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CaHDZ27, a Homeodomain-Leucine Zipper I Protein, Positively Regulates the Resistance to *Ralstonia solanacearum* Infection in Pepper

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Homeodomain-leucine zipper class I (HD-Zip I) transcription factors have been functionally characterized in plant responses to abiotic stresses, but their roles in plant immunity are poorly understood. Here, a HD-Zip I gene, *CaHZ27*, was isolated from pepper (*Capsicum annum*) and characterized for its role in pepper immunity. Quantitative real-time polymerase chain reaction showed that *CaHDZ27* was transcriptionally induced by *Ralstonia solanacearum* inoculation and exogenous application of methyl jasmonate, salicylic acid, or ethephon. The CaHDZ27-green fluorescent protein fused protein was targeted exclusively to the nucleus. Chromatin immunoprecipitation demonstrated that *CaHDZ27* bound to the 9-bp pseudopalindromic element (CAATAATTG) and triggered β-glucuronidase expression in a CAATAATTG-dependent manner. Virus-induced gene silencing of *CaHDZ27* significantly attenuated the resistance of pepper plants against *R. solanacearum* and downregulated defense-related marker genes, including *CaHIR1*, *CaACO1*, *CaPR1*, *CaPR4*, *CaPO2*, and *CaBPR1*. By contrast, transient overexpression of *CaHDZ27* triggered strong cell death mediated by the hypersensitive response and upregulated the tested immunity-associated marker genes. Ectopic *CaHDZ27* expression in tobacco enhances its resistance against *R. solanacearum*. These results collectively suggest that *CaHDZ27* functions as a positive regulator in pepper resistance against *R. solanacearum*. Bimolecular fluorescence complementation and coimmunoprecipitation assays indicate that CaHDZ27 monomers bind with each other, and this binding is enhanced significantly by *R. solanacearum* inoculation. We speculate that homodimerization of CaHZ27 might play a role in pepper response to *R. solanacearum*, further direct evidence is required to confirm it.

Plants encounter a vast array of potentially phytopathogenic pathogens in their natural environments and have developed sophisticated defense strategies during coevolution with these pathogens. These strategies generally include constitutive chemical and physical barriers along with inducible innate immunity, which includes pathogen-associated molecular pattern–triggered immunity and effector-triggered immunity (ETI) (Hein et al. 2009; Jones and Dangl 2006). Inducible innate immunity is largely regulated at the transcriptional level by the actions of many transcriptional factors (TFs), which are generally encoded by large gene families. TFs are important players in regulated gene expression networks. Some studies have suggested that TFs are promising biotechnological targets for engineering-improved plant disease resistance (Goossens et al. 2017; Wang et al. 2015; Xu et al. 2011). To fully exploit these immunity-related TFs, a deeper understanding of their functional roles and mechanisms is required.

HD-Zip proteins contain a DNA-binding homeodomain (HD) and the adjacent leucine zipper (Zip) (Ariel et al. 2007). HD-Zip proteins constitute a large family of plant-specific TFs, with the identification of 55 HD-Zip TFs in corn (Mao et al. 2016), 27 in citrus (Ge et al. 2015), 51 in tomato (Zhang et al. 2014b), 33 in peach (Zhang et al. 2014a), 88 in soybean (Chen et al. 2014), 63 in *Populus* spp. (Hu et al. 2012), and 47 in *Arabidopsis thaliana* (Henriksson et al. 2005). HD-Zip protein family members are classified into four subfamilies (HD-Zip I to HD-Zip IV) based on their sequence similarities (Ariel et al. 2007; Henriksson et al. 2005; Hu et al. 2016; Schena and Davis 1994). The majority of *HD-Zip* genes in different classes are responsible for modulating developmental processes, such as those of vascular tissue, trichomes, cuticle (Müller et al. 2016; Yan et al. 2017), embryogenesis (Roodbarkelari and Groot 2017), leaf polarity (Merelo et al. 2016), and flowering (Kovalchuk et al. 2016). *HD-Zip* genes also are involved in plant responses to drought (Chen et al. 2014; Harris et al. 2016; Zhao et al. 2014), salinity (Chen et al. 2014; Zhao et al. 2014), and pathogen infection (AbuQamar et al. 2006; Oh et al. 2013).

HD-Zip class I functions by binding to a 9-bp pseudopalindromic DNA sequence (CAATNATTG) in the promoters of their target genes (Johannesson et al. 2001; Palena et al. 1999). HD-Zip I proteins form homodimers and heterodimers with HD-Zip proteins in the same class (Harris et al. 2016; Johannesson et al. 2001; Meijer et al. 2000). Most plant stress-responsive HD-Zip proteins belong to class I. Functional studies of HD-Zip I members indicate that they have important roles in abiotic stress responses to frost (Kovalchuk et al. 2016), flooding (Cabello et al. 2016), and drought (Harris et al. 2016; Romani et al. 2016; Zhao et al. 2014). Several reports suggested that HD-Zip I members are involved in plant responses to pathogen infection. For example, overexpression of the HD-Zip I gene *ATHB13* in *Arabidopsis* leads to broad-spectrum disease resistance (Gao et al. 2014). The tomato HD-Zip I gene *H52* is upregulated in response to

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Pseudomonas syringae pv. *tomato* infection, resulting in an oxidative burst that normally precedes the hypersensitive response (HR) (Mayda et al. 1999). The sunflower HD-Zip I gene *HAHB4* is strongly induced by treatment with methyl jasmonate (MeJA) or ethylene (ET). *Arabidopsis* plants ectopically expressing HAHB4 are sensitive to bacterial infection and reduce levels of SA after infection with *Pseudomonas syringae* (Manavella et al. 2008). However, the involvement of HD-Zip I proteins in plant immunity is poorly understood.

Pepper (*Capsicum annuum*) is one of the most important vegetables worldwide. Pepper is a member of the *Solanaceae* family and is vulnerable to several soilborne diseases, such as bacterial wilt caused by *Ralstonia solanacearum*, which causes serious yield and economic losses (Flores-Cruz and Allen 2009). The most efficient and sustainable way to overcome pepper pathogenic diseases is to develop cultivars with disease resistance. A deeper understanding of the mechanisms involved in pepper immunity would facilitate the genetic improvement of pepper disease resistance. In the present study, we isolate and functionally characterize the *Capsicum annuum* HD-Zip I gene *CaHDZ27*. Our results indicate that *CaHDZ27* functions as a positive regulator of pepper's response to *R. solanacearum* infection.

RESULTS

Sequence analysis of CaHDZ27.

HD-Zip I proteins have been implicated in plant immunity, but no studies have been conducted on HD-Zip I proteins in pepper immunity. To isolate pepper genes associated with immunity, a cDNA library was constructed using RNA of pepper leaves inoculated with *R. solanacearum*. Random sequencing of this library identified a full-length cDNA containing an open reading frame (ORF) of 891 bp that harbored a homeodomain (HD) and an adjacent leucine zipper (Zip) domain without any other known domains in its deduced amino acid sequence (Fig. 1), suggesting that it was a pepper HD-Zip I protein (Ariel et al. 2007; Brandt et al. 2014). The deduced amino acid sequence of this cDNA was most similar to SlHZ27 (83.39%), an HD-Zip I protein family member in tomato (*Solanum lycopersicum*), we designated it as CaHDZ27. We performed a phylogenetic analysis using the full-length amino acid sequences of CaHDZ27, 22 HD-Zip I proteins from tomato (Zhang et al. 2014b), and 17 from *Arabidopsis* (Henriksson et al. 2005); the results indicated that CaHDZ27 was most closely related to SlHZ27 and belonged to a subgroup that included only three tomato proteins SlHZ27, SlHZ24, and SlHZ21 (Supplementary Fig. S1). Other subgroups included members from both tomato and *Arabidopsis*. These results were consistent with a previous report (Zhang et al. 2014b). Alignment analysis of the deduced amino acid sequence of CaHDZ27 and HD-Zip I proteins in other plant species showed the high sequence similarity of CaHDZ27 with other HD-Zip I proteins. Interestingly, we also found a conserved motif in which serine (S) and aspartate (D) are rich (Fig. 1). Therefore, CaHDZ27 is likely an HD-Zip I protein.

CaHDZ27 is transcriptionally upregulated by *R. solanacearum* inoculation and exogenous application of salicylic acid (SA), MeJA, or ethephon (ETH).

