The Pepper E3 Ubiquitin Ligase RING1 Gene, *CaRING1*, Is Required for Cell Death and the Salicylic Acid-Dependent Defense Response[1][C][W][OA]

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Ubiquitination is essential for ubiquitin/proteasome-mediated protein degradation in plant development and defense. Here, we identified a novel E3 ubiquitin ligase RING1 gene, *CaRING1*, from pepper (*Capsicum annuum*). In pepper, *CaRING1* expression is induced by avirulent *Xanthomonas campestris* pv *vesicatoria* infection. CaRING1 contains an amino-terminal transmembrane domain and a carboxyl-terminal RING domain. In addition, it displays in vitro E3 ubiquitin ligase activity, and the RING domain is essential for E3 ubiquitin ligase activity in CaRING1. CaRING1 also localizes to the plasma membrane. In pepper plants, virus-induced gene silencing of *CaRING1* confers enhanced susceptibility to avirulent *X. campestris* pv *vesicatoria* infection, which is accompanied by compromised hypersensitive cell death, reduced expression of *PATHOGENESIS-RELATED1*, and lowered salicylic acid levels in leaves. Transient expression of *CaRING1* in pepper leaves induces cell death and the defense response that requires the E3 ubiquitin ligase activity of CaRING1. By contrast, overexpression of *CaRING1* in Arabidopsis (*Arabidopsis thaliana*) confers enhanced resistance to hemibiotrophic *Pseudomonas syringae* pv *tomato* and biotrophic *Hyaloperonospora arabidopsidis* infections. Taken together, these results suggest that CaRING1 is involved in the induction of cell death and the regulation of ubiquitination during the defense response to microbial pathogens.

Plants continuously confront pathogen attacks using a variety of defense responses, including callose deposition (Gómez-Gómez et al., 1999), oxidative burst (Apel and Hirt, 2004), synthesis of pathogenesis-related (PR) proteins (van Loon et al., 2006), and localized cell death via the hypersensitive response (HR; Hwang and Hwang, 2011; Kim and Hwang, 2011). Hypersensitive cell death is a genetically controlled process that eliminates infected and neighboring cells using the product of a resistance (*R*) gene that can recognize a pathogen carrying the corresponding avirulence (Avr) effector (Dangl and Jones, 2001). During pathogen infection, plants mount a defense response by altering physiological and biological systems via proteomic plasticity through protein synthesis (transcription and translation) as well as remodeling and degradation processes (posttranslational modification; Stone and Callis, 2007). There are many mechanisms for protein modification, including phosphorylation, methylation, acetylation, myristoylation, glycosylation, and ubiquitination (Kwon et al., 2006). Among these, ubiquitination is essential for ubiquitin/proteasome-mediated protein degradation. In some cases, ubiquitin-mediated protein degradation can be as influential in proteome structure as protein production and modification (Callis and Vierstra, 2000; Dreher and Callis, 2007). The ubiquitination pathway is involved in plant development (e.g. embryogenesis, photomorphogenesis, hormone regulation, and senescence; Moon et al., 2004; Dreher and Callis, 2007) as well as in defense (Zeng et al., 2006). Ubiquitin contains 76 amino acids and it is covalently conjugated to Lys residues in substrate proteins (Smalle and Vierstra, 2004; Dreher and Callis, 2007). There are several types of ubiquitination with different outcomes. Polyubiquitination is required for degradation of the protein via the proteasome. Ubiquitin is attached to target proteins in a stepwise conjugation cascade. First, a ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner. Second, the activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) by E1 enzyme. Finally, E2 enzyme interacts with a specific E3 ubiquitin ligase (E3), leading to ubiquitination of the substrate protein or autoubiquitination of E3. In this pathway, E3 ubiquitin ligase plays a central role in selecting candidate proteins for ubiquitination (Ciechanover, 1998).

E3 ubiquitin ligases are classified into two groups based on the presence of a HECT (for Homology to E6-AP C Terminus) or RING (for Really Interesting New

Gene)/U box domain, which have different subunit compositions and mechanisms of action (Pickart, 2001). The RING domain has a consensus sequence containing Cys and His residues (Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys/His-X_2-Cys-X_{4-48}-Cys-X_2-Cys), which functions as a binding site for the ubiquitin-E2 intermediate that has a zinc-binding domain formed by conserved Cys and His residues. The RING domains of RING finger proteins can be divided into two types, C3-H-C4 and C3-H2-C3, according to the presence of Cys or His in the fifth position (Borden and Freemont, 1996). The E3 ubiquitin ligase activity of RING finger proteins can be detected through in vitro autoubiquitination. Among the 1,300 E3 ubiquitin ligase genes in the Arabidopsis (*Arabidopsis thaliana*) genome, more than 400 are predicted to belong to the RING finger type (Stone et al., 2005). However, the functions of only a few RING-type proteins have been characterized in vivo.

In plants, ubiquitination regulates endogenous signals in response to pathogen attack. Although plants contain many RING finger proteins, only some of them have been shown to be induced by biotic stresses (Dreher and Callis, 2007; Hong et al., 2007). These RING finger proteins are specifically induced in plants by pathogen attack and play an essential role in plant defense (Zeng et al., 2006). In Arabidopsis, the *ARABIDOPSIS TOXICOS EN LEVADURA* (*ATL*) gene family members *ATL2* and *ATL6* are rapidly upregulated in response to chitin, a basal defense elicitor associated with fungal cell walls and insect exoskeletons (Salinas-Mondragón et al., 1999). Defense-related genes such as *NON-EXPRESSER OF PATHOGENESIS-RELATED GENES1*, *PHENYLALANINE AMMONIA-LYASE*, and *CHALCONE SYNTHASE* are induced constitutively under normal growth conditions in Arabidopsis mutants that constitutively express *ATL2* (Serrano and Guzmán, 2004). The fungal elicitor *N*-acetylchitooligosaccharide induces the expression of rice (*Oryza sativa*) EL5 RING E3 ligase, which possesses in vitro ubiquitination activity (Takai et al., 2002). In Arabidopsis cell cultures and seedlings, the general bacterial flagellin peptide elicitor (flg22), which also stimulates basal defense responses, can induce a 2.5-fold change in the expression levels of over 250 genes (Felix et al., 1999). Among these up-regulated genes were genes encoding 10 putative RING finger E3 ligases, including RING-H2 FINGER A3b (RHA3b), RHA1b, RING MEMBRANE-ANCHOR1, and ATL6 (Navarro et al., 2004). RING finger proteins, such as the F-box proteins AVR9/CF-9 RAPIDLY ELICITED GENES189 (ACRE189; van den Burg et al., 2008) and ACRE132 (Durrant et al., 2000), are involved in gene-for-gene resistance-initiated responses. U-box E3 ligases are variants of RING finger proteins, and some have been implicated in plant disease resistance (Zeng et al., 2004; González-Lamothe et al., 2006; Yang et al., 2006), including ACRE74 and ACRE276 from tobacco (*Nicotiana tabacum*), which are essential for positive regulation of plant defense responses. By contrast, the pathogen-induced C3-H-C4-type RING finger protein

CaRFP1 is associated with disease susceptibility and osmotic stress tolerance in pepper (*Capsicum annuum*; Hong et al., 2007). The Arabidopsis E3 ubiquitin ligases PLANT U-BOX PROTEIN22 (PUB22), PUB23, and PUB24 also play roles in the negative regulation of pathogen-associated molecular patterns-triggered immunity (Trujillo et al., 2008).

In this study, we identified *CaRING1*, which encodes a novel E3 ubiquitin ligase RING1 protein in pepper. *CaRING1* expression is induced by avirulent *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) infection. We show that a RING-type protein is essential for HR production and resistance to infection with virulent (Ds1) and avirulent (Bv5-4a) strains of *Xcv*. *CaRING1* encodes a low-M_r protein that contains an N-terminal transmembrane (TM) domain and a C-terminal RING-H2 domain. CaRING1 displays in vitro E3 ubiquitin ligase activity and localizes to the plasma membrane. Virus-induced gene silencing (VIGS) and *Agrobacterium tumefaciens*-mediated transient expression in pepper were used to characterize the activities of CaRING1 in plant defense. *CaRING1*-silenced pepper plants are highly susceptible to infection by avirulent *Xcv* and show compromised or delayed reactive oxygen species (ROS) induction, HR, *PR* gene expression, and accumulation of free salicylic acid (SA) during infection. By contrast, transient *CaRING1* expression in pepper leaves triggers the hypersensitive cell death response, which requires the E3 ubiquitin ligase activity of CaRING1. Transgenic Arabidopsis plants overexpressing *CaRING1* also exhibit enhanced bacterial and fungal disease resistance, accompanied by the induction of SA-responsive *AtPR1* and ethylene/jasmonic acid (JA)-responsive *AtPDF1.2* (for *PLANT DEFENSIN1.2*) during *Pseudomonas syringae* pv *tomato* (*Pst*) infection. These findings suggest that during the defense response to microbial pathogens, CaRING1 is involved in the activation of cell death via autoubiquitination and the regulation of posttranslational modification.

RESULTS

CaRING1 Encodes a Protein Containing a Conserved TM Domain and a RING Domain

CaRING1 cDNA (GQ359822) was isolated from a cDNA library constructed from pepper leaves infected with the avirulent *Xcv* strain Bv5-4a, according to a macro-cDNA array method (Jung and Hwang, 2000). The *CaRING1* cDNA sequence contains 985 bp, with a predicted open reading frame of 591 bp (Supplemental Fig. S1). The predicted CaRING1 protein comprises 197 amino acids and has a calculated molecular mass of 20.566 kD and a pI of 8.68. The PROSITE and SMART programs (http://www.expasy.ch/prosite/ and http://smart.embl-heidelberg.de/, respectively) revealed a putative TM domain for subcellular localization at the N terminus (residues 33–55) and a RING domain in the C-terminal region (residues 107–148;

