Viral-Induced Systemic Necrosis in Plants Involves Both Programmed Cell Death and the Inhibition of Viral Multiplication, Which Are Regulated by Independent Pathways

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Resistant plants respond rapidly to invading avirulent plant viruses by triggering a hypersensitive response (HR). An HR is accompanied by a restraint of virus multiplication and programmed cell death (PCD), both of which have been observed in systemic necrosis triggered by a successful viral infection. Here, we analyzed signaling pathways underlying the HR in resistance genotype plants and those leading to systemic necrosis. We show that systemic necrosis in *Nicotiana benthamiana*, induced by *Plantago asiatica* mosaic virus (PIAMV) infection, was associated with PCD, biochemical features, and gene expression patterns that are characteristic of HR. The induction of necrosis caused by PIAMV infection was dependent on SGT1, RAR1, and the downstream mitogen-activated protein kinase (MAPK) cascade involving MAPKKK α and MEK2. However, although *SGT1* and *RAR1* silencing led to an increased accumulation of PIAMV, silencing of the MAPKKK α -MEK2 cascade did not. This observation indicates that viral multiplication is partly restrained even in systemic necrosis induced by viral infection, and that this restraint requires SGT1 and RAR1 but not the MAPKKK α -MEK2 cascade. Similarly, although both SGT1 and MAPKKK α were essential for the Rx-mediated HR to *Potato virus X* (PVX), SGT1 but not MAPKKK α was involved in the restraint of PVX multiplication. These results suggest that systemic necrosis and HR consist of PCD and a restraint of virus multiplication, and that the latter is induced through unknown pathways independent from the former.

Plants activate various highly regulated defense mechanisms in response to pathogen invasions. The most studied mechanism is that of resistance (*R*) gene-mediated defense responses. The *R*-gene-mediated defense is induced by a specific pathogenic determinant (elicitor) in the plant expressing the corresponding *R* gene, which confers resistance to the avirulent pathogen in a strain-specific manner (Chisholm et al. 2006;

Jones and Dangl 2006). When plants are attacked by pathogens, including fungi, bacteria, and viruses, *R* gene-mediated resistance usually causes a hypersensitive response (HR), defined as a “rapid death of plant cells in association with the restraint on pathogen growth” (Goodman and Novacky 1994), which confines the pathogen to the initial infection area.

In association with an HR, reactive oxygen species (ROS) and ion fluxes are generated (Morel and Dangl 1997) with the induction of defense-related genes, such as *Hin1*, *Hsr203J*, and pathogenesis-related (*PR*) genes (Gopalan et al. 1996; Yang et al. 1997; Pontier et al. 1998), and programmed cell death (PCD) occurs (Lam et al. 2001). Although the roles of PCD in an HR are not fully understood, host cell PCD during an HR is postulated to have a negative effect on the growth of biotrophic pathogens, including viruses, which require living host cells to establish a successful infection (Greenberg and Yao 2004; Lam et al. 2001). This idea is supported by many studies demonstrating that SGT1 and RAR1, which are required for many *R*-gene-mediated HR (Peart et al. 2002; Tornero et al. 2002), play an important role in the restraint of virus multiplication and the induction of PCD (Liu et al. 2002; Peart et al. 2002). However, some studies suggest that PCD may not play a primary role in restraining viruses. For example, Rx-mediated resistance does not involve PCD, which is a type of extreme resistance (ER) (Peterhansel et al. 1997; Bendahmane et al. 1999; Hajimorad and Hill 2001; Ghazala and Varrelmann 2007). Nevertheless, studies on Rx-mediated ER to *Potato virus X* (PVX) showed that ectopic expression of the Rx protein and the viral elicitor for ER, the PVX coat protein (CP), induces PCD, suggesting that ER is similar to HR in that its *R* protein has the potential to induce PCD (Bendahmane et al. 1999). Consequently, these ER results provide evidence that PCD and a restraint on pathogen multiplication can be induced through distinct pathways and that these pathways can be uncoupled (Soosaar et al. 2005).

This suggestion has been supported by a series of HR studies. For example, in the *HRT*-mediated HR to *Turnip crinkle virus* (TCV) in *Arabidopsis thaliana*, the *R*-gene signaling components EDS1, EDS5, PAD4, and SID2 are all involved in TCV restraint but not in activation of PCD (Chandra-Shekara et al. 2004). Additionally, a locus designated *CCD1* from *Nicotiana clevelandii* suppresses PCD but not the restraint on *Cauliflower mosaic virus* (CaMV), although the actual *CCD1* gene has not yet been identified (Cawly et al. 2005). However,

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despite all of these findings, the precise pathway leading to PCD or PCD-independent restraint on virus multiplication during virus-induced HR remains to be revealed, and the role of PCD in HR is still under debate (Greenberg and Yao 2004).

Several studies have shown that systemic necrosis, one of the most severe symptoms in susceptible plants that eventually results in plant death, has HR characteristics, such as the induction of PCD (Xu and Roossinck 2000; Yao et al. 2002; Dickman et al. 2001; Xu et al. 2004; Love et al. 2005). Although the genetic basis for the systemic necrosis induced by pathogen infection in susceptible plants has not been well studied, the HR induced by the avirulent pathogen in some *R*-genotype plants occasionally spreads to the entire plant, resulting in systemic HR (SHR) (Dinesh-Kumar et al. 2000; Hajimorad et al. 2005). This is usually caused by incomplete resistance due to some genetic and environmental perturbation of normal elicitors or *R*-gene product functions. A similarity between *R*-gene-mediated SHR and systemic necrosis is suggested by recent studies showing that some of the systemic necrosis regarded as pathogen-induced symptoms were also regulated by nucleotide binding site leucine-rich repeat (NBS-LRR) proteins typically encoded by the largest *R*-gene class (Seo et al. 2006; Lorang et al. 2007; Atsumi et al. 2009) as well as by its phenotypic resemblance. Although an HR normally inhibits pathogen invasion, whereas systemic necrosis cannot, both responses have similar phenotypic and molecular features; therefore, a comparison of an HR and systemic necrosis could reveal similarities and differences in both responses.

