The Hypersensitive Induced Reaction and Leucine-Rich Repeat Proteins Regulate Plant Cell Death Associated with Disease and Plant Immunity

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Pathogen-induced programmed cell death (PCD) is intimately linked with disease resistance and susceptibility. However, the molecular components regulating PCD, including hypersensitive and susceptible cell death, are largely unknown in plants. In this study, we show that pathogen-induced *Capsicum annuum* hypersensitive induced reaction 1 (*CaHIR1*) and leucine-rich repeat 1 (*CaLRR1*) function as distinct plant PCD regulators in pepper plants during *Xanthomonas campestris* pv. *vesicatoria* infection. Confocal microscopy and protein gel blot analyses revealed that *CaLRR1* and *CaHIR1* localize to the extracellular matrix and plasma membrane (PM), respectively. Bimolecular fluorescent complementation and coimmunoprecipitation assays showed that the extracellular *CaLRR1* specifically binds to the PM-located *CaHIR1* in pepper leaves. Overexpression of *CaHIR1* triggered pathogen-independent cell death in pepper and *Nicotiana benthamiana* plants but not in yeast cells. Virus-induced gene silencing (VIGS) of *CaLRR1* and *CaHIR1* distinctly strengthened and compromised hypersensitive and susceptible cell death in pepper plants, respectively. Endogenous salicylic acid levels and pathogenesis-related gene transcripts were elevated in *CaHIR1*-silenced plants. VIGS of *NbLRR1* and *NbHIR1*, the *N. benthamiana* orthologs of *CaLRR1* and *CaHIR1*, regulated *Bax*- and *avrPto*-*Pto*-induced PCD. Taken together, these results suggest that leucine-rich repeat and hypersensitive induced reaction proteins may act as cell-death regulators associated with plant immunity and disease.

Programmed cell death (PCD), a highly regulated and sophisticated cellular mechanism, has been implicated as a common feature in incompatible and compatible plant-microbe interactions (Greenberg and Yao 2004; Williams and Dickman 2008). In incompatible interactions, the hypersensitive response (HR), a type of plant PCD, occurs immediately after the contact of a

single bacterium with a plant cell, resulting in the activation of local and systemic acquired resistance (SAR) to protect plants from attacks by a wide range of pathogens (Choi et al. 2007; del Pozo et al. 2004; Durrant and Dong 2001; Jones and Dangl 2006; Park 2005). Significant progress in understanding the molecular mechanisms underlying the HR has been achieved through the cloning and characterization of plant resistance genes involved in the recognition of pathogen avirulence factors (Jones and Dangl 2006). Subsequent analyses to dissect genetic pathways mediating the HR have led to the isolation of lesion-mimic mutants, such as *accelerated cell death* (*acd*), *lesion-stimulating disease-resistance response* (*lsd*), and *dnd* (*defense, no death*) mutant plants (Balagué et al. 2003; Clough et al. 2000; Mühlenbock et al. 2008; Yao and Greenberg 2006; Yu et al. 1998). In compatible plant-microbe interactions, however, susceptible cell death occurs relatively late during the course of infection. There is an increasing body of evidence to indicate that host-controlled PCD is also intimately linked with the onset of susceptible cell death and symptom development in plants (Greenberg and Yao 2004; Hwang et al. 2008; Navarre and Wolpert 1999; Yao et al. 2002). In *Nicotiana benthamiana* plants, virus-induced gene silencing (VIGS) of a mitogen-activated protein kinase kinase kinase gene (*MAPKKKα*), a positive regulator of cell death, compromises disease-associated cell death accompanied by reduced growth of *Pseudomonas syringae* (del Pozo et al. 2004). This suggests that host-controlled cell death during a compatible interaction may be triggered by virulent *P. syringae*. However, the molecular components regulating susceptible cell death are poorly understood in plants.

The *CaHIR1* (*Capsicum annuum* hypersensitive induced reaction 1) gene was identified in a pepper cDNA library in an attempt to characterize host cellular factors that specifically interact with *CaLRR1* (*Capsicum annuum* leucine-rich repeat 1) (Jung and Hwang 2007; Jung et al. 2004). Ectopic expression of *CaHIR1* in *Arabidopsis* plants regulates the cell-death response to various pathogens as well as to osmotic stress, accompanied by changes in the induction of some defense-related genes and proteins (Jung et al. 2008). In an earlier study, tobacco NG1 cDNA was isolated during a functional search for genes whose products elicited the HR in tobacco (Karrer et al. 1998). *NG1* expression induces the formation of disease symptoms and expression of the PR-2 protein. The three maize hypersensitive induced reaction (HIR) genes (*Zm-hir1*, *Zm-hir2*, and *Zm-hir3*) were identified based on their amino acid homology to the tobacco NG1 sequence (Nadimpalli et al. 2000). Among them,

Nucleotide sequence data of *CaHIR1* and *CaLRR1* from this article can be found in the GenBank data libraries under accession number AY529867 and AY237117, respectively.

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the *Zm-hir3* gene is strongly induced in the maize lesion mimic mutant *Les9*, suggesting a possible role of maize *HIR* genes for HR cell-death execution. Nadimpalli and associates (2000) showed that maize *HIR* proteins along with prohibitins and stomatins form a PID (proliferation, ion, and death) protein superfamily. Interestingly, these PID proteins share common features in their SPFH (stomatins, prohibitins, flotillins, HflK/C) domains. Prohibitin proteins are involved in mitochondrial biogenesis and in the protection of plants against stress and senescence (Ahn et al. 2006), and stomatins are integral membrane proteins of red blood cells that function as negative regulators of cation permeability (Nadimpalli et al. 2000). The presence of the SPFH domain in *HIR* proteins and their structural similarity to prohibitins and stomatins suggest that they may function as ion channel regulators. However, the molecular function of *HIR* proteins in cell-death execution is unknown.

Leucine-rich repeat (LRR) proteins have diverse functions and cellular locations in plants and animals (Federici et al. 2006; Kobe and Kajava 2001; Padmanabhan et al. 2009; Shanmugam 2005). In animal systems, LRR proteins have been shown to regulate gene expression and apoptosis signaling (Kobe and Kajava 2001). In plant systems, LRR proteins also appear to be involved in protein-protein interactions and signal transduction pathways that regulate PCD (Padmanabhan et al. 2009). The presence of LRR domains in the majority of disease-resistance proteins and receptor-like kinases (RLK) underscores the pivotal roles of LRR domains in gene-for-gene resistance and intracellular signaling events. Genetic and molecular genetic analyses in *Arabidopsis* ecotypes Col-0 and Po-1 revealed that the LRR domain of RPS2 determines specific interactions of RPS2 with other host factors during the RPS2-mediated disease-resistance response (Banerjee et al. 2001). However, the role of small LRR proteins, which lack the cytoplasmic kinase domain, is largely unknown in plant defense. Recently, the small LRR tobacco protein NtLRP1 was demonstrated to negatively regulate PCD (Jacques et al. 2006). *Agrobacterium*-mediated transient expression of endoplasmic reticulum-located NtLRP1 suppressed PCD induced by the fungal elicitor β -megaspermin. These findings suggest that small LRR proteins along with R proteins and RLK are involved in the regulation of plant PCD through a specific interaction with pathogen-derived ligands or host proteins.