Figure 1. Amino acid sequence analysis of pepper CaRING1 and the RING-H2 finger domain. A, Alignment of deduced amino acid sequences of pepper CaRING1 and other RING-H2 finger proteins from Arabidopsis (accession nos. NP_173506, NP_177767, AAM_63811, and NP_195273) and field mustard (accession no. ABK56013). Identical amino acids are shown in black boxes, and conserved amino acids are shaded in gray. Black boxes indicate the TM and RING domains. B, Alignment of RING domains. Sequences include CaRING1, Arabidopsis ATL8 (accession no. NP_177767), rice EL5 (accession no. Q9LRB7; Takai et al., 2002), *Nicotiana benthamiana* ACRE132 (accession no. AAG43550; Durrant et al., 2000), Arabidopsis ATL2 (accession no. NP_188294; Serrano and Guzmán, 2004), and Arabidopsis ATL6 (accession no. NP_566249). Identical amino acids are shown in black boxes. Conserved Cys (C) and His (H) residues are indicated by asterisks.
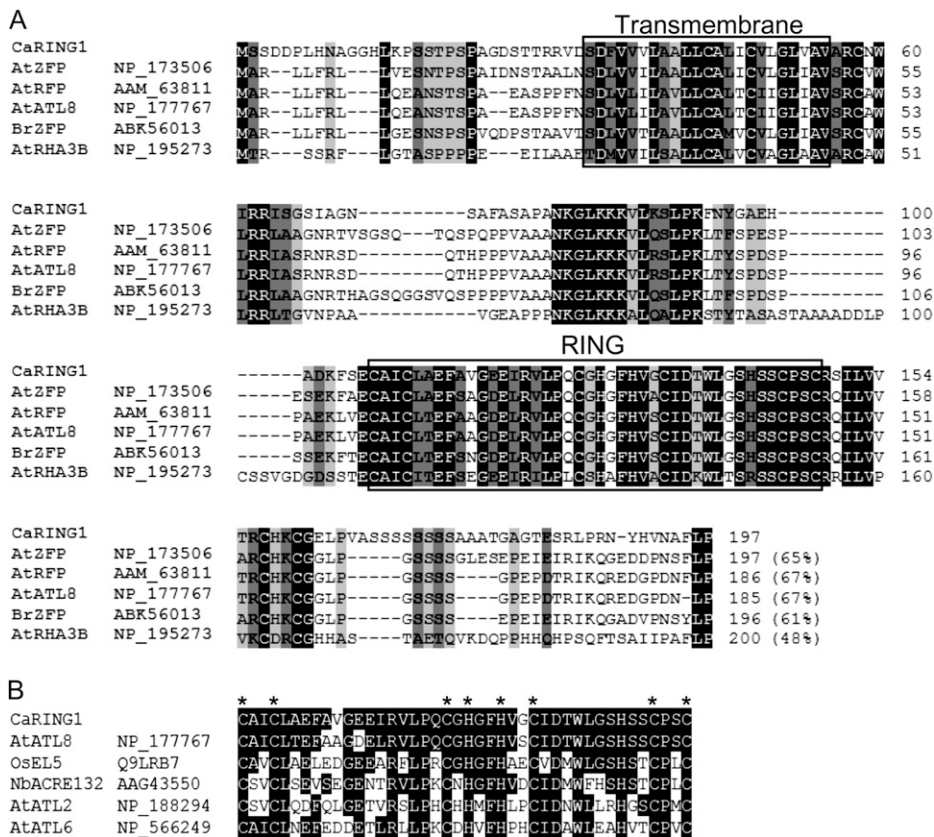
Fig. 1A). The RING domain belongs to the conserved C3H2C3-type RING-H2 group, which are essential for E3 ubiquitin ligase activity in the ubiquitin/26S proteasome system. CaRING1 shows relatively high amino acid sequence identity (48%–65%) to zinc finger or RING finger proteins in Arabidopsis and field mustard (*Brassica rapa*; Fig. 1A), which all contain highly conserved TM and RING domains. The RING domain of CaRING1 shares 55% to 90% identity with other plant defense-related RING finger proteins and contains eight conserved zinc-coordinating Cys and/or His residues that are essential for E3 ubiquitin ligase activity (Fig. 1B; Stone et al., 2005). Thus, domain analysis of the predicted CaRING1 sequence indicates that this protein may play a role in plant defense by modifying the proteome via ubiquitination.

Avirulent *Xcv* Infection Induces *CaRING1* Expression in Pepper Leaves
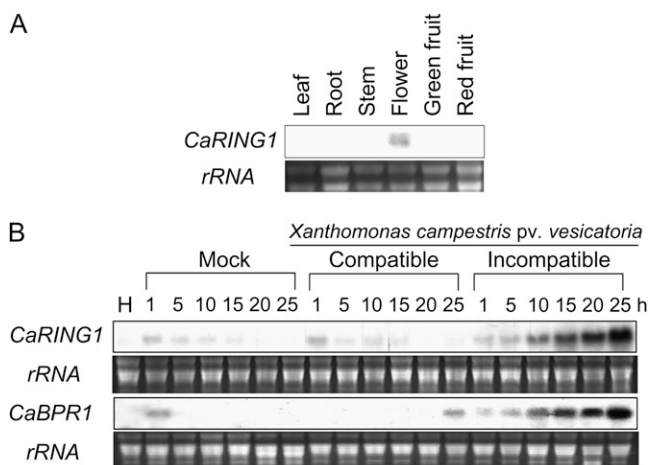
To ascertain whether *CaRING1* is expressed in specific plant tissues, RNA gel-blot analysis was used to examine *CaRING1* transcript levels in different plant tissues. *CaRING1* transcripts were detected in the flowers of healthy pepper plants but not in the leaves, stems, roots, or green/red fruits (Fig. 2A). This pattern indicates that, with the exception of flowers, *CaRING1* is not constitutively expressed in healthy plant organs.

To investigate *CaRING1* transcript levels induced during compatible and incompatible interactions with *Xcv*, pepper leaves were inoculated with virulent (compatible) and virulent (incompatible) strains (Ds-1 and Bv5-4a, respectively). RNA gel-blot analysis revealed rapid and strong *CaRING1* induction in leaves inoculated with *Xcv* Bv5-4a, with maximum levels achieved 25 h after inoculation (Fig. 2B). In contrast, mock-treated and virulent strain Ds1-inoculated leaves showed only negligible up-regulation of *CaRING1*. *CaBPR1*, which was used as a defense marker gene of pepper, was distinctly up-regulated during the incompatible interactions with *Xcv* (Fig. 2B). These findings indicate that *CaRING1* is induced specifically during the incompatible interaction between pepper plants and *Xcv*.

CaRING1 Functions as an E3 Ubiquitin Ligase

RING domain-containing proteins function as E3 ubiquitin ligases (Zhang et al., 2007; Lin et al., 2008), and the C terminus of CaRING1 contains a conserved C3H2C3-type RING-H2 domain (amino acids 107–148). The putative hydrophobicity of CaRING1 and a scheme for the construction of mutants are shown in Figure 3A. To test whether CaRING1 possesses E3 ubiquitin ligase activity, full-length CaRING1 was expressed in *Escherichia coli* (BL21) as a fusion protein with maltose-binding protein (MBP; Fig. 3, A and B). Following

A

CaRING1

rRNA

B

Xanthomonas campestris pv. *vesicatoria*

Mock Compatible Incompatible

H 1 5 10 15 20 25 | 1 5 10 15 20 25 | 1 5 10 15 20 25 h

CaRING1

rRNA

CaBPR1

rRNA

Figure 2. RNA gel-blot analysis of *CaRING1* expression in pepper plants. Samples were hybridized with a [^{32}P]dCTP-labeled *CaRING1* probe. Equal loading of total RNA (10 μg) was verified by visualizing rRNA on a gel stained with ethidium bromide. A, Organ-specific expression of *CaRING1* in pepper plants. B, Expression of *CaRING1* and *CaBPR1* in pepper leaves at various time points after inoculation at the six-leaf stage with 10^9 cfu mL^{-1} of the virulent *Xcv* strain Ds1 (compatible) or the avirulent *Xcv* strain Bv5-4a (incompatible) or 10 mM MgCl$_2$. H, Healthy leaves; Mock, Leaves treated with 10 mM MgCl$_2$.

cell lysis, affinity chromatography was used to purify MBP-CaRING1 from the soluble fraction containing total proteins. Yeast E1 (ubiquitin-activating enzyme 1 [UBA1]) and Arabidopsis E2 (ubiquitin-conjugating enzyme 10 [UBC10]) were used for the in vitro E3 ubiquitin ligase activity assay (Fig. 3C). Ubiquitination activity was detected using anti-ubiquitin antibody and anti-MBP antibody. MBP-CaRING1 displayed E3 ubiquitin ligase activity (polyubiquitinated smear ladders), whereas MBP did not demonstrate in vitro ubiquitination. MBP-CaRING1 did not display E3 ubiquitin ligase activity in the absence of E1, E2, E3, ATP, or ubiquitin. These data indicate that CaRING1 protein functions as an E3 ubiquitin ligase.

N- and C-terminal deletion mutants (MBP-CaRING1ΔTM and MBP-CaRING1ΔRING, respectively) as well as substitution mutants with changes to individual conserved amino acids were generated to determine whether the E3 ubiquitin ligase activity of CaRING1 requires a complete RING domain (Fig. 3D). Among all the MBP-CaRING1 mutants generated, only MBP-CaRING1ΔTM exhibited E3 ubiquitin ligase activity. This result indicates that a complete RING domain is essential for E3 ubiquitin ligase activity in CaRING1. Interestingly, however, MBP-CaRING1ΔTM displayed a decreased polyubiquitinated smear pattern on the immunoblot using the anti-MBP antibody (Fig. 3D; Supplemental Fig. S2), indicating that the TM region of CaRING1 also partly supports E3 ubiquitin ligase activity or may be ubiquitinated. The UbPred programs (http://ubpred.org/) predict that the TM region of CaRING1 contains a putative ubiquitination site at residue Lys-15. Thus, we further used

the MBP-CaRING1K15R mutant to determine if the TM region of CaRING1 is ubiquitinated (Supplemental Fig. S2). The MBP-CaRING1K15R mutant exhibited ubiquitination patterns similar to MBP-CaRING1 on the immunoblots using both anti-ubiquitin and anti-MBP antibodies, indicating that Lys-15 is not required for E3 ubiquitin ligase activity.

CaRING1 Localizes to the Plasma Membrane

CaRING1 is predicted to contain a TM domain (amino acids 33–55) in the N-terminal region. Fusion proteins between CaRING1 and soluble-modified (sm) GFP were used to determine the subcellular localization of CaRING1, and expression of the fusion proteins was driven by the cauliflower mosaic virus *35S* promoter (Fig. 4A). Following particle bombardment into onion (*Allium cepa*) epidermal cells, transient expression of *35S:CaRING1:smGFP* showed that CaRING1 localizes specifically to the plasma membrane (Fig. 4B). Plasmolysis confirmed that CaRING1 localizes to plasma membranes rather than cell walls.

Expression vectors containing genes encoding smGFP fusions to TM and RING domain deletion mutants (*35S:CaRING1ΔTM:smGFP* and *35S:CaRING1ΔRING*, respectively) were used to test the prediction that the putative TM domain functions in the subcellular localization of CaRING1 (Fig. 3A). Fluorescent signals from CaRING1-ΔTM:smGFP were detected in the cytosol and nuclei, whereas signals from CaRING1ΔRING:smGFP were found only at the plasma membrane (Fig. 4B). These results indicate that the TM domain of CaRING1 is essential for subcellular localization to the plasma membrane.