Here, we analyzed the systemic necrosis triggered by infection with a potyvirus, *Plantago asiatica mosaic virus* (PIAMV), in the experimental host *N. benthamiana*. The PIAMV Li1 isolate, which was originally from lilies (Komatsu et al. 2008), induces systemic necrosis in *N. benthamiana*, whereas the Li6 isolate infects asymptotically (Ozeki et al. 2006). We previously showed that RNA-dependent RNA polymerase (RdRp) amino acid no. 1154 of PIAMV contributes to systemic necrosis (Ozeki et al. 2006). In this study, we demonstrated that systemic necrosis in PIAMV-infected plants revealed several plant defense response features observed in an HR. In addition, we analyzed the pathways leading to this systemic necrosis and HR by comparing the systemic necrosis induced by PIAMV with the HR triggered by PVX in transgenic *N. benthamiana* plants expressing *Rx* (Bendahmane et al. 1999). To this end, we used a virus-induced gene silencing (VIGS) approach to downregulate the expression of genes required for the HR. We found that SGT1 and RAR1, as well as components of the mitogen-activated protein kinase (MAPK) cascade, which could act at the downstream of both factors, such as MEK2 and its possible upstream activator MAPK kinase kinase α (MAPKKK α) (del Pozo et al. 2004), would be essential for PCD in both the HR and systemic necrosis. However, SGT1 and

RAR1 are involved in the restraint of PIAMV multiplication, whereas MAPKKK α and MEK2 are not. Similarly, we showed that SGT1 but not MAPKKK α was involved in the restraint of PVX multiplication, suggesting that systemic necrosis and HR consist of PCD and a restraint on virus multiplication, and that the latter is induced through unknown pathways that are distinct from the former involving MAPKKK α .

RESULTS

Systemic necrosis is associated with HR characteristics.

To investigate whether systemic necrosis induced by PIAMV is accompanied by biochemical features and gene expression patterns that are characteristic of an HR, we used four PIAMV variants (Li1 and Li6 isolates and their respective mutants, Li1-1154Y and Li6-1154C) (Ozeki et al. 2006). All four viruses cause systemic infection in wild-type *N. benthamiana* but symptoms produced by these viruses can be classified into two typical types: Li1 and Li6-1154C develop systemic necrosis, whereas Li6 and Li1-1154Y cause no symptoms (latent infection) (Ozeki et al. 2006).

When each of the four PIAMV variants was agroinoculated into an *N. benthamiana* leaf, the Li1- and Li6-1154C-infiltrated areas exhibited necrosis, whereas no symptoms were visible in the Li6- and Li1-1154Y-infiltrated areas (Fig. 1A). These symptoms in the infiltrated areas were in accordance with those induced in systemic tissues described above. Typical defense responses induced by plant pathogens, such as cell death, the accumulation of ROS (e.g., hydrogen peroxide [H₂O₂]), and callose deposition, were examined by staining infiltrated tissues. Trypan blue was used to selectively stain dead cells. At 6 days post infiltration (dpi), blue staining was observed in the Li1/Li6-1154C-infiltrated areas of the leaf but not in the Li1-1154Y/Li6-infiltrated areas (Fig. 1B). 3,3'-Diaminobenzidine (DAB) staining was performed to detect H₂O₂ production (Thordal-Christensen et al. 1997). A characteristic brown color was detected in the Li1/Li6-1154C-infiltrated areas of the leaf, indicating H₂O₂ production (Fig. 1C). Callose deposition, a signal of a defense response against plant pathogen attack, was visualized as bright fluorescence under UV light after aniline blue staining (Rate et al. 1999). Specific callose deposition signals were detected in the Li1/Li6-1154C-infiltrated areas (Fig. 1D). The Li1-1154Y/Li6-infiltrated areas did not show cell death, H₂O₂ production, or callose deposition. We then monitored ion leakage as a quantitative measure of the extent of cell death (Pontier et al. 1998). At 4 dpi, high levels of ion leakage occurred in the Li1/Li6-1154C-infiltrated areas compared with those in the Li1-1154Y/Li6-infiltrated areas (Fig. 1E). Additionally, H₂O₂ production was seen in the noninoculated upper leaves and stems infected with Li1 but not with Li1-1154Y, suggesting that the defense response char-

Fig. 1. Systemic necrosis induced by *Plantago asiatica mosaic virus* (PIAMV) has characteristics of the hypersensitive response (HR). **A** through **D**, Typical HR biochemical features associated with PIAMV-induced systemic necrosis. HR markers were detected in the PIAMV-infiltrated area using several staining methods. Expanded *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* cultures expressing PIAMV viral RNA at a final turbidity of 0.5 (optical density at 600 nm). **A**, Typical symptoms in the infiltrated areas upon inoculation with Li1 (left upper), Li6 (right upper), Li1-1154Y (left bottom), and Li6-1154C (right bottom) at 6 days post infiltration (dpi). Red and blue circles represent necrosis and no symptoms, respectively. **B**, Detection of dead cells with trypan blue staining. **C**, Hydrogen peroxide (H₂O₂) accumulation with 3,3'-diaminobenzidine (DAB) staining. **D**, Callose deposition with aniline blue staining. In **B** through **D**, each of the four viruses was infiltrated at the same position as in **A**, and leaves were taken and analyzed at 6 dpi. **E**, Ion leakage assay of the PIAMV-infiltrated area. Leaf discs were excised and assayed for ion leakage at 4 dpi. In all figures showing ion leakage results, a minimum of five independent replicates was sampled, and the results are shown as the mean $\pm$ standard deviation. **F**, H₂O₂ accumulation with DAB staining in the noninoculated leaves and stems infected with Li1 (middle) and Li1-1154Y (right). Noninoculated control (β -glucuronidase [GUS]; plants infiltrated with pCAM-BIA1301) is shown on the left. Scale bars: 5 mm. **G**, Northern blots of the plant defense-related genes *PR-1a*, *Hsr203J*, and *Hin1* in both local and upper noninfiltrated leaves. The expression levels of defense-related genes in the noninfiltrated leaves (left) and upper noninoculated leaves (right) were determined at 3 and 11 dpi, respectively. Ethidium bromide staining of ribosomal RNA confirmed equal loading in each lane. **H**, Detection of nuclear DNA fragmentation with the transferase-mediated dUTP nick-end labeling (TUNEL) assay. PIAMV- and empty vector-infiltrated areas were assayed and observed at 4 dpi. Sections of an empty vector-infiltrated area were treated with DNase I (DNase I+) as a positive control. Scale bars: 50 μ m.