To test whether *CaHDZ27* is involved in pepper immunity, its transcriptional expression was examined by real-time

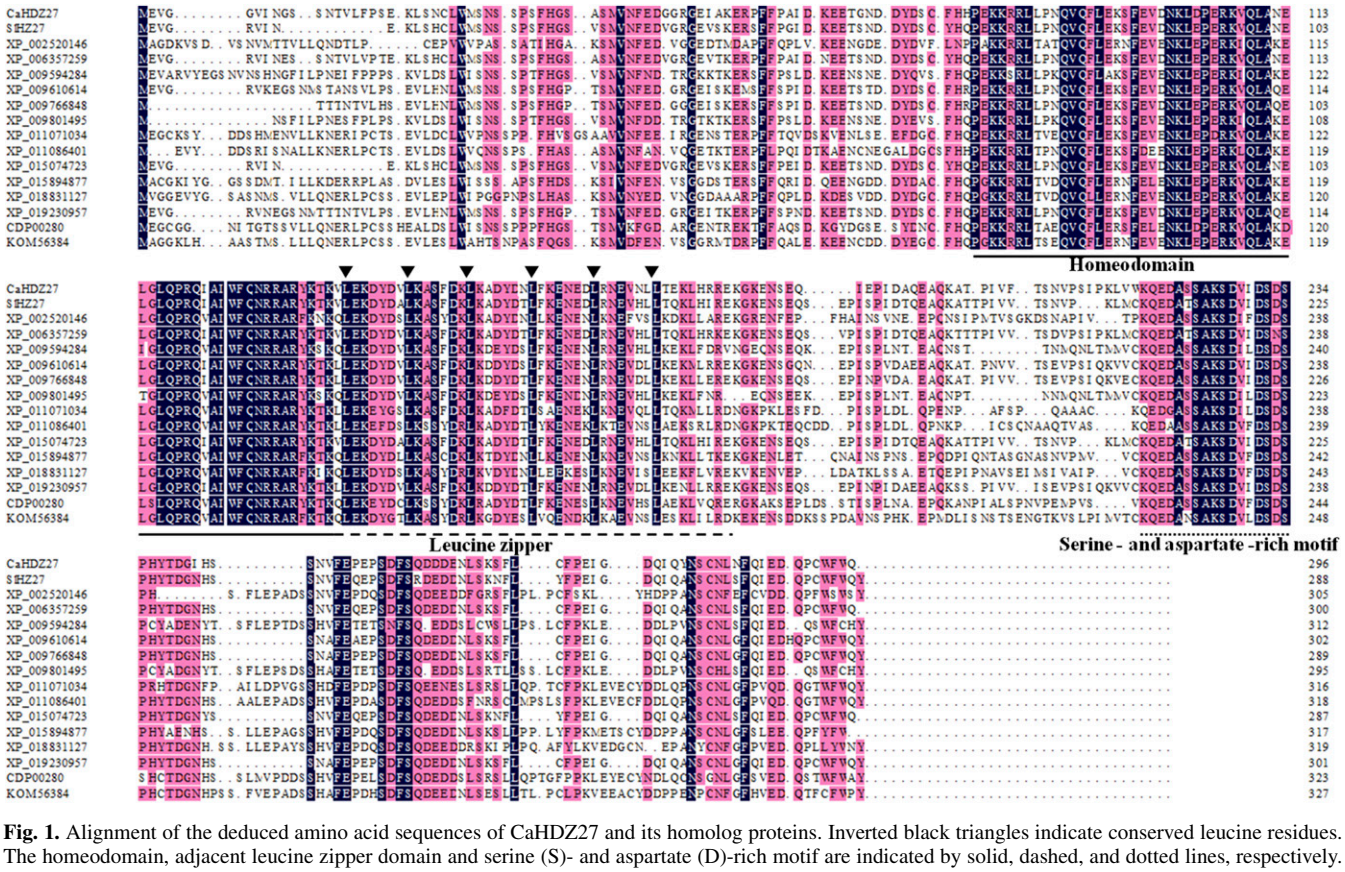

Fig. 1. Alignment of the deduced amino acid sequences of CaHDZ27 and its homolog proteins. Inverted black triangles indicate conserved leucine residues. The homeodomain, adjacent leucine zipper domain and serine (S)- and aspartate (D)-rich motif are indicated by solid, dashed, and dotted lines, respectively. CaHDZ27 homolog proteins were used from potato (*Solanum tuberosum*, XP_006357259, 88.00%), the stress-tolerant wild tomato (*Solanum pennellii*, XP_015074723, 83.67%), tomato (*Solanum lycopersicum*, XP_004238764, SlHZ27, 83.67%), *Nicotiana* spp. (*N. sylvestris*, XP_009766848, 81.94%; *N. tomentosiformis*, XP_009610614, 80.82%; *N. attenuata*, XP_019230957, 82.18%; *N. sylvestris*, XP_009801495, 59.42%, and *N. tomentosiformis*, XP_009594284, 58.81%), *Coffea canephora* (CDP00280, 58.41%), *Ziziphus jujuba* (XP_015894877, 57.05%), *Sesamum indicum* (XP_011086401, 55.28% and XP_011071034, 53.42%), *Ricinus communis* (XP_002520146, 53.59%), *Juglans regia* (XP_018831127, 52.34%) and *Vigna angularis* (KOM56384, 50.91%).

quantitative polymerase chain reaction (qPCR) analysis of pepper plants inoculated with *R. solanacearum*, the causal agent of bacterial wilt of pepper. The results showed that *CaHDZ27* transcript levels were upregulated significantly at 24 and 48 h postinoculation (Fig. 2A). SA, jasmonic acid (JA), and ET have been implicated as general defense signaling molecules in plant immunity; therefore, we tested the effects of exogenous application of SA, MeJA, or ETH on *CaHDZ27* transcript levels in pepper seedlings. Different exogenous hormone concentrations were first investigated to determine suitable concentrations; 1 mM SA, 100 μM MeJA, and 100 μM are the optimal concentrations for spraying (Supplementary Fig. S2). qPCR analysis showed that MeJA application increased *CaHDZ27* transcript levels at 6, 12, and 24 h posttreatment (hpt), with the peak at 24 hpt, compared with those in mock-treated control plants (Fig. 2B). SA application increased *CaHDZ27* transcript levels at 1 and 6 hpt, compared with those in mock-treated control plants (Fig. 2C). ETH application markedly increased *CaHDZ27* transcript levels at 6, 12, and 24 hpt, compared with those in mock-treated control plants (Fig. 2D). These combined results indicate that *CaHDZ27* transcription is modulated by *R. solanacearum* inoculation and exogenous application of SA, MeJA, and ETH. Therefore, *CaHDZ27* may have a functional role in pepper immunity against *R. solanacearum*.

CaHDZ27 protein is localized in the nucleus.

A nuclear localization sequence (NLS) at amino acid residue 78 to 86 (PEKKRRLLP, cNLS Mapper score 7) was determined using the open source software cNLS Mapper, suggesting that it is targeted to the nucleus. To determine the subcellular localization, *CaHDZ27* was fused with the green fluorescent protein (*GFP*) gene under the control of the 35S promoter. This construct (35S::CaHDZ27-GFP) was transformed into *Agrobacterium tumefaciens* GV3101 and was infiltrated by injection into *Nicotiana benthamiana* leaves. GFP expression was examined after 48 h, using a Leica confocal microscopy. GFP fluorescence was detected exclusively in the nuclei of cells transiently overexpressing *CaHDZ27-GFP*, which also were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Supplementary Fig. S3). By contrast, GFP fluorescence in control leaves, infiltrated with 35S::GFP, was observed throughout the cells. These results suggest that CaHDZ27 is localized in the nucleus.

CaHDZ27 has DNA-binding and transactivating activities.

HD-Zip I proteins act as TFs by binding to the pseudopalindromic sequence CAAT(A/T)ATTG in their target genes (Ariel et al. 2007). To test if CaHDZ27 can bind this *cis* element, we performed chromatin immunoprecipitation (ChIP) analyses. We constructed two β-glucuronidase (GUS) reporter vectors (2×*cis-p35Score*::*GUS* and 2×m*cis-p35Score*::*GUS*), which were driven by a synthetic promoter containing two copies of the *cis* element (*cis*, CAATAATTG) or its mutant (m*cis*, CAGGGGTTG) and an adjacent *Cauliflower mosaic virus* (CaMV) 35S core promoter sequence. The control vector construct (*p35Score*::*GUS*) contained only the CaMV 35S core promoter sequence (Fig. 3A). The effect vector (*35S*::*CaHDZ27-HA*) and reporter vectors were transformed into GV3101. Cells containing both effect and reporter vectors