Transient Expression of *CaRING1* Induces Cell Death in Pepper Leaves

Since avirulent *Xcv* infection strongly induces *CaRING1* expression in pepper leaves during HR, further transient expression experiments were performed to investigate the potential relationship between *CaRING1* expression and induction of cell death. *Agrobacterium*-mediated transient expression of *CaRING1* (*35S:CaRING1*) induced a much stronger cell death response in pepper leaves compared with transient expression of the control vector (*35S:00*; Fig. 5A). UV-fluorescing phenolic compounds accumulated in pepper leaves expressing *CaRING1* but were barely detected in leaves expressing the empty control vector. In tobacco, such UV-fluorescing compounds indicate an accumulation of the phenolic phytoalexins, such as scopoletin and its aglycone (Costet et al., 2002), which represent an indicator of the HR (Gachon et al., 2004; Fig. 5A). To test whether ubiquitination functions in inducing cell death, site-directed mutants *35S:CaRING1C110S* and *35S:CaRING1H131Y*, in which a Ser was substituted for a conserved Cys and a His was changed by Tyr in the RING domain of CaRING1, respectively, were transiently expressed in pepper leaves. These RING
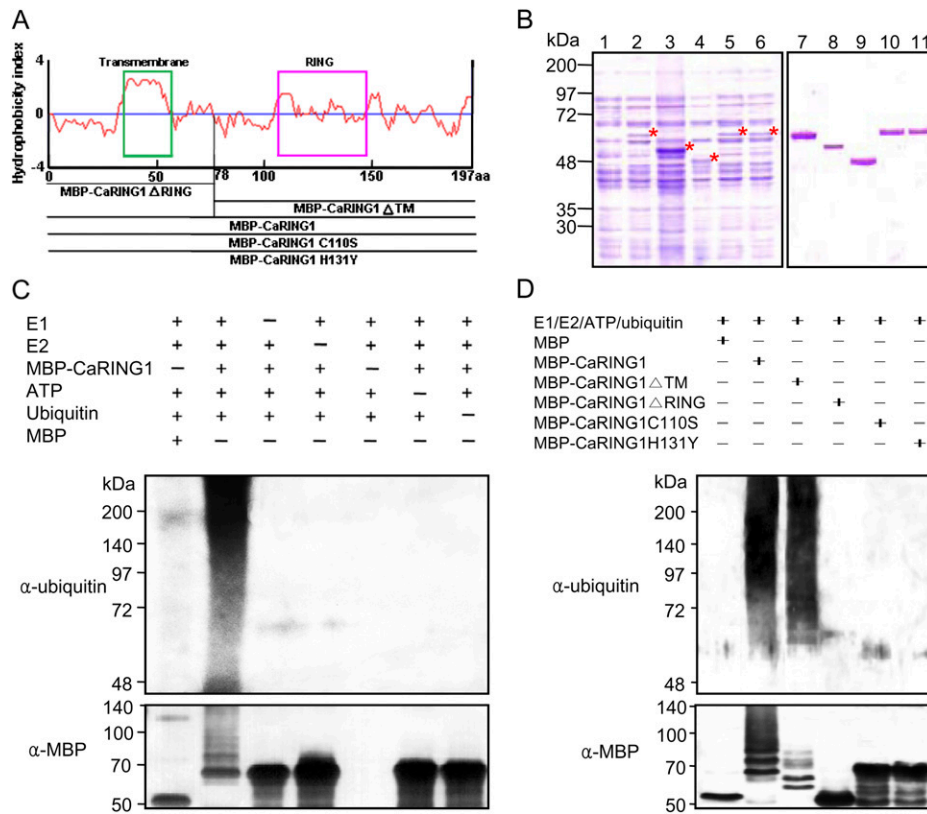
Figure 3. E3 ubiquitin ligase activity of CaRING1. A, Hydrophobicity index and expression constructs used for the in vitro ubiquitination assay. Recombinant fusion proteins are as follows: MBP-CaRING1, containing the full-length CaRING1; MBP-CaRING1ΔRING and MBP-CaRING1ΔTM mutants, with deletion of the C-terminal RING-H2 domain and N-terminal TM domain, respectively; and MBP-CaRING1C110S and MBP-CaRING1H131Y substitution mutants, with replacement of Cys-110 with Ser and His-131 with Tyr, respectively. The green box indicates the TM domain. B, SDS-PAGE of the recombinant MBP-CaRING1 and mutant variant proteins. Fusion constructs were transformed into *E. coli* BL21 (DE3). Cells were grown in LB medium, and expression of recombinant proteins was induced with 300 μM isopropyl-β-D-thiogalactopyranoside. Lane 1, Soluble fraction of uninduced *E. coli* MBP-CaRING1 extract. Lanes 2 to 6, Soluble fractions of induced *E. coli* MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1C110S, and CaRING1H131Y, respectively. Induced proteins are indicated by asterisks. Lanes 7 to 11, Purified MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1C110S, and CaRING1H131Y, respectively. C, E3 ubiquitin ligase activity assay of CaRING1. Recombinant MBP-CaRING1 fusion protein was incubated in the presence or absence of E1 (ScUBA1), E2 (AtUBC10), ATP, and/or ubiquitin. The reactions were analyzed with immunoblots using anti-ubiquitin antibodies (top panel) and anti-MBP antibodies (bottom panel). E3 ubiquitin ligase activity of MBP-CaRING1 was only detected in the presence of E1, E2, ATP, and ubiquitin. D, E3 ubiquitin ligase activity assay of CaRING1 mutant proteins. MBP-CaRING1 protein was used as a positive control. Only the mutant MBP-CaRING1ΔTM exhibited E3 ubiquitin ligase activity, indicating that the RING-H2 domain is essential for enzyme activity. [See online article for color version of this figure.]

domain mutants exhibited distinctly decreased cell death phenotypes compared with the wild-type *35S: CaRING1* (Fig. 5B). Compromised cell death phenotypes and accumulation of UV-fluorescing phenolic compounds were associated with the expression of these mutations. In agroinfiltrated pepper leaves, *35S: CaRING1*-induced cell death led to a significant increase in electrolyte leakage compared with empty vector controls (Fig. 5C). In contrast, the *CaRING1* mutants did not generate high levels of electrolyte leakage and ROS. Reverse transcription (RT)-PCR and immunoblot analyses showed that *35S:CaRING1* and the site-directed mutants *35:CaRING1C110S* and *35S: CaRING1H131Y* were transiently expressed at both transcript and protein levels in pepper leaves 24 and

48 h after agroinfiltration (Fig. 5, D and E). No significant differences between *35S:CaRING1* and the mutants were found in the transient expression levels of their transcripts and proteins. Taken together, these results indicate that expression and ubiquitination of CaRING1 are required to induce cell death effectively in pepper leaves.

CaRING1 Is Required for Resistance to *Xcv* Infection in Pepper Plants

The incompatible interaction with *Xcv* avirulent strain Bv5-4a rapidly and strongly induced *CaRING1* expression in pepper leaves. To determine the role played by CaRING1 in the basal defense response of pepper, VIGS
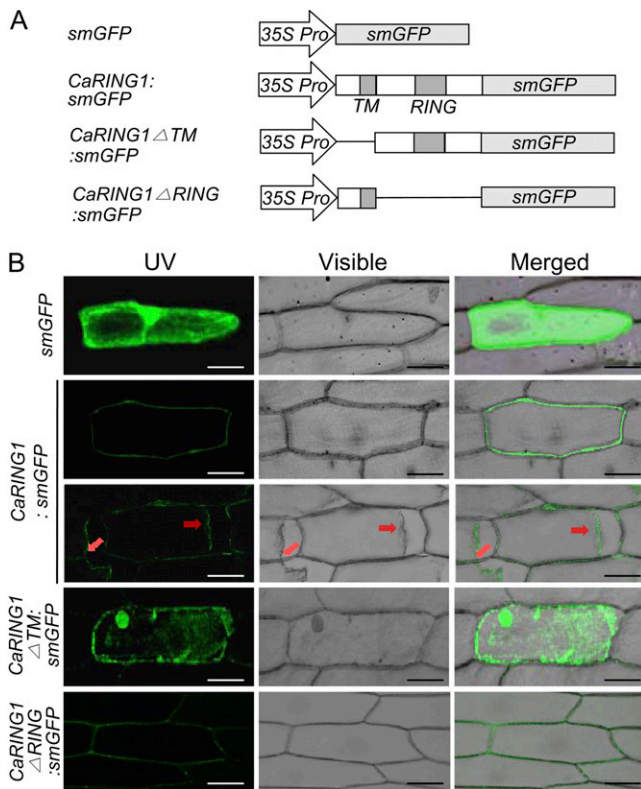
Figure 4. Subcellular localization of CaRING1 and its mutants using transient expression in onion epidermal cells. A, Schematic structure of *CaRING1* and the constructs used for subcellular localization analysis. Lines indicate deleted regions. The *smGFP* gene was fused to the 3' region of constructs. TM, TM domain; RING, RING-H2 finger domain. B, Transient expression of *smGFP* or *smGFP*-tagged constructs in onion epidermal cells 24 h after biolistic transformation, as detected by confocal laser-scanning microscopy. Constructs are as follows: *smGFP* (control), *CaRING1:smGFP* (wild type), *CaRINGΔTM:smGFP* (TM domain-deleted mutant), and *CaRINGΔRING:smGFP* (RING domain-deleted mutant). Arrows indicate the plasma membrane in a plasmolyzed cell. Bars = 200 μm.

was performed in pepper plants using *Tobacco rattle virus* (TRV; Liu et al., 2002; Choi et al., 2007). A 385-bp fragment from the 3' end of the *CaRING1* open reading frame was cloned into the TRV vector (TRV:*CaRING1*). Empty TRV vector (TRV:*00*) was used as a negative control. In comparison with pepper plants expressing the control vector, *CaRING1*-silenced (TRV:*CaRING1*) plants were highly susceptible to virulent *Xcv* infection, which was accompanied by an extensive suite of disease symptoms 6 d after inoculation. Little necrotic disease development was observed in *CaRING1*-silenced (TRV:*CaRING1*) pepper plants. However, the HR induced by avirulent *Xcv* infection was much weaker and delayed in *CaRING1*-silenced (TRV:*CaRING1*) pepper plants compared with plants expressing the empty TRV vector (TRV:*00*). Consistent with observations of transient CaRING1 expression in pepper leaves, UV-fluorescing phenolic compounds were induced as part of the defense response in plants expressing the empty TRV

vector (TRV:*00*; Fig. 6A). This HR marker was substantially reduced in *CaRING1*-silenced leaves. *CaRING1*-silenced leaves showed significantly higher levels of bacterial growth compared with leaves expressing the empty vector control (Fig. 6B). Electrolyte leakage was used to quantify differences in cell death between plants expressing the empty TRV vector (TRV:*00*) and *CaRING1*-silencing (TRV:*CaRING1*) vector during the HR. Avirulent *Xcv* infection induced earlier and more substantial levels of electrolyte leakage in leaves expressing empty TRV vector (TRV:*00*) than in *CaRING1*-silenced (TRV:*CaRING1*) leaves (Fig. 6E). Similarly, leaves expressing empty vector (TRV:*00*) showed stronger trypan blue staining than leaves in which *CaRING1* was silenced (Fig. 6C). In addition, *CaRING1*-silenced (TRV:*CaRING1*) plants exhibited significantly reduced accumulation of ROS (hydrogen peroxide [H_2O_2]) at 12 h after inoculation compared with plants with empty vector (Fig. 6D). Reduced H_2O_2 production in *CaRING1*-silenced (TRV:*CaRING1*) plants was also visualized by 3,3-diaminobenzidine staining (Fig. 6C). These results indicate that *CaRING1* expression plays a significant role in the induction and signal transduction of the HR during avirulent *Xcv* infection. However, the gene knockdown strategy such as VIGS may silence genes with sequence similar to the target gene. To reduce this nonspecific silencing effect, a 299-bp fragment from the 3' end of the *CaRING1* untranslated region (UTR) was cloned into the TRV2 vector (TRV2:*CaRING1UTR*) to specifically silence the *CaRING1* gene. Like *CaRING1*-silenced pepper plants (Fig. 6), *CaRING1* UTR-silenced (TRV:*CaRING1UTR*) pepper plants exhibited enhanced susceptibility to virulent and avirulent *Xcv* infection, which was accompanied by increased bacterial growth as well as reduced H_2O_2 accumulation, cell death, and electrolyte leakage (Supplemental Fig. S3).