acteristics of an HR were also induced in noninoculated systemic tissues showing necrotic symptoms (Fig. 1F).

The induction of defense-related genes (Gopalan et al. 1996; Yang et al. 1997; Pontier et al. 1998; Takabatake et al. 2007) was then investigated for PIAMV-induced systemic necrosis. Northern blots indicated that the levels of *PR-1a*, *Hsr203J*, and *Hin1* messenger (m)RNAs in the Li1/Li6-1154C-infiltrated area were markedly higher than in the Li1-1154Y/Li6- or empty vector-infiltrated areas (Fig. 1G). The same results were obtained in the noninoculated upper leaves of each of the

PIAMV-infected plants (Fig. 1G). The presence of the four PIAMV variants in all of the analyzed upper leaves in Figure 1G was confirmed by Northern blotting using a PIAMV CP-specific probe (data not shown).

PIAMV-infiltrated areas were analyzed using the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to investigate whether PCD was actually involved in the necrosis induced by Li1/Li6-1154C (Xu and Roossinck 2000; Yao et al. 2002). Positive green fluorescence indicated that DNA fragmentation had occurred in both the positive control sections treated with DNase I and the sections from the Li1/Li6-1154C-infiltrated areas of the leaf but not in sections from the Li1-1154Y/Li6-infiltrated areas (Fig. 1H).

Both SGT1 and RAR1 are critical for the PCD of systemic necrosis elicited by PIAMV.

Although the systemic necrosis triggered by viruses possesses several features characteristic of an HR, the pathway leading to the induction of systemic necrosis has not been characterized. We investigated whether SGT1 and RAR1 are also involved in the induction of systemic necrosis by PIAMV. To knock down the expression of each gene, we performed *Tomato rattle virus* (TRV)-based VIGS in *N. benthamiana* (Ratcliff et al. 2001). Plants were inoculated with the TRV vector carrying a partial fragment of either the endogenous *NbSGT1* or *NbRAR1*. To confirm that either gene was indeed knocked down, Northern blotting or semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using total RNA extracted from noninoculated upper leaves of the plants at 21 dpi. The results showed significant knockdown of the respective gene transcripts (Fig. 2A and B).

Li1 and Li1-1154Y were inoculated on *NbSGT1*- and *NbRAR1*-silenced and nonsilenced control plants by agroinfiltration to examine the responses of *NbSGT1*- and *NbRAR1*-silenced plants following PIAMV infection. In the Li1-infiltrated area of the nonsilenced plants, characteristic necrotic symptom was observed and H_2O_2 was detected by DAB staining at 7 dpi (Fig. 2C). However, no necrosis or H_2O_2 production was seen in the Li1-infiltrated area of the *NbSGT1*- and *NbRAR1*-silenced plants. Necrosis induction and H_2O_2 production did not occur in the Li1-1154Y-infiltrated area of both silenced and nonsilenced plants. The suppression of Li1-induced necrosis in the *NbSGT1*- and *NbRAR1*-silenced plants was confirmed by measuring ion leakage; the level of ion leakage in the Li1-infiltrated area of both *NbSGT1*- and *NbRAR1*-silenced plants was considerably lower than that in the nonsilenced plants (Fig. 2D). These results

Fig. 2. SGT1 and RAR1 silencing compromises the programmed cell death of *Plantago asiatica mosaic virus* (PIAMV)-induced systemic necrosis. **A**, Confirmation of *NbSGT1* silencing. Northern blots were performed to detect *NbSGT1* in the total RNA from upper noninoculated leaves at 21 days after *NbSGT1* silencing was induced. Two replicates for each virus-induced gene silencing (VIGS) construct are shown. Ethidium bromide staining of ribosomal RNA is shown as a loading control. **B**, Confirmation of the silencing of *NbRAR1*. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed to detect *NbRAR1* at 21 days after *NbRAR1* silencing was induced. Ubiquitin (UBI) messenger RNA was used as an internal control, and lanes 1 through 5 correspond to products from PCR cycles 25, 29, 33, 37, and 41, respectively. Lane M is the 100-bp ladder marker. **C**, Symptoms in the Li1- or Li1-1154Y-infiltrated areas of nonsilenced and *NbSGT1*- and *NbRAR1*-silenced plants (left column) and 3,3'-diaminobenzidine (DAB) staining of the same leaves (right column). Photographs were taken at 7 days post infiltration (dpi). **D**, Ion leakage assay in the Li1-infiltrated area at 7 dpi in nonsilenced and *NbSGT1*- or *NbRAR1*-silenced plants. The bars "VIGS vector" and "VIGS vector*" represent the controls for *NbSGT1*- and *NbRAR1*-silenced plants, respectively.

strongly suggest that Li1-mediated systemic necrosis was induced in a manner dependent on both SGT1 and RAR1.

The MAPKKK α -MEK2 cascade is critical for the PCD of systemic necrosis elicited by PIAMV.

