Running Head: E3 Ubiquitin Ligase in Plant Cell Death and Defense

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The Pepper E3 Ubiquitin Ligase RING1 Gene, CaRING1, Is Required for Cell Death and the Salicylic Acid-Dependent Defense Response

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Abstract
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 (Xcv) infection. CaRING1 contains an N-terminal transmembrane domain and a C-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 (VIGS) of CaRING1 confers enhanced susceptibility to avirulent Xcv infection, which is accompanied by compromised hypersensitive cell death, reduced expression of PR1 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 confers enhanced resistance to hemibiotrophic Pseudomonas syringae pv. tomato and biotrophic Hyaloperonospora arabidopsidis infections. Taken together, these results suggest that CaRING1 is involved in induction of cell death and regulation of ubiquitination during the defense response to microbial pathogens.

INTRODUCTION
Plants continuously confront pathogen attacks using a variety of defense responses, including callose deposition (Gomez-Gomez et al., 1999), oxidative burst (Apel and Hirt, 2004), synthesis of pathogenesis-related (PR) proteins (van Loon and Pieterse, 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 neighbouring 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 (post-translational 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 Viestra, 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 lysine (Lys) residues in substrate proteins (Smalle and Vierstra, 2004; Dreher and Callis, 2007). There are several types of ubiquitination with different outcome. 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 a 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 (Homology to E6-AP C-Terminus) or RING (Really Interesting New Gene)/U-box domains, which have different subunit compositions and mechanisms of action (Picart, 2001). The RING domain has a consensus sequence containing cysteine (Cys) and histidine (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, i.e., C3-H-C4 and C3-H2-C3, according to presence of cysteine or histidine in the fifth position (Borden and Freemont, 1996). The E3 ubiquitin ligase activity of RING finger proteins can be detected through \textit{in vitro} autoubiquitination. Among the 1300 E3 ubiquitin ligase genes in the Arabidopsis 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 \textit{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 \textit{ATL2} and \textit{ATL6}, are rapidly up-regulated in response to chitin, a basal defense elicitor associated with
fungal cell walls and insect exoskeletons (Salinas-Mondragon et al., 1999). Defense-related genes such as NPR1, PAL and CHS, are induced constitutively under normal growth conditions in Arabidopsis mutants that constitutively express ATL2 (Serrano and Guzman, 2004). The fungal elicitor N-acetylchitooligosaccharide induces expression of rice 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 upregulated genes, were genes encoding 10 putative RING finger E3 ligases, including RHA3b, RHA1b, RMA1 and ATL6 (Navarro et al., 2004). RING finger proteins, such as the F-box proteins 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; Gonzalez-Lamothe et al., 2006; Yang et al. 2006), including ACRE74 and ACRE276 from tobacco, 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 (Hong et al., 2007). The Arabidopsis E3 ubiquitin ligases PUB22, PUB23 and PUB24, also play roles in the negative regulation of PAMP-triggered immunity (Trujillo et al., 2008).

In this study, we identified CaRING1, which encodes a novel E3 ubiquitin ligase RING1 protein in pepper (Capsicum annuum). 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 molecular weight protein that contains an N-terminal transmembrane domain and 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-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 ROS induction, HR, PR gene expression and accumulation of free SA during infection. By contrast, transient CaRING1 expression in pepper leaves triggers the hypersensitive cell death response that requires the E3 ubiquitin ligase activity of CaRING1. Transgenic Arabidopsis plants overexpressing (OX) CaRING1 also exhibit enhanced bacterial and fungal disease resistance, accompanied by induction of salicylic acid-responsive AtPR1 and ethylene/jasmonic acid-responsive AtPDF1.2 during Pseudomonas syringae pv. tomato infection. The 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 post-translational modification.