Using quantitative RT-PCR, *PR* marker gene transcript levels were assayed in empty vector control (TRV:*00*) and *CaRING1*-silenced pepper leaves before and 12 and 24 h after infection with virulent and avirulent strains of *Xcv* (Fig. 7A). *CaRING1* expression was very low in *CaRING1*-silenced pepper leaves, suggesting that this gene was silenced effectively. Three pepper *PR* genes were assayed: *CaBPR1* (basic PR1; SA-dependent marker), *CaDEF1* (defensin), and *CaPO2* (peroxidase; positive regulator of HR). In comparison with plants expressing the empty vector control (TRV:*00*), significantly lower levels of *CaBPR1* induction were detected in *CaRING1*-silenced (TRV:*CaRING1*) leaves 24 h after infection with the avirulent *Xcv* strain. This finding suggests that CaRING1 may play a positive regulatory role in *CaBPR1* expression. Similarly, induction of the pepper *PR* genes *CaDEF1* and *CaPO2* was also reduced by silencing of *CaRING1* (Fig. 7A). Like *CaRING1*-silenced pepper leaves, *CaRING1* UTR-silenced leaves exhibited significantly reduced induction of defense-related genes *CaBPR1*, *CaDEF1*, and *CaPO2* during *Xcv* infection compared with empty vector control leaves, especially in the incompatible interactions (Supplemental Fig. S4).
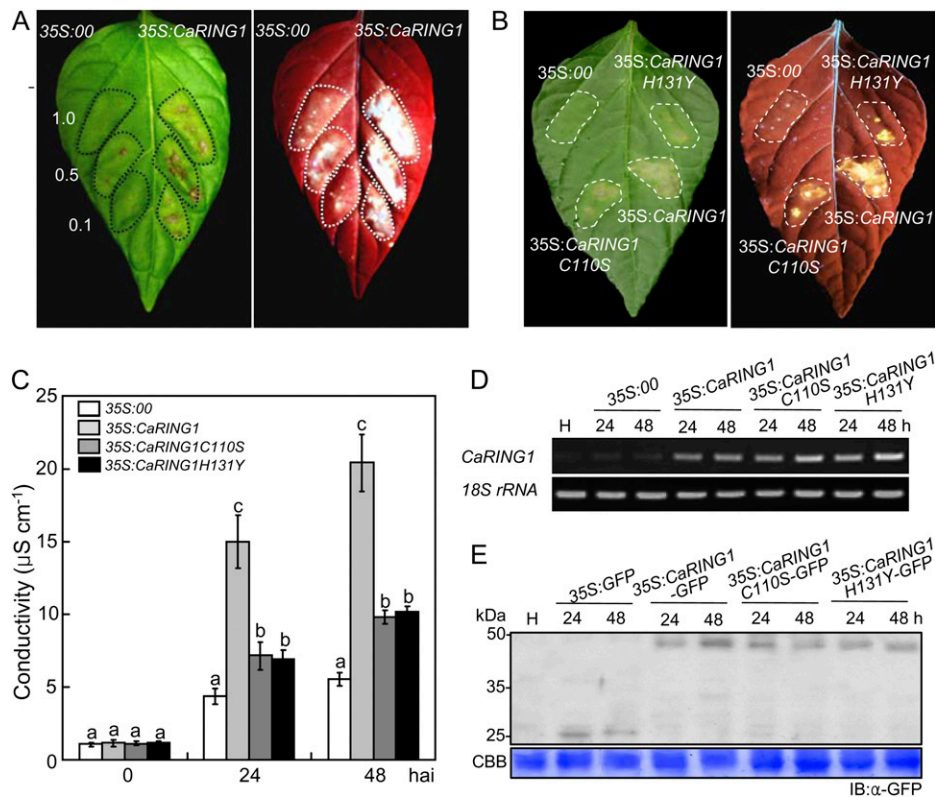
Figure 5. Induction of cell death by transient expression of *CaRING1* in pepper leaves. A, Cell death phenotypes in pepper leaves transiently expressing *35S:CaRING1* at 7 d after *Agrobacterium*-mediated transformation with the concentrations indicated (OD$_{600}$ = 1.0, 0.5, and 0.1). UV-fluorescing phenolic compounds associated with cell death are indicated in the same leaves. B, Site-directed CaRING1 mutations cause reduced cell death. Reduced-cell-death phenotypes are shown in pepper leaves infiltrated with *Agrobacterium* strains (OD$_{600}$ = 1.0) carrying the *35S:CaRING1C110S* or *35S:CaRING1H131Y* mutant. The marked regions on each leaf indicate the area infiltrated with *Agrobacterium* strains (OD$_{600}$ = 1.0). Photographs were taken 3 d after infiltration. UV-fluorescing phenolic compounds on the same leaf are shown in the right panels. C, Quantification of electrolyte leakage from pepper leaf discs transiently expressing empty vector control (*35S:00*, *35S:CaRING1*, *35S:CaRING1C110S*, or *35S:CaRING1H131Y* at various time points after infiltration (OD$_{600}$ = 1.0). The data represent means ± SD from three independent experiments. D, Transient expression of empty vector control (*35S:00*) and *35S:CaRING1*, *35S:CaRING1C110S*, or *35S:CaRING1H131Y* in pepper leaves 24 and 48 h after infiltration (OD$_{600}$ = 1.0). Gene expression was analyzed by quantitative RT-PCR. E, Immunoblot analysis of *35S:CaRING1* and *35S:CaRING1C110S* expression in pepper leaves 24 and 48 h after infiltration with *Agrobacterium* carrying *35S:CaRING1:GFP*, *35S:CaRING1C110S:GFP*, or *35S:CaRING1H131Y:GFP*. Total protein was extracted from mature leaves and used to detect GFP-tagged protein. Coomassie blue (CBB) staining confirmed equal protein loading. H, Healthy leaves.

Since *CaBPR1* expression was reduced in *CaRING1*-silenced pepper plants, it is possible that the synthesis and/or recognition of SA is also affected. Free SA and glucoside-conjugated SA (SAG) levels were determined in *CaRING1*-silenced (TRV:*CaRING1*) plants infected with virulent and avirulent strains of *Xcv*. Significant accumulation of free SA was detected in plants expressing the empty TRV vector (TRV:*00*), whereas *CaRING1*-silenced (TRV:*CaRING1*) plants showed, on average, a 2-fold reduction in free SA accumulation 24 h after infection with the avirulent *Xcv* strain (Fig. 7B). By contrast, *CaRING1*-silenced (TRV:*CaRING1*) plants exhibited reduced free SA but highly elevated SAG accumulation during infection with the avirulent strain of *Xcv* (Fig. 7B). *CaRING1*-silenced (TRV:*CaRING1*) leaves accumulated approx-imately 2-fold more SAG than wild-type leaves at 24 h after avirulent *Xcv* infection. These findings indicate that CaRING1 negatively regulates the transformation of SAG to SA. In addition, the reduction in free SA levels in *CaRING1*-silenced leaves supports the hy-pothesis that CaRING1 modulates the expression of SA-dependent *CaBPR1* as well as resistance to *Xcv* infection in pepper.

Overexpression of *CaRING1* Enhances Disease Resistance in Arabidopsis

Since transformation of pepper plants is difficult, *CaRING1* was overexpressed in Arabidopsis to assess the in vivo effects of *CaRING1*. Among 16 T2 lines of *CaRING1*-overexpressing (OX) plants, lines 5, 13, and
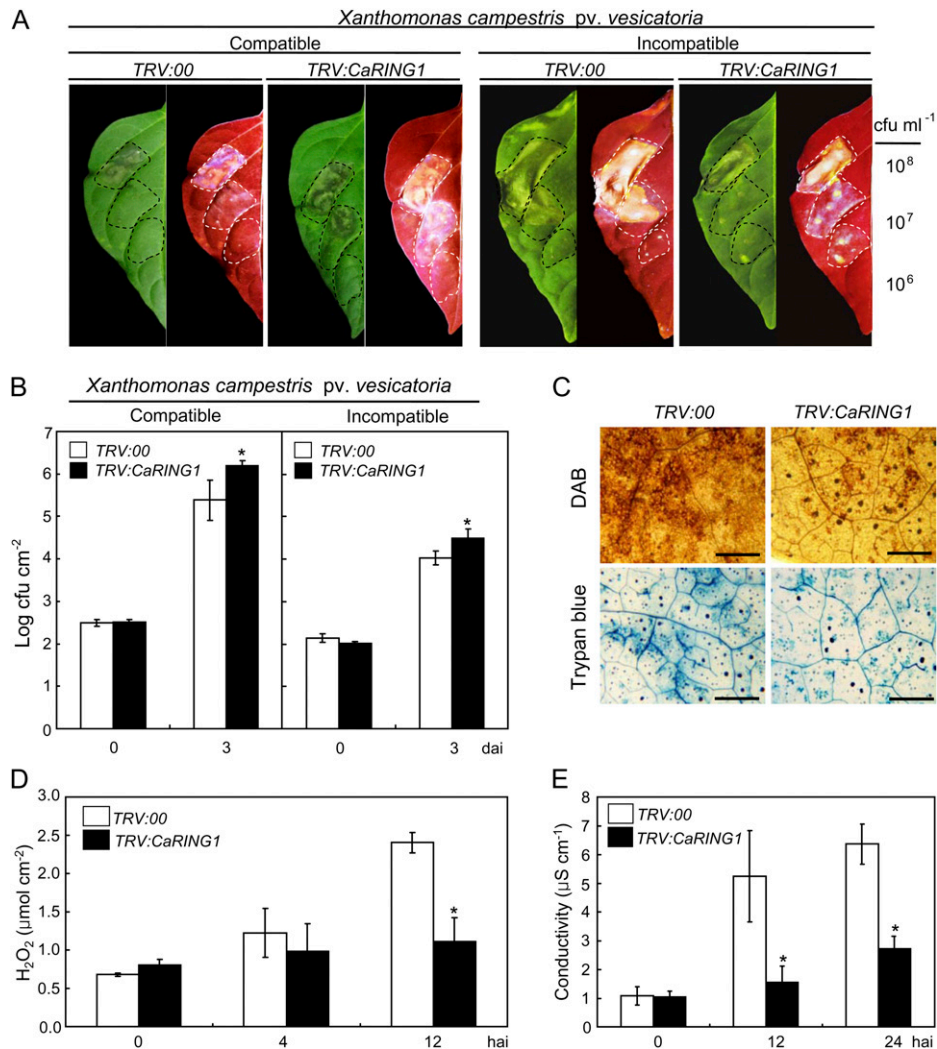
Figure 6. Enhanced susceptibility of *CaRING1*-silenced pepper plants to infection with the virulent *Xcv* strain Ds1 and the avirulent *Xcv* strain Bv5-4a. A, Disease symptoms developed on empty vector control (TRV:*00*) or *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves 6 and 3 d after inoculation with the virulent (Ds1; compatible) and avirulent (Bv5-4a; incompatible) strains, respectively. Highlighted areas of leaves show areas inoculated with the bacterial concentrations indicated. Infected leaves with UV-fluorescing phenolic compounds associated with susceptibility to infection and HR cell death are shown in the right panels. B, Growth of the virulent *Xcv* strain Ds1 and avirulent *Xcv* strain Bv5-4a in empty vector control (TRV:*00*) or *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves at 0 or 3 d after inoculation (5×10^4 cfu mL^{-1}). Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1* gene-silenced pepper plants as determined by Student's *t* test ($P < 0.05$). C, Reduced H_2O_2 production and cell death in *CaRING1*-silenced leaves infected with the avirulent *Xcv* strain Bv5-4a (10^7 cfu mL^{-1}). *Xcv*-infected leaves were harvested and stained with 3,3-diaminobenzidine (DAB) and trypan blue at 12 and 24 h after inoculation, respectively. Bars = 500 μm. D, Production of H_2O_2 in leaf discs from empty vector control (TRV:*00*) and *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves at different time points after inoculation with 10^7 cfu mL^{-1} of the avirulent *Xcv* strain Bv5-4a. Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1*-silenced pepper plants as determined by Student's *t* test ($P < 0.05$). E, Cell death was monitored by electrolyte leakage (conductivity) from empty vector control (TRV:*00*) and *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves challenged with the avirulent *Xcv* strain. Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1*-silenced pepper plants as determined by Student's *t* test ($P < 0.05$).