In a previous gain-of-function study, overexpression of the *CaHIR1* gene in *Arabidopsis* was found to result in the spontaneous cell-death phenotype, which was accompanied by increased K⁺ efflux and salicylic acid (SA) pathway-dependent *PR* gene induction (Jung and Hwang 2007). Moreover, co-overexpression of both *CaLRR1* and *CaHIR1* in transgenic *Arabidopsis* plants compromised *CaHIR1*-mediated *PR* gene expression and the cell-death phenotype. Thus, we hypothesized that the *CaLRR1* protein specifically interacts with the *CaHIR1* protein and suppresses cell death induced by *CaHIR1*. In this study, we used the *Tobacco rattle virus* (TRV)-based VIGS technique to further define the functional roles of *CaLRR1* and *CaHIR1* in disease resistance and cell death of pepper plants. It is time-consuming and very difficult to generate transgenic pepper plants. The VIGS technique not only provided a fast and effective means to generate loss-of-function phenotypes but also allowed us to identify the functional roles of *CaLRR1* and *CaHIR1* for cell death and defense responses of pepper plants. *CaLRR1*- and *CaHIR1*-silenced pepper plants exhibited enhanced and reduced cell-death phenotypes during *Xanthomonas campestris* pv. *vesicatoria* infection, respectively. However, the enhanced cell-death response of *CaLRR1*-silenced pepper plants did not significantly promote disease resistance. In contrast, silencing of *CaHIR1* significantly compromised hypersensitive and susceptible cell deaths, which were accompanied by elevated SA levels

and *PR*-gene expression. We discuss LRR- and *HIR*-mediated cell death in comparison to the cell death and HR-based innate immune response of *N. benthamiana* plants following *Agrobacterium*-mediated transient expression of *Bax*, *AvrPto*, and *AvrPto/Pto*.

RESULTS

Extracellular *CaLRR1* interacts with membrane-associated *CaHIR1* to form membrane microdomains.

Computational analyses of the predicted protein sequences revealed that *CaLRR1* possesses four LRR domains and that *CaHIR1* has an SPFH domain (SMART database) that contains putative transmembrane helices between amino-acid residues 30 and 49 (TMpred database) (Fig. 1A). To investigate the subcellular localization and distribution of *CaLRR1* and *CaHIR1*, we used a biolistic transformation system in onion epidermal cells. As shown in Figure 1B, soluble-modified green fluorescent protein (smGFP)-tagged *CaLRR1* and *CaHIR1* proteins were localized in small patches at the plasma membrane (PM). This distinct punctate localization along the PM is characteristic of various membrane microdomain proteins (Latif et al. 2003; Solis et al. 2007). Using different constructs, we next identified the *CaHIR1* domains involved in punctate localization in the PM. Interestingly, smGFP-tagged ΔC consisting of the SPFH domain but not smGFP-tagged Δ SPFH exhibited punctate localization that is similar to that of *CaHIR1*. Furthermore, the smGFP-tagged transmembrane domain of *CaHIR1*, which contains the 20 amino-acid residues of putative transmembrane helices, was localized to the PM, without punctate characteristics. Intriguingly, the PM of onion epidermal cells expressing the *CaHIR1:smGFP* construct but not the *CaLRR1:smGFP* construct was detached from the cell wall, leaving a visible gap, which is a characteristic of apoptotic-like PCD (Reape and McCabe 2008).

To determine whether *CaLRR1* and *CaHIR1* are extracellular or membrane-associated proteins, total and extracellular proteins were extracted from pepper leaves 6 h after avirulent *X. campestris* pv. *vesicatoria* infection. Total, soluble, microsomal, and extracellular proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were immunoblotted using anti-*CaLRR1* and anti-*CaHIR1* polyclonal antibodies (Fig. 1C). The *CaLRR1* protein was enriched in the extracellular protein extract, suggesting that it localizes to the extracellular matrix. However, the *CaHIR1* protein was detected in the separated microsomal fraction, indicating that it is a membrane-associated protein.

CaHIR1 was initially identified as a molecular partner of *CaLRR1* by yeast two-hybrid screening (Jung and Hwang 2007). We used the bimolecular fluorescence complementation (BiFC) technique to examine heterodimerization of *CaLRR1* with *CaHIR1* in pepper leaves (Fig. 1D) (Walter et al. 2004). Most of the fluorescence signals induced by heterodimerization of *CaLRR1* with *CaHIR1* were located in the punctate site of the PM and the peripheral region of pepper epidermal cells. However, we could not detect fluorescence signals in pepper epidermal cells infiltrated with single plasmids or empty vectors (Supplementary Fig. 1). Coimmunoprecipitation experiment also revealed the specific interaction of *CaHIR1* with *CaLRR1* (Fig. 1E). Taken together, these findings suggest that extracellular *CaLRR1* specifically interacts with PM-associated *CaHIR1* to form membrane microdomains, which may function as platforms for compartmentalizing various cellular processes, such as membrane transport, protein sorting, calcium homeostasis, and signal transduction (Browman et al. 2007).

Agroexpression of *CaLRR1* and *CaHIR1* alters defense responses of pepper plants.

Overexpression of *CaLRR1* suppresses *CaHIR1*-induced cell death in *Arabidopsis* and tobacco (*Nicotiana tabacum* cv. Xanthi) plants (Jung and Hwang 2007). Because transformation is very difficult in pepper plants, an *Agrobacterium*-mediated transient expression (agroexpression) system was used to gain insight into the in vivo function of *CaLRR1* and *CaHIR1* in pepper plants. Transient expression of *CaHIR1* in pepper leaves strongly induced cell death 4 days after agroinfiltration; however, coexpression of *CaLRR1* and *CaHIR1* significantly suppressed cell death in these tissues (Fig. 2A and B). Measurements of electrolyte leakage supported significantly attenuated or strengthened cell death in *CaLRR1*- or *CaHIR1*-expressing pepper leaf tissues, respectively (Fig. 2C). These findings suggest that overexpression of *CaLRR1* suppresses *CaHIR1*-induced cell death in pepper plants, as observed in *Arabidopsis* and tobacco plants (Jung and Hwang 2007).