RESULTS

CaRING1 Encodes a Protein Containing a Conserved Transmembrane 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 (ORF) of 591 bp.
(Supplemental Fig.1). The predicted CaRING1 protein comprises 197 amino acids
and has a calculated molecular mass of 20.566 kDa 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 transmembrane domain for
subcellular localization at the N-terminus (residues 33-55) and a RING domain in the
C-terminal region (residues 107-148) (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 (UPS). CaRING1 shows relatively
high amino acid sequence identity (48–65%) to zinc finger or RING finger proteins in
Arabidopsis and field mustard (Fig. 1A), which all contain highly-conserved
transmembrane and RING domains. The RING domain of CaRING1 shares 55–90%
identity with other plant defense-related RING-finger proteins and contains eight
conserved zinc-coordinating Cys and/or His residues which 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 Xanthomonas campestris pv. vesicatoria (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 transcripts 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 the 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 (PR 1) that was used as a defense-marker gene of pepper was distinctly upregulated 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 a 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 scheme for 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 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 a 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 in the immunoblot using the anti-MBP antibody (Fig. 3D; Supplemental Fig. 2), indicating that the TM region of CaRING1 also partly supports E3 ubiquitin ligase activity or may be ubiquitinated. The UbPred programs (http://ubpred.org/) predicts that the TM region of CaRING1 contains a putative ubiquitination site at the residue K15. Thus, we further used the MBP-CaRING1K15R mutant to determine if the TM region of CaRING1 is ubiquitinated (Supplemental Fig. 2). The MBP-CaRING1K15R mutant exhibited the ubiquitination patterns similar to the MBP-CaRING1 in the immunoblottings using both anti-ubiquitin and anti-MBP antibodies, indicating that
K15 is not required for E3 ubiquitin ligase activity.

**CaRING1 Localizes to the Plasma Membrane**

CaRING1 is predicted to contain a transmembrane domain (amino acids 33–55) in the N-terminal region. Fusion proteins between CaRING1 and soluble-modified green fluorescent fusion protein (smGFP) were used to determine the subcellular localization of CaRING1, and expression of the fusion proteins was driven by the cauliflower mosaic virus (CaMV) 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 transmembrane and RING domain deletion mutants (35S:CaRING1ΔTM:smGFP and 35S:CaRING1ΔRING, respectively) were used to test the prediction that the putative transmembrane domain functions in subcellular localization of CaRING1 (Fig. 3A). Fluorescent signals from CaRING1ΔTM: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 transmembrane 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 to 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-fluorescent compounds indicate 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 serine was substituted for a conserved cysteine and a histidine was changed by tyrosine in the RING domain of CaRING1, respectively, was transiently expressed in pepper leaves. These Ring domain mutants exhibited distinctly decreased cell death phenotypes compared to the wild-type 35S:CaRING1 (Fig. 5B). Compromised cell death phenotypes and accumulation of UV-fluorescing phenolic compounds were associated with expression of these mutations. In agroinfiltrated pepper leaves, 35S:CaRING1-induced cell death led to a significant increase in electrolyte leakage compared to empty vector controls (Fig. 5C). In contrast, the CaRING1 mutants did not generate high levels of electrolyte leakage and ROS. RT-PCR and immunoblotting analyses showed that 35S:CaRING1 and the site-directed mutants 35:CaRING1C110S or 35S:CaRING1H131Y were transiently expressed at both transcript and protein levels in pepper leaves 24 and 48 h after agroinfiltration (Fig. 5D and 5E). 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 is 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 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 ORF was cloned into the TRV vector (TRV:CaRING1). Empty TRV vector (TRV:00) was used as a negative control. In comparison to 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 days 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 to 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 (H₂O₂) at 12 h after inoculation compared to plants with empty vector (Fig. 6D). Reduced H₂O₂ production in CaRING1-silenced (TRV:CaRING1) plants was also visualized by DAB staining (Fig. 6C). These results indicate that CaRING1 expression plays a significant role in induction and signal transduction of the HR during avirulent Xcv infection. However, the gene knock-down strategy such as VIGS may silence genes with the sequence similar to the target gene. To reduce this nonspecific silencing effect, a 299-bp fragment from the 3' end of the CaRING1 UTR was cloned into the TRV2 vector (TRV2:CaRING1UTR) to specifically silence the CaRING1 gene. Like CaRING1-silenced pepper plants (Figure 6), CaRING1 UTR-silenced (TRV:CaRING1UTR) pepper plants exhibited enhanced susceptibility to virulent and avirulent Xcv infection, which was accompanied by the increased bacterial growth, as well as reduced H₂O₂ accumulation, cell death and electrolyte leakage (Supplemental Fig. 3).

Using quantitative RT-PCR, PR marker gene transcript levels were assayed in empty vector control (TRV:00) and CaRING1-silenced pepper leaves before, 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 to 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 positively 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 to empty vector control leaves, especially in the incompatible interactions (Supplemental Fig. 4).