16 displayed strong *CaRING1* expression levels. These lines were selected for further study after confirmation of *CaRING1* transcript levels using RT-PCR (Fig. 8A). No apparent phenotypic differences were observed between the wild type and *CaRING1*-OX lines.

To determine the role played by *CaRING1* in pathogen resistance, wild-type and *CaRING1*-OX lines were inoculated with *Pst* DC3000 and disease development was observed (Fig. 8B). Six days after inoculation of leaves with *Pst* DC3000, wild-type plants showed

severe chlorotic symptoms, whereas *CaRING1*-OX transgenic lines exhibited only slight chlorotic symptoms. Consistent with a reduction in visible disease symptoms, *CaRING1* transgenic lines exhibited significantly lower bacterial growth 3 d after inoculation than wild-type plants (Fig. 8C). In Arabidopsis, enhanced disease resistance is often associated with elevated expression of several marker *PR* genes. Quantitative RT-PCR was used to examine the expression of *AtPR1*, which is associated with the SA-dependent pathway, *AtPDF1.2*,
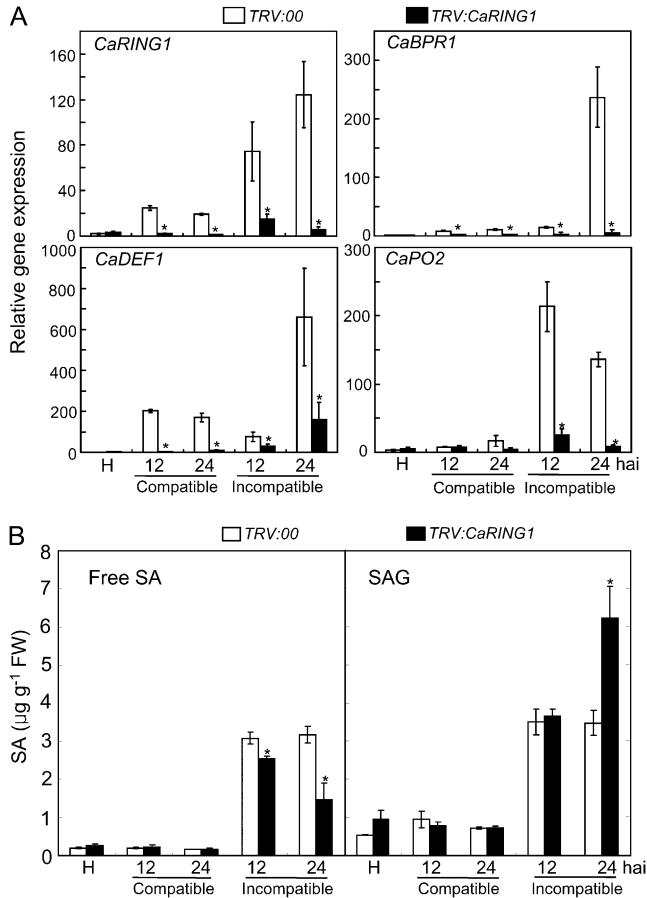
Figure 7. Expression of pepper defense-related maker genes and SA accumulation in *CaRING1*-silenced pepper leaves infected with *Xcv*. A, Expression of *CaRING1* and pepper defense-related marker genes in empty vector control (TRV:*00*) and CaRING1-silenced (TRV:*CaRING1*) pepper plants 12 and 24 h after inoculation with virulent and avirulent strains of *Xcv*. Gene expression was analyzed using quantitative RT-PCR and normalized using constitutively expressed 18S *rRNA*. Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1*-silenced pepper plants as determined by Student's *t* test (*P* < 0.05). H, Healthy leaves. B, Free SA and total SA levels in empty vector control (TRV:*00*) or *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves 12 and 24 h after inoculation (10^7 cfu mL^{-1}) with the virulent *Xcv* strain Ds1 (compatible) and the avirulent *Xcv* strain Bv5-4a (incompatible). Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1*-silenced pepper plants as determined by Student's *t* test (*P* < 0.05). FW, Fresh weight.
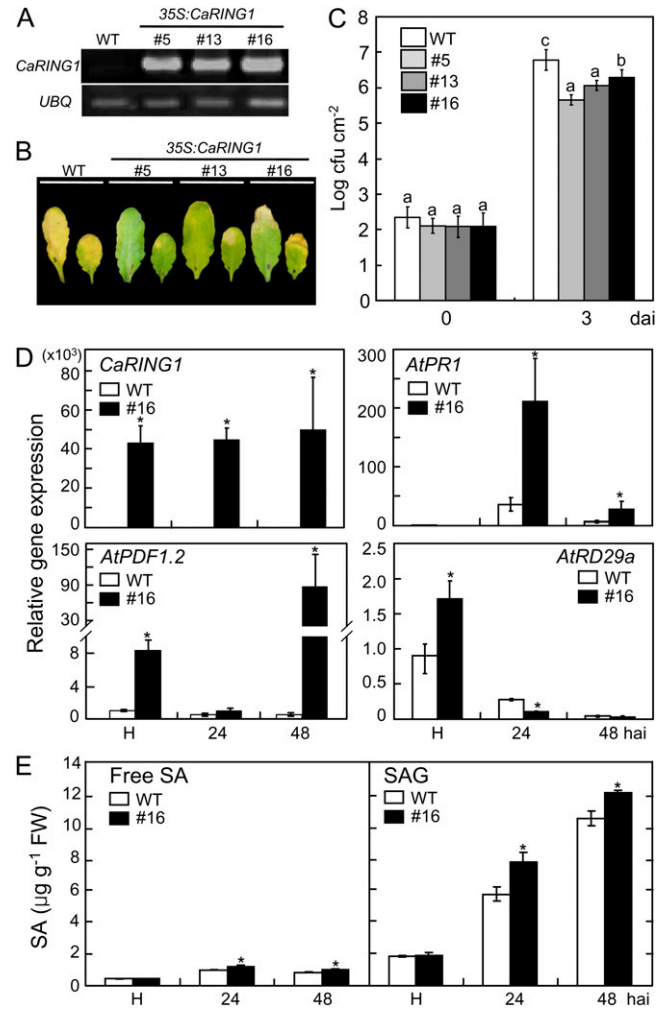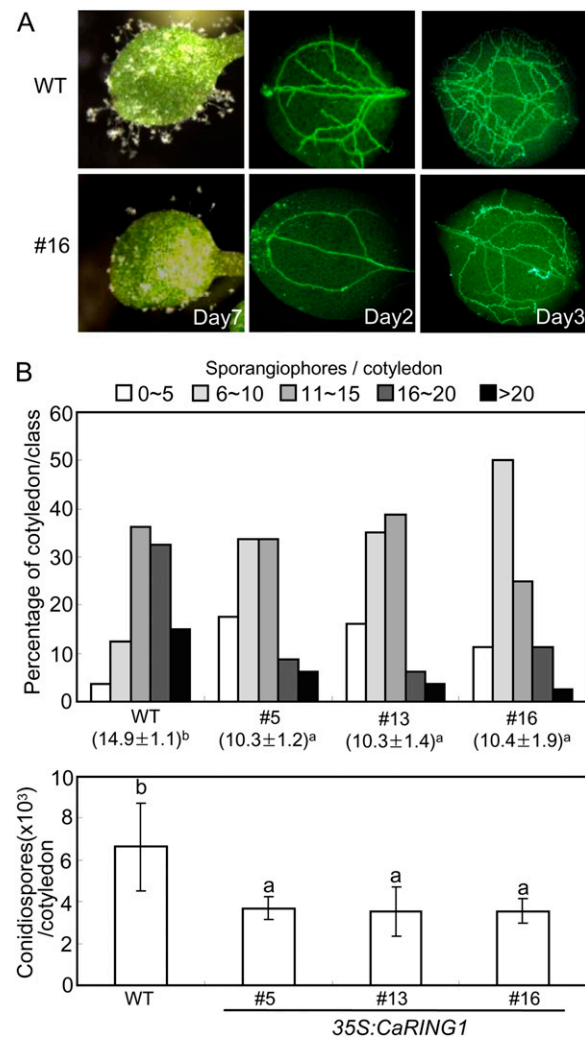

Figure 8. *CaRING1*-OX transgenic Arabidopsis plants exhibit enhanced resistance to *Pst* DC3000 infection. A, RT-PCR analysis of *CaRING1* expression in wild-type (WT) and *35S:CaRING1* transgenic lines. Expression of the ubiquitin (*UBQ*) gene was used as a control. B, Disease symptoms on the leaves of wild-type or transgenic plants 6 d after infection with *Pst* DC3000 (10^6 cfu mL^{-1}). C, Bacterial growth in wild-type and transgenic plant leaves at 0 or 3 d after inoculation with *Pst* DC3000 (10^5 cfu mL^{-1}). Data represent means ± SD from three independent experiments. Statistically significant differences between means were determined using Fisher's LSD test (*P* < 0.05). D, Expression of *CaRING1*, *AtPR1*, *AtPDF1.2*, and *AtRD29a* in wild-type and transgenic plants (T3) at 24 and 48 h after infiltration with *Pst* DC3000. Quantitative analysis was performed using RT-PCR, and relative gene expression levels were normalized using the constitutively expressed gene *AtACT1*. Data represent means ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and *CaRING1*-silenced pepper plants as determined by Student's *t* test (*P* < 0.05). E, Free SA and SAG levels in wild-type and transgenic plants 24 and 48 h after infiltration with *Pst* DC3000. Data represent means ± SD from two independent experiments. Asterisks indicate significant differences between wild-type and transgenic plants as determined by Student's *t* test (*P* < 0.05). FW, Fresh weight. [See online article for color version of this figure.]