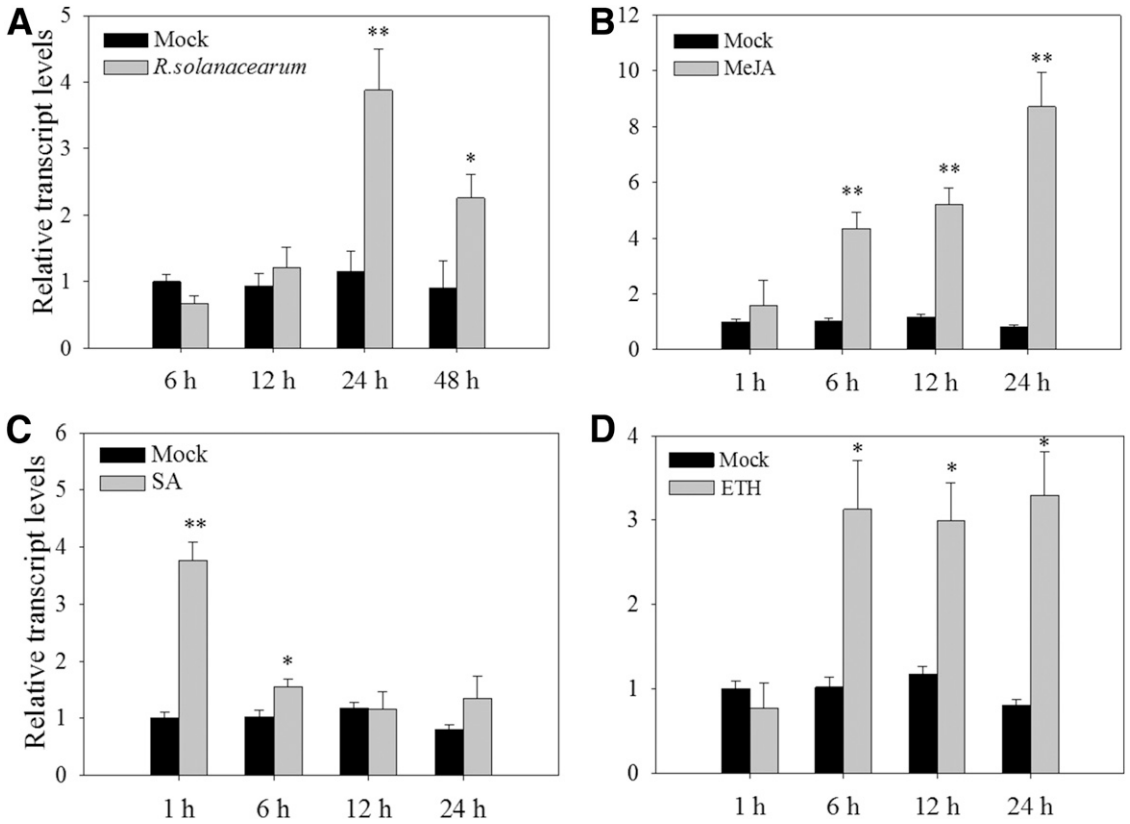

Fig. 2. Quantitative real time polymerase chain reaction analysis of relative *CaHDZ27* transcript levels in pepper treated with *Ralstonia solanacearum* or exogenously applied phytohormones. **A,** *CaHDZ27* transcript levels in pepper leaves were assessed at different timepoints after *R. solanacearum* inoculation. **B to D,** *CaHDZ27* transcript levels were examined in pepper leaves at various timepoints after treatment with methyl jasmonate (MeJA, 100 μM), salicylic acid (SA, 1 mM), or ethephon (ETH, 100 μM). Error bars indicate standard error. Experiments were repeated three times along with at least three independent repetitions of the biological experiments. Asterisks (** for $P < 0.01$ and * for $P < 0.05$ indicate significant differences compared with the treatment and mock control, as determined by Student's *t* test.

were mixed at a 1:1 ratio and were infiltrated into pepper leaves, which were harvested after 48 h and were used for ChIP analyses. The results revealed that CaHDZ27 bound to the synthetic promoter containing the CAATAATTG *cis* element but did not bind to the promoter containing the m*cis* element (CAGGGGTTG) or only the CaMV 35S core promoter sequence (Fig. 3B). We analyzed GUS activities in pepper leaves at 48 or 72 h after infiltration with GV3101 cells carrying effect and reporter vectors. The results showed that GUS activities were significantly higher after infiltration with 2×*cis-p35Score*::*GUS* compared with 2×m*cis-p35Score*::*GUS* or *p35Score*::*GUS* (Fig. 3C). These results indicate that CaHDZ27 can activate GUS expression by binding to the *cis* element (CAATAATTG).

CaHDZ27 silencing significantly impairs pepper resistance against *R. solanacearum* infection.

The obtained results suggest that *CaHDZ27* might function as a TF and is upregulated by *R. solanacearum* infection. Therefore, we performed a gene silencing analysis using virus-induced gene silencing (VIGS) and a *Tobacco rattle virus* (TRV) vector containing a 185-bp fragment in the 3′ untranslated region (UTR) of *CaHDZ27*. Three independent experiments were performed, and each experiment included approximately 100 TRV::*00* and 100 TRV::*CaHDZ27* plants. The control plants expressed a pepper phytoene desaturase gene (TRV::*CaPDS*), which enabled visual inspection of gene silencing based on photobleaching (Supplementary Fig. S4). Reverse transcription (RT)-PCR demonstrated that the

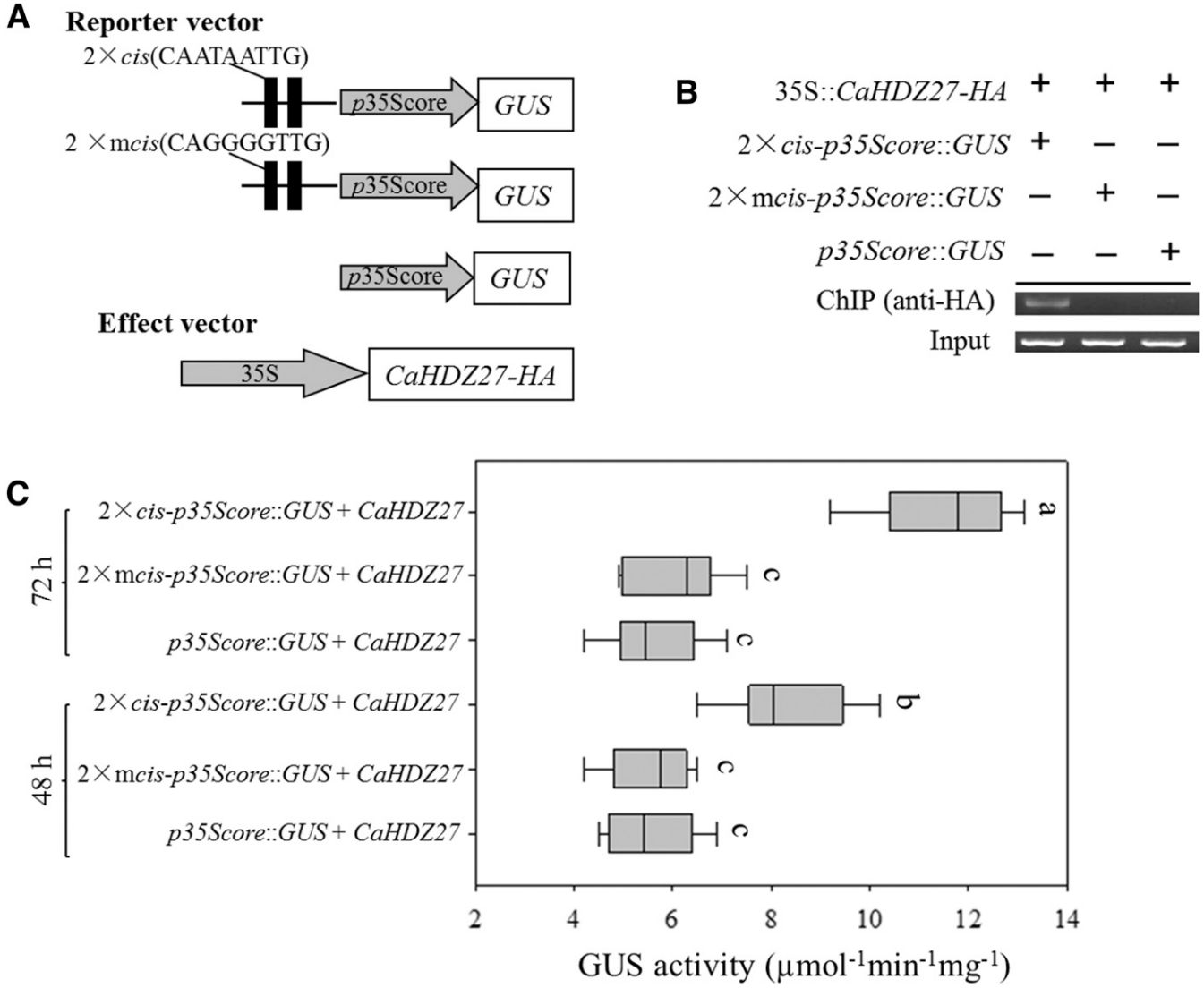

Fig. 3. CaHDZ27 binds the *cis* element (CAATAATTG) and triggers β-glucuronidase (GUS) expression in a CAATAATTG-dependent manner. **A,** Schematic diagram of the reporter and effect vectors used for the chromatin immunoprecipitation (ChIP) assay. The 2×*cis-p35Score*::*GUS* vector contains two tandem copies of the conserved HD-Zip I protein-binding *cis* element (CAATAATTG), whereas the 2×m*cis-p35Score*::*GUS* vector contains two tandem copies of mutant *cis* element (CAGGGGTTG), and *p35Score*::*GUS* contains only the *Cauliflower mosaic virus* 35S core promoter sequence. **B,** ChIP assay shows that CaHDZ27 binds the *cis* element (CAATAATTG). *Agrobacterium tumefaciens* GV3101 cells containing *35S*::*CaHDZ27-HA* and 2×*cis-p35Score*::*GUS*, 2×m*cis-p35Score*::*GUS*, or *p35Score*::*GUS* constructs were coinfiltrated into pepper leaves, which were harvested after 48 h and were fixed with 1% formaldehyde. The input protein was immunoprecipitated with anti-hemagglutinin (anti-HA) antibodies. Acquired DNAs were adjusted to the same concentrations, and polymerase chain reaction–amplified using attB-specific primers. Input, total DNA-protein complex; ChIP (anti-HA), DNA-protein complex immunoprecipitated with anti-HA antibody. **C,** CaHDZ27 triggers GUS expression in a CAATAATTG-dependent manner. Pepper leaves were coinfiltrated with *A. tumefaciens* GV3101 cells carrying 2×*cis-p35Score*::*GUS*, 2×m*cis-p35Score*::*GUS*, *p35Score*::*GUS*, and 35S::*CaHDZ27*, and leaves were harvested after 48 or 72 h. GUS activity was determined using a microplate reader. Bars represent medians, and boxes represent the 25th and 75th percentiles. Different letters represent significant differences ($P < 0.05$, least significant difference test).

transcript level of *CaHDZ27* is reduced in most of the TRV::*CaHDZ27*-infiltrated plants, as compared with TRV::*00*-infiltrated plants (Supplementary Fig. S5). Subsequently, the C*aHDZ27* silenced plants and TRV::*00*-infiltrated plants were inoculated by infiltrating leaves with an injection of 20 µl of *R. solanacearum* FJC100301 cells (10^8 CFU/ml). The result of qPCR analysis showed that *CaHDZ27* transcript levels during *R. solanacearum* infection in TRV::*CaHDZ27*-infiltrated plants were significantly down-regulated compared with TRV::*00* control plants (Fig. 4A).