Components of MAPK cascades play a central role in many R-gene-mediated defense responses to plant pathogens (Nakagami et al. 2005; Pedley and Martin 2005). They have been proposed to act at the downstream of the elicitor recognition step that involves SGT1 and RAR1 (Liu et al. 2004b; Takabatake et al. 2007). MEK2 is a MAPK kinase required for N-mediated resistance to *Tobacco mosaic virus* (TMV) (Jin et al. 2003), and MAPKKK α is a possible activator of the downstream MEK2-SIPK pathway (del Pozo et al. 2004). To examine whether the MAPKKK α -MEK2 cascade is involved in PIAMV-mediated systemic necrosis, VIGS of *NbMAPKKK α* and *NbMEK2* was performed using a TRV-based vector. Knockdown of these genes was confirmed by Northern blotting or semiquantitative RT-PCR using total RNA extracted from noninoculated upper leaves (Fig. 3A and B).

PIAMV-Li1 and Li1-1154Y were inoculated by agroinfiltration onto the *NbMAPKKK α* - and *NbMEK2*-silenced plants and on nonsilenced control plants. No necrotic symptoms occurred, and H₂O₂ production was suppressed in the PIAMV-Li1-infiltrated area of the *NbMAPKKK α* - and *NbMEK2*-silenced plants (Fig. 3C). However, a characteristic necrotic symptom was observed in the corresponding area of the nonsilenced plants, together with a strong H₂O₂ signal. Necrosis induction and H₂O₂ production were not detected in the Li1-1154Y-infiltrated area of the *NbMAPKKK α* - and *NbMEK2*-silenced and nonsilenced plants. These observations were supported by significantly reduced ion leakage in the PIAMV-Li1-infiltrated area of the *NbMAPKKK α* - and *NbMEK2*-silenced plants compared with that in nonsilenced plants (Fig. 3D).

Moreover, we also investigated the effect of *NbSGT1*, *NbRAR1*, *NbMAPKKK α* , and *NbMEK2* silencing on Li1 infection in noninoculated systemic leaves. Upper noninoculated leaves and stems of nonsilenced plants inoculated by Li1 developed necrotic symptoms at 10 dpi. However, no visible or significantly reduced necrotic symptoms were observed in the Li1-infected upper leaves and stems of *NbSGT1*-, *NbRAR1*-, *NbMAPKKK α* -, and *NbMEK2*-silenced plants. In addition, PIAMV RNA was detectable by RT-PCR analysis in the upper noninoculated leaves of all silenced and nonsilenced plants (Supplementary Fig. 1).

SGT1 and RAR1 but not the MAPKKK α -MEK2 cascade are critical for the restraint of virus multiplication during systemic necrosis elicited by PIAMV.

SGT1, RAR1, and the MAPKKK α -MEK2 cascade are critical components of PIAMV-induced necrosis; however, whether they affect virus multiplication is not clear. We investigated the effect of *NbSGT1* and *NbRAR1* silencing on the accumulation of PIAMV using Northern blotting. Total RNA was extracted from the PIAMV-Li1/Li1-1154Y-infiltrated areas in the *NbSGT1*- and

Fig. 3. Mitogen-activated protein kinase kinase kinase α (MAPKKK α) and MEK2 silencing compromises the programmed cell death of *Plantago asiatica* mosaic virus (PIAMV)-induced systemic necrosis. **A**, Confirmation of *NbMAPKKK α* silencing. Northern blots were performed to detect *NbMAPKKK α* in the total RNA from the upper noninoculated leaves at 21 days after *NbMAPKKK α* silencing was induced. Two replicates for each construct are shown. Ethidium bromide staining of ribosomal RNA is presented as a loading control. **B**, Confirmation of *NbMEK2* silencing. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed to detect *NbMEK2* at 21 days after *NbMEK2* silencing was induced. Ubiquitin (UBI) messenger RNA was used as an internal control, and lanes 1 through 3 correspond to products from PCR cycles 28, 32, and 36, respectively. Lane M is the 100-bp ladder marker. **C**, Symptoms in the Li1- and Li1-1154Y-infiltrated areas of nonsilenced, *NbMAPKKK α* -silenced, and *NbMEK2*-silenced plants (left column) and 3,3'-diaminobenzidine (DAB) staining of the same leaves (right column). Photographs were taken at 7 days post infiltration (dpi). **D**, Ion leakage assay in the Li1-infiltrated area at 7 dpi in nonsilenced and *NbMAPKKK α* - or *NbMEK2*-silenced plants. The bars "VIGS vector" and "VIGS vector*" represent the controls for *NbMAPKKK α* - and *NbMEK2*-silenced plants, respectively.

NbRAR1-silenced plants and used for analysis. At 4 dpi, PIAMV-Li1 accumulation was significantly higher in the *NbSGT1*- and *NbRAR1*-silenced plants than in the nonsilenced plants but no difference was observed in Li1-1154Y accumulation between silenced and nonsilenced plants (Fig. 4A).

We further investigated the effect of *NbMAPKKKα* and *NbMEK2* silencing on the accumulation of PIAMV. Total RNA from the PIAMV-Li1/Li1-1154Y-infiltrated areas in the *NbMAPKKKα*- and *NbMEK2*-silenced plants was used for Northern blotting. At 4 dpi, the level of PIAMV-Li1 RNA in both silenced plants was nearly the same as that in the nonsilenced plants (Fig. 4B). Moreover, no difference was detected in Li1-1154Y accumulation between silenced and nonsilenced plants.

The above results were further confirmed by monitoring PIAMV RNA accumulation using real-time RT-PCR. PIAMV-Li1 viral RNA accumulated to higher levels in the *NbSGT1*-silenced plants than the nonsilenced plants, whereas no signifi-

cant differences in Li1-1154Y viral RNA accumulation were observed between *NbSGT1*-silenced and nonsilenced plants (Fig. 4C). In contrast, no differences in accumulation of either Li1 or Li1-1154Y were seen between nonsilenced and *NbMAPKKKα*-silenced plants (Fig. 4C).

MAPKKKα is critical for PCD but not for the restraint of virus multiplication in the *Rx*-mediated HR elicited by PVX.