UV illumination of pepper leaves 4 days after agroinfiltration revealed that transient activation of *CaLRR1* and *CaHIR1* strongly induced and caused accumulation of defense-related fluorescent phenolic compounds (blue), compared with the empty-vector control, which induced yellowish chlorosis (Fig. 2A). Transient *CaLRR1* expression strongly induced H₂O₂ accumulation (Supplementary Fig. 2) and common defense-related genes, including *CaHIR1*, *CaBPR1*, *CaPOA1*, *CaPR10*, and *CaDEF1*; however, transient *CaHIR1* expression did not induce these defense-related genes (Fig. 2E and F). Interestingly, agroexpression of both *CaLRR1* and *CaHIR1* in pepper leaves strongly induced most of the analyzed defense-related genes, with the exception of *CaPOA1*. Aniline blue staining of pepper leaves revealed attenuated and strengthened callose deposition in pepper leaves after agroexpression of *CaLRR1* and *CaHIR1*, respectively, compared with the empty-vector control leaves (Fig. 2B and D). Callose or callose synthase have been demonstrated to negatively regulate the SA pathway

Fig. 1. Structure, subcellular localization, and bimolecular fluorescence complementation (BiFC) visualization of *CaLRR1* and *CaHIR1*. **A**, Domain structures of the *CaLRR1* and *CaHIR1* proteins and constructs. Numbers refer to encoded amino acids. LRR = leucine-rich repeat domain, SPFH = stomatin, prohibitin, flotillins, HflK/C domain, TM = transmembrane domain, and C = uncharacterized C-terminal region. **B**, Punctate localization of soluble-modified green fluorescent protein (smGFP)-tagged *CaLRR1* and *CaHIR1* proteins in the plasma membrane (PM) of onion (*Allium cepa* L.) epidermal cells. P = PM in plasmolyzed cells and Control = 35S:smGFP. The SPFH domain of *CaHIR1* is sufficient for the punctate localization of *CaHIR1* in the PM. Bars = 100 μ m. **C**, Immunological detection of the subcellular localization of *CaLRR1* and *CaHIR1* in pepper leaves infected with *Xanthomonas campestris* pv. *vesicatoria*. *CaLRR1* and *CaHIR1* are extracellular and membrane-bound proteins, respectively. Membranes were probed with polyclonal antibodies raised against *CaLRR1* or *CaHIR1* in rabbits. Protein loading is shown by Coomassie brilliant blue (CBB) staining. **D**, BiFC visualization of *CaLRR1* and *CaHIR1* heterodimerization in pepper leaves. Confocal images of yellow fluorescent protein (YFP) signals (Fluorescence) induced by interaction of *CaLRR1* with *CaHIR1* and bright field images (Visible) of pepper epidermal leaf cells. Bars = 100 μ m. **E**, Coimmunoprecipitation of *CaLRR1* and *CaHIR1* in pepper leaves. *CaLRR1*-cMyc and *CaHIR1*-hemagglutinin (HA) transgenes were expressed in pepper leaves by agroexpression. Total proteins were extracted from leaves and were subjected to immunoprecipitation (IP) with anti-HA antibody-conjugated beads. Total and immunoprecipitated proteins were immunoblotted with the indicated antibodies.

in the *Arabidopsis* powdery mildew resistant 4 (*pmr4*) mutant, which does not deposit callose (Nishimura et al. 2003). Thus, strengthened callose deposition by transient expression of *CaHIR1* may reflect the suppression of the SA-dependant defense signaling pathway in pepper plants.

SA accumulation, SA-dependent cell death, and *PR* gene expression have been proposed to be intimately correlated with disease resistance in higher plants (Vlot et al. 2009). We analyzed whether endogenous SA is involved in the *CaLRR1*- and *CaHIR1*-mediated defense responses, including cell death and *PR* gene expression (Fig. 2G). SA accumulation was significantly enhanced and suppressed in transiently *CaLRR1*- and

CaHIR1-expressing pepper leaves, respectively, compared with the *Agrobacterium* empty vector-expressing leaves. Furthermore, coexpression of *CaLRR1* with *CaHIR1* compromised *CaLRR1*-induced SA accumulation in pepper leaves. These results suggest that *CaLRR1* and *CaHIR1* positively and negatively regulate SA accumulation in pepper plants, respectively.

VIGS of *CaLRR1* and *CaHIR1* alters the defense responses of pepper plants.

The TRV-based VIGS technique has been used as an efficient reverse genetics approach to define the functions of defense-related genes involved in the disease resistance of pepper

Fig. 2. Effects of agroexpression of *CaLRR1* and *CaHIR1* on cell death and defense responses of pepper leaves. **A**, Cell-death response caused in the pepper leaf by transient expression of *CaHIR1* (left panel). Chlorophyll autofluorescence (red), chlorosis (yellow), and accumulation of defense-related phenolic compounds (blue) were visualized by UV illumination (right panel) 4 days after inoculation. The blue and red circles indicate the absence and presence of cell death, respectively. EV = empty vector. **B**, Trypan blue and aniline blue staining of pepper leaves 48 h after agroexpression with the indicated transgenes. Bars = 500 μ m. **C**, Ion leakage from leaf disks of pepper plants 0, 12, 24, 48, and 72 h after inoculation with *Agrobacterium tumefaciens* carrying the indicated transgenes. **D**, Number of callose foci in pepper leaves 48 h after agroexpression of *CaLRR1* and *CaHIR1*. Callose deposition was quantified from more than ten leaf regions. **E** and **F**, Real-time reverse transcription-polymerase chain reaction analysis of expression of the *CaLRR1*, *CaHIR1*, *CaBPR1* (pepper basic PR1), *CaPR10* (pepper PR10), *CaPOA1* (pepper ascorbate peroxidase), and *CaDEF1* (pepper defensin) genes in pepper leaves 24 h after agroexpression of the indicated transgenes. Expression of the pepper 18S rRNA gene served as a control. **G**, Salicylic acid accumulation in pepper leaves 24 h after inoculation with *A. tumefaciens* carrying the indicated transgenes. The data in **C**, **D**, **E**, **F**, and **G** are the mean $\pm$ standard deviation from four independent experiments. Statistically significant differences between means were determined by employing Fisher's least significant difference test. Different letters indicate significant differences at $P = 0.05$. E = empty vector; L = 35S:*CaLRR1*; H = 35S:*CaHIR1*; and L/H = 35S:*CaLRR1* and 35S:*CaHIR1*.

plants (Choi et al. 2007, 2008; Lee et al. 2008; Liu et al. 2002). During infection with the avirulent *X. campestris* pv. *vesicatoria* Bv5-4a, VIGS of *CaLRR1* and *CaHIR1* remarkably compromised the accumulation of their transcripts and proteins