Wounded root inoculations were performed by pouring 10 ml of the 10^8 CFU/ml suspensions into each pot. TRV::*CaHDZ27* plants displayed leaf wilting at 10 days after root inoculation, and this progressed until complete leaf wilting was observed approximately 12 days after root inoculation. By contrast, plants inoculated with TRV::*00* displayed delayed symptom development at 10 days after root inoculation (Fig. 4B; Supplementary Table S1) and symptom progression (leaf wilting) significantly differed in TRV::*CaHDZ27* and control TRV::*00* plants (Fig. 4C).

To investigate whether *CaHDZ27* silencing affects HR-mediated cell death in response to *R. solanacearum* infection, we stained leaves of TRV:*00* and TRV::*CaHDZ27* plants with diaminobenzidine (DAB) (indicator of H_2O_2 accumulation) and trypan blue (indicator of cell death or necrosis) 48 h after *R. solanacearum* inoculation. The results showed that DAB and trypan blue staining intensities were higher in TRV::*00* control leaves than in TRV::*CaHDZ27* leaves (Fig. 4D). Consistently, electrolyte leakage levels were significantly higher in TRV::*00* leaves than in TRV::*CaHDZ27* leaves at 48 or 72 h after *R. solanacearum* infection (Fig. 4E). Notably, H_2O_2 accumulation and cell death during *R. solanacearum* infection were significantly attenuated in *CaHDZ27*-silenced pepper leaves.

To confirm these phenotypic results and to investigate the mechanism of *CaHDZ27* activity in pepper immunity, we performed qPCR analysis of the following pepper defense-related gene expression levels in TRV::*CaHDZ27* and TRV::*00* control plants challenged with *R. solanacearum*: the HR marker gene *CaHIR1* (Choi et al. 2011), the ET biosynthesis–associated gene *CaACO1* (Cai et al. 2015), ET/JA-responsive gene *CaPR4* (Cai et al. 2015), the reactive oxygen species (ROS) detoxification–associated gene *CaPO2* (Choi et al. 2007), the SA-responsive gene *CaPR1* (Jung and Hwang 2000), and the basal defense–related gene *CaBPR1* (An et al. 2008). The results showed that transcript levels of *CaHIR1*, *CaACO1*, *CaPR4*, *CaPO2*, *CaPR1*, and *CaBPR1* were significantly reduced in *CaHDZ27*-silenced leaves compared with the levels in control plants with or without *R. solanacearum* infection (Fig. 4F). These combined results suggest that *CaHDZ27* silencing directly or indirectly downregulates the expression of these defense-related genes during *R. solanacearum* infection, and enhances the susceptibility of pepper plants to *R. solanacearum* infection.

Transient *CaHDZ27* expression triggers cell death and upregulates immunity-associated marker genes in pepper leaves.

We performed gain-of-function analysis to further confirm the role of *CaHDZ27* in pepper immunity. For these experiments, we tested the transient expression of *CaHDZ27*, using *Agrobacterium* infiltration into pepper leaves, and monitored *CaHDZ27* expression by qPCR analysis. The results showed that *CaHDZ27* transcript levels were substantially higher in leaves transiently overexpressing *CaHDZ27* (35S::*CaHDZ27*) compared with those in control leaves (35S::*00*) (Fig. 5A).

HR-mediated cell death is a hallmark indicator of ETI. To determine whether HR-mediated cell death is triggered by transient *CaHDZ27* overexpression, the infiltrated leaves were stained with trypan blue and DAB. The results showed that transient *CaHDZ27* overexpression resulted in more intense trypan blue and DAB staining, indicating that infiltrated leaves transiently overexpressing *CaHDZ27* have higher levels of cell death and H_2O_2 accumulation than control leaves (35S::*00*) (Fig. 5B). Higher levels of electrolyte leakage also occurred in leaves expressing 35S::*CaHDZ27* than in control leaves (35S::*00*) at 48 and 72 h after infiltration (Fig. 5C). We also performed a qPCR analysis of defense-marker gene expression, and the results showed higher expression levels of *CaHIR1*, *CaACO1*, *CaPR1*, *CaPR4*, *CaPO2*, and *CaBPR1* in leaves transiently expressing *CaHDZ27* than in control leaves (Fig. 5D). These combined results indicate that CaHDZ27 has a role in regulating HR- and defense-associated gene expression during pepper defense responses.

Ectopic *CaHDZ27* overexpression confers *R. solanacearum* resistance in transgenic tobacco.

Stable transformation of pepper plants is still technically challenging. Therefore, we generated transformed tobacco (*Nicotiana tabacum*) plants overexpressing *CaHDZ27*, using *A. tumefaciens*–mediated leaf disc transformation. We acquired a number of homozygous T_3 lines that did not exhibit significant morphological or developmental differences from wild-type plants. Three lines (#3, #5, and #8) were randomly selected for further functional characterization of *CaHDZ27* (Supplementary Fig. S6). By inoculating roots with *R. solanacearum*, leaf-wilting symptoms were observed in wild-type plants at 12 days after inoculation, whereas *CaHDZ27*-overexpressing transgenic plants (lines #3, #5, and #8) exhibited only minimal leaf-wilting symptoms (Fig. 6A). The temporal progression of symptoms significantly differed between wild-type and *CaHDZ27*-overexpressing transgenic plants (Fig. 6B; Supplementary Table S2).

Next, we investigated H_2O_2 accumulation and HR-mediated cell death in response to *R. solanacearum* infiltration into leaf veins. After 48 h, the infiltrated leaves were stained with DAB and trypan blue. The results showed that H_2O_2 accumulation and cell death were higher in the *CaHDZ27*-overexpressing line #3 challenged with *R. solanacearum* (Fig. 6C). Consistently, bacterial growth was significantly reduced in leaves of line #3 compared with that in wild-type plants (Fig. 6D). These results indicate that *CaHDZ27* overexpression enhances the resistance of tobacco plants against *R. solanacearum* infection. The qPCR analysis indicated that transcript levels of HR marker genes (*NtHSR201* [Sohn et al. 2007], ET biosynthesis-associated *NtACC oxidase* [Chen et al. 2003], and SA- or JA-associated *NtPRa/c*, *NtPR2*, *NtPR3*, and *NtPR4* [Brogue et al. 1991; Ward et al. 1991; Dang et al. 2014]) were significantly higher in *CaHDZ27*-overexpressing plants with or without *R. solanacearum* inoculation than in wild-type tobacco plants (Fig. 6E).

CaHDZ27 can form homodimers.

To test whether CaHDZ27 can form homodimers, we performed bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (CoIP) analyses. For the BiFC assay, *CaHDZ27* was fused to the N- and C-terminal ends of yellow fluorescent protein (YFP) to generate *CaHDZ27-nYFP* and *CaHDZ27-cYFP*, respectively. Each construct was introduced into *A. tumefaciens* GV3101, and cells carrying different constructs were simultaneously infiltrated into *N. benthamiana* leaves. The infiltrated leaves were examined by confocal microscopy after 48 h. YFP fluorescence was observed exclusively in nuclei in leaves infiltrated with both *CaHDZ27-nYFP* and *CaHDZ27-cYFP*, whereas no YFP fluorescence was observed in negative controls (coinfiltration of *nYFP* and *CaHDZ27-cYFP*, or *cYFP* and *CaHDZ27-nYFP*) (Fig. 7A). We also examined CaHDZ27 homodimerization using CoIP. FLAG-tagged CaHDZ27 and hemagglutinin (HA)-tagged CaHDZ27 were transiently coexpressed in *N. benthamiana* leaves by infiltration. After 48 h, total protein was