To evaluate the role of MAPKKKα in the *R*-gene-mediated HR against viruses, we focused on the HR induced by PVX in the plants carrying the *Rx* gene, which confers resistance to PVX. Note that both PVX and PIAMV belong to the genus *Potexvirus*. Although transgenic *N. benthamiana* plants expressing *Rx* (*Rx-N. benthamiana*) show ER without PCD to mechanically inoculated PVX, the ectopic expression of PVX CP (the viral elicitor) by agroinfiltration induces *Rx*-dependent PCD (Bendahmane et al. 1999). When we inoculated PVX

Fig. 4. Accumulation of *Plantago asiatica* mosaic virus (PIAMV) RNA in *NbSGT1*-, *NbRAR1*-, *NbMAPKKKα*-, and *NbMEK2*-silenced plants. **A** and **B**, Northern blots were performed to detect the accumulation of viral RNA in the Li1- and Li1-1154Y-infiltrated areas of nonsilenced plants and **A**, *NbSGT1*- or *NbRAR1*-silenced plants and **B**, *NbMAPKKKα*- or *NbMEK2*-silenced plants. Northern blots were performed at 4 days post infiltration (dpi) and two replicates for each detection are shown. Results presented are representative of three independent experiments. Ethidium bromide staining of ribosomal RNA is shown as a loading control. **C**, Quantitative real-time reverse-transcriptase polymerase chain reaction was used to analyze PIAMV RNA accumulation in the Li1- and Li1-1154Y-infiltrated areas of nonsilenced, *NbSGT1*-silenced, and *NbMAPKKKα*-silenced plants. The PIAMV RNA value was normalized relative to the EF1A messenger RNA value in each leaf tissue sample. Data are shown as the means $\pm$ standard error of four leaf tissues.

into *Rx-N. benthamiana* by agroinfiltration at a high bacterial concentration, we found that PVX induced a characteristic HR with PCD. However, ER without PCD was induced at low bacterial concentrations (data not shown). A possible explanation for this observation is that, following agroinfiltration at a high bacterial concentration, PVX CP may have been overexpressed by replication-competent PVX to levels high enough to induce PCD. In contrast, at a low bacterial concentration, PVX CP may not have accumulated sufficiently to elicit PCD, which is a situation similar to that of leaves mechanically inoculated with PVX.

We knocked down *NbMAPKKK α* in *Rx-N. benthamiana* by TRV-based VIGS and subsequently inoculated green fluorescent protein (GFP)-tagged PVX (PVX-GFP) by agroinfiltration at PCD-inducing bacterial concentrations. PCD was induced upon agroinfiltration of PVX-GFP in nonsilenced *Rx-N. benthamiana* (Fig. 5A) but no PCD occurred in *NbMAPKKK α* -silenced *Rx-N. benthamiana* following PVX-GFP infection. Consistent with this observation, the PVX-GFP-infiltrated area of the

NbMAPKKK α -silenced *Rx-N. benthamiana* had a considerably lower level of ion leakage than the nonsilenced plants (Fig. 5B).

To investigate the effect of a loss of MAPKKK α function on the restraint on PVX multiplication in the *Rx*-mediated HR, we conducted PVX-GFP infiltration at a lower bacterial concentration, which can only induce ER without PCD in *Rx-N. benthamiana*. No GFP fluorescence occurred and no CP was detected by Western blotting in the PVX-GFP-infiltrated area of *NbMAPKKK α* -silenced *Rx-N. benthamiana* (Fig. 5C and D). In contrast, as already shown in the previous report (Peart et al. 2002), both GFP fluorescence and CP were detected in the PVX-GFP-infiltrated area in the *NbSGT1*-silenced *Rx-N. benthamiana* (Fig. 5C and D).

To eliminate the possibility that MAPKKK α was a critical factor for PVX accumulation, we agroinfiltrated PVX-GFP into nontransgenic *N. benthamiana* plants in which *NbSGT1* or *NbMAPKKK α* had been silenced; similar levels of PVX-CP accumulation occurred in both *NbSGT1*- and *NbMAPKKK α* -silenced nontransgenic plants (Fig. 5E).

Fig. 5. Silencing of mitogen-activated protein kinase kinase kinase α (MAPKKK α) in *Rx*-transgenic *Nicotiana benthamiana* compromises the programmed cell death (PCD) but not the restraint of virus multiplication of the *Rx*-mediated hypersensitive response (HR) induced by *Potato virus X* (PVX). **A**, Effects of *NbMAPKKK α* silencing on the PCD induced by PVX-green fluorescent protein (GFP) in *Rx*-transgenic *N. benthamiana*. A high concentration (optical density at 600 nm [OD₆₀₀] = 0.05) of *Agrobacterium*-expressing PVX-GFP, which induces the *Rx*-mediated HR, was infiltrated, and symptoms in the PVX-GFP-infiltrated area of nonsilenced (left) and *NbMAPKKK α* -silenced (right) *Rx*-transgenic plants were photographed at 7 days post infiltration (dpi). Red and blue circles represent necrosis and no symptoms, respectively. **B**, Ion leakage in the PVX-GFP infiltrated area of nonsilenced (virus-induced gene silencing [VIGS] vector) and *NbMAPKKK α* -silenced (VIGS-*NbMAPKKK α*) *Rx*-transgenic plants at 9 dpi. **C**, Effects of MAPKKK α silencing on the restraint of virus multiplication induced by PVX-GFP in *Rx*-transgenic *N. benthamiana*. A low concentration (OD₆₀₀ = 0.001) of *Agrobacterium*-expressing PVX-GFP, which induces *Rx*-mediated ER, was infiltrated in nonsilenced and *NbMAPKKK α* -silenced plants. PVX-GFP accumulation was monitored by GFP fluorescence under UV light at 5 dpi in nonsilenced (left column), *NbSGT1*-silenced (middle column), and *NbMAPKKK α* -silenced (right column) *Rx*-transgenic plants. Photographs of the same leaves taken under normal light are shown in the lower panels. **D**, Immunoblots of PVX coat protein (CP) in the PVX-GFP-infiltrated areas of nonsilenced (lanes 1 and 2), *NbSGT1*-silenced (lanes 3 and 4), and *NbMAPKKK α* -silenced (lanes 5 and 6) *Rx*-transgenic plants. Two replicates are shown for each VIGS construct. Coomassie blue staining was performed to confirm equal loading in each lane. **E**, PVX CP immunoblots in the PVX-GFP-infiltrated area of nontransgenic *N. benthamiana* plants. Figure layout and experimental procedures are the same as in D.

DISCUSSION

In this study, we analyzed the pathway leading to systemic necrosis induced by PIAMV infection and that leading to the *Rx*-mediated HR to PVX. Silencing of *SGT1*, *RAR1*, *MAPKKK α* , and *MEK2* resulted in significant suppression of systemic necrosis upon PIAMV infection, suggesting that these host factors constitute the PCD-inducing pathway for systemic necrosis. Previous studies have revealed that *SGT1* is required for PCD in the *Rx*-mediated HR to PVX (Peart et al. 2002; Lu et al. 2003). In this study, we demonstrated that *MAPKKK α* is also essential for PCD in the *Rx*-mediated HR to PVX. Therefore, we propose that *SGT1*, *RAR1*, and a MAPK signaling cascade, including *MAPKKK α* , are involved in the induction of PCD in both PIAMV-induced systemic necrosis and PVX-induced HR.

We also investigated the effect of host factor silencing on virus multiplication in systemic necrosis and HR. *SGT1* and *RAR1* silencing has previously been shown to not only suppress PCD but also compromise the *R*-gene-mediated restraint on virus multiplication (Peart et al. 2002; Lu et al. 2003; Liu et al. 2004a). In this study, we revealed that the PIAMV systemic necrosis-inducing isolate (Li1) accumulated to higher levels in *SGT1*- and *RAR1*-silenced plants than in nonsilenced plants (Fig. 4), suggesting that PIAMV multiplication was partially suppressed by an *SGT1*- and *RAR1*-dependent pathway. In contrast, *MAPKKK α* and *MEK2* silencing had no effect on PIAMV multiplication. Similar results were obtained in the *Rx*-mediated HR to PVX; the silencing of *MAPKKK α* significantly compromised PCD in the HR, whereas it had no effect on the restraint of PVX multiplication (Fig. 5). These results suggest that *SGT1* and *RAR1* are involved in the restraint of virus multiplication in both the systemic necrosis induced by PIAMV and the *Rx*-mediated HR induced by PVX but their downstream MAPK cascade, including *MAPKKK α* and *MEK2*, is not. Taken together, these results reveal the following. First, the PCD-independent restraint on virus multiplication is induced even in PIAMV-triggered systemic necrosis when the viruses are not completely arrested within the inoculated leaves and can infect the plant systemically and, second, the restraint on virus multiplication in both systemic necrosis and HR seems to be activated via an unidentified pathway that is dependent on *SGT1* and *RAR1* but independent of *MAPKKK α* and *MEK2* (Supplementary Fig. 2).

The role of PCD in *R*-gene-mediated virus resistance is not fully understood. To properly evaluate the role of PCD in virus resistance, we must identify the components or pathways specifically involved in either PCD or the restraint of virus multiplication. Thus far, one of the most promising candidate factors for selective functioning in PCD is a protein that possesses caspase activity, which plays an essential role in executing PCD in animal cells (Greenberg and Yao 2004). However, plant vacuolar protease (VPE), which exhibits caspase-like activity, is required for both PCD and virus restraint in the *N*-gene-mediated HR against TMV infection (Hatsugai et al. 2004). Moreover, transgenic tobacco plants expressing p35, a caspase inhibitor from baculovirus, disrupt both PCD and the restraint of virus multiplication in the *N*-mediated HR to TMV (del Pozo and Lam 2003). In our study, we demonstrated that the MAPK signaling cascade, including *MAPKKK α* and *MEK2*, was specifically involved in PCD during both *Rx*-mediated HR triggered by PVX and systemic necrosis induced by PIAMV infection. To our knowledge, this is the first report of a plant pathway selectively required for the PCD in HR and systemic necrosis. *MAPKKK α* is a plant MAPKKK essential for PCD that is associated with resistance against various fungal and bacterial pathogens, and downstream of which *MEK2*

acts to induce PCD (del Pozo et al. 2004). Note, however, that *MEK2* silencing in *N*-transgenic plants leads to the loss of PCD and allows for a greater TMV RNA accumulation, suggesting that *MEK2* is involved in the pathways leading to both PCD and the restraint of virus multiplication in the *N*-TMV system (Jin et al. 2003). Similarly, silencing studies have revealed that other MAPK cascade components, two MAPK (SIPK and WIPK) and another MAPKKK (NPK1), are also involved in both PCD and the restraint of virus multiplication in the *N*-mediated HR to TMV (Jin et al. 2002, 2003). These apparently different roles of the MAPK cascade between our results and those demonstrated in the *N*-TMV system can be explained partly by variations in the role of each MAPK cascade in PCD and the restraint of virus multiplication. In addition to the *MAPKKK α* -*MEK2* pathway selectively involved in PCD, other MAPK cascades may be involved in the restraint of virus multiplication in the *Rx*-mediated HR to PVX and in PIAMV-induced systemic necrosis. They can also be explained by the relatively strong correlation of PCD with the restraint of virus multiplication in the *N*-mediated HR against TMV infection and the critical role of PCD in its resistance. Indeed, although our results revealed that PCD does not contribute to virus resistance in the *Rx*-mediated HR to PVX and PIAMV-induced systemic necrosis, this does not mean that PCD could not confer resistance in other plant-virus systems.

Similar to our findings, PCD and the restraint on virus multiplication have been found to be controlled by separate pathways in the *HRT*-mediated HR to TCV (Chandra-Shekara et al. 2004). Genetic analyses revealed that restraint of virus multiplication is conferred by a single recessive gene designated *rrt* in the *HRT*-mediated HR to TCV in *Arabidopsis* (Kachroo et al. 2000). Moreover, restraint of TCV multiplication requires signaling components such as *EDS1*, *EDS5*, *PAD4*, and *SID1*, which are not involved in PCD (Chandra-Shekara et al. 2004). Although the *HRT*-mediated HR is independent of *SGT1* and *RAR1*, determining whether MAPK cascades, such as the *MAPKKK α* -*MEK2* pathway, can act downstream of both factors and also function selectively in PCD will be of interest.

In this study, we investigated the mechanisms by which PIAMV induced systemic necrosis and demonstrated that it is induced through the pathway composed of PCD as well as the restraint of virus multiplication. In agreement with this result, we showed that the systemic necrosis caused by PIAMV infection in *N. benthamiana* was accompanied by host nuclear DNA fragmentation, H_2O_2 production, ion leakage, callose deposition, and the induction of defense gene expression, confirming that systemic necrosis has characteristic HR phenotypes (Fig. 1). Consistent with this observation, both H_2O_2 production and an upregulation of defense-related gene expression such as *PR-1a*, a well-known indicator of plant HR, were reported in systemic necrosis of tomato induced by *Cucumber mosaic virus* (CMV)/D satellite RNA infection and of *Arabidopsis* induced by CaMV (Xu et al. 2003; Love et al. 2005). Moreover, NBS-LRR-type R proteins are involved in some systemic necrosis associated with virus disease susceptibility (Seo et al. 2006; Atsumi et al. 2009). Involvement of *SGT1* and *RAR1*, which are both required for the function of many R proteins (Hubert et al. 2003; Liu et al. 2004a), strongly suggests the presence of unidentified NBS-LRR-type R proteins in PIAMV-induced systemic necrosis. Our data also support the argument that systemic necrosis, which is usually considered a symptom in susceptible plants, is molecularly and functionally similar to *R*-gene-dependent SHR.

Although our results suggest that HR and systemic necrosis are induced through the same pathway, significant differences remain to be addressed: *R*-gene-mediated HR can usually arrest virus accumulation at the site of infection, whereas systemic

necrosis or SHR cannot, and allows systemic spread of a virus. Although the precise mechanism underlying these differences remains unresolved, a mutation in either an elicitor protein or an R protein is implicated in these differences. For example, previous studies on the *N*-mediated HR to TMV and the *RCY1*-mediated HR to CMV have shown that several single-amino-acid substitutions in the R protein allow viruses to move systemically, causing an SHR instead of an HR (Dinesh-Kumar et al. 2000; Sekine et al. 2006). Moreover, a single-amino-acid mutation introduced into the elicitor RdRp of an avirulent CMV strain that elicits HR on cowpea also results in an SHR (Kim and Palukaitis 1997). These phenomena can be explained by a model in which quantitative and qualitative variation in virus multiplication restraint, which depends on the timing and the level of the elicitor–R protein interaction, could lead to an interaction between plants and pathogens which ranges from ER to an SHR (Bendahmane et al. 1999). This model indicates that systemic necrosis can be caused when the restraint of virus multiplication is weak or late and not sufficiently strong to restrict virus propagation. In this study, we experimentally validated this model by demonstrating that the restraint of virus multiplication occurs even during PIAMV-induced systemic necrosis, indicating that the restraint is not sufficient to inhibit systemic virus spread.

In summary, our study demonstrated that a pathway including SGT1 and RAR1 is involved in PCD induced by a plant virus systemic infection, as well as in PCD caused during *R*-gene-mediated resistance. Moreover, restraint of virus multiplication is induced in both systemic necrosis and the HR through pathways distinct from those leading to PCD. In the future, elucidating how various host factors essential to *R*-gene-mediated HR also function in the necrosis induced by systemic viral infection and whether they are involved in the restraint on virus multiplication will be important. Future comprehensive studies to address this question will reveal cascades leading to either PCD or the restraint of virus multiplication, molecular mechanisms underlying the control of them, and their precise contributions to resistance.

MATERIALS AND METHODS

Plant material, viral isolates, and agroinfiltration.

Transgenic *N. benthamiana* plants carrying the *Rx* resistance gene, TRV vectors for VIGS, and PVX-GFP were provided by D. Baulcombe (University of Cambridge, U.K.). Wild-type (nontransgenic) and transgenic *N. benthamiana* plants were grown in a growth chamber at 25°C. PIAMV and PVX-GFP viral cDNAs were cloned into the *Agrobacterium* binary *Ti* plasmid vector pCAMBIA1301 as previously described (Ozeki et al. 2006). These plasmid vectors were transformed into *Agrobacterium tumefaciens* strain EHA105. Agroinfiltration was performed as described (Senshu et al. 2009) at a final turbidity of 0.5 (optical density of 600 nm) in cell death assays or 0.05 in analyses of the effect of VIGS in a sequential inoculation with potexviruses.

Cell death assays.