Fig. 3. Effects of virus-induced gene silencing of *CaLRR1* or *CaHIR1* on the defense responses of pepper plants. **A**, Reverse transcription-polymerase chain reaction (RT-PCR) analyses of expression of the *CaLRR1*, *CaHIR1*, and *CaBPR1* genes in gene-silenced pepper leaves 6 h after inoculation (hpi) with the virulent (C = compatible) Ds1 and avirulent (I = incompatible) Bv5-4a strains of *Xanthomonas campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). **B**, Real-time RT-PCR analyses of expression of the *CaLRR1* and *CaHIR1* genes in gene-silenced pepper leaves 12 and 24 hpi with *X. campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). Expression of the pepper 18s rRNA gene served as a control. H = uninoculated healthy leaves. **C**, Immunological detection of *CaLRR1* and *CaHIR1* in gene-silenced pepper leaves 6 hpi with the *X. campestris* pv. *vesicatoria* avirulent strain Bv5-4a (10^7 CFU ml^{-1}). Membranes were probed with polyclonal antibodies raised against *CaLRR1* or *CaHIR1* in rabbits. Protein loading is shown by Ponceau S staining. **D**, Disease symptoms on gene-silenced pepper leaves 0, 3, and 5 days after inoculation (dpi) with the virulent strain of *X. campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). **E**, Hypersensitive response phenotype. **F**, trypan blue staining, and **G**, Ion leakage measurement of gene-silenced pepper leaves inoculated with avirulent *X. campestris* pv. *vesicatoria* (10^7 CFU ml^{-1}). **H**, Bacterial growth in gene-silenced pepper leaves 0 and 3 dpi with virulent and avirulent *X. campestris* pv. *vesicatoria*. The data in **B**, **G**, and **H** are the means $\pm$ standard deviation from four independent experiments. Statistically significant differences between means were determined by employing Fisher's least significant difference test. Different letters indicate significant differences at $P = 0.05$.

compared with the effect of the unsilenced empty vector in control leaves (TRV:00); however, *CaHIR1* transcripts and proteins remained at a slightly visible level in *CaHIR1*-silenced pepper leaves (Fig. 3A, B, and C). This result suggests that silencing was effective for *CaLRR1* and *CaHIR1*. Interestingly, VIGS of *CaLRR1* significantly suppressed *CaHIR1* expression in both healthy and avirulent *X. campestris* pv. *vesicatoria*-inoculated pepper leaves. These findings suggest that *CaHIR1* expression may be involved in the *CaLRR1*-dependent signaling pathway. In contrast, VIGS of *CaHIR1* significantly enhanced *CaLRR1* expression in avirulent *X. campestris* pv. *vesicatoria*-inoculated pepper leaves, suggesting that *CaHIR1* may negatively regulate *CaLRR1* expression.

The biological functions of *CaLRR1* and *CaHIR1* during infection were evaluated in gene-silenced pepper leaves inoculated with the virulent strain Ds1 or the avirulent strain Bv5-4a of *X. campestris* pv. *vesicatoria*. *CaLRR1*-silenced leaves exhibited enhanced susceptibility-induced chlorosis and cell death upon virulent and avirulent *X. campestris* pv. *vesicatoria* infection, respectively (Fig. 3D and E). However, silencing of *CaLRR1* did not significantly enhance bacterial growth in leaves (Fig. 3H). In contrast, silencing of *CaHIR1* compromised susceptibility-induced cell death during *X. campestris* pv. *vesicatoria* infection. Typical chlorotic susceptible disease symptoms were not observed in *CaHIR1*-silenced leaves 5 days after virulent *X. campestris* pv. *vesicatoria* inoculation (Fig. 3D; Supplementary Fig. 3). Trypan blue staining and ion leakage measurement revealed extensively strengthened and attenuated cell death in *CaLRR1*- and *CaHIR1*-silenced leaves, respectively, compared with empty-vector control leaves after inoculation with avirulent *X. campestris* pv. *vesicatoria* (Fig. 3F and G). *CaHIR1*-silenced leaves exhibited significantly enhanced resistance to both virulent and avirulent *X. campestris* pv. *vesicatoria*, as observed by bacterial growth (Fig. 3H). Together, these findings suggest that *CaHIR1*-mediated cell death is dependent upon disease susceptibility (Clough et al. 2000; Greenberg et al. 2000).

To determine whether silencing of *CaLRR1* or *CaHIR1* affects the expression of genes involved in disease resistance, we further analyzed the transcript levels of several defense-related pepper genes by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 4A). Silencing of *CaHIR1* remarkably induced *CaBPR1*, *CaPOA1*, *CaPR10*, and *CaDEF1* in pepper leaves, compared with expression in empty-vector control leaves during infection with virulent and avirulent *X. campestris* pv. *vesicatoria*. Furthermore, a high level of *CaPOA1* transcripts was expressed in *CaHIR1*-silenced leaves uninoculated with *X. campestris* pv. *vesicatoria*. In contrast, silencing of *CaLRR1* distinctly compromised expression of *CaHIR1*, *CaPOA1*, *CaPR10*, and *CaDEF1* in leaves infected by avirulent *X. campestris* pv. *vesicatoria*. We next determined SA levels in *CaLRR1*- and *CaHIR1*-silenced leaves 1 and 3 days after inoculation with virulent and avirulent *X. campestris* pv. *vesicatoria* (Fig. 4B). Silencing of *CaHIR1* but not of *CaLRR1* significantly enhanced SA levels in pepper leaves during *X. campestris* pv. *vesicatoria* infection, especially in the incompatible interactions. This indicates that *CaHIR1*-mediated cell death does not induce SA accumulation in pepper leaves. It is also noteworthy that a high level of SA in pepper leaves is required for the *CaLRR1*-mediated resistance response.

Silencing of *NbLRR1* and *NbHIR1* alters PCD in *N. benthamiana* leaves.

Ectopic or transient overexpression of *CaHIR1* induced the cell-death response in *Arabidopsis*, tobacco (*N. tabacum* cv. Xanthi), and pepper plants (Fig. 2A) (Jung and Hwang 2007).

Thus, we hypothesized that *CaHIR1* may act as a trigger of plant cell-death execution. Transient expression of *CaHIR1*, but not *CaLRR1*, in *N. benthamiana* induced the cell-death phenotype 8 days after agroinfiltration (Fig. 5A and B). To determine whether *CaHIR1*-mediated cell death affects HR-based immunity in *N. benthamiana*, we used the *avrPto/Pto*-mediated PCD system. The signaling components necessary for *Pto*-mediated PCD are conserved in *N. benthamiana* (Sessa et al. 2000). As expected, *Agrobacterium*-transient expression of *avrPto* and *avrPto/Pto* induced chlorosis and HR-like cell death, respectively (Fig. 5C). Surprisingly, transient coexpression of *CaLRR1* or *CaHIR1* with *avrPto* compromised or strengthened, respectively, *avrPto*-mediated chlorosis.

The nucleotide sequences of *CaLRR1* and *CaHIR1* share 62 and 87% identities with putative *N. benthamiana* *LRR1* (*NbLRR1*; accession number CK291392) and *N. benthamiana* *HIR1* (*NbHIR1*; accession number CK292032), respectively (Supplementary Fig. 4). To determine whether the *NbLRR1* and *NbHIR1* genes are involved in cell-death regulation, we silenced these genes in *N. benthamiana* plants using TRV2:*CaLRR1* and TRV2:*CaHIR1* constructs. The efficiency of VIGS of *NbLRR1* and *NbHIR1* was examined by RT-PCR analysis (Fig. 5D). The silenced leaves lacked endogenous *NbLRR1* and *NbHIR1* transcripts, indicating that silencing was effective for *NbLRR1* and *NbHIR1*.

N. benthamiana leaves silenced for *NbLRR1* and *NbHIR1* genes were infiltrated with *Agrobacterium* strains carrying the following transgenes expressed by the *CaMV35S* promoter: the tomato *R* gene *Pto*, the *Pseudomonas syringae* avirulence gene *avrPto*, and the mouse proapoptotic effector gene *Bax* (Fig. 5E, F, and G) (Lacomme and Santa Cruz 1999). In empty-vector control leaves (TRV:00), PCD occurred in areas expressing *Bax* and *avrPto/Pto*. The *avrPto/Pto*-induced PCD phenotypes were much stronger than the *Bax*-induced phenotypes. As expected, transient expression of *avrPto* alone triggered the chlorosis phenotype in the empty-vector control leaves, whereas *Pto* expression had no effect in control or silenced leaves. *Bax*-mediated PCD was more pronounced in *NbLRR1*-silenced leaves compared with empty-vector control leaves. More importantly, *Agrobacterium*-mediated transient expression of *Bax*, *avrPto/Pto*, or *avrPto* induced distinct cell-death phenotypes in *NbLRR1*-silenced leaves. In contrast, silencing of *NbHIR1* compromised *Bax*- and *avrPto/Pto*-mediated PCD in *N. ben-*

thamiana leaves. Measurements of electrolyte leakage supported significantly strengthened or attenuated cell death in *NbLRR1*- or *NbHIR1*-silenced leaf tissues, respectively, following *Agrobacterium*-mediated transient expression of *avrPto*, *avrPto/Pto*, and *Bax* (Fig. 5G). Taken together, these findings support the notion that *LRR1* (*CaLRR1* or *NbLRR1*) and *HIR1* (*CaHIR1* or *NbHIR1*) may act as negative and positive regulators of cell death in plants, respectively.

HIR protein is a plant-specific modulator of cellular defense and cell death.

To investigate whether *CaHIR1* acts as a general component of eukaryotic cell-death execution, we expressed the gene in yeast cells. However, we failed to detect *CaHIR1*-induced cell death in yeast cells (Supplementary Fig. 5). Our phylogenetic analyses of 56 members of the SPFH superfamily revealed that 16 HIR proteins from diverse plant species constitute one family that is distinguished from other SPFH proteins from diverse phyla (Fig. 6).

DISCUSSION

CaHIR1 acts as a positive regulator of PCD associated with plant immunity and disease.

Transient expression of *CaHIR1* triggered cell death in pepper and *N. benthamiana* plants. In contrast, VIGS of *CaHIR1* suppressed not only disease progression but also hypersensitive cell death in response to virulent and avirulent *X. campestris* pv. *vesicatoria* infection, respectively. In addition, silencing of *NbHIR1*, a *N. benthamiana* ortholog of *CaHIR1*, compromised *Bax*- and *avrPto/Pto*-induced PCD in *N. benthamiana* plants. Collectively, these findings support the notion that *CaHIR1* acts as a positive regulator of PCD associated with plant immunity and disease susceptibility. In terms of the primary and secondary predicted protein structures and hydropathy profiles, plant HIR proteins are proposed to belong to a PID (proliferation, ion, and death) protein superfamily that also includes prohibitins and stomatins (Nadimpalli et al. 2000). Members of these gene families exhibit diverse expression patterns under stressful conditions, suggesting pivotal roles in plant defense responses (Ahn et al. 2006; Nadimpalli et al. 2000; Rostoks et al. 2003). More recently, proteomics analysis of PM proteins revealed a differential accumulation of *OsHIR1* in

Fig. 4. Effects of virus-induced gene silencing of *CaLRR1* or *CaHIR1* on pepper defense-related gene expression and salicylic acid (SA) accumulation. **A**, Real-time reverse transcription-polymerase chain reaction analyses of expression of pepper defense-related genes in gene-silenced pepper leaves 12 and 24 h after inoculation (hai) with the virulent (compatible) Ds1 and avirulent (incompatible) Bv5-4a strains of *Xanthomonas campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). Expression of the pepper 18S rRNA gene served as a control. **B**, SA accumulation in *CaLRR1*- and *CaHIR1*-silenced pepper leaves 1 and 3 days after inoculation (dpi) with *X. campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). H = Noninoculated healthy leaves. The data in **A** and **B** are the means $\pm$ standard deviation from four independent experiments. Statistically significant differences between means were determined by employing Fisher's least significant difference test. Different letters indicate significant differences at $P = 0.05$.

rice suspension cells during infection with *Xanthomonas oryzae* pv. *oryzae* (Chen et al. 2007). These findings, together with our agroexpression and VIGS studies, provide in vivo evidence for a functional role of HIR proteins in the execution of plant PCD.

Membrane-bound CaHIR1 interacts with extracellular CaLRR1.

The *CaLRR1* and *CaHIR1* genes encode LRR and SPFH domain-containing proteins, respectively, for which biological functions have not been fully defined. Confocal microscopy and protein gel-blot analyses revealed that CaLRR1 and CaHIR1 are extracellular and PM-associated proteins, respectively. BiFC and coimmunoprecipitation assays showed that extracellular CaLRR1 is capable of forming a complex with the PM-associated, SPFH domain-containing CaHIR1 protein in pepper plants. Strikingly, both smGFP-tagged CaLRR1 and CaHIR1 proteins exhibited punctate localization along the PM of onion epidermal cells. This pattern may be due to the presence of the SPFH domain within the protein, which is essential for triggering cell death by forming a microdomain. The punctate localization of both CaLRR1 and CaHIR1 in the PM of onion cells indicates that these proteins may physically bind to each other on the PM of pepper cells. These findings are well supported by recent observations that the SPFH domain-containing microdomain protein flotillin/reggie in vertebrates is punctately localized along the PM (Langhorst et al. 2007, 2008). Punctate localization of smGFP-tagged CaLRR1 in onion epidermal cells also raises the possibility that onion cells

may contain functional CaHIR1 homologues that can bind CaLRR1 to form a microdomain in the membrane (Zhou et al. 2009). These microdomain proteins may be involved in various cellular signaling pathways, such as neural regeneration, cell proliferation, clathrin- and caveolin-independent endocytosis, and T-cell activation (Babuke and Tikkanen 2007; Browman et al. 2007; Rivera-Milla et al. 2006). Recently, direct interaction between OsLRR1 and OsHIR1, which are rice orthologs of CaLRR1 and CaHIR1, was demonstrated by yeast two-hybrid and in vitro pull-down assays (Zhou et al. 2009). The LRR1 and HIR1 orthologs was shown to be highly conserved among different plant species, including *Arabidopsis* plants. Our findings combined with these ortholog data support strong evidence that the defense-related LRR1 and HIR1 microdomain may be conserved in plant species.

Negative feedback regulation of *CaLRR1* by *CaHIR1* determines endogenous SA levels in pepper.

Our interesting finding concerning *CaHIR1* silencing in pepper leaves is that bacterial growth is significantly reduced in silenced pepper leaves infected by virulent *X. campestris* pv. *vesicatoria* as well as avirulent *X. campestris* pv. *vesicatoria*, despite the absence of the HR. This raises the possibility that the expression of *CaHIR1* suppresses the defense responses of pepper plants to promote *X. campestris* pv. *vesicatoria* infection. Real-time RT-PCR analyses revealed that induction of the *CaLRR1* and *CaHIR1* genes by *X. campestris* pv. *vesicatoria* infection distinctly regulates the downstream SA-dependent defense marker genes *CaBPR1*, *CaPR10*, and *CaPOA1*. The

Fig. 5. Effects of virus-induced gene silencing of *NbLRR1* and *NbHIR1* on *Bax*- and *Pto/AvrPto*-induced cell death and *AvrPto*-induced chlorosis. **A**, *CaHIR1*-induced cell death in leaves. Pictures were taken 8 days after agroexpression. **B**, Immunological detection of CaLRR1-cMyc and CaHIR1-hemagglutinin proteins, using the indicated antibodies in leaves 2 days after inoculation with *Agrobacterium tumefaciens* carrying the indicated transgenes. Protein loading is shown by Ponceau S staining. **C**, Responses of *Nicotiana benthamiana* leaves to agroexpression of the indicated transgenes. Pictures were taken 4 days after agroexpression. **D**, Reverse transcription-polymerase chain reaction analyses of the *NbLRR1* and *NbHIR1* genes in *NbLRR1*- or *NbHIR1*-silenced leaves. **E**, Effects of *NbLRR1* or *NbHIR1* silencing on *Bax*- and *avrPto/Pto*-induced cell death and *avrPto*-induced chlorosis in leaves. The blue, yellow, and red circles indicate no cell death, chlorosis, and cell death, respectively. **F**, Cell-death scores for agroexpression of *Bax*, *avrPto*, *Pto*, and *avrPto/Pto* in *NbLRR1*- or *NbHIR1*-silenced leaves. **G**, Quantification of electrolyte leakage from disks of *NbLRR1*- or *NbHIR1*-silenced leaves 0, 24, 48, and 72 h after inoculation with *A. tumefaciens* carrying the indicated transgenes. Statistically significant differences between means were determined by employing Fisher's least significant difference test. Different letters indicate significant differences at $P = 0.05$.

altered expression of these SA-dependent genes in pepper leaves may lead to subsequent changes in the levels of endogenous SA, an important signaling molecule for defense response in plants (Durrant and Dong 2001). Importantly, transient expression of *CaLRR1* was very effective in enhancing endogenous SA levels in pepper plants, suggesting that *CaLRR1* positively regulates SA-dependent defense signaling in pepper plants. However, we detected only slight decreases in SA levels of *CaLRR1*-silenced plants infected by *X. campestris* pv. *vesicatoria*. In contrast, transient expression or silencing of *CaHIR1* drastically reduced or enhanced SA levels in pepper leaves, respectively, indicating a negative role for *CaHIR1* in SA accumulation. Taken together, our data suggest that *CaLRR1* and *CaHIR1* manipulate the endogenous SA-dependent signal-

ing pathway to positively and negatively regulate plant cell death and defense responses, respectively.

A key role of SA is proposed to be the induction of *PR* gene expression and disease resistance that is frequently associated with HR cell death in plants (Vlot et al. 2009). It is interesting to note complex roles of SA in regulation of cell death. In *Arabidopsis* lesion-mimic mutants, micro-cell death phenotypes were correlated with spatial and temporal patterns of SA accumulation (Mühlenbock et al. 2008; Yao and Greenberg 2006). However, some of lesion-mimic mutants, such as *defense no death 1* (*dnd1*), *HR-like lesion mimic 1* (*hlm1/dnd2*), and *hypersensitive response-like lesions 1* (*hrl1*) mutants retain characteristics of gene-for-gene resistance, such as strong SA accumulation and the ability to limit pathogen growth, without the HR (Clough et al. 2000; Balagué et al. 2003; Devadas and Raina 2002; Yu et al. 1998). This raises the possibility that SA accumulation occurs as a result of cell death and the cell death is not influenced by SA levels. Furthermore, constitutively elevated endogenous SA levels in uninfected *dnd1*, *hlm1/dnd2*, and *hrl1* mutants support an important role of SA in negative feedback regulation of HR-associated cell death (Balagué et al. 2003; Devadas and Raina 2002; Yu et al. 1998). Consistent with this hypothesis, pretreatment with SA and benzothiadiazole significantly suppressed HR against avirulent pathogen infection in wild-type *Arabidopsis* plants but not in *npr1* and *nahG* plants (Devadas and Raina 2002; Vlot et al. 2009). Interestingly, endogenous SA levels were constitutively elevated in *HIR1*-silenced plants. These findings support the notion that the preexisting SA-induced SAR of *HIR1*-silenced plants may serve as one form of negative feedback loop to regulate HR-associated cell death, as observed in *dnd1*, *hlm1/dnd2*, and *hrl1* mutants (Devadas and Raina 2002). Furthermore, transcript levels of SA-inducible *POA1*, encoding an important reactive oxygen species (ROS)-detoxifying enzyme (Do et al. 2003), also significantly increased in *HIR1*-silenced pepper plants noninoculated or inoculated with avirulent *X. campestris* pv. *vesicatoria*. Therefore, the enhanced ROS-scavenging capacity of *HIR1*-silenced plants may attenuate ROS-triggered cell-death signals during the incompatible interaction.

Real-time RT-PCR analyses revealed that *CaLRR1* upregulated endogenous *CaHIR1* expression, whereas *CaHIR1* down-regulated endogenous *CaLRR1* expression at the transcription

Fig. 6. An unrooted neighbor-joining dendrogram depicting the relationship among 56 representatives of the SPFH (stomatin, prohibitin, flotillins, HflK/C) superfamily and hypersensitive-induced reaction (HIR) proteins. Plant HIR proteins are clearly partitioned by families within the SPFH protein superfamily. The scale value of 0.1 represents 0.1 amino-acid substitutions per site.

Fig. 7. Proposed model for *CaLRR1*- and *CaHIR1*-mediated defense responses and cell death during the compatible and incompatible interactions of *Xanthomonas campestris* pv. *vesicatoria* with pepper plants. ETI = effector-triggered immunity and PTI = pathogen-associated molecular pattern-triggered immunity.

level. These findings raise the possibility that *CaLRR1* expression is regulated in a negative feedback manner by the induction of *CaHIR1*. Expression of *CaLRR1* enhances basal defense through the SA-dependent signaling pathway, simultaneously leading to the induced expression of *CaHIR1*. Expression of *CaHIR1* may negatively regulate SA-dependent defense signaling by suppressing *CaLRR1* expression to evade uncontrolled or excessive activation of defense responses.

Proposed model for the regulation of cell death and defense responses by *CaLRR1* and *CaHIR1*.

Combining the data we present here, we propose a working model for *CaLRR1*- and *CaHIR1*-mediated cell death and defense responses in pepper plants (Fig. 7). *PR* genes, such as *CaBPR1*, *CaPOA1*, *CaPRI0*, and *CaDEF1*, are differentially regulated downstream of the SA-dependent pathway, of which the signaling intensity is stronger for effector-triggered immunity (ETI) than for pathogen-associated molecular pattern-triggered immunity (PTI) (Jones and Dangl 2006). During the compatible interaction of pepper plants with virulent *X. campestris* pv. *vesicatoria*, *CaLRR1* and *CaHIR1* proteins may be induced by PTI. Expression of *CaLRR1* contributes to the establishment of basal defense through SA-dependent signaling pathways. Induction of *CaHIR1* triggers cell-death signals, in which signal intensities are not strong enough to exceed the threshold of the HR in the compatible interaction. Consequently, the signals triggered by *CaHIR1* may lead to susceptible cell death during compatible interactions. In contrast, *CaHIR1* induction that is strengthened and prolonged by ETI negatively regulates *CaLRR1* expression during incompatible interactions of pepper plants with avirulent *X. campestris* pv. *vesicatoria*. Consequently, the signals triggered by strong induction of *CaHIR1* may lead to hypersensitive cell death during the incompatible interaction. However, the absence of catalytic motifs, such as the kinase domain and others, in *CaHIR1* proteins makes it difficult to define the role of this protein in regulation of cell death and defense responses. Further studies of the novel *CaHIR1*-interacting proteins, the molecular mechanisms underlying strengthened and prolonged induction of *CaHIR1* by avirulent *X. campestris* pv. *vesicatoria*, and the antagonistic effects of *CaHIR1* on SA-mediated defense signaling are required to gain insights into its role in the regulation of cell death and defense responses.

MATERIALS AND METHODS

Plant materials and pathogen inoculation.

Seeds of pepper (*Capsicum annuum* L. cv. Nockwang) and *Nicotiana benthamiana* were germinated and grown in pots containing steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, vol/vol/vol) and loam soil (1:1, vol/vol) at 25°C with a 16-h light photoperiod cycle at a light intensity of 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Choi et al. 2007). Pepper plants were inoculated with virulent strain Ds1 and the avirulent strain Bv5-4a of *Xanthomonas campestris* pv. *vesicatoria* as previously described (Chung et al. 2007; Kim et al. 2007).

RT-PCR and real-time PCR analyses.

Total cellular RNA was isolated from plant leaves using TRIzol reagent (Invitrogen, San Diego, CA, U.S.A.) and was treated with RNase-free DNase (Roche, Indianapolis, IN, U.S.A.). First-strand cDNA synthesis and real-time PCR experiments were performed as previously described (Choi et al. 2007). Quantitative RT-PCR data were normalized with the level of the *Capsicum annuum* 18S rRNA internal transcript control. The gene-specific oligonucleotides used for real-time RT-PCR are listed in Supplementary Table 1.

Coimmunoprecipitation and immunoblotting.

CaLRR1 and *CaHIR1* cDNAs were cloned into the vector pET32a (Novagen, Madison, WI, U.S.A.). Each construct for protein expression was transformed into *Escherichia coli* BL21 (Invitrogen), and transformants were grown overnight in 5 ml of Luria-Bertani (LB) broth at 37°C. An aliquot of the culture was added to 50 ml of LB broth and was grown at 37°C to an optical density at 600 nm (OD_{600}) = 0.5. Protein expression was induced with 100 μM isopropyl- β -D-thio-galactoside for 3 h at 28°C. The His-tagged *CaLRR1* and *CaHIR1* proteins were purified using the Ni-NTA purification system kit (Invitrogen). The purified proteins were subjected to SDS-PAGE, were further purified by excising the bands, and were used for the generation of immune sera in rabbits (LabFrontier, Seoul, Korea). Specific binding of immune sera against *CaLRR1* or *CaHIR1* was confirmed and was used for Western blot analysis.

Total protein extracts were prepared by grinding 1 g of leaf tissue of pepper or *N. benthamiana* in 2 ml of grinding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1% SDS, 5 mM dithiothreitol (DTT), and complete protease inhibitor cocktail [Roche, Branchburg, NJ, U.S.A.]), followed by pelleting insoluble debris by centrifugation at $20,000 \times g$ for 15 min at 4°C. To separate soluble and microsomal fractions, total proteins were centrifuged for 1 h at $100,000 \times g$. To extract extracellular proteins, pepper leaf disks were vacuum-infiltrated with extraction buffer (10 mM phosphate, 0.1% sodium metabisulphite, pH 6.8) and were washed three times with sterile water at 4°C. Discs were dried on filter paper and were centrifuged to collect the extracellular fluid. Protein concentrations were determined by the Bradford protein assay (Bradford 1976). Western blotting was done by a standard method. Equal loading of proteins were ensured by Coomassie blue staining of SDS-PAGE gels or Ponceau S staining of polyvinylidene difluoride (PVDF) membranes.