Since CaBPR1 expression was reduced in CaRING1-silenced pepper plants, it is possible that synthesis and/or recognition of SA is also affected. Free and SAG (glucoside-conjugated SA) 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 avirulent strain of Xcv (Fig. 7B). CaRING1-silenced (TRV:CaRING1) leaves accumulated approximately 2-fold more SAG than wild-type leaves at 24 h after avirulent Xcv infection. These findings indicate that CaRING1 negatively regulates
transformation of SAG to SA. In addition, the reduction in free SA levels in *CaRING1*-silenced leaves supports the hypothesis that CaRING1 modulates 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 T<sub>2</sub> lines of *CaRING1*-overexpressing (OX) plants, lines #5, #13 and #16, displayed strong levels *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 *P. syringae pv. tomato* 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 days 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*, which is related to the ethylene/JA-dependent pathway and *AtRD29a*, a known regulator of ABA metabolism (Fig. 8D). *Pst* DC3000 infection induced stronger expression of *AtPR1* and *AtPDF1.2* in transgenic plants than in 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 ABA-responsive transcript *AtRD29a* were reduced in wild-type and *CaRING1*-OX transgenic plants 24 h after inoculation with *Pst* DC3000, but the downregulation is more pronounced in *CaRING1*-OX line compared to that of wild-type plants. As compared to 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 induced accumulation of SA for resistance to *Pst* DC3000 infection.

To determine whether or not *CaRING1* overexpression alters resistance to a biotrophic oomycete pathogen, 7-day-old cotyledons of transgenic *CaRING1*-OX were sprayed with a conidiospore suspension (5 x 10⁴ mL⁻¹) of *Hyaloperonospora arabidopsidis* isolate Noco2 (Fig. 9). Transgenic *CaRING1*-OX plants not only exhibited reduced formation of sporangiophores compared to 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 were reduced significantly in *CaRING1* transgenic lines compared to wild-type plants (Fig. 9B). These observations indicate that *CaRING1* overexpression suppresses *Hp. 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 hypersensitive response (HR) cell death against Xanthomonas campestris pv. vesicatoria (Xcv) infection. CaRING1 contains an N-terminal transmembrane 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 posttranslational 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).

The maltose binding fusion protein 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 transmembrane 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 ubiquitin 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 lysine (K) 15 residue in the TM region did not abrogate ubiquitination of CaRING1 K15R (Supplemental Fig. 2). 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 on many angstroms (Å) 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 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 transfer of ubiquitins from the E2 to substrates on CaRING1 leading to the normal ubiquitination. However, the precise mechanism underlying ubiquitination of CaRING1 remains to be investigated.

Following transient expression of CaRING1 in onion epidermal cells, CaRING1 localized to the plasma membrane and its transmembrane 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 accumulation of UV-fluorescing phenolic compounds, an ROS (H2O2) 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 activity 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* knock-down 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 to 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 or not 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 activation of plant defense and its accumulation is necessary for the induction of the HR and 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 (Lee et al., 2007; Zhou et al., 1998). 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 production of SA synthesis but rather 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 conversion of SA to SAG during infection with avirulent Xcv strains remain 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 Hwawng, 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 crosstalk 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 Hyaloperonospora arabidopsidis (Fig. 9). Similar to our findings, enhanced resistance to TMV and P. syringe 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 non-host 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 L., cv. Nockwang) seeds were planted in a plastic
tray (55 x 35 x 15 cm) containing soil mix (perlite, vermiculite and 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 x 15 x 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 Col-0 and CaRING1-overexpression (OX) lines were surface-sterilized and planted on growth media (1x Murashige and Skoog [MS] and 1% [w/v] sucrose) and kept at 4°C for a minimum of 3 days 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 light per day; 130 µmol photons m⁻² s⁻¹) and 60% humidity.