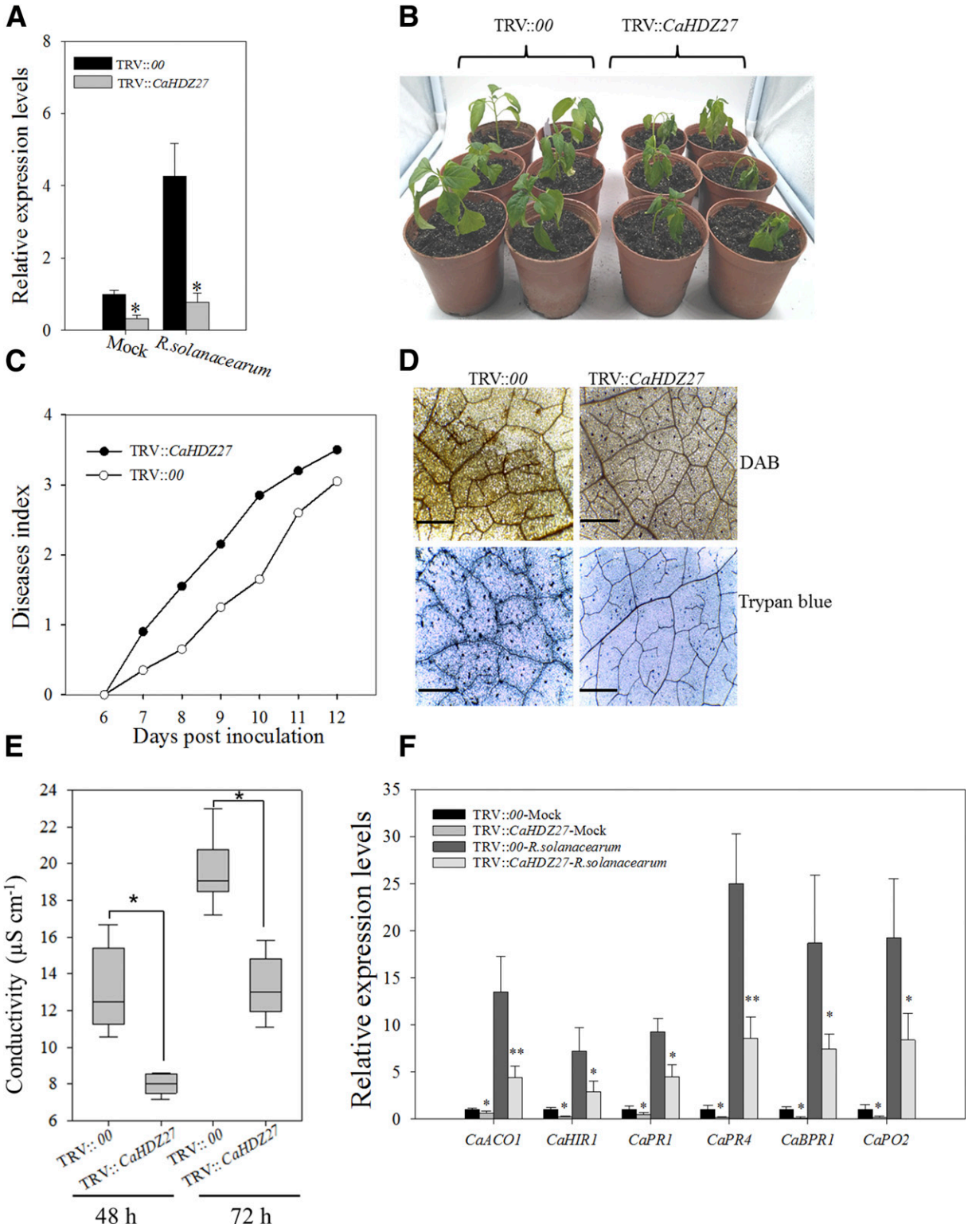

Fig. 4. Virus-induced gene silencing of *CaHDZ27* in pepper plants enhances susceptibility to *Ralstonia solanacearum* infection. **A,** Quantitative polymerase chain reaction analysis of *CaHDZ27* transcript levels in TRV::*00* or TRV::*CaHDZ27* plants at 48 h after leaf inoculation with *R. solanacearum*. **B,** CaHDZ27 silencing in TRV::*CaHDZ27* plants attenuated resistance to *R. solanacearum* infection. The wounded roots of pepper plants, silenced for 4 to 5 weeks, were inoculated with *R. solanacearum*. Several representative plants are shown at 10 days after inoculation. **C,** Disease index score was recorded from 7 to 12 days after wounded root inoculation with *R. solanacearum*. Disease index score was determined according to the percentage of wilted leaves, with values defined as 0 (no symptoms), 1 (0 to 25% wilted leaves), 2 (25 to 50% wilted leaves), 3 (50 to 75% wilted leaves), and 4 (75 to 100% wilted leaves). Each point represents the mean disease index of 30 inoculated plants. The disease index score of TRV::*CaHDZ27* plants was lower than TRV::*00* plants throughout the entire experimental period. **D,** Visualization of diaminobenzidine (DAB) and trypan blue staining 48 h after leaf inoculation with *R. solanacearum*. The experiment was performed three times with similar results. Bars = 500 μm. **E,** Electrolyte leakage was measured in TRV::*00* and TRV::*CaHDZ27* leaves inoculated with *R. solanacearum*. Bars represent medians, and boxes represent the 25th and 75th percentiles. **F,** Transcript levels of defense-related genes were determined by quantitative reverse transcription-polymerase chain reaction in TRV::*CaHDZ27* and TRV::*00* pepper plants after inoculating leaves with *R. solanacearum.* Error bars show the mean of the standard deviation of replicate samples ($n = 6$). Asterisks (** for $P < 0.01$ and * for $P < 0.05$) indicate significant differences between TRV::*00* and TRV::*CaHDZ27* plants, as determined by Student's *t* test.

extracted and immunoprecipitated with FLAG-antibody–conjugated agarose beads, and the isolated proteins were then analyzed with HA antibody. The results show that CaHDZ27 can bind to itself to form homodimers (Fig. 7B).

CaHDZ27 dimerization is enhanced by *R. solanacearum* infection.

Next, we tested whether CaHDZ27 dimerization was affected by *R. solanacearum* infection by performing floating-leaf luciferase (LUC) complementation imaging using Gateway-compatible split-LUC destination vectors (Gehl et al. 2011). We generated *35S::CaHDZ27-NLUC*, *35S::CaHDZ27-CLUC*, and *pEXP-GUS* (control) transient overexpression constructs and transformed them into *A. tumefaciens* GV3101 cells. Then, pepper leaves were coinfiltrated with the transient overexpression constructs. GUS activity was measured and used to normalize total protein levels, and dynamic protein-protein interactions were detected by luminescence activity measurement assays. The negative controls (coinfiltration of *CaHDZ27-NLUC* and *CLUC*, or *CaHDZ27-CLUC* and *NLUC*) had lower luminescence activity than leaves coinfiltrated

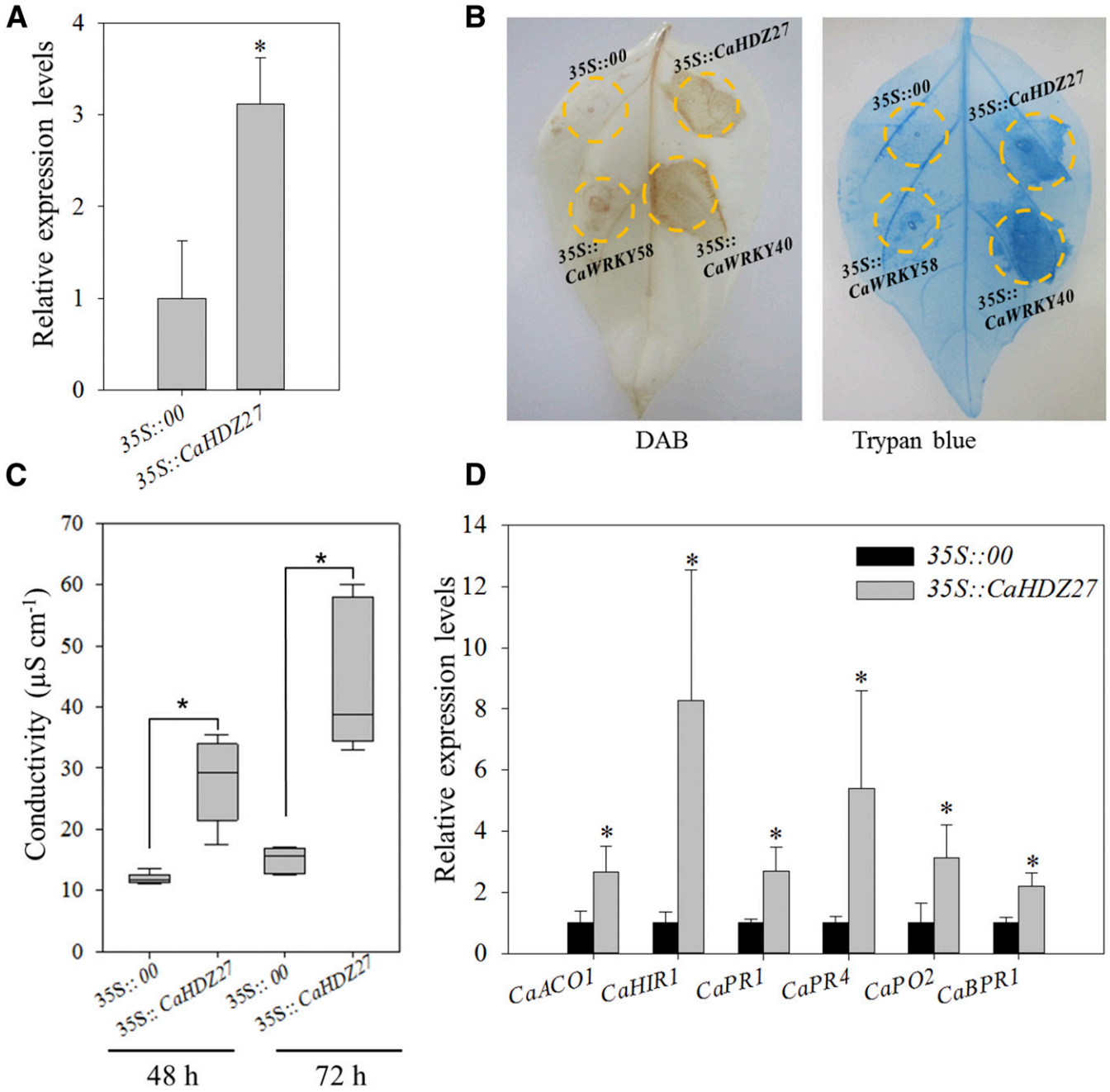

Fig. 5. Transient *CaHDZ27* overexpression in pepper leaves triggers immunity. **A,** Transient *CaHDZ27* overexpression was determined by quantitative reverse transcription-polymerase chain reaction analysis. **B,** Transient *CaHDZ27* expression induces the cell-death response as visualized with diaminobenzidine (DAB) and trypan blue staining 48 h after infiltration. *CaWRKY40* (Dang et al. 2013) and *CaWRKY58* (Wang et al. 2013) were used as positive and negative controls, respectively. The experiment was performed three times with similar results. **C,** Quantification of electrolyte leakage to assess the cell-death response in pepper leaves transiently overexpressing *CaHDZ27*. Bars represent medians, and boxes represent the 25th and 75th percentiles. **D,** Transient *CaHDZ27* overexpression upregulates the expression of defense-related marker genes compared with that in control plants at 48 h after infiltration. Data represent the mean ± standard deviation ($n = 6$). One asterisk (* for $P < 0.05$) indicates significant differences between *35S::00* and *35S::CaHDZ27* plants as determined by Student's *t* test.

