Carr 2009).

Third, a moderate and sustained titer of a protective virus is necessary to trigger cross-protection. The type IV TuMV mutants have low titers in infected plants that cannot trigger cross-protection (*data not shown*). This is consistent with findings from a protection examination of ZYMV GAB (ZGAB) that has a titer too low to trigger cross-protection in zucchini squash (Lin et al. 2007b). In contrast, Tu-GK has a moderate titer that can sustain viral survival in the host to trigger cross-protection. This was also shown in GAC-mediated incomplete protection in zucchini squash before 5 dpi, in which the inhibition of challenge virus replication by GAC depends on the titer (Lin et al. 2007b). In addition, our data suggest that the moderate titer of Tu-GK triggers strong innate immune responses against infection by a challenge virus. As further support of this notion, P1 of the genus *Potyvirus* interferes SA-dependent defense pathway, and low titers of a mutant *Plum pox virus* with the N-terminal P1 deleted are correlated with higher expression levels of SA-associated genes in infected plants (Pasin et al. 2014).

Finally, the mild strain should produce stable mild symptoms and yet should not cause severe adverse effects on the protected host. Tu-GK showed stable mild symptoms in *N. benthamiana* and was symptomless in *Arabidopsis* for more than four passages (*data not shown*). The same phenomenon was observed for ZYMV mild strains in zucchini squash (Gal-On 2000; Lin et al. 2007b).

Recent studies suggest that the PTGS machinery crosstalks with various gene regulatory pathways, such as autophagy and alternative splicing (Derrien et al. 2012; Gibbings et al. 2012; Morlando et al. 2008). Several lines of evidence also suggest that SA-dependent defense pathways crosstalk with PTGS (Garcia-Ruiz et al. 2010; Rakhshandehroo et al. 2009; Yang et al. 2004) and that DCL2 and DCL4 are involved in SA-dependent and -independent defense pathways. Recently, Pasin and associates (2014) demonstrated that P1 has modulates SA-dependent pathway. These findings implicate crosstalk between the PTGS machinery and innate immunity as a new direction of research in the future.

DCL2/DCL4-dependent and -independent vsiRNA generation.

Fig. 7. Proposed model of Tu-GK-induced cross-protection. The replicating Tu-GK produces double-stranded viral RNAs (dsRNAs) that serve as a pathogen-associated molecular pattern. The DCL2 and DCL4 recognize the dsRNAs, triggering RNA-mediated silencing (DCL2/DCL4-dependent and -independent RNA degradation). The HC-Pro^K in Tu-GK is compromised in suppression of DCL-mediated post-transcriptional gene silencing (PTGS), resulting in low titers of the Tu-GK virus in the host cells that are sufficient to maintain RNA silencing. Tu-GK infection triggers strong salicylic acid (SA)-dependent and SA-independent innate immunity responses. In addition, Tu-GK may provide protein-mediated resistance, including competition for viral proteins and sequestration of essential host factors to suppress the replication of the challenge virus. DCL2/4-dependent RNA silencing pathways may crosstalk with basal innate immunity pathways in host defense and in cross-protection. The dotted line represents defective suppression of PTGS by HC-Pro^K. Gray lines and arrowheads indicate the suggested pathways or mechanisms for cross-protection.

A working model for cross-protection.

In Figure 7, we propose a working model for Tu-GK-induced cross-protection. In early infection of Tu-GK, no RNA-silencing is induced. However, the dsRNAs of Tu-GK produced during replication are recognized by DCL2/DCL4 or other RNase III proteins to trigger DCL2/DCL4-dependent and -independent gene silencing. This RNA-mediated silencing targets the genomic RNA of the challenge virus for degradation. During Tu-GR-mediated ETI, HC-Pro^R suppresses DCL2/DCL4-dependent gene silencing, resulting in susceptibility to infection. However, HC-Pro^K of the Tu-GK mutant is incapable of fully suppressing the DCL2/DCL4-mediated pathway in the host, thus resulting in a moderate titer of Tu-GK. The moderate virus titer and the inability to interfere with miRNA biogenesis result in symptomless infection in Tu-GK-infected Col-0 plants. Because Tu-GK continues to exist in the host, protein-mediated resistance for sequestering the essential host or virus factors might play a role to suppress the challenge virus infection through the CP of Tu-GK or through sequestration of essential host factors by Tu-GK. In addition, the Tu-GK infection also triggers SA-dependent and -independent defense pathways to provide a basal defense response to the challenge virus. However, the basal innate immunity alone may not be responsible for conferring complete cross-protection because this response has no specificity. Finally, we found that DCL2 and DCL4 can crosstalk with innate immunity, defining a new direction for further study on virus-induced secondary signaling for acquired resistance. Further studies will determine the extent to which innate immunity pathways participate in or interact with RNA silencing in cross-protection. In summary, our working model suggests multiple and likely interactive mechanisms of resistance in viral cross-protection.

MATERIALS AND METHODS

Plant materials and growth conditions.

A. thaliana and *N. benthamiana* plants were sown directly on Florabella potting compost/sand mix (3:1) and maintained in a growth room (16 h of light and 8 h of darkness at 20 to 25°C). The *Arabidopsis dcl2/4* double mutant, derived from *dcl2-4* (FLAG_451B03) × *dcl4-1* (FLAG_330A04), was provided by H. Vaucheret (Bouché et al. 2006).

Infectious clones and virus inocula.

The Taiwan strain of the TuMV infectious clone (p35STuMV-GFP) includes a 35S promoter, *GFP* gene, and the full-length cDNA of the TuMV YC5 strain (Chen et al. 2003). The *GFP* gene was inserted between the *NIb* and the *CP* genes to generate chimeric Tu-GR (Lin et al. 2009; Niu et al. 2006). Tu-GR was used as a surrogate wild-type virus and a backbone for the construction of various recombinant viruses described herein. PCR-mediated mutagenesis was used to generate serial single mutations individually, to substitute Arg182 in HC-Pro, and to introduce a restriction enzyme site for screening the clones. For example, to generate Arg182Lys mutation, the primer sets PTuHCSphI-F (5'-CAAGCATGCAGTGCAGATAC-3')/MTuHCBamHI-A (5'-CTTGTTAAGAAAGGACTTCAAGGATCCCC-3') and PTuHCBamHI-A (5'-GGGATCCTTGAAGTCCCTTTCTTAACAAG-3')/MTuHCSphI-R (5'-GGGCATGCTTTTGCACATGGCAG-3') were used to change the amino acid Arg182 to Lys182 (underlined) and to include two nucleotide mutations (bold) to generate a unique *Bam*HI restriction site. The other single mutations were produced similarly. The mutated PCR fragments were digested with *Sph*I, and the resulting fragments were used to replace the wild-type HC-Pro region of the p35STuMVGFP to generate recombinant viruses (Fig. 1A). The recombinant viruses are named Tu-GX, in which X refers to the introduced amino acid (Fig. 1A).

Challenge inoculation with recombinant viruses and cross-protection assays.

All recombinant viruses were propagated from DNA-infectious clones. Aliquots of 1 µg of DNA in sterilized water (20 µl) were applied onto Carborundum-dusted leaves of *Chenopodium quinoa* with a sterilized glass spatula. At 6 dpi, establishment of infection of each recombinant virus on the inoculated leaves was examined based on GFP signal under a fluorescent microscope. The virus was then isolated from a single lesion and transferred to *N. benthamiana* plants for amplification and evaluation of viral pathogenicity at 7 dpi. Twenty *N. benthamiana* plants were used for each experiment. Results from triplicates of each experiment were used to make conclusions.

In cross-protection assays, Tu-GK was used as the protective virus and YC5 was used as the challenge virus. The 2.5-week-old *Arabidopsis* seedlings were mechanically inoculated with Tu-GK, which was prepared from 0.5 g of infected *N. benthamiana* leaves using 2 ml of 0.01 M sodium phosphate buffer, pH 7.2. At 7 days after the protective virus inoculation, two fully expanded leaves of a plant were mechanically challenged with YC5, which was prepared from 0.5 g of *N. benthamiana* leaves using 2 ml of 0.01 M sodium phosphate buffer, pH 7.2. The protection was evaluated by examining the development of symptoms in the plants. The presence of challenge virus was monitored by indirect ELISA and real-time RT-PCR at 14 days after challenge inoculation, as described below.

Transgenic *Arabidopsis*

for P1/HC-Pro^R and P1/HC-Pro^K expression.

The *P1/HC-Pro^R* and *P1/HC-Pro^K* fragments were amplified from the Tu-GR- and Tu-GK-infectious clones, respectively, by PCR with primer pair PTuP1-Nco (5'-GCGCCATGGCAGTAGTTACATTCGCATCGGCT-3') and MTuHC-Pac (5'-CTAGTTAATTAATTATCCGACACGGTAGTGTTTTAAGCT-3'), which contain *Nco*I and *Pac*I (underlined), respectively, and cloned into the pBCo vector (Wu et al. 2010) to generate pBCo-P1/HC^R and pBCo-P1/HC^K, respectively. The pBCo binary vector contains a double 35S promoter to express the *P1/HC-Pro* coding sequence.

Plants of the *Arabidopsis* L1 line (Elmayan et al. 1998), which carries a silenced *GUS* gene, were transformed with the above binary vectors via a floral-dip method (Clough and Bent 1998; Zhang et al. 2006). Seed from the infiltrated plants were selected on standard Murashige and Skoog medium (Murashige and Skoog 1962) containing the appropriate selective agents. The growth and developmental phenotypes were analyzed for T2 transformants.

Confocal microscopy.

GFP fluorescence at 10 dpi of Tu-GR- or Tu-GK-infected *Arabidopsis* leaves was monitored using a Leica TCS SP5 II confocal laser-scanning microscope that was equipped with a multiline argon laser and a filter set for a GFP fluorescence filter (excitation filter Acousto-optic Tunable filter 488, emission bandwidth 496 to 574 nm). All images were obtained with PMT2 offset (-1.0)/grain (895.3), and graphically arranged using Adobe Photoshop CS3 software (Adobe Systems Inc., Mountain View, CA, U.S.A.). The GFP fluorescence intensity was calculated by LAS AF (Leica, Ryswyck, The Netherlands).

ELISA.

Nine leaf disks, approximately 0.5 cm each in diameter, were punched from three different upper leaves of each infected plant at 0, 5, 10, 15, and 20 dpi. The disks from each plant were combined into triplicate samples and assayed by indirect ELISA using anti-TuMV CP antiserum as the primary

antibody (Lin et al. 2013). The results were recorded as absorbance at 405 nm using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Real-time RT-PCR.

Total RNA was extracted from 0.1 g of plant tissue using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RT was performed using the SuperScript III first-strand synthesis system (Invitrogen) following the manufacturer's instructions. Real-time PCR was performed using a LightCycler 480 instrument (Roche, Indianapolis, IN, U.S.A.) using three sets of primers: PGFP (5'-GGACGAGCTGTACAAGGCTA-3') and MTuCP1 (5'-TTTGCTCTCTCGTTCCTTT-3') for the amplification of the GFP/CP region of Tu-GR or Tu-GK, PTuN1b (5'-AAGGAACCAGCTCAAGAGGA-3') and MTuCP1 for the amplification of the N1b/CP region of YC5, and UBQ-F1 (5'-CACTTCACCTGGTCTTGCGT-3'); and UBQ-R1 (5'-TATCCTGGATCTTGGCCTTC-3') for the detection of the *ubiquitin* gene. The Tu-GR, Tu-GK, YC5, and *ubiquitin* transcripts were quantified via a relative cycle threshold (Ct) method. The primers for analyzing the expression of SA-dependent and -independent-associated genes were the same as those reported by Ho and associates (2013). All experiments were performed with three independent replicates to compensate for possible loading errors. The relative expression levels were calculated based on the $\Delta\Delta C_t$ value, and each sample was normalized according to the expression levels of *ubiquitin*. At 0 and 5 dpi for the viral RNA level evaluation, the virus-inoculated leaves were collected for detecting the host genes. At 10 dpi, the systemic leaves of the virus-infected plants were collected for detection.

High-throughput deep sequencing of small RNAs.

The small RNA profiles in Tu-GR- and Tu-GK-infected *Arabidopsis* Col-0 and *dcl2/4* plants were analyzed by deep sequencing. Total RNA was extracted from TuMV-infected plants with Trizol (Invitrogen) at 15 dpi, following the manufacturer's protocols, and 10 μ g of total RNA from each sample was used to generate small RNA libraries for sequencing with Illumina HiSeq2000 by Genomics BioSci & Tech Co. A high-throughput data analysis was performed using the CLC Genome Workbench 5.1.

The total reads for each sample underwent quality trimming and size selection (21, 22, and 24 nt). The read counts for the selected samples of size k (Fig. 3E) were calculated using the following formula: total reads for the sample of size k / (total reads for *dcl2/4* plants / total reads for Col-0 plants).

The denominator of the above formula was used to adjust for different throughput numbers under two conditions. The reads per kilobase of exon model per million mapped reads (RPKM) values for the selected samples of size k (Fig. 3E) were calculated using the following equation: $RPKM = 10^9 \times [(total\ sample\ reads) / (total\ reads\ of\ size\ k) / k]$.

To test whether the total number of reads for a given sample was significantly different from that for the Col-0 plant, we performed a two-sample Poisson test using the "poisson.test" function in the R software package (R Development Core Team 2012). The significance parameters based on the Poisson test P values are marked in Figure 4A.

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