For trypan blue staining (Rate et al. 1999), inoculated leaves were boiled for 3 min in alcoholic lactophenol (2:1, 99% ethanol/lactophenol) containing trypan blue and kept at room temperature overnight. Stained leaves were decolorized in a chloral hydrate solution containing 1 kg of chloral hydrate in 400 ml of H₂O. For DAB staining (Thordal-Christensen et al. 1997) to detect H₂O₂ production, leaves were vacuum infiltrated for 2 min with DAB solution at 1 mg/ml, incubated for 2 h, and decolorized in 99% ethanol. H₂O₂ was visualized as a reddish-brown color. For aniline blue staining (Rate et al. 1999) to

detect callose deposition, leaves were boiled in alcoholic lactophenol for decolorization and incubated in 0.05% aniline blue in 0.15 M K₂HPO₄ for 1 h. Callose is observed as a white luminescence under UV light. For the trypan blue, DAB, and aniline blue stainings, inoculated leaves were analyzed at 6 dpi. For the ion leakage assays (Lopez-Solanilla et al. 2004), two 7-mm-diameter leaf discs from the same leaf were collected at 4 dpi, placed in a tube with 3 ml of H₂O, and then infiltrated with H₂O by applying a vacuum using a syringe. After 3 h of gentle shaking at room temperature, conductivity was measured using a conductivity meter B-173 (Horiba, Kyoto, Japan). Data presented are the means of at least five replicates and error bars represent the standard deviation. The TUNEL assay was performed at 4 dpi using the *in situ* cell death detection kit-fluorescein (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Before detection, PIAMV isolates or the empty vector pCAMBIA1301 (expressing β -glucuronidase)-infiltrated leaves were fixed, embedded in Paraplast, and sectioned horizontally to the leaf surface. Sections (10- μ m) were cut on glass slides, dewaxed in xylene, rehydrated, and then pretreated with proteinase K at 20 μ g/ml. Slides treated with 5 μ g of DNaseI in 50 mM Tris-HCl (pH 7.5) for 20 min at room temperature served as positive controls.

Northern blotting and RT-PCR to detect viral and plant genes.

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's protocol. Total RNA (5 μ g) was used for Northern blot analyses using the digoxigenin system (Roche Diagnostics) following the manufacturer's protocols. Probes were prepared by amplifying the full-length PIAMV CP sequence (Ozeki et al. 2009), *PR-1a* cDNA fragments (with the primers 5'-AAG AAT TCG AAC CTT TGA CCT GGG ACG A-3' and 5'-TTG AAT TCA AGC TTC GAG TTA CGC CAA ACC ACC T-3'), *Hsr203J* (with the primers 5'-AGG TAC ACA CTA AAC AAA CTT C-3' and 5'-CGT GAC AAT CAA GAC GGT ACA T-3'), *Hin1* (with the primers 5'-CCG AAT TCA TGC CGG AAT CCA ACT TGA A-3' and 5'-GGC TCG AGC TAC CAA TCA AAA TGG CAT CT-3'), *NbSGT1* (with the primers 5'-ATG GCG TCC GAT CTG GAG A-3' and 5'-TGC TAA ACT AGC ACC AGT TTC C-3'), and *NbMAPKKK α* (with the primers 5'-GGC CAT GGC AAA ATG GAA GAA AGG CAA GCT C-3' and 5'-CCC ACG TGT CAT TGG TTT TCA CAA AAG GGT G-3') to detect PIAMV viral RNA, *PR-1a*, *Hsr203J*, *Hin1*, *NbSGT1*, and *NbMAPKKK α* , respectively. Total RNA (0.5 μ g) was subjected to semiquantitative RT-PCR to confirm *NbRAR1* and *NbMEK2* silencing using primers 5'-CCA TCG ATA TGG AGA GCT TCG TTG CCA GA-3' and 5'-CGG GAT CCG TGA TCA GAA CAA AAG AAT CCC-3' for *NbRAR1* and 5'-GGC CAT GGC AAA ATG GAA GAA AGG CAA GCT C-3' and 5'-CCC ACG TGT CAT TGG TTT TCA CAA AAG GGT G-3' for *NbMEK2*. Ubiquitin mRNA was used as an internal constitutively expressed control.

Real-time PCR.

Total RNA isolated was treated with RNase-free DNase I (TaKaRa, Shiga, Japan), and 2 μ g of the total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer's protocols. One microliter of 50-fold diluted cDNA was used for real-time PCR using the Thermal Cycler Dice real-time PCR system (TaKaRa) with SYBR Premix ExTaq (TaKaRa). The primers used for real-time PCR were PIAMV-F (5'-CAT TTC CCT CGC CCA AAT CGC-3') and PIAMV-R (5'-CAG ATG ATT TTG GCG TAG AAG

CGG-3') for PIAMV and EF1U (5'-TGG TGT CCT CAA GCC TGG TAT GGT TG-3') and EF1D (5'-ACG CTT GAG ATC CTT AAC CGC AAC ATT CTT-3') for *EF1A*.

VIGS constructs and VIGS.

A 500-bp fragment of *NbSGT1*, a 336-bp fragment of *NbRAR1*, a 322-bp fragment of *NbMAPKKKα*, and a 349-bp fragment of *NbMEK2* were amplified and introduced into the pTV:00 (binary TRV RNA2 vector) in the antisense direction to generate pTV:*NbSGT1*, pTV:*NbRAR1*, pTV:*NbMAPKKKα*, and pTV:*NbMEK2*, respectively. The primer pairs used to amplify these genes were 5'-CGC AAG CTT CCC GGG ACT TAG TGT TAG CAT TGA TGT G-3' and 5'-GCG ACT AGT GGA TCC CTA GAT TTC CCA TTT CTT CAG C-3' for *NbSGT1*, 5'-CCA TCG ATG GTT CAC AAC CCA GAG AAG T-3' and 5'-GGG ATC CTT AGG ACG CTG GGC TGG C-3' for *NbRAR1*, 5'-TTT GGT ACC GTC GAC ATG CCT GCT TGG TGG GGA A-3' and 5'-TTT GGA TCC AAG CTT CAG GCC CAT CAG ATG ATC C-3' for *NbMAPKKKα*, and 5'-CCA TCG ATA AGC TTC GTT TTA GGT TCG GAG AAA TTT-3' and 5'-GGA CTA GTG GAT CCG CAG CTA AAT AAG TCC ATG AC-3' for *NbMEK2*. VIGS was performed as described (Peart et al. 2002) using pTV:00 derivatives along with pBintra6 (binary TRV RNA1 vector).

Immunoblotting.

N. benthamiana leaves were collected at 6 dpi and total protein was extracted to determine PVX-CP levels. Protein extraction and immunoblotting using the PVX-CP antibody were performed as described (Kagiwada et al. 2005).

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