which is related to the ethylene/JA-dependent pathway, and *AtRD29a*, a known regulator of abscisic acid metabolism (Fig. 8D). *Pst* DC3000 infection induced stronger expression of *AtPR1* and *AtPDF1.2* in transgenic plants than in the wild type. In particular, *CaRING1*-OX transgenic plants exhibited a close relationship between *AtPR1* and *AtPDF1.2* induction upon *Pst* DC3000 infection. After inoculation with *Pst* DC3000, the expression levels of *AtPR1* and *AtPDF1.2* transcripts in *CaRING1*-OX transgenic plants peaked at 24 and 48 h, respectively. These results indicate that *CaRING1* may effectively regulate cross talk between SA- and ethylene/JA-dependent pathways in the defense response of Arabidopsis. However, levels of the abscisic acid-responsive transcript *AtRD29a* were reduced in wild-type and *CaRING1*-OX transgenic plants 24 h after inoculation with *Pst* DC3000, but the downregulation was more pronounced in the *CaRING1*-OX line compared with the wild-type plants. As compared with the wild-type plants inoculated with *Pst* DC3000, accumulation of both free SA and SAG was significantly enhanced in *CaRING1*-OX transgenic plants 24 and 48 h after inoculation with *Pst* DC3000 (Fig. 8E). These findings support the possibility that the increased expression of *AtPR1* in transgenic plants is intimately associated with the induced accumulation of SA for resistance to *Pst* DC3000 infection.

To determine whether *CaRING1* overexpression alters resistance to a biotrophic oomycete pathogen, 7-d-old cotyledons of transgenic *CaRING1*-OX plants were sprayed with a conidiospore suspension (5×10^4 mL^{-1}) of *Hyaloperonospora arabidopsidis* isolate Noco2 (Fig. 9). Transgenic *CaRING1*-OX plants not only exhibited reduced formation of sporangiophores compared with wild-type plants but also showed retarded hyphal growth in cotyledons (Fig. 9A). Consistent with these visually observed phenotypes, the number of sporangiophores and spores was reduced significantly in *CaRING1* transgenic lines compared with wild-type plants (Fig. 9B). These observations indicate that *CaRING1* overexpression suppresses *H. arabidopsidis* sporulation and induces a defense response against the infection. Taken together, these results suggest that *CaRING1* overexpression enhances basal defense to hemibiotrophic bacterial and biotrophic oomycete pathogens in Arabidopsis plants.

DISCUSSION

In this study, we have found that the pepper E3 ubiquitin ligase RING1 (*CaRING1*) gene is required for disease resistance and for the HR cell death against *Xcv* infection. CaRING1 contains an N-terminal TM domain and C-terminal region RING domain (Fig. 1). The presence of a RING domain in CaRING1 indicates that this protein may possess E3 ubiquitin ligase activity (i.e. the ability to transfer ubiquitin to specific substrates in the ubiquitination machinery; Fig. 3). Ubiquitination is a unique eukaryotic posttransla-

Figure 9. *CaRING1*-OX transgenic Arabidopsis plants show enhanced resistance to *H. arabidopsidis* isolate Noco2 infection. A, Disease symptoms and aniline blue-stained mycelia on cotyledons of wild-type (WT) and transgenic Arabidopsis plants at 7 d and 2 to 3 d after spray inoculation with *H. arabidopsidis* isolate Noco2 (5×10^4 spores mL^{-1}), respectively. B, Production of sporangiophores per cotyledon of wild-type and transgenic plants 6 d after inoculation with *H. arabidopsidis*. Single cotyledons from over 60 seedlings per genotype were analyzed with the microscope and categorized into one of five categories of sporangiophore production (0–5, 6–10, 11–15, 16–20, or >20, depending on the number of sporangiophores observed). Average numbers of newly formed sporangiophores on cotyledons of wild-type and transgenic lines are indicated below each of the lines analyzed. C, Sporulation of *H. arabidopsidis* isolate Noco2 per cotyledon at 8 d after inoculation. Each experiment contained average spore counts from 50 inoculated cotyledons of wild-type and transgenic lines. Statistically significant differences between means were determined using Fisher's LSD test ($P < 0.05$). [See online article for color version of this figure.]

tional modification system that has been shown to play a significant role in the recognition and induction of various signals by modulating the stability of proteins involved in signal perception or responses (Smalle and Vierstra, 2004; Kwon et al., 2006; Dreher

and Callis, 2007). In plants, various physiological and biochemical pathways are regulated by ubiquitin-mediated degradation of specific target proteins in response to biotic and abiotic stresses (Kwon et al., 2006; Dreher and Callis, 2007). To date, RING domains have been identified in several defense-related proteins induced by pathogen infection (Hong et al., 2007; Stone and Callis, 2007; Liu et al., 2008). The E3 ubiquitin ligase activity of RING-type proteins can be predicted by in silico analysis of amino acid sequences (Stone et al., 2005). This study has shown that bacterial pathogens can induce *CaRING1* expression and that CaRING1 is important for production of the HR and restriction of pathogen growth, both of which are controlled by E3 ubiquitin ligases (Devoto et al., 2003).

MBP-CaRING1 displayed E3 ubiquitin ligase activity, in which E1, E2, and ATP were required for in vitro ubiquitination. Analysis of deletion and site-directed mutants revealed that the CaRING1 RING domain is essential for in vitro ubiquitination (Fig. 3). However, immunoblotting with anti-MBP antibody determined that the E3 ubiquitin ligase activity of MBP-CaRING1ΔTM was significantly lower than that of MBP-CaRING1 (Fig. 3D). Previously, it has been suggested that MBP is ubiquitinated by E3 ubiquitin ligases (Matsuda et al., 2001; Takai et al., 2002). Therefore, the reduced level of ubiquitination may be due to deletion of the TM domain (residues 1–78), and UbPred programs (http://ubpred.org/) reveal that this region of CaRING1 contains a predicted ubiquitination site (residue 15). BCA1, a RING-type E3 ligase in human cells, is known to regulate its own stability by autoubiquitination (Amemiya et al., 2008). Unexpectedly, however, the mutation of CaRING1 at the Lys-15 residue in the TM region did not abrogate the ubiquitination of CaRING1 K15R (Supplemental Fig. S2). However, the MBP-CaRING1ΔTM deletion mutant was impaired, suggesting an involvement of this TM domain at least in in vitro ubiquitination. Transfer of ubiquitins from the E2 to substrates, which is mediated by RING E3s, is known to require the substrate-docking site on RING E3s, which is located many angstroms from the predicted location of the docked E2-bound ubiquitin (Zheng et al., 2000; Deshaies and Joazeiro, 2009). Thus, decreased ubiquitination by MBP-CaRING1ΔTM may be associated with a change in space between the MBP (substrate) and the E2 on CaRING1 caused by the TM deletion. Taken together, these results suggest that the TM region of CaRING1 may be involved in the transfer of ubiquitins from the E2 to substrates on CaRING1, leading to the normal ubiquitination. However, the precise mechanism underlying the ubiquitination of CaRING1 remains to be investigated.

Following transient expression of *CaRING1* in onion epidermal cells, CaRING1 localized to the plasma membrane and its TM domain was essential for this subcellular localization (Fig. 4). The E3 ubiquitin ligases Arabidopsis RING1 and rice EL5 are rapidly induced as part of the disease resistance process, and these proteins have recently been detected in the plasma membrane (Koiwai et al., 2007; Lin et al., 2008). In particular, Arabidopsis RING1 is associated with plasma membrane lipid rafts, and since similar assemblages have been implicated in signaling pathways and apoptosis of animal lymphocytes, this protein may be induced for programmed cell death in Arabidopsis (Lin et al., 2008). These data suggest that localization of CaRING1 to the plasma membrane may be important for signal transduction of hypersensitive cell death during infection.

Agrobacterium-mediated *CaRING1* expression significantly induced a cell death phenotype in pepper leaves (Fig. 5). Markers for hypersensitive cell death include the accumulation of UV-fluorescing phenolic compounds, a ROS (H_2O_2) burst, and increased electrolyte leakage. These characteristic markers were detected at areas of localized cell death in leaves transiently expressing *CaRING1*. The findings described above strongly support the notion that *CaRING1* expression is required for HR-like cell death. The E3 ubiquitin ligase activities of Arabidopsis PLANT U-BOX17 and its functional tobacco homolog ACRE276 are involved in cell death and defense (Yang et al., 2006). *ACRE276* was identified as a potential E3 ubiquitin ligase, since it was rapidly induced in *R* gene *Cf9*-carrying tomato plants by infection with the fungal pathogen *Cladosporium fulvum* expressing the elicitor Avr9 (*Avr9/Cf9*). Although *ACRE276* does not encode a RING-type E3 ubiquitin ligase, such as CaRING1, *ACRE276* knockdown compromised HR triggered by various elicitors (Yang et al., 2006). More importantly, *Agrobacterium*-mediated expression of the site-directed mutants *35S:CaRING1C110S* and *35S:CaRING1H131Y* led to the distinctly decreased cell death and early defense responses compared with the wild-type *35S:CaRING1* (Fig. 5B). Together with E3 ubiquitin ligase activity assay data of these CaRING1 mutant proteins (Fig. 3D), these findings strongly support the notion that E3 ubiquitin ligase activity of CaRING1 is required for the induction of cell death in pepper plants.

CaRING1-silenced pepper plants exhibited enhanced susceptibility to virulent and avirulent *Xcv* infection (Figs. 6 and 7). This finding suggests that CaRING1 induction confers enhanced resistance to *Xcv* infection in pepper plants. *CaRING1*-silenced pepper plants displayed reduced induction of phenolic compounds, ROS burst, *PR* gene expression, and the HR, all of which are crucial components for effective resistance to avirulent *Xcv* infection. Expression of the SA-dependent *CaBPR1*, which is an essential *PR* gene during the HR, is severely compromised in the response of *CaRING1*-silenced pepper plants to avirulent *Xcv* infection (Fig. 8). Free SA and total SA were quantified to determine whether the reduced *CaBPR1* expression correlated with SA accumulation. Consistent with the previous finding, *CaRING1*-silenced leaves showed an approximately 2-fold reduction in avirulent *Xcv*-induced free SA. However, SAG levels were significantly higher in *CaRING1* gene-silenced leaves. In many plants, free SA represents a key compound for the activation of

plant defense, and its accumulation is necessary for the induction of the HR and the expression of marker *PR* genes (Lee et al., 1995; Alvarez, 2000). In general, enhanced production of free SA induces SAG accumulation in the defense response, whereas reduced expression of free SA is accompanied by decreased accumulation of SAG (Zhou et al., 1998; Lee et al., 2007). Since high concentrations of free SA are phytotoxic, a large proportion of the SA in plants is present in conjugated, inactive forms that are glucosylated for storage. Moreover, SAG is not exuded from infected leaves and does not induce *PR* gene expression (Lee et al., 1995). These data support the suggestion that the decrease in free SA in *CaRING1* gene-silenced leaves enhances plant susceptibility to avirulent *Xcv* infection. Thus, it is possible that CaRING1 may not modulate the production of SA synthesis but rather the conversion of SA to SAG, since the accumulation of total SA was similar in *CaRING1*-silenced and empty vector control plants. It is clear that the relationship between *CaRING1* expression and the mechanisms underlying the conversion of SA to SAG during infection with avirulent *Xcv* strains remains to be elucidated.