Pathogens and Inoculation Procedures

The bacterial strains Xanthomonas campestris pv. vesicatoria (Xcv) 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 analyses. Pseudomonas syringae pv. tomato (Pst) 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 10⁵ cfu mL⁻¹ Pst DC3000 using a needleless syringe. The infected
plants were incubated at 26°C and infected leaves were 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 (5x10^4 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 days 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://www.ncbi.nlm.gov/BLAST/) 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, Ipswich, MA) was used to
construct expression vectors containing fusions with CaRING1, RING-H2 domain-deleted CaRING1ΔRING, transmembrane domain-deleted CaRING1ΔTM and the site-directed mutants CaRING1K15R, CaRING1C110S and CaRING1H131Y. The expression constructs were transformed into E. coli BL21 (DE2) cells to produce maltose-binding protein (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 OD₆₀₀ was 0.4–0.6. Expression was then induced by the addition of 300 µM isopropyl-β-D-thiogalactopyranoside (IPTG) 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 13,000 g 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 ubiquitin-conjugating enzyme 10 (UBC10, At5g53300) was obtained from the ABRC (http://www.Arabidopsis.org) and the fragment encoding UBC10 was ligated into pET28a (Invitrogen, Carlsbad, CA). The 6x His-UBC10 fusion protein was transformed into E. coli BL21 (DE2) and expressed as described above. Harvested cells were resuspended in 1x native purification buffer (50 mM NaH₂PO₄ [pH 8.0], 0.5M NaCl) and 6x His-UBC10 was purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) 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 bovine ubiquitin (Sigma-Aldrich, St Louis, MS), 0.1 µg yeast E1 (Boston Biochem, Cambridge, MA), 0.2 µg purified Arabidopsis E2 6x His-UBC10 and 0.2 µg purified E3 (MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1K15R, CaRING1C110S or CaRING1H131Y) in ubiquitination buffer (40 mM Tris-HCl, [pH 7.5], 5 mM MgCl₂, 2 mM ATP and 2 mM DL-dithiothreitol [DTT]). After incubation at 30°C for 3 h, the reaction was stopped with 2x SDS-PAGE loading buffer (20 mM Tris-HCl [pH 7.5], 20% glycerol, 5% SDS, 2 mM EDTA, 200 mM DTT and 0.02% bromophenol 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 CaMV 35 promoter was used to drive expression of gene fusions (CaRING1-smGFP, the RING-domain deletion CaRING1ΔRING-smGFP or the transmembrane domain deletion CaRING1ΔTM-smGFP) carried in the binary vector p326GFP. Plasmids were purified using QIAGEN plasmid maxi kits (Qiagen, Valencia, CA) and particle bombardment assays were performed using a Bio-Rad He/1000 particle delivery system (Bio-Rad, Hercules, CA). Bombarded cells were incubated for 24 h on 1x MS agar and GFP fluorescence was observed using a LSM 5 Exciter microscope (Carl-Zeiss, Oberkochen, Germany) 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, Bedford, MA) 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). EcoR1 restriction was used to release the cDNA fragment containing CaRING1 from the TOP blunt vector. The fragment was then randomly labeled using [γ-32P]dCTP, heat-denatured, added to hybridization buffer and incubated with blocked membranes overnight at 65°C. Membranes were washed once with 2x SSC, 0.1% SDS at room temperature, twice with 0.1x SSC, 0.1% SDS at 65°C and then exposed to X-ray film.

Virus-Induced Gene Silencing

In this study, the tobacco rattle virus (TRV) vectors pTRV1 and pTRV2 were used for virus-induced gene silencing (VIGS; Liu et al., 2002). TOP blunt vector (Enzynomix, Seoul) containing full-length CaRING1 cDNA (385 bp) was digested with EcoRI and the resulting fragment was inserted into the same site in pTRV2. The 3’ region of CaRING UTR was PCR-amplified using specific primers of CaRING1UTR (forward, 5’-GAATCGAGTTGCCGTTAGCTCCTCAT-3’; reverse, 5’-GAATCCACAAATTCCATTAATCCAAAC-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 *A. tumefaciens* strain GV3101 containing each construct were grown for 16 h at 28°C. *Agrobacterium* cultures were pelleted, resuspended in infiltration buffer (10 mM 4-morpholineethanesulfonic acid [MES] and 10 mM MgCl₂, [pH 5.7]) and adjusted to an O.D₆₀₀=1.0. Cells were incubated in 200 µM acetosyringone at room temperature for 1–2 h before use. An equal volume of pTRV1 *Agrobacterium* culture was mixed with one of the pTRV2 cultures before infiltration (OD₆₀₀=0.2). *Agrobacterium* cultures were infiltrated into the cotyledons of pepper seedlings (Hong et al., 2008). After 24-48 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 was 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 15000 g for 10 min at 4°C. The supernatant was transferred to a tube and the remaining pellet was re-extracted in 1 mL absolute methanol. The supernatant fractions were combined and dried in a speed vacuum under heat. The pellet was resuspended in 1 ml of 5% trichloroacetic acid (TCA) and sonicated for 10 min. Organic extraction of free SA was performed by adding 1 mL ethylacetate:
cyclopentane: isopropanol (50:50:1). The upper phase containing free SA was transferred to a tube and the aqueous phase was then re-extracted. Supernatants were dried under nitrogen gas and then suspended in 0.5 mL of the HPLC mobile phase (55% acetonitrile: 45% dH₂O with 4% [v/v] acetic acid). The resulting solution was filtered and separated on a C₁₈ analytical column (J’sphere ODS-H80, 150 x 4.6 mm) (YMC, Kyoto, Japan) using HPLC (Waters) and detected with a fluorescence detector (excitation 305 nm and emission 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 (glucose-conjugated SA) 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’-TCTAGAATGAGTAGTGATGATCCTTTACAT-3’ and reverse, 5’-GAGCTCTTACGGCAGAAATGC-3’. Fragments were cloned into the TOP blunt vector (Enzynomix, Seoul, Korea). The resulting plasmid was digested with *Xba1/Sac1* and then the *CaRING1* gene fragment was ligated into pBIN35S. To generate transgenic Arabidopsis plants, the construct was transformed into *Agrobacterium tumefaciens* 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, Haarlem, The Netherlands).
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 extraction buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 4M urea, 0.2% NP-40, and plant protease inhibitor cocktail [Roche]). The lysates were centrifuged at 15,000 g 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, Santa-Cruz, CA).

**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: *CaRING1* forward, 5'-ATGAGTAGTGATGATCCTTTACATAACG-3’ and reverse, 5’-TTACGGCAGAA ATGCATTTACAT-3’; Arabidopsis *UBQ* forward, 5’-CAAGACAGGAGAAATA TGTCTCG-3’ and reverse, 5’-ATCCTTTCTTAGGCATAG-3’; *CaBPR1* forward, 5’-CAGGATGCAACACTCTGGTGG-3’ and reverse, 5’-ATCAAAGGCCC GGTTGGTC-3’; *CaDEF1* forward, 5’-
CAAGGGAGTATGTGCTAGTGAGAC-3' and reverse, 5'-TGCACAGCACTATCATTGCATAC-3'; CaPO2 forward, 5'-GCAGAAACGGGTA'TCTCCCTG-3' and reverse, 5'-CTCCCATTCTAATCATGGCAG-3'; and pepper 18S rRNA forward, 5'-AAACGGGTACCACATCCAG-3' and reverse, 5'-ACCCATCAGGAAGGTTCAACT-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 days after infiltration. *Agrobacterium* cultures were grown overnight at 28°C in 10 mL LB media containing 50 µg mL\(^{-1}\) kanamycin and 50 µg mL\(^{-1}\) rifampicin. Cultures were pelleted and resuspended in induction media (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 to 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 (DAB)
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 lactic acid, 10 mL glycerol,
10 g phenol and 10 mg trypan blue in 10 mL 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, Japan).

**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 non-ionic 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, Loveland, CO).

**Quantification of \(\text{H}_2\text{O}_2\) using the Xylenol Orange Assay**

\(\text{H}_2\text{O}_2\) 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\text{SO}_4 and 2.5 M \text{H}_2\text{SO}_4) and reagent B (125
\(\mu\)M 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\(^{-1}\) of avirulent *Xcv* strain Bv5-4a. Leaf discs
were removed using a cork borer and then floated on 1 mL distilled water. To quantify
the H$_2$O$_2$, 100 µL of supernatant was added immediately to 1 mL xylenol orange mix and then incubated for 30 min at room temperature. H$_2$O$_2$ production was determined spectrophotometrically by measuring absorbance at 560 nm (A$_{560}$). A standard H$_2$O$_2$ solution was used for calibration.

**Sequence Data**

Sequences for genes used in this study can be found in the EMBL/GenBank 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 3.** Enhanced susceptibility of CaRING1 UTR-silenced pepper plants to Xanthomonas campestris pv. vesicatoria infection.

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FIGURE LEGENDS

Figure 1. Amino acids 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 thaliana (accession nos. NP_173506, NP_177767, AAM_63811 and NP_195273) and field mustard (Brassica rapa, accession no. ABK56013). Identical amino acids are shown in black boxes, and conserved amino acids are shaded in gray. Black boxes indicate the transmembrane and RING domains. B, Alignment of RING domains. Sequences include CaRING1, A. thaliana ATL8 (accession no. NP_177767), Oryza sativa EL5 (accession no. Q9LRB7; Takai et al. 2002), Nicotiana benthamiana ACRE132 (accession no. AAG43550; Durrant et al. 2000), A. thaliana ATL2 (accession no. NP_188294; Serrano and Guzmán, 2004) and A. thaliana ATL6 (accession no. NP_566249). Identical amino acids are shown in black boxes. Conserved cysteine (C) and histidine (H) residues are indicated by asterisks (*).
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 Xanthomonas campestris pv. vesicatoria (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$_2$.

Figure 3. E3 ubiquitin ligase activity of CaRING1. A, Hydrophobicity index and expression constructs used for the in vitro ubiquitination assay. Recombinant fusion proteins: 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 transmembrane domain, respectively; and MBP-CaRING1C110S and MBP-CaRING1H131Y substitution mutants with replacement of Cys110 with Ser, and His131 with Tyr respectively. Green box indicates transmembrane 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 recombinant proteins was induced with 300 µM IPTG. 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 were indicated by asterisks (*). Lanes 7 to 11, purified MBP-CaRING1, CaRING1ΔTM, CaRING1ΔTM, CaRING1C110S and CaRING1H131Y, respectively.
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 (upper panel) and anti-MBP antibodies (lower panel). E3 ubiquitin ligase activity of MBP-CaRING1 was only detected in the presence of the 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.

**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, Transmembrane 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. smGFP (control), CaRING1:smGFP (wild-type), CaRINGΔTM:smGFP (transmembrane domain-deleted mutant) and CaRINGΔRING:smGFP (RING domain-deleted mutant). Arrows indicate the plasma membrane in a plasmolyzed cell. Bar=200µm.

**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 7 days 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 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 days after infiltration. UV-fluorescing phenolic compounds on the same leaf are shown in the right panel. 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 the mean ±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 was used to detect GFP-tagged protein. Coomassie blue staining confirmed equal protein loadings. H: Healthy leaves.

**Figure 6.** Enhanced susceptibility of CaRING1-silenced pepper plants to infection with the virulent Xanthomonas campestris pv. vesicatoria (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 days after inoculation with the virulent (Ds1, compatible) and avirulent (Bv5-4a, incompatible) strains, respectively. Highlighted areas of leaves show areas inoculated with 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 days after inoculation (5 X 10⁴ cfu mL⁻¹). Data represent the mean ±SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1 gene-silenced pepper plants as determined by the Student’s t test (P < 0.05). C, Reduced H₂O₂ production and cell death in CaRING1-silenced leaves infected with the avirulent Xcv strain Bv5-4a (10⁷ cfu mL⁻¹). Xcv-infected leaves were harvested and stained with 3,3-diaminobenzidine (DAB) and trypan blue, 12 and 24 h after inoculation, respectively. Scale bars = 500 μm. D, Production of H₂O₂ in leaf discs from empty vector control (TRV:00) and CaRING1-silenced (TRV:CaRING1) pepper leaves at different time points after inoculation with 10⁷ cfu mL⁻¹ of the avirulent Xcv strain Bv5-4a. Data represent the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants as determined by the 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 the mean ±SD from three independent experiments.
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**Figure 7.** Expression of pepper defense-related maker genes and SA accumulation in CaRING1-silenced pepper leaves infected with *Xanthomonas campestris* pv. *vesicatoria* (*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 the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants as determined by the Student’s t test ($P < 0.05$). 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 the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants as determined by the Student’s t test ($P < 0.05$).

**Figure 8.** CaRING1-OX transgenic Arabidopsis plants exhibit enhanced resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 infection. A, RT-PCR analysis of CaRING1 expression in wild-type 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 days after infection with Pst DC3000 (10^5 cfu mL^-1). C, Bacterial growth in wild-type and transgenic plant leaves at 0 or 3 days after inoculation with Pst DC3000 (10^5 cfu mL^-1). Data represent the mean ±SD from three independent experiments. Statistically significant differences between means were determined using Fisher’s least significant difference test (P < 0.05). D, Expression of the CaRING1, AtPR1, AtPDF1.2 and AtRD29a in wild-type and transgenic plants (T3) 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 the mean ±SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants, as determined by the 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 the mean ± SD from two independent experiments. Asterisks indicate significant differences between wild-type and transgenic plants, as determined by the Student’s t test (P < 0.05). WT, Col-0 plants. WT, Col-0 plants.

**Figure 9.** CaRING1-OX transgenic Arabidopsis plants show enhanced resistance to Hyaloperonospora arabidopsis isolate Noco2 infection. A, Disease symptoms and aniline blue-stained mycelia on cotyledons of wild-type and transgenic Arabidopsis plants at 7 days and 2 to 3 days after spray inoculation with *H. arabidopsis* isolate Noco2 (5 x 10^4 spores mL^-1), respectively. B, Production of sporangiophores per cotyledon of wild-type and transgenic plants, 6 days after inoculation with *H.
arabidopsisidis. Single cotyledons from over 60 seedlings per genotype were analyzed under the microscope and categorized into one of five categories of sporangiophore production (0 to 5, 6 to 10, 11 to 15, 16 to 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. arabidopsisidis isolate Noco2 per cotyledon at 8 days 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 least significant difference test (P < 0.05).

Supplemental Figure 1. Nucleotide and predicted amino acid sequences of pepper CaRING1 cDNA, which encodes a RING-H2 finger protein. The deduced amino acid sequences are below the nucleotide sequences. The transcriptional start site is shown in bold type and the termination codon is marked by an asterisk (*).

Supplemental Figure 2. A, SDS-PAGE of the recombinant MBP-CaRING1 and mutant variant proteins. Lane 1, protein markers; Lane 2, soluble fraction of uninduced E. coli MBP-CaRING1 extract; lanes 3 to 8: soluble fractions of induced E. coli MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1K15R, CaRING1C110S and CaRING1H131Y, respectively. Induced proteins were indicated by asterisks (*). Lanes 9 to 14: purified MBP-CaRING1, CaRING1ΔTM, CaRING1ΔRING, CaRING1K15R, CaRING1C110S and CaRING1H131Y, respectively. B, E3 ubiquitin ligase activity assay of the recombinant MBP-CaRING1
Supplemental Figure 3. Enhanced susceptibility of CaRING1 UTR-silenced pepper plants to infection with the virulent Xanthomonas campestris pv. vesicatoria (Xcv) strain Ds1 and the avirulent Xcv strain Bv5-4a. A, Disease symptoms developed on empty vector control (TRV:00)- or silenced (TRV:CaRING1 UTR) pepper leaves 6 and 3 days after inoculation with the virulent (Ds1, compatible) and avirulent (Bv5-4a, incompatible) strains, respectively. Highlighted areas of leaves show areas inoculated with bacterial concentrations indicated. Inoculated leaves with UV-fluorescing phenolic compounds associated with susceptibility to infection and HR-cell death, are shown on the right panels. B, Growth of the virulent Xcv strain Ds1 and avirulent Xcv strain Bv5-4a in empty vector control or CaRING1 UTR-silenced pepper leaves 0 and 3 days after inoculation (5 x 10^4 cfu mL^-1). Data represent the mean ±SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1 UTR-silenced pepper plants as determined by the Student’s t test (P < 0.05). C, Diminished H_2O_2 burst and cell death in CaRING1 UTR-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 12 and 24 h after inoculation, respectively. Bars = 500 µm. D, Measurement of H_2O_2 in leaf discs from empty vector control and CaRING1 UTR-silenced pepper plants 0, 4 and 12 h after inoculation with the avirulent Xcv strain Bv5-4a (10^7 cfu mL^-1). Data represent the mean ±SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1 UTR-silenced pepper plants as determined by
the Student’s t test ($P < 0.05$). E, Electrolyte leakage from empty vector control and CaRING1 UTR-silenced pepper leaves inoculated with the avirulent Xcv strain Bv5-4a. Data represent the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1 UTR-silenced pepper plants as determined by the Student’s t test ($P < 0.05$).

Supplemental Figure 4. Expression of CaRING1 and pepper defense-related marker genes in empty vector control (TRV:00) and CaRING1 UTR-silenced (TRV:CaRING1 UTR) pepper plants 12 and 24 h after inoculation with the virulent (Ds1, compatible) and avirulent (Bv5-4a, incompatible) strains of Xanthomonas campestris pv. vesicatoria (Xcv). Gene expression was analyzed using quantitative RT-PCR and normalized using constitutively-expressed 18S rRNA. Data represent the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1 UTR-silenced pepper plants as determined by the Student’s t test ($P < 0.05$).
Figure 1. Amino acids 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 thaliana* (accession nos. NP_173506, NP_177767, AAM_63811 and NP_195273) and field mustard (*Brassica rapa*, accession no. ABK56013). Identical amino acids are shown in black boxes, and conserved amino acids are shaded in gray. Black boxes indicate the transmembrane and RING domains. B, Alignment of RING domains. Sequences include CaRING1, *A. thaliana* ATL8 (accession no. NP_177767), *Oryza sativa* EL5 (accession no. Q9LRB7; Takai et al. 2002), *Nicotiana benthamiana* ACRE132 (accession no. AAG43550; Durrant et al. 2000), *A. thaliana* ATL2 (accession no. NP_188294; Serrano and Guzmán, 2004) and *A. thaliana* ATL6 (accession no. NP_566249). Identical amino acids are shown in black boxes. Conserved cysteine (C) and histidine (H) residues are indicated by asterisks (*").
Figure 2. RNA gel blot analysis of CaRING1 expression in pepper plants. Samples were hybridized with a 32P-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 Xanthomonas campestris pv. vesicatoria (Xcv) strain Ds1 (compatible) or the avirulent Xcv strain Bv5-4a (incompatible), or 10 mM MgCl₂. H, Healthy leaves; Mock, Leaves treated with 10 mM MgCl₂.
Figure 3. E3 ubiquitin ligase activity of CaRING1. A, Hydrophobicity index and expression constructs used for the in vitro ubiquitination assay. Recombinant fusion proteins: 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 transmembrane domain, respectively; and MBP-CaRING1C110S and MBP-CaRING1H131Y substitution mutants with replacement of Cys110 with Ser, and His131 with Tyr respectively. Green box indicates transmembrane 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 recombinant proteins was induced with 300 µM IPTG. 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 were 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 (upper panel) and anti-MBP antibodies (lower panel). E3 ubiquitin ligase activity of MBP-CaRING1 was only detected in the presence of the 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.
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, Transmembrane 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. smGFP (control), CaRING1:smGFP (wild-type), CaRINGΔTM:smGFP (transmembrane domain-deleted mutant) and CaRINGΔRING:smGFP (RING domain-deleted mutant). Arrows indicate the plasma membrane in a plasmolyzed cell. Bar=200µm
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Figure 7. Expression of pepper defense-related maker genes and SA accumulation in CaRING1-silenced pepper leaves infected with Xanthomonas campestris pv. vesicatoria (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 the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants as determined by the Student’s t test (P < 0.05). 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 the mean ± SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants as determined by the Student’s t test (P < 0.05).
A. Relative gene expression of CaRING1, AtPR1, AtRD29a, AtPDF1.2, and SAG in WT and transgenic lines across different time points.

B. Image showing leaf samples from different lines.

C. Bar graph showing log cfu cm⁻² across different time points.

D. Dendrogram showing relative gene expression of CaRING1, AtPR1, AtRD29a, AtPDF1.2, and SAG in WT and transgenic lines across different time points.

E. Graph showing Free SA and SAG levels in WT and transgenic lines across different time points.

*Note: Images and graphs are placeholders for actual visual content.*
Figure 8. CaRING1-OX transgenic Arabidopsis plants exhibit enhanced resistance to Pseudomonas syringae pv. tomato (Pst) DC3000 infection. A, RT-PCR analysis of CaRING1 expression in wild-type 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 days after infection with Pst DC3000 (10^6 cfu mL^{-1}). C, Bacterial growth in wild-type and transgenic plant leaves at 0 or 3 days after inoculation with Pst DC3000 (10^5 cfu mL^{-1}). Data represent the mean ±SD from three independent experiments. Statistically significant differences between means were determined using Fisher's least significant difference test (P < 0.05). D, Expression of the CaRING1, AtPR1, AtPDF1.2 and AtRD29a in wild-type and transgenic plants (T3) 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 the mean ±SD from three independent experiments. Asterisks indicate significant differences between empty vector control and CaRING1-silenced pepper plants, as determined by the 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 the mean ± SD from two independent experiments. Asterisks indicate significant differences between wild-type and transgenic plants, as determined by the Student's t test (P < 0.05). WT, Col-0 plants. WT, Col-0 plants.
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