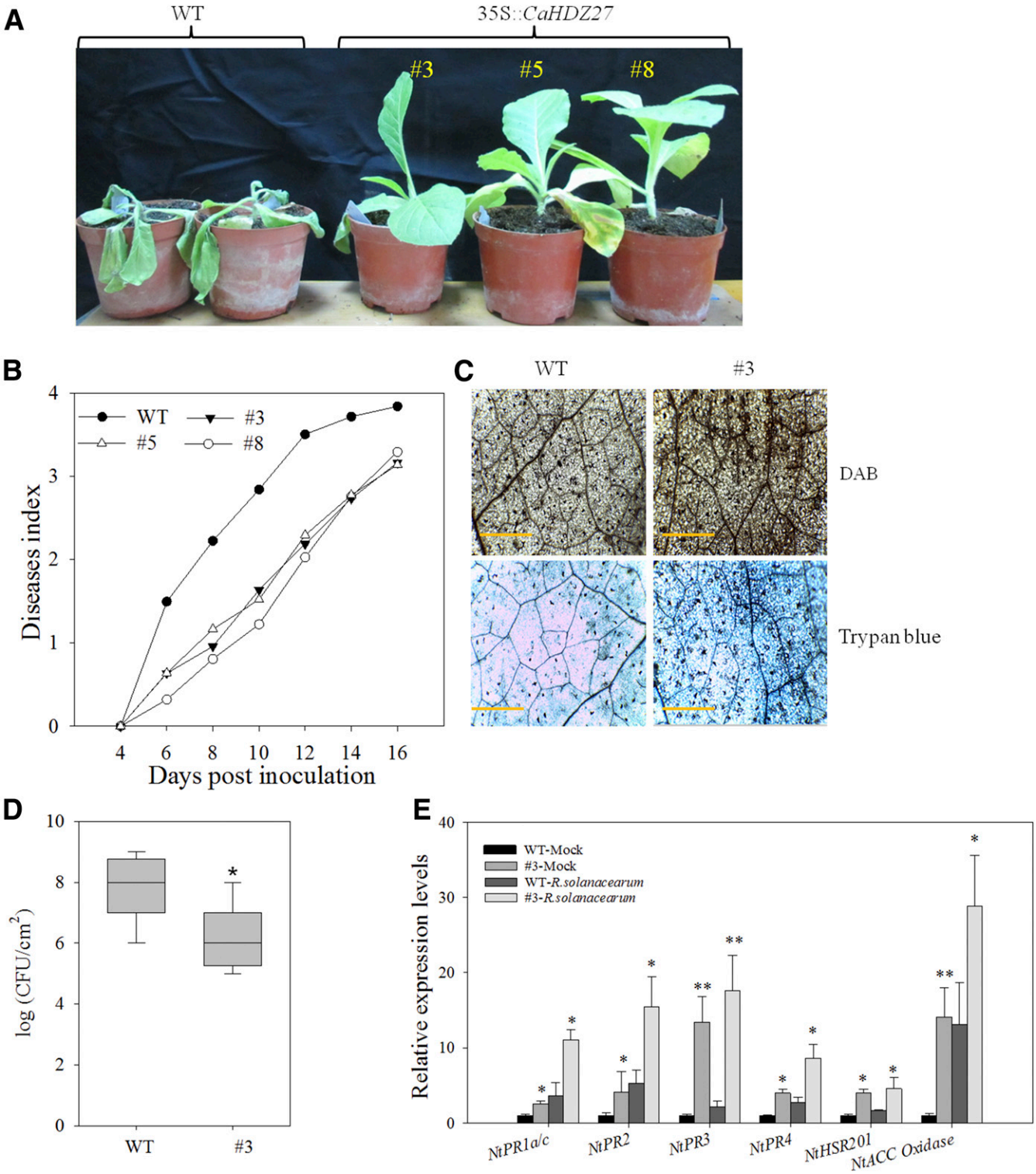

Fig. 6. Ectopic *CaHDZ27* expression in tobacco plants enhances resistance against *Ralstonia solanacearum* infection. **A,** Transgenic *CaHDZ27*-overexpressing tobacco plants (6 weeks old) had higher resistance to *R. solanacearum* infection than wild-type (WT) plants at 12 days after inoculation of wounded roots. **B,** Disease index scores were recorded from wild-type and transgenic tobacco plants every 2 days from 6 to 16 days after inoculation of wounded roots with *R. solanacearum*. **C,** Hypersensitive response–mimicking cell death was visualized by diaminobenzidine (DAB) and trypan blue staining in transgenic *CaHDZ27*-overexpressing plants (line #3) inoculated with *R. solanacearum*; DAB and trypan blue staining were more intense in *CaHDZ27*-overexpressing plants than in wild-type plants. Bars = 500 µm. **D,** Growth of *R. solanacearum* in transgenic *CaHDZ27*-overexpressing plants (line #3) was significantly lower than in wild-type plants at 48 h after leaf inoculation. Bars represent medians, and boxes represent the 25th and 75th percentiles. **E,** Transcript levels of defense-related genes were determined by quantitative polymerase chain reaction and were significantly higher in *CaHDZ27*-overexpressing plants than in wild-type plants inoculated with *R. solanacearum*. Data represent the mean ± standard deviation ($n = 6$). Asterisks (** for $P < 0.01$ and * for $P < 0.05$) indicate significant differences between wild-type and transgenic plants (line #3) as determined by Student's *t* test.

with *CaHDZ27-NLUC* and *CaHZ27-CLUC*. These results suggest that CaHDZ27 can form homodimers. The luminescence activity in pepper leaves coinfiltrated with *CaHDZ27-NLUC* and *CaHDZ27-CLUC* was significantly increased at 1 day after *R. solanacearum* inoculation (Fig. 8), suggesting that CaHDZ27 homodimerization was enhanced by *R. solanacearum* infection.

DISCUSSION

HD-Zip proteins are unique TFs in plants, and HD-Zip I proteins have been implicated in abiotic stress responses (Harris et al. 2011; Zhang et al. 2014b; Zhao et al. 2014). Several HD-Zip I members have important roles in biotic stress responses, such as ATHB13 in *Arabidopsis* (Gao et al. 2014), H52 in tomato (Mayda et al. 1999), and HAHB4 in sunflower (Manavella et al. 2008). Information is lacking about pepper HD-Zip I members, and their biological functions are unknown. This study performed a functional genetic analysis of the CaHDZ27 HD-Zip I protein of *Capsicum annuum*. The results indicate that CaHDZ27 functions as a positive regulator of *R. solanacearum* infection in pepper.

The deduced amino acid sequence of *CaHDZ27* contains a HD and an adjacent Zip domain and exhibits the highest sequence similarity with SlHZ27, among all HD-Zip I proteins in tomato and *Arabidopsis*. CaHDZ27 also has high sequence similarities with homologs from other species, including potato, tomato, and tobacco. We also show that CaHDZ27 binds to the 9-bp pseudopalindromic DNA sequence (CAATNATTG), which is specifically bound by HD-Zip class I proteins (Johannesson et al. 2001; Palena et al. 1999), and activates GUS expression in a CAATAATTG-dependent manner. These results suggest that CaHDZ27 is an HD-Zip class I protein in pepper. This conclusion was further supported by the observation that CaHDZ27-GFP expression was observed exclusively in the nuclei, which is frequently observed in other HD-Zip I proteins (Cao et al. 2016; Zhao et al. 2014).

Real-time qPCR analysis indicated that *CaHDZ27* transcription was significantly induced by *R. solanacearum* inoculation, implying an involvement in pepper response to *R. solanacearum*. Plant genes that are upregulated in response to pathogen attack frequently have important roles in plant immunity (Cai et al. 2015; Dang et al. 2013, 2014; Shen et al. 2016a and b). Loss-of-function VIGS silencing experiments in pepper plants confirmed this hypothesis, because *CaHDZ27* silencing increased susceptibility to *R. solanacearum* infection and downregulated the defense-related marker genes *CaHIR1* (Choi et al. 2011, 2013; Jung and Hwang 2007; Jung et al. 2008), *CaACO1* (Dong et al. 2014; Cheng et al. 2017), *CaPR1* (Yi et al. 2013; Kim et al. 2014), *CaPR4* (Dong et al. 2014),