CaRING1-OX Arabidopsis plants exhibited significantly enhanced resistance to *Pst* DC3000 infection, which was accompanied by rapid induction of *AtPR1*, increased accumulation of SA and *AtPDF1.2*, but not *AtRD29a* (Fig. 8). These enhanced defense responses may be activated by various signal transduction pathways, which can be modulated by plant hormones such as SA and JA/ethylene (Lee and Hwang, 2005; Pieterse et al., 2009; Choi and Hwang, 2011). In Arabidopsis, *AtPR1* induction depends upon the SA-dependent pathway (Glazebrook et al., 1996; Rogers and Ausubel, 1997), whereas *AtPDF1.2* is a JA/ethylene-responsive marker (Penninckx et al., 1998). Our findings support the suggestion that CaRING1 is necessary for the coordination of SA- and JA/ethylene-responsive gene expression. However, further study will be required to develop a full understanding of the mechanisms underlying cross talk between SA- and JA/ethylene-dependent signaling pathways in *CaRING1*-OX Arabidopsis plants during the defense response. In addition, CaRING1 overexpression also enhanced resistance to infection with the biotrophic pathogen *H. arabidopsidis* (Fig. 9). Similar to our findings, enhanced resistance to TMV and *P. syringae* pv *tabaci* infection has recently been demonstrated in tobacco plants overexpressing the E3 ubiquitin ligase *OsBIRF1* (Liu et al., 2008).

In conclusion, it is clear that the E3 ubiquitin ligase function of pepper CaRING1 is critical for the plant defense response. Moreover, overexpression of *CaRING1* can confer nonhost resistance to infection with bacterial and fungal pathogens. Taken together, our results suggest that CaRING1 is a positive regulator of cell death in pepper and requires E3 ubiquitin ligase activity for its function. It is also likely that CaRING1 functions as a novel positive regulator of cell death by degrading negative regulators of cell death. Thus, CaRING1 may

be important for timely activation of the plant defense response, accompanied by HR. Further identification of CaRING1 substrate proteins will be required for complete elucidation of the roles played by this E3 ubiquitin ligase in the plant defense response.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pepper (*Capsicum annuum* 'Nockwang') seeds were planted in a plastic tray (55 × 35 × 15 cm) containing soil mix (perlite:vermiculite:loam soil, 1:1:3, v/v/v) and grown at 25°C under a 16-h day and light intensity of 65 μmol photons m^{-2} s^{-1}. At the two-leaf stage, three seedlings were transplanted to a plastic pot (5 × 15 × 10 cm) containing the same soil mix. Pepper plants at the six-leaf stage were used for pathogen inoculation or other treatments.

Seeds of Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) and *CaRING1*-OX lines were surface sterilized, planted on growth medium (1× Murashige and Skoog [MS] and 1% [w/v] Suc), and kept at 4°C for a minimum of 3 d to overcome dormancy. Seedlings at the two-leaf stage were transplanted to pots containing vermiculite, perlite, and loam soil (1:1:2, v/v/v) and then grown in a climate chamber at 24°C under long-day conditions (16 h of light per day; 130 μmol photons m^{-2} s^{-1}) and 60% humidity.

Pathogens and Inoculation Procedures

The bacterial strains *Xanthomonas campestris* pv *vesicatoria* Ds1 (virulent) and Bv5-4a (avirulent) were grown overnight in yeast-nutrient broth (5 g L^{-1} yeast extract, 8 g L^{-1} nutrient broth) at 28°C. A needleless syringe was used to infiltrate bacterial suspensions into the abaxial side of fully expanded leaves at the six-leaf stage. Infected leaves were harvested at various time points to determine bacterial growth and perform RNA gel-blot analysis. *Pseudomonas syringae* pv *tomato* DC3000 was grown overnight in King's B medium containing 50 mg mL^{-1} rifampicin and 50 mg mL^{-1} kanamycin. To measure bacterial growth, leaves from 4-week-old wild-type and *CaRING1*-OX Arabidopsis transgenic T3 plants were infiltrated with 10^5 colony-forming units (cfu) mL^{-1} *Pst* DC3000 using a needleless syringe. The infected plants were incubated at 26°C and harvested at various time points to determine bacterial growth. One-week-old seedlings of wild-type (Col-0) and *CaRING1*-OX transgenic plants were spray inoculated with conidiospores (5 × 10^4 spores mL^{-1}) of *Hyaloperonospora arabidopsidis* isolate Noco2. The infected plants were incubated at 17°C in a controlled-environment chamber. Sporangiophores and conidiospores were counted at 6 and 8 d after inoculation, respectively.

Isolation and Sequence Analysis of CaRING1

Previously, leaves were inoculated with the avirulent *Xcv* strain Bv5-4a, and following isolation of total mRNA, cDNAs were amplified for use as probes to screen a cDNA library established from elicited pepper cells (Jung and Hwang, 2000). Differential hybridization was then used to isolate the 594-bp full-length *CaRING1* cDNA. Among the cDNA clones tested, *CaRING1* cDNA hybridized strongly to cDNA probes from leaves infected with the avirulent *Xcv* strain Bv5-4a.

Sequence analysis was performed using BLAST (Altschul et al., 1997; http://blast.ncbi.nlm.nih.gov/), and homologous proteins were identified by searching with the CaRING1 protein sequence. TMHMM (Krogh et al., 2001) and SMART (http://smart.embl-heidelberg.de/) Web servers were used for the identification of protein domains. A phylogenetic tree was constructed by the neighbor-joining method using ClustalW (http://www.ch.embnet.org/software/ClustalW.html).

Expression and Purification of Recombinant Proteins

The vector pMAL-c4X (New England Biolabs) was used to construct expression vectors containing fusions with *CaRING1*, RING-H2 domain-deleted *CaRING1ΔRING*, TM domain-deleted *CaRING1ΔTM*, and the site-directed mutants *CaRING1K15R*, *CaRING1C110S*, and *CaRING1H131Y*. The expression constructs were transformed into *Escherichia coli* BL21 (DE2) cells to produce MBP fusion proteins. As a negative control, pMAL-c4x vector was

used to express MBP alone. Overnight cultures grown at 37°C were used to inoculate fresh Luria-Bertani (LB) medium (50 μg mL^{-1} kanamycin), and then cells were incubated at 37°C until the optical density at 600 nm (OD$_{600}$) was 0.4 to 0.6. Expression was then induced by the addition of 300 μM isopropyl-β-D-thiogalactopyranoside, and cultures were grown for a further 3 h at 37°C. Cells were harvested and resuspended in column buffer (20 mM Tris-HCl [pH 7.4] and 0.2 M NaCl), followed by sonication and centrifugation at 13,000g for 15 min at 4°C. Expressed MBP fusion proteins were purified by amylase affinity chromatography, according to the manufacturer's instructions (New England Biolabs).

A clone encoding the full-length Arabidopsis *UBC10* (At5g53300) was obtained from the Arabidopsis Biological Resource Center (http://www.Arabidopsis.org), and the fragment encoding UBC10 was ligated into pET28a (Invitrogen). The 6xHis-UBC10 fusion protein was transformed into *E. coli* BL21 (DE2) and expressed as described above. Harvested cells were resuspended in 1× native purification buffer (50 mM NaH$_2$PO$_4$ [pH 8.0] and 0.5 M NaCl), and 6xHis-UBC10 was purified by affinity chromatography using nickel-nitrilotriacetic acid agarose resin, according to the manufacturer's instructions (Invitrogen).

E3 Ubiquitin Ligase Activity Assay

For the E3 ubiquitin ligase activity assay, each reaction (30 μL final volume) contained 10 μg of bovine ubiquitin (Sigma-Aldrich), 0.1 μg of yeast E1 (Boston Biochem), 0.2 μg of purified Arabidopsis E2 6xHis-UBC10, and 0.2 μg of purified E3 (MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1K15R, CaRING1C110S, or CaRING1H131Y) in ubiquitination buffer (40 mM Tris-HCl [pH 7.5], 5 mM MgCl$_2$, 2 mM ATP, and 2 mM DL-dithiothreitol). After incubation at 30°C for 3 h, the reaction was stopped with 2× SDS-PAGE loading buffer (20 mM Tris-HCl [pH 7.5], 20% glycerol, 5% SDS, 2 mM EDTA, 200 mM DL-dithiothreitol, and 0.02% bromphenol blue) at 80°C for 7 min. Aliquots (10 μL) for each reaction were separated by electrophoresis using 8% SDS-PAGE gels. Immunoblot analyses were performed using anti-ubiquitin antibody (Sigma-Aldrich) and anti-MBP antibody (New England Biolabs) raised in rabbits.

Subcellular Localization of CaRING1

For transient expression in onion (*Allium cepa*) epidermal cells, the cauliflower mosaic virus *35* promoter was used to drive the expression of gene fusions (*CaRING1-smGFP*, the RING domain deletion *CaRING1ΔRING-smGFP*, or the TM domain deletion *CaRING1ΔTM-smGFP*) carried in the binary vector p326GFP. Plasmids were purified using Qiagen plasmid maxi kits, and particle bombardment assays were performed using a Bio-Rad He/1000 particle delivery system. Bombarded cells were incubated for 24 h on 1× MS agar, and GFP fluorescence was observed using a LSM 5 Exciter microscope (Carl-Zeiss) with a 488-nm filter. To confirm that smGFP was anchored to the cell membrane, plasmolyzed cells were observed.

RNA Gel-Blot Analysis

TRIzol (Invitrogen) was used to isolate total RNA from healthy pepper tissues (leaves, stems, roots, flowers, and fruits) and leaves infected with the virulent *Xcv* strain Ds1 and the avirulent *Xcv* strain Bv5-4a. The RNA was separated by electrophoresis on 1.2% formaldehyde-agarose gels, blotted onto Hybond N$^+$ (Pall), and then incubated at 65°C for 1 h in hybridization buffer (5% [w/v] dextran sulfate, 0.25 M disodium phosphate [pH 7.2], 7% [w/v] SDS, and 1 mM EDTA). *Eco*RI restriction was used to release the cDNA fragment containing *CaRING1* from the TOP blunt vector. The fragment was then randomly labeled using [γ-^{32}P]dCTP, heat denatured, added to hybridization buffer, and incubated with blocked membranes overnight at 65°C. Membranes were washed once with 2× SSC, 0.1% SDS at room temperature, twice with 0.1× SSC, 0.1% SDS at 65°C, and then exposed to x-ray film.