For immunoprecipitations, 1 g of leaf tissues of pepper plants infiltrated with *Agrobacterium tumefaciens* GV3101 carrying each construct were ground in liquid nitrogen with a pestle and mortar and were homogenized in 2 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and complete protease inhibitor cocktail). Insoluble material was pelleted by centrifugation at $20,000 \times g$ for 20 min at 4°C. The supernatant (500 μl) was combined with 100 μl of resuspended anti-hemagglutinin affinity matrix (Roche). After incubation at 4°C overnight, the resin was washed four times with 1 ml of lysis buffer and was subjected to centrifugation at $15,000 \times g$ at 4°C. Electrophoresis sample buffer (200 μl) (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5% SDS, 0.02% bromophenol blue, 20% glycerol, and 200 mM DTT) was added to the washed pellets, and the mixture was boiled for 5 min and, then, centrifuged at $15,000 \times g$ at 4°C. Supernatant (30 μl) was subjected to SDS-PAGE and transferred to PVDF membrane for immunoblotting using anti-cMyc antibody (Sigma, St. Louis).

Particle bombardment.

The coding regions of the *CaLRR1* and *CaHIR1* genes were cloned between the *CaMV35* promoter and the *smGFP* region of the binary vector p326GFP to generate 3' fusions of *smGFP* to the *CaLRR1* and *CaHIR1* genes. To identify the *CaHIR1* domains involved in punctate localization in the PM, truncated forms of *CaHIR1* were also cloned in p326GFP. Particle bombardment experiments were conducted as previously described (Choi et al. 2007).

BiFC.

BiFC experiments were conducted as previously described by Walter and associates (2004). The coding regions of the

CaLRR1 and *CaHIR1* genes were cloned into pSPYNE-35S and pSPYCE-35S. *Agrobacterium tumefaciens* GV3101 carrying each construct was infiltrated into the abaxial side of pepper leaves ($OD_{600} = 0.5$ for each construct). The epidermal cell layers of pepper leaves were observed by confocal laser scanning microscopy (LSM 5 Exciter) 1 and 2 days after infiltration.

Agrobacterium-mediated transient expression assay.

The agroexpression assay was performed as previously described (Choi et al. 2009; Jung and Hwang 2007). The coding regions of *CaHIR1*, *CaLRR1*, *Pto*, *AvrPto*, and *Bax* were cloned between the *CaMV35* promoter and the *nos* terminator region in the binary vector pBIN35S. *A. tumefaciens* GV3101 containing each construct was grown overnight at 28°C in LB broth containing 50 µg of kanamycin per milliliter and 100 µg of rifampicin per milliliter. Bacterial cells were collected by centrifugation and were suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM morpholineethanesulfonic acid, pH 5.7, 200 µM acetosyringone) to a final OD_{600} of 0.2. Coexpression experiments were performed by mixing the *Agrobacterium* suspensions at a 1:1 ratio (final $OD_{600} = 0.2$ for each construct).

VIGS.

The TRV-based VIGS system was used for gene silencing in pepper and *N. benthamiana* (Liu et al. 2002). The full-length coding regions of *CaLRR1* and *CaHIR1* were amplified by PCR and were cloned into the vector pTRV2 to yield pTRV2: *CaLRR1* and pTRV2: *CaHIR1*. *A. tumefaciens* GV3101 strains carrying pTRV1 or pTRV2 constructs were coinfiltrated into the fully expanded cotyledons of pepper and first leaves of *N. benthamiana* plants (final $OD_{600} = 0.2$ for each construct). Plants were placed in a growth room at 25°C with a 16-h-light and 8-h-dark photoperiod cycle to allow growth and viral spread. Plants silenced for 4 to 5 weeks were used for pathogen inoculation.

Histochemistry.

Diaminobenzidine and trypan blue staining was performed as previously described (Choi et al. 2007). Aniline blue staining was performed to detect callose deposition as described by Adam and Somerville (1996). Pepper leaves were completely cleared in 20 ml of alcoholic lactophenol (1 ml of lactic acid, 1 ml of glycerol, 1 g of phenol, and 1 ml of H_2O), were rinsed in 50% ethanol and water, and were then stained for 30 min in 150 mM K_2HPO_4 (pH 9.5) containing 0.05% aniline blue. Samples were mounted in 25% glycerol and were observed with a microscope equipped with a UV lamp (Olympus, Tokyo).

Ion leakage measurement.

Six leaf disks of defined area (0.6 cm in diameter) were removed and washed three times with distilled water following infiltration and were incubated in 3 ml of distilled water at room temperature. The conductivity of the incubation medium was recorded at various timepoints with a conductivity meter.

Determination of SA.

SA was measured as previously described (Aboul-Soud et al. 2004). Pepper leaf tissues (1 g) were homogenized in liquid nitrogen and were extracted with 1 ml of 90% methanol and were then disrupted by sonication for 15 min. After centrifugation, the pellet was re-extracted with 1 ml of absolute methanol. As an internal standard for SA recovery, 20 µg of 3-hydroxy benzoic acid was added to the samples. The combined supernatants were dried in a speed vacuum. The residue was resuspended in 1 ml of 5% trichloroacetic acid and was sonicated for 10 min. Organic extraction of free SA was performed by adding 1 ml of ethylacetate/cyclopentane/isopropanol

(50:50:1). After two-phase separation, the aqueous phase that contained the conjugated SA was acidified with HCl to pH 1 and boiled for 30 min and, then, was extracted with the organic mixture to release free SA from acid-labile conjugated forms. The organic phase containing free SA was transferred to a 2-ml tube and dried under nitrogen gas. The dried extract was suspended in 0.5 ml of high-pressure liquid chromatography (HPLC) mobile phase (55% acetonitrile [ACN], 45% dH_2O with 4% [vol/vol] acetic acid) and was filtered and analyzed by HPLC (Waters, Milford, MA, U.S.A.) equipped with a C_{18} analytical column (J'sphere ODS-H80, 150 × 4.6 mm) (YMC, Kyoto, Japan) and fluorescence detector (excitation 305 nm and emission 405 nm) (Waters). The HPLC was programmed for gradient elution (55% ACN, 45% dH_2O with 4% [vol/vol] acetic acid to 80% ACN, 20% dH_2O with 4% acetic acid [vol/vol] over 10 min) at a flow rate of 1.0 ml min^{-1} . SA and glucoside-conjugated SA levels were quantified by area integration of the HPLC peaks.

Statistical analysis.

Data for each experiment were analyzed using the SAS 8.2 statistical package (SAS Institute, Cary NC, U.S.A.). Analysis of variance was carried out using the SAS general linear modeling procedure. Means were compared using Fisher's least significant difference tests, and different characters in the figures indicate significant differences.

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SMART database: smart.embl-heidelberg.de

TMPred database: www.ch.embnet.org/software/TMPRED_form.html