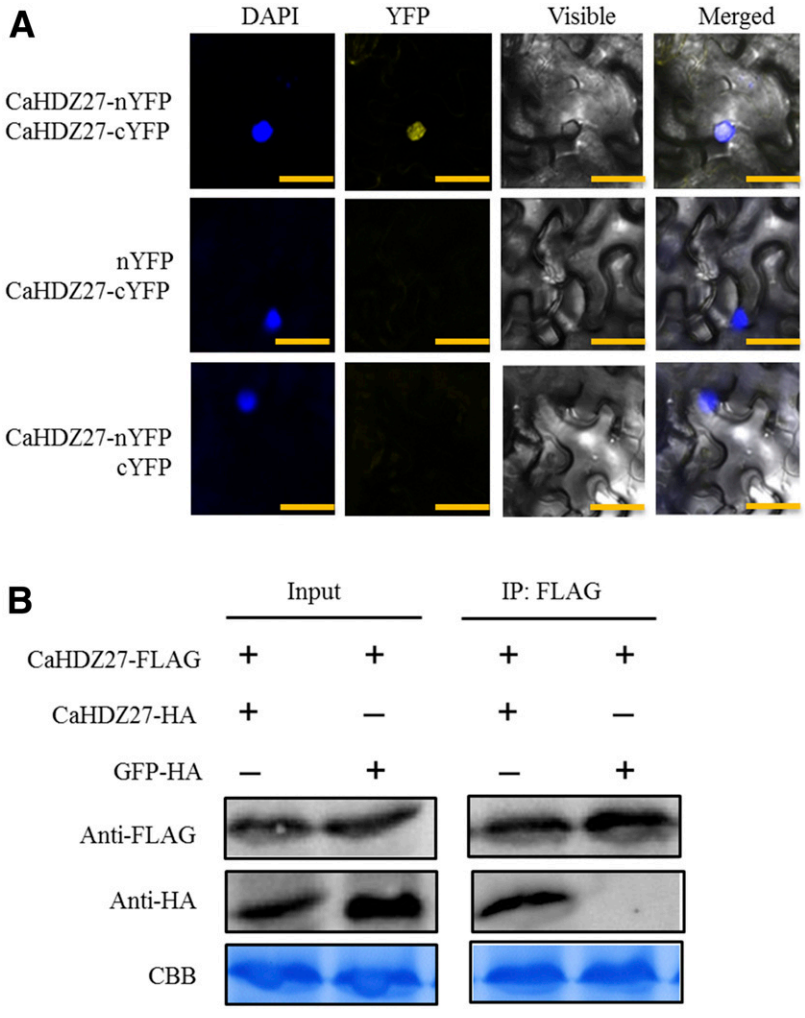

Fig. 7. Bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (Co-IP) assays indicate that CaHDZ27 binds to itself to form homodimers. **A,** BiFC analysis of *Nicotiana benthamiana* leaves transiently overexpressing CaHDZ27 indicate that CaHDZ27 forms homodimers. Bars = 50 µm. **B,** Co-IP analysis of *N. benthamiana* leaves, transiently overexpressing CaHDZ27-FLAG and CaHDZ27-HA, confirms that CaHDZ27 forms homodimers. Leaves were harvested 48 h after infiltration, total protein was isolated, and soluble protein extracts before (input) and after immunoprecipitation (IP), with beads conjugated with anti-FLAG antibody, were detected by Western blotting with anti-HA (anti-hemagglutinin) antibody.

CaPO2 (Choi and Hwang 2012; Choi et al. 2007), and *CaBPR1* (Hong et al. 2005; Sarowar et al. 2005). By contrast, transient *CaHDZ27* overexpression in pepper plants increased HR-mimicking cell death and upregulated defense-related marker genes. Consistently, ectopic *CaHDZ27* overexpression in transgenic tobacco enhanced resistance against *R. solanacearum* infection and induced H_2O_2 accumulation and defense-related gene expression of *NtHSR201* (Czernic et al. 1996; Qiu et al. 2016; Takahashi et al. 2004), *NtACC* oxidase (Avni et al. 1994; Fammartino et al. 2010; Lasserre et al. 1997), *NtPR1a/c* (Jamir et al. 2004; Matsuoka et al. 1987; Zhang et al. 2012b), *NtPR2* (Ghanta et al. 2011; Naoumkina et al. 2008; Wang et al. 2013), *NtPR3* (Zhang et al. 2012a), and *NtPR4* (Li et al. 2015; Maimbo et al. 2007). These combined results indicate that *R. solanacearum* infection enhances *CaHDZ27* expression and enhanced *CaHDZ27* expression in turn activates HR- or immunity-associated gene expression, leading to enhanced disease resistance.

The plant hormones SA, JA, and ET have key roles in regulating defense signaling networks that are activated in response to pathogen invasion (Pieterse et al. 2012). SA is primarily involved in biotrophic pathogen defense signaling, whereas JA and ET are primarily involved in necrotrophic pathogen defense signaling (Glazebrook 2005; Spoel et al. 2007). The production of SA, JA, and ET is frequently coupled with ETI or pathogen-triggered immunity (PTI) (Tsuda and Katagiri 2010), and the defense phytohormone levels depend on the recognized pathogen (De Vos et al. 2005) and the invading population density (Pétriacq et al. 2016). The defense phytohormones can act synergistically or antagonistically, depending on their concentration levels (Kunkel and Brooks 2002; Mur et al. 2006). Recent work reported that SA and JA/ET are synergistically related in PTI but compensatorily related in ETI (Tsuda et al. 2009). The present study found that the *CaHDZ27* transcriptional response to exogenous application of MeJA, SA, and ETH was consistent with SA-, JA-, and ET-responsive pathogenesis-related marker gene expression in pepper and tobacco plants overexpressing *CaHDZ27*. The expression of *CaPO2*, a marker gene for ROS-dependent resistance, also was upregulated by transient *CaHDZ27* overexpression in pepper. These combined results indicate that *R. solanacearum* infection in pepper upregulates *CaHDZ27* expression, which in turn synergistically boosts SA-, JA-, and ET-mediated defense responses.

HD-Zip proteins form homodimers and heterodimers with other HD-Zip proteins in the same family (Johannesson et al. 2001; Meijer et al. 2000). Our results showed that CaHZ27 forms homodimers in pepper and this homodimeriztion was found to be enhanced by *R. solanacearum* infection, indicating that some unidentified signaling components might be activated by *R. solanacearum,* which potentiate homodimeriztion of CaHZ27. We speculate that the homodimeriztion of CaHZ27 might play a role in resistance of pepper to *R. solanacearum* infection; to confirm this speculation, further investigation and more evidence is required.

In summary, our data indicate that CaHDZ27 is a member of the HD-Zip I protein family in pepper. *CaHDZ27* is transcriptionally upregulated by *R. solanacearum* infection, thereby integrating SA-, JA-, and ET-dependent defense signaling. CaHDZ27 may act as a positive regulator of defense responses against *R. solanacearum* infection.

MATERIALS AND METHODS

Plant materials and growth conditions.

Seeds of pepper (*Capsicum annuum* L. cv. Fj8) were obtained from the pepper breeding group in Fujian Agriculture and Forestry University (Fuzhou, Fujian, China). Tobacco (*Nicotiana tabacum* L. cv. Honghuadajinyuan) was supplied by the tobacco breeding group in Fujian Agriculture and Forestry University. Pepper and tobacco plants were grown in steam-sterilized soil mix in plastic pots at 25°C under a 16-h day and 8-h night photoperiod.

Pathogens and inoculation procedures.

Ralstonia solanacearum FJC100301 was cultured in potato sucrose agar (medium overnight at 28°C, according to the method of Dang et al. (2013). The bacterial culture density was measured as 10^8 CFU/ml (optical density at 600 nm [OD_{600}] = 0.8) from serial dilutions in 10 mM $MgCl_2$. Wounded root inoculation was

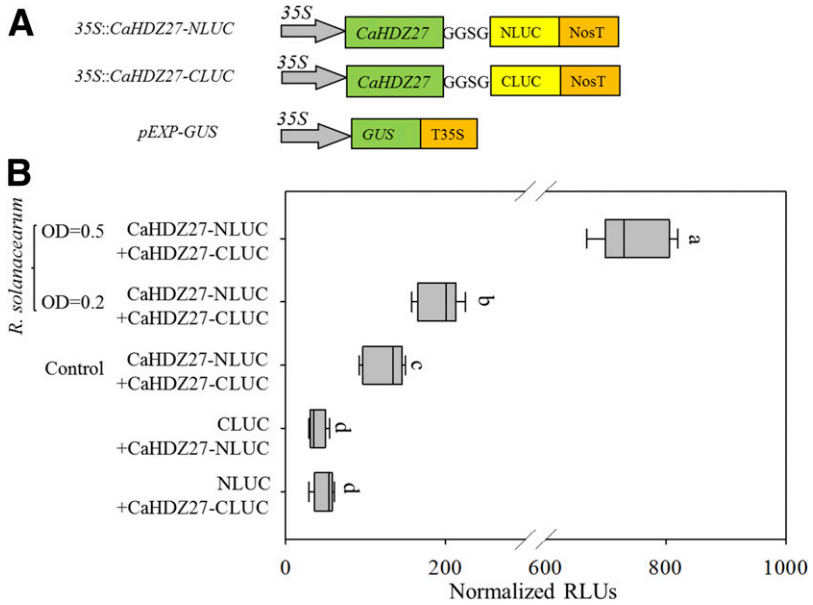

Fig. 8. Luminescence activity measurement assay to quantify the strength of CaHDZ27 homodimerization in pepper leaves inoculated with *Ralstonia solanacearum*. **A,** Schematic representation of 35S::CaHDZ27-NLUC and 35S::CaHDZ27-CLUC constructs for analysis of CaHDZ27 homodimerization. The pEXP-GUS construct was used for β-glucuronidase activity measurement, which was used to normalize the total protein concentration. **B,** Luminescence activity measurement analysis of CaHDZ27 homodimers in pepper leaves responding to *R. solanacearum* inoculation. Different letters represent significant differences as determined by the least significant difference test ($P < 0.05$). Bars represent medians, and boxes represent the 25th and 75th percentiles.

performed by pouring 10 ml of the 10^8-CFU/ml bacterial suspension into each pot. Before inoculation, roots were slightly damaged by making three holes in the soil. Then, the pots were kept in a growth room at 28°C with soil moisture >90%.