Virus-Induced Gene Silencing

In this study, the TRV vectors pTRV1 and pTRV2 were used for VIGS (Liu et al., 2002). TOP blunt vector (Enzynomix) containing full-length *CaRING1* cDNA (385 bp) was digested with *Eco*RI, and the resulting fragment was inserted into the same site in pTRV2. The 3' region of the *CaRING* UTR was PCR amplified using specific primers of *CaRING1UTR* (forward, 5'-GAATTC-

GAGTTGCCGGTAGCTAGCTCCTCAT-3'; reverse, 5'-GAATTCACAAATT-CACATTAATCCAAAC-3') and inserted into pTRV2 to generate pTRV2:CaRING1UTR. pTRV1, pTRV2:00, pTRV2:CaRING1, and pTRV2:CaRING1UTR constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. Cultures of *Agrobacterium* strain GV3101 containing each construct were grown for 16 h at 28°C. *Agrobacterium* cultures were pelleted, resuspended in infiltration buffer (10 mM MES, 10 mM MgCl [pH 5.7]), and adjusted to an OD$_{600}$ of 1.0. Cells were incubated in 200 μM acetosyringone at room temperature for 1 to 2 h before use. An equal volume of pTRV1 *Agrobacterium* culture was mixed with one of the pTRV2 cultures before infiltration (OD$_{600}$ = 0.2). *Agrobacterium* cultures were infiltrated into the cotyledons of pepper seedlings (Hong et al., 2008). After 24 to 48 h at 17°C in a growth chamber, infected plants were grown under a 16/8-h light/dark cycle at 25°C. At 4 and 5 weeks after infiltration, the upper leaves of the infected plants were used for RT-PCR analysis and various disease assays.

Measurement of SA and SAG

SA extraction and quantification were performed as described by Aboul-Soud et al. (2004) and Choi et al. (2011). Pepper and Arabidopsis leaf tissues (0.5 g) were ground to a powder using liquid nitrogen and extracted in 1 mL of 90% methanol. 3-Hydroxy benzoic acid (50 μg) in 100% methanol was added to each sample as an internal standard. Samples were vortexed, sonicated for 15 min, and centrifuged at 15,000g for 10 min at 4°C. The supernatant was transferred to a tube, and the remaining pellet was reextracted in 1 mL of absolute methanol. The supernatant fractions were combined and dried in a speed vacuum under heat. The pellet was resuspended in 1 mL of 5% TCA and sonicated for 10 min. Organic extraction of free SA was performed by adding 1 mL of ethylacetate:cyclopentane:isopropanol (50:50:1). The upper phase containing free SA was transferred to a tube, and the aqueous phase was then reextracted. Supernatants were dried under nitrogen gas and then suspended in 0.5 mL of the HPLC mobile phase (55% acetonitrile:45% deionized water with 4% [v/v] acetic acid). The resulting solution was filtered and separated on a C$_{18}$ analytical column (J'sphere ODS-H80, 150 × 4.6 mm; YMC) using HPLC (Waters) and detected with a fluorescence detector (excitation at 305 nm, emission at 405 nm; Waters). The HPLC was programmed for isocratic conditions (80% methanol) and a flow rate of 1.0 mL min^{-1}. The aqueous phase containing SAG was acidified to pH 1 with HCl and then boiled for 30 min to release SA from any acid-labile conjugated forms. The released SA was then extracted with the organic mixture and treated as described above. SA and SAG were quantified by area integration of HPLC peaks.

Generation of Transgenic Arabidopsis Plants

Full-length *CaRING1* cDNA was PCR amplified in the sense and antisense directions using the following primers: CaRING1 forward (5'-TCTAGAATGAG-TAGTGATGATCCTTTACAT-3') and reverse (5'-GAGCTCTTACGGCAGAAA-TGC-3'). Fragments were cloned into the TOP blunt vector (Enzynomix). The resulting plasmid was digested with *XbaI/SacI*, and then the *CaRING1* gene fragment was ligated into pBIN35S. To generate transgenic Arabidopsis plants, the construct was transformed into *Agrobacterium* strain GV3101 using the floral dipping method (Clough and Bent, 1998). Putative transgenic Arabidopsis plants harboring the *35S:CaRING1* construct were selected on MS plates (Duchefa) containing 50 mg L^{-1} kanamycin.

Protein Gel-Blot Analysis

Pepper leaves were infiltrated with *Agrobacterium* carrying *CaRING1-GFP*, *CaRING1C110S-GFP*, or *CaRING1H131Y-GFP*. Total proteins were extracted from the pepper leaves transiently expressing these constructs. For immunoblotting, harvested pepper leaves (0.5 g) were ground in liquid nitrogen and homogenized in 500 μL of extraction buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 4 M urea, 0.2% Nonidet P-40, and plant protease inhibitor cocktail [Roche]). The lysates were centrifuged at 15,000g for 30 min at 4°C. Supernatants were collected and quantified by Bradford assay. Proteins were analyzed using immunoblots probed with anti-GFP antibody (Santa-Cruz Biotechnology).

RT-PCR and Real-Time RT-PCR

TRIzol reagent (Invitrogen) was used to extract total RNA from the leaves of pepper and Arabidopsis plants, according to the manufacturer's

recommendations. First-strand cDNA was synthesized from total RNA (2 μg) using Moloney murine leukemia virus reverse transcriptase (Enzynomics). Real-time PCR was performed using the SYBR Green Supermix (Bio-Rad). The following gene-specific primer pairs were used: for *CaRING1*, forward (5′-ATGAGTAGTGATGATCCTTTACATAACG-3′) and reverse (5′-TTACG-GCAGAAATGCATTTACAT-3′); for Arabidopsis *UBQ*, forward (5′-CAA-GACAGGAGAAATATGTCTCG-3′) and reverse (5′-ATCCTTTCTTAGGCA-TAG-3′); for *CaBPR1*, forward (5′-CAGGATGCAACACTCTGGTGG-3′) and reverse (5′-ATCAAAGGCCGGTTGGTC-3′); for *CaDEF1*, forward (5′-CAAG-GGAGTATGTGCTAGTGAGAC-3′) and reverse (5′-TGCACAGCACTATC-ATTGCATAC-3′); for *CaPO2*, forward (5′-GCAGAAACGGATCTCCCTG-3′) and reverse (5′-CTCCCATTCTAATCATGGCAG-3′); and for pepper *18S rRNA*, forward (5′-AAACGGCTACCACATCCAAG-3′) and reverse (5′-ACC-CATCCCAAGGTTCAACT-3′).

Agrobacterium-Mediated Transient Expression in Pepper Leaves

For analysis of cell death caused by *CaRING1* overexpression, *Agrobacterium* strain GV3101 containing empty control vector (*35S:00*) or binary vectors expressing *CaRING1* (*35S:CaRING1*) or the site-directed CaRING1 mutants (*35S:CaRING1C110S* and *35S:CaRING1H131Y*) was infiltrated into pepper leaves at the eight-leaf stage (Choi and Hwang, 2011). The development of cell death was observed at 3 and 7 d after infiltration. *Agrobacterium* cultures were grown overnight at 28°C in 10 mL of LB medium containing 50 μg mL^{-1} kanamycin and 50 μg mL^{-1} rifampicin. Cultures were pelleted and resuspended in induction medium (10 mM MES [pH 5.6], 10 mM MgCl$_2$ and 200 mM acetosyringone) at OD$_{600}$ = 2 and then left at room temperature for 2 to 3 h prior to infiltration. *Agrobacterium* cultures (OD$_{600}$ = 0.1–1.0) were infiltrated into leaves between the lateral veins, and then plants were incubated and observed at room temperature for 2 weeks. Autofluorescence was detected under UV illumination.

Histochemical Staining

For visualization of H$_2$O$_2$, inoculated leaves were detached at various time points and dipped into a solution containing 1 mg mL^{-1} 3,3-diaminobenzidine solution. Samples were stained for 15 h and then cleared in 95% ethanol. For detection of cell death, infected leaves were sampled at the time points indicated and then stained with lactophenol-trypan blue solution (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue in 10 mL of distilled water). Leaves were boiled briefly in staining solution and then cleared in chloral hydrate solution (2.5 g mL^{-1} chloral hydrate). Samples were mounted in 60% glycerol, and representative phenotypes were photographed with a light microscope (Olympus).

Measurement of Electrolyte Leakage

To assay cell death, electrolyte leakage was measured from six leaf discs. Leakage was compared between wild-type plants and lines transiently expressing *CaRING1* or empty vector control as well as CaRING1-silenced plants. Leaf discs were immersed in 30 mL of nonionic distilled water and then shaken slowly at room temperature. After incubation, the conductivity of the bathing solution was measured using a Crison conductivity meter (Hach).

Quantification of H$_2$O$_2$ Using the Xylenol Orange Assay

H$_2$O$_2$ production was measured in wild-type and *CaRING1*-OX transgenic plants using freshly prepared xylenol orange reagents. Two reagents were prepared: reagent A [25 mM FeSO$_4$, 25 mM (NH$_4$)$_2$SO$_4$, and 2.5 M H$_2$SO$_4$] and reagent B (125 μM xylenol orange [Sigma-Aldrich] and 100 mM sorbitol). The xylenol orange reaction mixture comprised 0.1 mL of reagent A and 10 mL of reagent B. Pepper leaves were infiltrated with 10^7 cfu mL^{-1} avirulent *Xcv* strain Bv5-4a. Leaf discs were removed using a cork borer and then floated on 1 mL of distilled water. To quantify the H$_2$O$_2$, 100 μL of supernatant was added immediately to 1 mL of xylenol orange mix and then incubated for 30 min at room temperature. H$_2$O$_2$ production was determined spectrophotometrically by measuring A_{560}. A standard H$_2$O$_2$ solution was used for calibration.

Sequences for genes used in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: GQ359822 (*CaRING1*), AF053343 (*CaBPR1*), AF442388 (*CaDEF1*), DQ489711 (*CaPO2*), At2G14610 (*AtPR1*), At5g44420 (*AtPDF1.2*), D13044 (*AtRD29a*), and At3g62250 (*AtUBQ5*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Nucleotide and predicted amino acid sequences of pepper *CaRING1* cDNA, which encodes a RING-H2 finger protein.

Supplemental Figure S2. SDS-PAGE and E3 ubiquitin ligase activity assay of the recombinant MBP-CaRING1 and mutant variant proteins.

Supplemental Figure S3. Enhanced susceptibility of *CaRING1UTR*-silenced pepper plants to *Xcv* infection.

Supplemental Figure S4. Expression of *CaRING1* and pepper defense-related marker genes in empty vector control (TRV:*00*) and *CaRING1UTR*-silenced (TRV:*CaRING1UTR*) pepper plants 12 and 24 h after inoculation with the virulent (Ds1; compatible) and avirulent (Bv5-4a; incompatible) strains of *Xcv*.

ACKNOWLEDGMENTS

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