For RNA extraction, trypan blue and DAB staining, and ion conductivity experiments, plants were inoculated by infiltrating bacterial suspension (OD_{600} = 0.8) into the third leaf from the apical meristem, using a syringe without a needle, and mock control plants were inoculated with sterile 10 mM $MgCl_2$.

Treatment of plants with exogenous hormones.

Pepper plants at the six-leaf stage were used to investigate *CaHDZ27* expression patterns in response to exogenous application of hormones or growth regulators. Plants were sprayed with 0.1, 1, or 10 mM SA (dissolved in 10% ethanol), 10, 100, or 1,000 μM MeJA (dissolved in 10% ethanol), or 10, 100, or 1,000 μM ETH (dissolved in sterile distilled and deionized H_2O). Mock control plants were sprayed with 10% ethanol or sterile distilled and deionized H_2O.

Vector construction.

Vectors were constructed using a Gateway cloning technique (Invitrogen, Carlsbad, CA, U.S.A.), and a series of Gateway-compatible destination vectors were employed. The ORF of *CaHDZ27*, with or without the stop codon, was initially amplified by PCR with its specific primer pair (5′-ATGGAAGTTG GAGGAGTTATT-3′ and 5′-CTATTGCCAGAACCAACAAGG-3′) flanked with attB for Gateway cloning and GXL DNA polymerase (Takara, Osaka, Japan). The amplified product was confirmed by sequencing. The *CaHDZ27* ORF was cloned into the entry vector pDONR207, using the BP reaction and was then cloned into destination vectors such as pMDC83, pK7WG2, and pEarleyGate201, using the LR reaction for subcellularr localization, transient overexpression, or ChIP analysis, respectively.

DNA fragments, containing two copies of conserved HD-Zip I protein-binding *cis* element (CAATAATTG), or two copies of mutant element (CAGGGGTTG) and the 54-bp minimal CaMV 35S promoter flanked with *attB*, were synthesized by Convenience Corporation (Suzhou, China). These were cloned into the pMDC163 destination vector by sequential BP and LR reactions for promoter expression assays of the GUS reporter gene in pepper plants.

To construct the gene-silencing vectors for VIGS analysis, a fragment (approximately 185 bp) of the 3′ UTR of *CaHDZ27* was amplified by PCR with a specific primer pair (5′-TTCCA CAAGAGAATAGTG-3′ and 5′-GGAACAAAGCTAATAAA-3′). The fragment was cloned into the VIGS vector TRV2 (PYL279), using the Gateway cloning technique, as described above.

For construction of the BiFC vector, the *CaHDZ27* ORF without the stop codon was cloned into pDONR207, using the BP reaction. Then, the LR reaction was used to recombine the entry plasmids into the Gateway binary destination vectors puc-SPYNE^{GW} and puc-SPYCE^{GW}, which contain the N-terminal or C-terminal fragment of YFP (nYFP or cYFP, respectively). Thus, the CaHDZ27 protein was independently tagged with nYFP or cYFP at either the N or C terminus.

Split-LUC vectors were constructed by amplifying the *attB*-flanked *CaHDZ27* ORF without stop codon, using gene-specific primers. The PCR products were introduced into the satellite vector pDONR207. The *CaHDZ27* entry vector was transformed into the destination vectors (pDEST-^{GW}NLUC and pDEST-^{GW}CLUC) to generate *35S::CaHDZ27-NLUC* and *35S::CaHDZ27-CLUC*, respectively.

Transient expression of *CaHDZ27* in pepper leaves.

The 35S::*CaHDZ27* vector was transformed into *A. tumefaciens* GV3101. GV3101 cells carrying the *35S::CaHDZ27* vector were grown overnight in YEP medium with appropriate antibiotics. Cells were suspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 200 μM acetosyringone). The cell suspension (OD_{600} = 0.8) was infiltrated into leaves of pepper plants at the eight-leaf stage, using a syringe without a needle. The plants were kept in a growth room at 25°C, 60 to 70 mmol photons/m/s, 70% relative humidity, and a 16-h light and 8-h dark photoperiod, for 2 days before injected leaves were used for assays.

Tobacco transformation.

Transgenic tobacco plants overexpressing *CaHDZ27* were generated using *Agrobacterium*-mediated tobacco leaf disc transformation (Oh et al. 2005). Eight kanamycin-resistant lines of transgenic tobacco plants harboring the *35S::CaHDZ27* construct were selected and were confirmed with RT-PCR analysis. Seeds of T_1 plants were collected from regenerated T_0 plants, and seedlings of T_2 and T_3 lines were further selected on Murashige-Skoog agar plates containing 50 μg of kanamycin per milliliter. T_3 plants were used for analyses.

Subcellular localization of CaHDZ27.

The 35S::*CaHDZ27-GFP* and 35S::*GFP* constructs were transformed into *A. tumefaciens* GV3101 and were grown overnight. Then, GV3101 cells were suspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 200 μM acetosyringone) and were infiltrated into *N. benthamiana* leaves, using a syringe without a needle. For DAPI staining, *N. benthamiana* leaves were immersed in DAPI solution (0.1% DAPI and 5% dimethyl sulfoxide [DMSO]) and were incubated at 37°C for 1 h, before observation. GFP fluorescence was imaged using a laser scanning confocal microscope (TCS SP8; Leica, Solms, Germany) with an excitation wavelength of 488 nm and a 505 to 530 nm band-pass emission filter.

VIGS of *CaHDZ27* in pepper plants.

VIGS of *CaHDZ27* was performed according to a previously described method (Dang et al. 2013). GV3101 cells harboring *TRV1* (PYL192) with *TRV2* (PYL279) and *TRV2*::*CaHDZ27* (OD_{600} = 0.8) were mixed at a 1:1 ratio, and the mixtures were coinfiltrated into cotyledons of 2-week-old pepper seedlings. The seedlings were incubated at 16°C for 56 h and were then grown at 25°C.

Histochemical staining and ion conductivity measurement assay.

Histochemical staining and ion conductivity measurements were conducted using previously published methods (Choi et al. 2012). *Agrobacterium*-infiltrated leaves were stained with DAB for H_2O_2 detection and with trypan blue for cell-death detection. To quantify cell death in pepper leaves, ion conductivity was measured, using a Mettler Toledo 326 (Mettler, Zurich, Switzerland), as described previously (Cai et al. 2015).

Real-time qPCR.

The qPCR analysis was performed, using a Bio-Rad real-time PCR system (Foster City, CA, U.S.A.) and the SYBR Premix Ex Taq II system (Takara), as described previously (Cai et al. 2015; Dang et al. 2013). The expression of *CaHDZ27* and defense-related genes in pepper or tobacco plants were examined, using the primers listed in Supplementary Tables S3 and S4. Relative transcript levels were normalized with respect to the levels of *CaActin* and *18srRNA* in pepper or *NtActin* and *NtEE1α* in tobacco. Data were calculated by the by the $2^{-\Delta\Delta CT}$ method, as described previously (Livak and Schmittgen 2001). In each case, three technical replicates were performed for at least three independent biological replicates.

ChIP analysis.

ChIP assays were performed as described previously (Cai et al. 2015). The leaves of pepper plants at the eight-leaf stage were

infiltrated with *A. tumefaciens* GV3101 cells carrying the effect vector (35S::*CaHDZ27-HA*) and reporter vectors (2×*cis-p35Score*::*GUS*, 2×m*cis-p35Score*::*GUS*, and *p35Score*::*GUS*). T-DNA insertion into the pepper genome was confirmed. After 48 h, approximately 2 g of leaves were harvested and treated with either 10 mM bithionol sulfoxide or DMSO (solvent control) for 16 h and were then fixed with 1.0% formaldehyde for 5 min. The chromatin was isolated and sheared to an average length of 500 bp. Chromatin fragments that bound to CaHDZ27 were immunoprecipitated using the HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The resulting DNA fragments were isolated and were used as templates for PCR analysis with *attB*-specific primer pairs.

BiFC analysis.

BiFC was performed as described previously (Liu et al. 2016). *CaHDZ27-nYFP* and *CaHDZ27-cYFP* were introduced into *A. tumefaciens* GV3101, and cells carrying the BiFC constructs were infiltrated into *N. benthamiana* leaves. After 48 h, leaf fluorescence was analyzed using a confocal microscope.

CoIP assay.

The *35S*::*CaHDZ27-FLAG* and *35S*::*CaHDZ27-HA* constructs were transformed into *A. tumefaciens* GV3101, and cells carrying *35S::CaHDZ27-FLAG* or *35S::CaHDZ27-HA* were coinfiltrated into *N. benthamiana* leaves at a ratio of 1:1. The Co-IP assay was performed as described previously (Liu et al. 2016).

LUC activity determination.

GV3101 cells carrying the appropriate split-LUC constructs were to infiltrate pepper leaves. For coexpression analyses, cells carrying the different vectors were mixed before infiltration. GV3101 cells carrying the appropriate split-LUC constructs were infiltrated by injecting 5-week-old pepper leaves. After 1 day, the infiltrated leaves were inoculated with 20 µl of *R. solanacearum* cells, and leaves were harvested for LUC activity assay after 1 day. LUC activity reconstituted from the *35S*::*CaHDZ27-NLUC* and *35S*::*CaHDZ27-CLUC* vectors was determined as described previously (Liu et al. 2016).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

cNLS Mapper open source software:
http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi