CaWRKY28 Cys249 is Required for Interaction with CaWRKY40 in the Regulation of Pepper Immunity to *Ralstonia solanacearum*

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WRKY transcription factors have been implicated in plant response to pathogens but how WRKY-mediated networks are organized and operate to produce appropriate transcriptional outputs remains largely unclear. Here, we identify a member of the WRKY family from pepper (*Capsicum annuum*), CaWRKY28, that physically interacts with CaWRKY40, a positive regulator of pepper immunity and thermotolerance. We confirmed CaWRKY28–CaWRKY40 interaction by coimmunoprecipitation, bimolecular fluorescence complementation, and microscale thermophoresis. Our findings supported the idea that CaWRKY28 is a nuclear protein that acts as positive regulator in pepper responses to infection by the pathogenic bacterium *Ralstonia solanacearum*. It performs its function not by directly modulating the W-box containing immunity-related genes but by promoting CaWRKY40 via physical interaction to bind and activate its immunity-related target genes, including *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI*, but not its thermotolerance-related target gene, *CaHSP24*. All of these data indicate that CaWRKY28 interacts with and potentiates CaWRKY40 in regulating immunity against *R. solanacearum* infection but not thermotolerance. Importantly, we discovered that CaWRKY28 Cys249, shared by CaWRKY28 and its orthologs probably only in the family Solanaceae, is crucial for the CaWRKY28–CaWRKY40 interaction. These results highlight how CaWRKY28 associates with CaWRKY40 during the establishment of WRKY networks, and how CaWRKY40

achieves its functional specificity during pepper responses to *R. solanacearum* infection.

Keywords: *Capsicum annuum*, CaWRKY28, CaWRKY40, defense signaling pathways, pathogens, *Ralstonia solanacearum*, RSI, transpiration

Numerous studies have demonstrated that the plant immune response against pathogens is regulated largely at the transcriptional level and relies on the action of various transcription factors (TFs). By targeting specific promoters and regulating the underlying loci, TFs activate a massive transcriptional reprogramming leading to an effective immune response. To generate appropriate immune outputs, TFs form highly interconnected transcriptional networks (Birkenbihl et al. 2017b; Tsuda and Somssich 2015). However, how these networks are organized and operate in response to different pathogens remains poorly understood.

WRKY TFs constitute one of the largest families of plant TFs, and have been intensively studied over the past two decades (Amorim et al. 2017; Birkenbihl et al. 2017b; Pandey and Somssich 2009). Members of this family contain one or two WRKY domains, consisting of the highly conserved motif WRKYGQK at their N terminus, together with a novel zinc-finger-like motif (Eulgem et al. 2000; Zhang and Wang 2005). WRKY TFs bind a highly conserved cis-regulatory element, W-box (TTGACC/T), in the promoters of their target genes, thereby regulating a wide array of biological processes including growth, development, and defense response to environmental stresses. The functional specificities of WRKY TFs are determined, on the one hand, by their structures—indeed, variations in the conserved WRKY domains (such as WRKYGEK and WRKYGKK) or in the zinc-finger-like motifs (Eulgem et al. 2000; Zhang and Wang 2005)—and, on the other hand, by the structure of DNA motifs (Kanofsky et al. 2017; Liu et al. 2015; Machens et al. 2014) or their adjacent bases (Ciolkowski et al. 2008). In addition, the specific targeting and, thus, the functions of WRKY TFs may also be modulated by other proteins, including WRKY TFs, via protein–protein interaction (Alves et al. 2014; Chi et al. 2013). Collectively, the above factors all contribute to the complex regulation of the functional specificity associated with a given WRKY TF, which remains poorly understood.

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One of the most essential roles of WRKY TFs is the regulation of plant defense in response to pathogen infection. Most genes encoding WRKY TFs identified in the genome of a given plant species are induced at the transcriptional level by the attack of a single pathogen (Dong et al. 2003; Pandey and Somssich 2009; Segonzac et al. 2017). These WRKY TFs are believed to assemble WRKY networks in defense signaling (Eulgem and Somssich 2007), although only a few cases have been described to date (Abeyasinghe et al. 2019; Liu et al. 2012; Xu et al. 2006). Relying on a complex network of proteins rather than a single linear pathway may grant the plant immune system a greater resistance against suppression by effectors. However, several WRKY TFs may be protected in some host plants by quickly evolving resistance proteins (Le Roux et al. 2015; Sarris et al. 2015). How these WRKY networks are organized and operate is currently largely unclear.

The Solanaceae family comprises 3,000 to 4,000 species classified into approximately 90 genera, including economically important crops such as tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and pepper (*Capsicum annuum*) (Gebhardt 2016). These crop species are very often exposed to, and infected by, soilborne pathogens such as the bacterium *Ralstonia solanacearum*, which causes plant wilt resulting in substantial production loss (Lebeau et al. 2011; Mamphogoro et al. 2020; Thera et al. 2010). Despite differences in the evolution of their chromosomes, genomes (Wu and Tanksley 2010), and transcriptomes (Rensink et al. 2005), most genes exhibit a high degree of sequence conservation within the Solanaceae family (Rensink et al. 2005). We previously determined that a subset of WRKY TFs was induced in pepper plants in response to *R. solanacearum* infection in a deep sequencing of the transcriptome (RNA-sequencing) dataset (data in the China National GeneBank). In particular, the pepper WRKY family members *CaWRKY6*, *CaWRKY22*, *CaWRKY27*, *CaWRKY40*, *CaWRKY40b*, and *CaWRKY58* are involved in the immune response of pepper plants to *R. solanacearum*, during which they act as positive or negative regulators (Cai et al. 2015; Dang et al. 2013; Hussain et al. 2018; Ifnan Khan et al. 2018; Wang et al. 2013). *CaWRKY40*, a positive regulator in pepper response to *R. solanacearum* infection (RSI) that is targeted and transcriptionally regulated by *CaWRKY6*, *CabZIP63*, and *CaWRKY40b*, positively regulates *CaWRKY22* and *CaWRKY27* and forms positive feedback loops with *CaCDPK15* and *CabZIP63* (Shen et al. 2016a, b). In addition, *CaWRKY40* exhibits an autoregulation capability by targeting the double-W box, which is highly conserved in the promoter of *CaWRKY40* and its orthologs in other plant species, during response to pathogen attack (Liu et al. 2020). All of these results highlight a role of *CaWRKY40* as central node in the WRKY TF networks and in the regulation of transcriptional reprogramming in pepper immunity against *R. solanacearum* infection. Interestingly, the majority of these WRKY TFs also participate in pepper responses to high-temperature and high-humidity stress (HTHH) (Cai et al. 2015; Dang et al. 2013; Hussain et al. 2018; Ifnan Khan et al. 2018; Wang et al. 2013), suggesting a close link between pepper resistance to *R. solanacearum* infection and HTHH. We speculate that WRKYs may interact with other proteins to achieve their specific functions in distinct contexts. In the present study, we characterized a set of proteins that interact with pepper *CaWRKY40*, which acts as a critical node in the WRKY network involved in responses to both *R. solanacearum* infection and thermotolerance under high humidity (Dang et al. 2013). These putative interaction partners were isolated by pull-down assays, followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The other pepper WRKY TF, *CaWRKY28*, is one such *CaWRKY40* interactor and is the focus of the present study. We further reveal that Cys249 in *CaWRKY28* is critical for its

interaction with and activation of *CaWRKY40* and, specifically, for the regulation of pepper immunity against *R. solanacearum*.

RESULTS

CaWRKY28 interacts with CaWRKY40 in pepper plants challenged by *R. solanacearum* infection.

CaWRKY40 is a positive regulator of pepper responses to *R. solanacearum* infection as well as to thermotolerance in high humidity conditions (Dang et al. 2013). Because the molecular underpinnings of plant immunity and thermotolerance are likely to be different, we speculated that *CaWRKY40* function might be modulated by specific interaction partners in order to achieve its role in response to *R. solanacearum* infection. We undertook a pull-down approach to identify *CaWRKY40* interactors; fusion protein *CaWRKY40*-glutathione-S-transferase (GST) was expressed from *Escherichia coli* BL21 (DE3), which was mixed with the proteins isolated from *R. solanacearum*-inoculated pepper leaves. The mixed proteins were purified using BeaverBeads GSH and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate and examine the possible interacting proteins of WRKY40. The possible interactors were cut off for the LC-MS/MS assay (Pennington et al. 2016). Among candidate interacting proteins, we identified a putative WRKY protein, whose deduced amino acid sequence (GenBank ID: KAF3622752.1) exhibited the highest similarity to WRKY28 in *Arabidopsis thaliana*. Therefore, we designated the encoding gene *CaWRKY28* (renamed from *WRKY23* from the pepper genome project). *CaWRKY28* also showed high sequence similarity with putative orthologs in other Solanaceae species, including *NtWRKY71* in tobacco, *SlWRKY71* in tomato, *StWRKY71* in potato (*S. tuberosum*), and *NsWRKY71* in *N. sylvestris*. On the basis of the presence of a single and highly conserved WRKY domain and zinc-finger domain (C-X4-C-X23-H-X1-H), *CaWRKY28* is a member of the group II WRKY proteins in pepper. However, the presence of a conserved Cys249 residue in *CaWRKY28* and its Solanaceae orthologs, but not in other plant species such as *AtWRKY28* or *OsWRKY28*, also gives *CaWRKY28* and Solanaceae orthologs attributes of group III WRKY members (Supplementary Fig. S1).

Confirmation of the CaWRKY28–CaWRKY40 interaction.

To confirm the interaction between *CaWRKY28* and *CaWRKY40*, we first performed a coimmunoprecipitation (Co-IP) assay. For the Co-IP assay, the total protein was isolated from pepper leaves coinfiltrated with *Agrobacterium tumefaciens* strain GV3101 cells containing 35S:*CaWRKY28*-FLAG and 35S:*CaWRKY40*-HA (at a 1:1 ratio) at 48 h postinfiltration (hpi), and was incubated with antibody of HA to immunoprecipitate *CaWRKY40*-HA. The presence of *CaWRKY28*-FLAG in copurified proteins was tested by immunoblotting with an anti-FLAG antibody. This analysis confirmed that *CaWRKY28* interacts with *CaWRKY40* (Fig. 1A). In addition, we determined whether *CaWRKY28* and *CaWRKY40* interacted in planta by a bimolecular fluorescence complementation (BiFC) assay, for which *CaWRKY28* was fused to the N terminus of the yellow fluorescent protein (YFP) (*CaWRKY28*-YFP^N), while *CaWRKY40* was fused to the C terminus of YFP (*CaWRKY40*-YFP^C). We also generated the reciprocal set of constructs (*CaWRKY28*-YFP^C and *CaWRKY40*-YFP^N). We tested for the interaction between the fusion proteins in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression at 48 hpi. Indeed, we detected YFP fluorescence in plants coexpressing both *CaWRKY28* and *CaWRKY40* fusion proteins but not in control plants. In addition, we observed YFP fluorescence only in the nucleus, as expected for TFs (Fig. 1B). In a final experiment, we further confirmed the interaction between *CaWRKY28*

Fig. 1. CaWRKY28 interacts with CaWRKY40 in vivo and in vitro. **A**, Interaction between CaWRKY28 and CaWRKY40 in vivo, as determined by a coimmunoprecipitation assay. Proteins were isolated from pepper leaves transiently overexpressing *CaWRKY28-Myc* and *CaWRKY40-FLAG*, and *CaWRKY40-FLAG* was immunoprecipitated with anti-FLAG antibody. **B**, Bimolecular fluorescence complementation confirmation of the interaction between CaWRKY28 and CaWRKY40 in *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* strain GV3101 strain cells bearing *CaWRKY28-YFP^C* + *CaWRKY40-YFP^N* or *CaWRKY28-YFP^N* + *CaWRKY40-YFP^C* constructs. YFP = yellow fluorescent protein. Yellow fluorescence, visible light, and merged images were taken by a confocal microscope at 48 h postinfiltration. Bars = 25 μ m. **C**, Analysis of the interaction in vitro between CaWRKY28 and CaWRKY40 by microscale thermophoresis. Green fluorescent protein (GFP) or CaWRKY28-GFP:target, CaWRKY40-glutathione-S-transferase (GST):ligand. Mixtures of GFP and CaWRKY40-GST or CaWRKY28-GFP and CaWRKY40-GST were loaded onto Monolith NT.115 Capillaries and were measured using 50% IR laser power and an LED excitation source with λ = 470 nm at ambient temperature.

and CaWRKY40 by performing a microscale thermophoresis (MST) assay. We fused green fluorescent protein (GFP) to the C-terminus of CaWRKY28 to provide the fluorescent label for the method (CaWRKY28-GFP), and purified the fusion protein from protein extracts of pepper leaves transiently overexpressing *CaWRKY28-GFP* via *Agrobacterium*-mediated infiltration. In parallel, we purified CaWRKY40-GST from *E. coli* strain BL21 (DE3) soluble extracts. We serially diluted the purified CaWRKY40-GST fusion proteins, then mixed them with a constant amount of CaWRKY28-GFP before subjecting the mixtures to the MST assay. Based on the data from the serial dilution assay, the dissociation constant (Kd) of the CaWRKY40-GST/CaWRKY28-GFP was calculated. The CaWRKY28–CaWRKY40 pair produced a clear binding curve, with a K_d of 6.45221×10^{-09} mol/liter (Fig. 1C). Collectively, these results strongly argue that CaWRKY28 interacts with CaWRKY40.

Induction of *CaWRKY28* expression by *R. solanacearum* infection and a subset of phytohormones in a CaWRKY40 dependent manner.

We have previously shown that CaWRKY40 is transcriptionally upregulated by *R. solanacearum* infection and by exogenous application of salicylic acid (SA), methyl jasmonate (MeJA), ethephon (ETH), or abscisic acid (ABA), and acts as positive regulator in pepper responses to *R. solanacearum* infection and to thermotolerance at high humidity (Dang et al. 2013). Because CaWRKY28 interacts with CaWRKY40, we speculated that CaWRKY28 might behave similarly to CaWRKY40 in response to the same stimuli. To test this possibility, we measured *CaWRKY28* transcript levels in pepper plants challenged with *R. solanacearum* infection or subjected to an exogenous application of SA, MeJA, ETH, or ABA by real-time quantitative PCR (RT-qPCR). *CaWRKY28* transcript levels rose upon *R. solanacearum* infection, as well as following SA and ETH treatment, as previously observed for *CaWRKY40*. However, we detected no changes after the exogenous application of MeJA or ABA, indicating that *CaWRKY28* and *CaWRKY40* expression patterns do not always respond to the same stimuli in the same manner (Supplementary Fig. S2). Because W-box elements are present in the promoters of both *CaWRKY28* and *CaWRKY40*, we performed a chromatin immunoprecipitation (ChIP)-PCR assay to determine whether CaWRKY28 and CaWRKY40 might regulate the expression of their encoding genes, reciprocally or not. We used chromatin purified from pepper leaves transiently overexpressing *CaWRKY28-HA* or *CaWRKY40-HA* with an anti-HA antibody, and discovered that CaWRKY40 directly bound to the *CaWRKY28* promoter; however, the converse was not true (Supplementary Fig. S4A). We then measured *CaWRKY28* and *CaWRKY40* transcript levels in pepper leaves transiently overexpressing *CaWRKY28* or *CaWRKY40* by RT-qPCR. *CaWRKY28* was upregulated by *CaWRKY40* transient overexpression but *CaWRKY40* was not transcriptionally regulated by *CaWRKY28* transient overexpression (Supplementary Fig. S4B), indicating that *CaWRKY28* is upregulated by *R. solanacearum* in a CaWRKY40-dependent manner, because *CaWRKY40* is also induced by *R. solanacearum* infection (Dang et al. 2013).

Nuclear protein CaWRKY28 acts as a positive regulator in pepper immunity against *R. solanacearum* infection.

We transiently overexpressed *CaWRKY28-GFP* in *N. benthamiana* leaves by *Agrobacterium*-mediated infiltration. We visualized GFP fluorescence at 48 hpi using a confocal microscope (TCS SP8; Leica Microsystems, Germany). We observed free GFP, derived from 35S::GFP, in the cytoplasm and nuclei of infiltrated epidermal cells, whereas GFP fluorescence for CaWRKY28-GFP was restricted to the nucleus (Supplementary Fig. S3), indicating that CaWRKY28 is a nuclear protein.

Because *CaWRKY28* is regulated by *R. solanacearum* infection, we reasoned that it might be involved in pepper immunity against the bacterium. To test this hypothesis, we studied the effect of virus-induced *CaWRKY28* silencing on pepper immunity against RSI. We constructed a vector with a specific fragment in the 3' untranslated region (UTR) of *CaWRKY28* (approximately 200 bp in length), whose specificity was confirmed by blasting against the genome of pepper. With this vector, we successfully and specifically silenced *CaWRKY28* in pepper plants, achieving an over 90% reduction in *CaWRKY28* transcript levels, while maintaining normal transcript levels of *CaWRKY71*, which exhibits the highest sequence similarity to *CaWRKY28* among all of the pepper WRKY genes (Fig. 2A). Upon challenge by *R. solanacearum* infection, *CaWRKY28*-silenced pepper plants exhibited an increased susceptibility to the *R. solanacearum*, as indicated by the more severe bacterial wilt symptoms and greater *R. solanacearum* population growth (in CFU) in these silenced plants as compared with the control plants. Consistent with these results, genes encoding pathogenesis-related (PR) proteins such as *CaPR1* (Pan et al. 2010; Zhang et al. 2012), NONEXPRESSOR OF PR GENES 1 (*CaNPR1*) (Chen et al. 2009; Zhang et al. 2012), DEFENSIN 1 (*CaDEF1*) (Choi et al. 2008, 2015; Kim et al. 2015; Zhang et al. 2019), and ABSCISIC ACID-RESPONSIVE 1 (*CaABR1*) (Choi and Hwang 2011) showed much lower expression levels in *CaWRKY28*-silenced pepper plants relative to mock-treated

control plants (Fig. 2). The SRDX domain was used to transform *CaWRKY28* into dominant-negative repressor versions and has been widely used to assess the roles of TFs (Grunewald et al. 2013; Takada 2013). In comparison, we characterized the effects associated with the transient overexpression of *35S:CaWRKY28-HA* or its chimeric repressor version *35S:CaWRKY28-SRDX-HA* on hypersensitive reaction (HR)-like cell death and the transcript levels of immunity-associated genes. We infiltrated *A. tumefaciens* strain GV3101 cells bearing the *35S:CaWRKY28-HA* or *35S:CaWRKY28-SRDX-HA* construct (*35S:HA* acting as negative control) into the leaves of pepper plants. We first confirmed that the constructs, indeed, led to the accumulation of the tagged *CaWRKY28* by performing RT-qPCR (for the transcripts) and immunoblot analysis with protein extracts from pepper leaves transiently overexpressing *CaWRKY28-HA* or *CaWRKY28-SRDX-HA* using an anti-HA antibody (for the proteins). Both *CaWRKY28-HA* and *CaWRKY28-SRDX-HA* appeared to successfully accumulate in pepper leaves (Supplementary Fig. S5A). Overexpression of *CaWRKY28-HA* caused extensive HR-like cell death, accompanied by increased leaf ion leakage, as illustrated by higher conductivity. By contrast, neither *CaWRKY28-SRDX-HA* nor the control triggered cell death (Supplementary Fig. S5B to D). In agreement, the transient overexpression of *CaWRKY28-HA* significantly raised the transcript levels of *CaPR1*, *CaNPR1*, *CaDEF1*, and *CaABR1*, whereas the transient overexpression of *CaWRKY28-SRDX-HA* had the opposite effect, when compared

Fig. 2. Effect of *CaWRKY28* silencing on the response of pepper plants to *Ralstonia solanacearum* infection. **A**, Relative transcript levels of *CaWRKY28* and *CaWRKY71* in TRV:CaWRKY28 pepper plants challenged with *R. solanacearum* infection (RSI). **B**, Effect of *CaWRKY28* silencing on *R. solanacearum* growth, shown as CFU; hpi = hours postinfiltration. **C**, Bacterial wilt symptoms of pepper plants (*CaWRKY28*-silenced and control) challenged by RSI at 7 days postinoculation (dpi). **D**, Relative transcript levels of the immunity-associated genes *CaPR1*, *CaNPR1*, *CaDEF1*, and *CaABR1* in pepper plants (*CaWRKY28*-silenced and control) challenged by RSI. Data are shown as means $\pm$ standard error of four replicates (A, B, and D). Different uppercase letters above the bars indicate significant differences between means ($P < 0.01$) by Fisher's protected least significant difference test.

with the control plants (Supplementary Fig. S5E). All of these results indicate that CaWRKY28 acts positively in pepper responses to *R. solanacearum* infection.

Dual transient overexpression of *CaWRKY28* and *CaWRKY40* additively triggers defense response in pepper leaves.

To determine the effect of the interaction between CaWRKY28 and CaWRKY40 on pepper immunity, we transiently overexpressed both *CaWRKY28-HA* and *CaWRKY40-HA* in pepper plant leaves, and recorded the consequences of their dual transient overexpression on pepper defense response. We first confirmed that the two proteins successfully accumulated by immunoblot analysis with an anti-HA antibody (Supplementary Fig. S6A), and that the cotransient accumulation of *CaWRKY28* and *CaWRKY40* induced a stronger HR-like cell death response (Supplementary Fig. S6B), more pronounced ion leakage as measured by conductivity (Supplementary Fig. S6C), as well as higher transcript levels of *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI*, when compared with the transient accumulation of *CaWRKY28* or *CaWRKY40* alone (Supplementary Fig. S6D). Therefore, these results suggest that CaWRKY28 and CaWRKY40 synergistically act in pepper immunity.

CaWRKY40 silencing blocks *CaWRKY28*-mediated immunity but the converse is not true.

To further catalog the effects linked to the functional association of CaWRKY28 and CaWRKY40 during pepper immunity, we measured the effects of silencing *CaWRKY40* on the HR-like cell death triggered by transient overexpression of *CaWRKY28-HA*. We first determined that CaWRKY28 successfully accumulated during *CaWRKY28-HA* transient overexpression in pepper leaves and that *CaWRKY40* was efficiently silenced by virus-induced gene silencing (VIGS), by carrying out an immunoblot analysis with an anti-HA antibody (for the detection of CaWRKY28 protein) and by RT-qPCR (for *CaWRKY28* transcripts) (Fig. 3A and C). The transient overexpression of *CaWRKY28-HA* in leaves triggered intensive HR-like cell death in the control plants (with normal *CaWRKY40* levels) but not in the *CaWRKY40*-silenced plants (Fig. 3B). Consistent with these observations, transient overexpression of *CaWRKY28-HA* in the leaves of control plants led to upregulations of the immunity-related genes *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI*; however, these upregulations were almost completely abolished upon *CaWRKY40* silencing (Fig. 3C). Taking a reciprocal approach, silencing *CaWRKY28* significantly attenuated but did not completely block CaWRKY40 function in pepper immunity (Fig. 3B and C). These results indicate that CaWRKY28 functions in pepper immunity in a CaWRKY40-dependent manner, while the function of CaWRKY40 is potentiated by CaWRKY28.

CaWRKY28 targets and regulates *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI* in planta in a CaWRKY40-dependent manner.

Because CaWRKY28 acts as a regulator of the expression of *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI* (Supplementary Figs. S5E and S6D), whose promoters all contain more than one W-box, it stands to reason that CaWRKY28 might regulate these genes directly. To test this possibility, we performed a ChIP-PCR assay using chromatin isolated from pepper leaves infiltrated with *A. tumefaciens* strain GV3101 containing 35S:*CaWRKY28-HA* at 48 hpi and appropriate primers of W-box containing promoter fragments. All primer pairs produced clear PCR products, while primer pairs designed against another promoter fragment lacking the W-box did not result in a product (Fig. 3D), indicating that CaWRKY28 likely binds to the promoters of the four immunity-related genes tested here in a W-box-dependent manner. To

determine whether CaWRKY40 might contribute to the targeting of these target genes by CaWRKY28, we isolated chromatin from *CaWRKY40*-silenced pepper leaves transiently overexpressing *CaWRKY28-HA* for use as template for ChIP-PCR. Satisfyingly, *CaWRKY40* silencing almost completely blocked the enrichment of CaWRKY28 at the tested promoters. Using the reciprocal approach, we discovered that silencing *CaWRKY28* significantly decreased but did not block the enrichment of CaWRKY40 at the promoters of the tested *PR* genes, whether or not pepper plants were challenged by *R. solanacearum* infection (Fig. 3E and F). Based on these results, we hypothesize that CaWRKY28 may fulfill its function in pepper immunity by potentiating CaWRKY40 to directly target and regulate immunity-related genes such as *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI*.

Cys249 is required for the function of CaWRKY28 in HR-like cell death induction.

Although it belongs to the group II WRKY proteins, CaWRKY28 also shares characteristics of group III WRKYs due to the presence of the Cys249 residue, which is found only in CaWRKY28 orthologs from the Solanaceae family, and is replaced by isoleucine (Ile) or threonine (Thr) in its *Arabidopsis* and rice orthologs, respectively. This conserved residue piqued our interest. To test whether this residue played a role in CaWRKY28 function during the regulation of pepper immunity, we replaced Cys249 (UGC) with Thr, as in *Arabidopsis*, generating CaWRKY28^{C249W} (Fig. 4A). We infiltrated pepper leaves with *A. tumefaciens* strain GV3101 cells carrying 35S:*CaWRKY28-HA*, 35S:*CaWRKY28^{C249W}-HA*, or 35S:*HA* (as control) constructs. The transient overexpression of *CaWRKY28-HA* triggered intensive HR-like cell death, as expected, whereas neither *CaWRKY28^{C249W}-HA* transient overexpression nor the control resulted in cell death (Fig. 4B). Just like CaWRKY28, the mutated version CaWRKY28^{C249W} accumulated in the nucleus, indicating that Cys249 does not contribute to the subcellular localization of CaWRKY28 (Fig. 4C). In addition, we tested the binding of CaWRKY28^{C249W} to the W-box in an electrophoretic mobility shift assay (EMSA) by using a CaWRKY28^{C249W}-GST (Fig. 4D), wild-type probe, mutated probe, and unlabeled probe (Fig. 4E). We observed a clear interaction (as indicated by the mobility shift) between CaWRKY28-GST or CaWRKY28^{C249W}-GST and the W-box-containing probe but not to the mutated probe (Fig. 4F), indicating that CaWRKY28^{C249W} retains the ability to bind the W-box in vitro. To further characterize the role of the Cys249 residue in the binding of CaWRKY28 to the W-box in planta, we performed a ChIP-PCR assay to compare the binding affinity of CaWRKY28 and CaWRKY28^{C249W} to the *CaPRI*, *CaNPRI*, *CaDEF1*, and *CaABRI* promoters, using chromatin isolated from pepper leaves transiently overexpressing *CaWRKY28* or *CaWRKY28^{C249W}*. Primer pairs for all four tested genes produced clear PCR products when using chromatin isolated from pepper leaves by transient overexpression of *CaWRKY28* but not *CaWRKY28^{C249W}*, indicating that CaWRKY28^{C249W} cannot bind to the W-box in vivo (Supplementary Fig. S7A). In agreement with this observation, transient overexpression of *CaWRKY28* increased the transcript levels of the four immunity-related genes but transient overexpression of *CaWRKY28^{C249W}* did not (Supplementary Fig. S7B). Together, these data indicate that Cys249 plays a role in the function of CaWRKY28 during pepper immunity.

CaWRKY28–CaWRKY40 interaction, mediated by Cys249, prevents CaWRKY28 from binding the W-box in planta.

To further clarify the possible role of Cys249 in CaWRKY28 function in the context of pepper immunity, we assayed the

Fig. 3. CaWRKY28 and CaWRKY40 both contribute to regulating pepper immunity. **A**, Accumulation of CaWRKY28-HA and CaWRKY40-HA in pepper leaves transiently overexpressing CaWRKY28-HA or CaWRKY40-HA, in the context of wild-type plants (pTRV:00) or plants silenced for CaWRKY28 (pTRV:CaWRKY28) or CaWRKY40 (pTRV:CaWRKY40), by immunoblot analysis with anti-HA antibody. EV = empty vector. **B**, Effect of CaWRKY28 or CaWRKY40 silencing on hypersensitive reaction-like cell death phenotypes induced by transient overexpression of CaWRKY28 or CaWRKY40. **C**, Effect of CaWRKY28 or CaWRKY40 silencing in pepper plants transiently overexpressing CaWRKY28 or CaWRKY40 on the relative transcript levels of immunity-related genes. **D**, Enrichment of CaWRKY28 at the promoters of immunity-related marker genes, as determined by chromatin immunoprecipitation PCR. **E**, Effect of CaWRKY28 silencing on the enrichment of CaWRKY40 at the promoters of immunity-related marker genes. **F**, Effect of CaWRKY40 silencing on the enrichment of CaWRKY28 at the promoters of immunity-related marker genes. Enrichment of the control was set to 1 after normalization by input (E and F). Data are shown as means $\pm$ standard error of four replicates (C, E, and F). Different uppercase letters above the bars indicate significant differences between means ($P < 0.01$) by Fisher's protected least significant difference test.

interaction between CaWRKY28^{C249W} or CaWRKY28 and CaWRKY40 by BiFC assay. CaWRKY28 interacted with CaWRKY40, while CaWRKY28^{C249W} did not (Fig. 5A). To further confirm this result, we performed an MST assay with CaWRKY40-GST purified from *E. coli* strain BL21 (DE3) and CaWRKY28-GFP or CaWRKY28^{C249W}-GFP (isolated from the leaves of pepper plants transiently overexpressing CaWRKY28-GFP or CaWRKY28^{C249W}-GFP, respectively, by immunoprecipitation with an anti-GFP antibody). Our results showed that CaWRKY28-GFP and CaWRKY40-GST produced a clear binding curve, consistent with physical interaction of the two proteins, but CaWRKY28^{C249W}-GFP and CaWRKY40-GST did not (Fig. 5B). These data indicate that Cys249 is crucial for the interaction of CaWRKY28 and CaWRKY40.

To determine whether CaWRKY40 might influence the DNA binding activity of CaWRKY28 by the formation of a CaWRKY28–CaWRKY40 complex, we turned to EMSA with CaWRKY40m, a mutant version of CaWRKY40 that cannot bind to the W-box (Shen et al. 2020). Using CaWRKY28^{C249W}-GST purified from *E. coli* strain BL21 (DE3) and the W-box-containing wild-type probe, we detected a mobility shift when CaWRKY28^{C249W}-GST was incubated with excess CaWRKY40, indicating that CaWRKY28^{C249W} can bind to the W-box (Fig. 5C), although it does not interact with CaWRKY40 (Fig. 5B). By contrast, we failed to detect a mobility shift when CaWRKY28 was presented with the W-box-containing wild-type probe and incubated with excess CaWRKY40m, although both recombinant proteins do interact (Fig. 5C; Supplementary Fig. S8). These results indicate that the interaction between CaWRKY28 and CaWRKY40, which is

determined by Cys249 in CaWRKY28, prevents CaWRKY28 from binding to the W-box containing DNA sequences. To test the effect of the CaWRKY28–CaWRKY40 interaction on the DNA binding potential of CaWRKY28, we next compared the effect of CaWRKY28 (or CaWRKY28^{C249W}) transient overexpression with or without CaWRKY40 transient overexpression in CaWRKY40-silenced pepper leaves on the enrichment of CaWRKY40 at the *CaPRI*, *CaNPR1*, *CaDEF1*, and *CaABR1* promoters by ChIP-qPCR. Note that the VIGS construct targets 3'UTR of *CaWRKY40*; therefore, it is possible to overexpress CaWRKY40 from a heterologous construct bearing a foreign transcription terminator. The enrichment of CaWRKY28 at the promoters of the four tested genes was increased by cotransient overexpression of CaWRKY40 and CaWRKY28 but not by cotransient overexpression of CaWRKY40 and CaWRKY28^{C249W} (Fig. 5D), indicating that the targeting of CaWRKY28 to the four tested immunity-related genes is potentiated by CaWRKY40 when the two proteins interact.

Modulation of thermotolerance-related *CaHSP24* by CaWRKY40 is not regulated by CaWRKY28.

Our results thus far show that CaWRKY40 acts as a positive regulator of pepper immunity and thermotolerance (Dang et al. 2013); CaWRKY40 function during infection by *R. solanacearum* is positively modulated by CaWRKY28, inviting the following question: does CaWRKY28 also modulate the function of CaWRKY40 in the regulation of thermotolerance? Our results showed that transient overexpression of CaWRKY28-HA did not alter the expression of the small heat shock protein gene *CaHSP24* (Supplementary Fig. S9A). Conversely, the silencing

Fig. 4. Cys249 in CaWRKY28 is necessary for the regulation of pepper immunity. **A**, Cys249 (codon UGC) in CaWRKY28 was mutated to Trp249 (codon UGG) (CaWRKY28^{C249W}). **B**, Hypersensitive reaction-like cell death phenotype triggered by transient overexpression of CaWRKY28 and CaWRKY28^{C249W} at 48 h postinfiltration. EV = empty vector. **C**, Nuclear localization of the CaWRKY28^{C249W} in *Nicotiana benthamiana* epidermal cells. GFP = green fluorescent protein and DAPI = 4',6-diamidino-2-phenylindole. **D**, Wild-type probe and its mutant version for electrophoretic mobility shift assay (EMSA). **E**, Purification of CaWRKY28-glutathione-S-transferase (GST) and CaWRKY28^{C249W}-GST protein. **F**, Binding of CaWRKY28 and CaWRKY28^{C249W} to W-box-containing probe by EMSA.

of *CaWRKY28* did not affect the upregulation of *CaHSP24* by transient overexpression of *CaWRKY40-HA* (Supplementary Fig. S9B). We also did not observe any synergistic effect on *CaHSP24* transcript levels when both *CaWRKY28-HA* and *CaWRKY40-HA* were transiently overexpressed, relative to *CaWRKY40-HA* transient overexpression alone (Supplementary Fig. S9C). These data indicate that *CaWRKY28* does not potentiate *CaWRKY40* in its modulation of *CaHSP24* expression.

DISCUSSION

The majority of WRKY family members have been implicated in immune responses against attacks from various pathogens in different plant species (Dong et al. 2003; Pandey and Somssich 2009; Segonzac et al. 2017). WRKYs have been suggested to

organize into interconnected WRKY networks (Eulgem and Somssich 2007). However, how these WRKY TFs build such networks and how these networks operate to produce appropriate transcriptional outputs remain poorly understood. In the present study, we provide evidence that, by interacting with *CaWRKY40*, *CaWRKY28* acts as a positive regulator in pepper response to *R. solanacearum* infection by potentiating the targeting and regulation of immunity-related genes by *CaWRKY40*, rather than by directly targeting immunity genes on its own. Moreover, Cys249 in *CaWRKY28* is critical for its interaction with *CaWRKY40*.

CaWRKY28 acts as positive regulator in pepper immunity during *R. solanacearum* infection.

The evidence that *CaWRKY28* acts as a positive regulator in pepper immunity in response to *R. solanacearum* infection

Fig. 5. *CaWRKY28* Cys249 is critical for the protein's interaction with *CaWRKY40*, DNA binding, and transcriptional activation of target genes. **A**, Bimolecular fluorescence complementation assay of the interaction between *CaWRKY28* or *CaWRKY28*^{C249W} and *CaWRKY40* in *Nicotiana benthamiana* leaves. YFP = yellow fluorescent protein. **B**, Microscale thermophoresis assay of the interaction between *CaWRKY28* or *CaWRKY28*^{C249W} and *CaWRKY40* in solution. Green fluorescent protein (GFP), *CaWRKY28*-GFP, *CaWRKY28*^{C249W}-GFP; targets: *CaWRKY40*-glutathione-S-transferase (GST):ligand. **C**, Electrophoretic mobility shift assay of the binding of *CaWRKY28*-GST and *CaWRKY28*^{C249W}-GST to the W-box-containing probe, with or without *CaWRKY40*-m (Shen et al. 2020). **D**, Enrichment of *CaWRKY28*-GFP or *CaWRKY28*^{C249W}-GFP at the promoters of immunity-related marker genes by chromatin immunoprecipitation quantitative PCR (qPCR) assay in pepper plants silenced for *CaWRKY40* and transiently overexpressing *CaWRKY28*-GFP or *CaWRKY28*^{C249W}-GFP, with or without cotransient overexpression of *CaWRKY40*-GFP. For qPCR assays, *CaWRKY28*-GFP (or *CaWRKY28*^{C249W}-GFP) was cotransiently overexpressed with *CaWRKY40*-GFP (or empty vector as control) in the leaves of *CaWRKY40*-silenced pepper plants. The enrichment of the control was set to 1 after normalization by input. Data are shown as means $\pm$ standard error of four replicates. Different uppercase letters above the bars indicate significant differences between means ($P < 0.01$) by Fisher's protected least significant difference test.

comes from the following observations. First, *CaWRKY28* is upregulated by *R. solanacearum* infection and by exogenous applications of SA and ETH. The signaling role mediated by SA or ET during plant immunity has been well established (Boller and Felix 2009; Cui et al. 2015; Tsuda and Katagiri 2010). Second, the silencing of *CaWRKY28* significantly raised the susceptibility of pepper plants to *R. solanacearum* infection. By contrast, transient overexpression of *CaWRKY28* triggered HR-like cell death, consistent with an increased resistance to pathogen attack. Third, transcript levels of the immunity-associated genes *CaPRI* (Pan et al. 2010; Zhang et al. 2012), *CaNPR1* (Chen et al. 2009; Zhang et al. 2012), *CaDEF1* (Choi et al. 2008, 2015; Kim et al. 2015; Zhang et al. 2019), and *CaABR1* (Choi and Hwang 2011) were significantly downregulated by *CaWRKY28* silencing, while upregulated by *CaWRKY28* transient overexpression in pepper plants. We may speculate that, upon *R. solanacearum* infection, *CaWRKY28* expression is upregulated, thereby activating immunity-associated genes leading to enhanced resistance to *R. solanacearum* infection. Thus far, a subset of WRKY TFs such as *CaWRKY6* (Cai et al. 2015), *CaWRKY22* (Hussain et al. 2018), *CaWRKY27* (Dang et al. 2014), *CaWRKY40* (Dang et al. 2013), *CaWRKY40b* (Ifnan Khan et al. 2018), and *CaWRKY58* (Wang et al. 2013) have been implicated in pepper immune responses to *R. solanacearum* infection, acting as positive or negative regulators. Their individual silencing produced distinctive phenotypes related to immune responses to *R. solanacearum* infection that are reminiscent of pathogen-associated molecular pattern-triggered immunity, with synergistic relationships between the signaling components (Tsuda et al. 2009).

CaWRKY28 interacts with and potentiates CaWRKY40 in regulating immunity against *R. solanacearum* infection but not thermotolerance.

Previous work has shown that the functions of WRKY TFs can be modulated by other proteins, including WRKYs, by direct protein–protein interaction (Chi et al. 2013), defining WRKY networks in plant defense signaling (Eulgem and Somssich 2007). An interaction between WRKY TFs might be necessary for their cooperative or antagonistic transcriptional regulation, by binding to closely spaced or distantly separated W-boxes in the promoters of their target genes, which is consistent with the observation that, thus far, only group II and group III WRKY proteins (with a single WRKY domain) have been shown to interact with other WRKYs (Chi et al. 2013). Herein, we described a novel type of WRKY interaction between *CaWRKY28* and *CaWRKY40*, which was originally discovered by pull-down followed by LC-MS/MS and further confirmed by Co-IP, BiFC, and MST assays. Although *CaWRKY28* can bind to the W-box in vitro, as indicated by results from EMSA (Fig. 5C), this binding might be prevented by *CaWRKY40* via interaction with *CaWRKY28* in vivo (Fig. 5C). We also revealed that the targeting and the regulation of immunity-related genes (*CaPRI*, *CaNPR1*, *CaDEF1*, and *CaABR1*) by *CaWRKY40* was enhanced by interaction with *CaWRKY28*, whereas the regulation of the thermotolerance-related gene *CaHSP24* was not affected by *CaWRKY28* (Dang et al. 2013). Because WRKYs such as *CaWRKY40* have been frequently found to be involved in regulating several seemingly disparate processes (Nuruzzaman et al. 2013; Rushton et al. 2010), we speculate that these TFs might achieve their specificity of function via a similar mechanism, through an interaction with appropriate transcriptional regulators such as *CaWRKY28*. Noticeably, although *CaWRKY28* and *CaWRKY40* transcript levels responded similarly to exogenous application of SA and ETH (Dang et al. 2013), they differed in their response to exogenously applied ABA and MeJA. This difference might provide the *CaWRKY28*–*CaWRKY40*

module with great regulatory adaptability against different internal and external stimuli.

Cys249 in CaWRKY28 is critical for the interaction of CaWRKY28 with CaWRKY40.

In previous studies, protein–protein interactions between WRKY proteins were thought to be mediated by their leucine zipper domain (Chi et al. 2013). However, our results indicate that the Cys249 residue within *CaWRKY28* is critical for its interaction with *CaWRKY40*, because *CaWRKY28*^{C249W} lost its ability to interact with *CaWRKY40*, thereby losing its W-box binding in vivo as well as its ability to induce the immune response. Interestingly, we determined that *CaWRKY28*^{C249W} was able to bind W-box-containing promoters from immunity-related genes in vitro but not in vivo. Because *CaWRKY28*^{C249W} cannot interact with *CaWRKY40*, we can exclude any blocking effect of *CaWRKY40* on the binding of *CaWRKY28*^{C249W} to W-box containing promoters in vivo. Therefore, we speculate that interacting proteins other than *CaWRKY40* might prevent *CaWRKY28*^{C249W} from binding to W-box containing promoters. In addition, it appears that the interaction between WRKY28 and WRKY40 family members is unique to the Solanaceae family, because the Cys249 residue is shared only across *CaWRKY28* orthologs from the Solanaceae species tobacco, tomato, and potato but not from other plant species such as rice or *Arabidopsis*. It is generally accepted that a given gene and its orthologs, as well as the underlying protein–protein interactions between WRKY TFs, can vary across plant species (Abeyasinghe et al. 2019; Chakraborty et al. 2018; Dang et al. 2013). For example, whereas WRKY40 facilitates powdery mildew infection in *Arabidopsis* (Pandey et al. 2010) and acts as a negative regulator to prevent exaggerated defense responses (Birkenbihl et al. 2017a), its orthologs in pepper and in chickpea (*Cicer arietinum*) act as positive regulators of the immune response to different pathogens (Chakraborty et al. 2018; Dang et al. 2013). We speculate that the specific presence of Cys249 in *CaWRKY28*, and the specific interaction between WRKY28 and WRKY40, might contribute to the different roles of WRKY40 TFs in different plant species.

Collectively, our data indicate that, by interacting with *CaWRKY40*, *CaWRKY28* acts as a positive regulator in pepper response to *R. solanacearum* infection. *CaWRKY28* does not directly target promoters or regulate gene expression but, rather, potentiates the direct regulation of immunity-related genes by *CaWRKY40*, with Cys249 being a critical component of the interaction between *CaWRKY28* and *CaWRKY40*.

MATERIALS AND METHODS

Plant materials and growth conditions.

The pepper inbred line HN42 and *N. benthamiana* plants were grown in sterilized soil in square plastic pots (7 by 7 cm) in a growth room at 25°C and 60% humidity, with a light intensity of 60 to 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and a photoperiod of 16 h of light and 8 h of darkness.

Vector construction.

We cloned the full-length open reading frames (ORFs) of *CaWRKY28* and *CaWRKY40* into the entry vector pDONR207 by BP reaction. After confirmation by sequencing, we recombined the ORFs into the destination vectors pEarleyGate103 (for C-terminal GFP fusions), pDEST-15 (for N-terminal GST fusions), or pEarleyGate201/202 (for overexpression) by LR reaction, using Gateway cloning techniques (Invitrogen, Carlsbad, CA, U.S.A.). To construct vectors for VIGS-mediated gene silencing, we PCR amplified a specific 300- to 400-bp fragment in the 3'UTRs of *CaWRKY28* or *CaWRKY40*, whose sequence

specificity was confirmed by Basic Local Alignment Tool (BLAST) search against the pepper genome, with specific primer pairs, using genomic DNA from pepper accession Zunla-1 as template. There are numerous CaWRKY members but they have great differences in 3'UTR fragments. In order to ensure the specificity of silencing, we chose to design primers in 3'UTR. We cloned the PCR products into pDONR207 by BP reaction and confirmed the identity of the clones by sequencing before further subcloning into the PYL279 (pTRV2) vector, resulting in pTRV2:CaWRKY28, or pTRV2:CaWRKY40. All vectors were introduced into *A. tumefaciens* strain GV3101.

VIGS.

A. tumefaciens strain GV3101 cells harboring pTRV1, pTRV2:00, pTRV2:CaPHYTOENE DESATURASE (*PDS*), pTRV2:CaWRKY28, or pTRV2:CaWRKY40 were grown overnight in Luria-Bertani (LB) medium supplemented with appropriate antibiotics, then pelleted and resuspended to a final cell density of optical density at 600 nm (OD_{600}) = 0.8 in infiltration medium (10 mM morpholineethanesulfonic acid, 10 mM MgCl₂, and 200 μ M acetosyringone; pH 5.4). *A. tumefaciens* strain GV3101 cells harboring pTRV1 were mixed with the cells containing pTRV2:00, pTRV2:CaPDS, pTRV2:CaWRKY28, or pTRV2:CaWRKY40 in a 1:1 ratio and infiltrated into the cotyledons of 2-week-old pepper seedlings. The plants were placed in a growth chamber at 16°C in the dark for 56 h, then transferred to a growth room at 25°C and 60% humidity, with a light intensity of 60 to 70 μ mol photons m⁻² s⁻¹ and a photoperiod of 16 h of light and 8 h of darkness, until the plants infiltrated with pTRV2:CaPDS exhibited a bleached phenotype, indicative of successful silencing of the *PDS* gene.

Transient overexpression of CaWRKY28-GFP or CaWRKY40-GFP in pepper leaves.

For transient overexpression analysis, *A. tumefaciens* strain GV3101 cells harboring the 35S:CaWRKY28-GFP or 35S:CaWRKY40-GFP constructs (and 35S:GFP as control) were grown overnight in LB medium with appropriate antibiotics, then pelleted and resuspended in infiltration medium to a final cell density corresponding to OD_{600} = 0.8. We infiltrated approximately 100 μ l of the cell suspension per infiltration site in the leaves of pepper plants at the eight-leaf stage using a syringe. After infiltration, we brought the plants to a growth room under the same conditions described above, until harvesting of the infiltrated leaves at the indicated time points for further use.

R. solanacearum inoculation.

Plants were inoculated with *R. solanacearum* strain FJC100301 as described previously (Qiu et al. 2018; Shen et al. 2016a). *R. solanacearum* was grown in sucrose peptone agar liquid medium at 28°C. Cells were then pelleted and resuspended in a 10 mM MgCl₂ solution to a final concentration of 10⁸ CFU/ml, and used to inoculate pepper plants via root irrigation.

Subcellular localization and BiFC assay.

We determined the subcellular localization of CaWRKY28 as described previously (Shen et al. 2016a). *A. tumefaciens* strain GV3101 cells harboring the 35S:CaWRKY28-GFP construct were infiltrated into *N. benthamiana* leaves; and the GFP signal was detected 48 hpi. BiFC assays were performed as described previously (Choi et al. 2012) and the fluorescent signal in the cells of the *Agrobacterium*-infiltrated leaves was observed at 48 hpi. GFP fluorescence was collected on a confocal microscope.

Prokaryotic expression of fusion proteins in *E. coli*.

To purify soluble CaWRKY28-GST or CaWRKY40-GST protein, we introduced a pDEST-17 plasmid harboring the

full-length ORFs of CaWRKY28 or CaWRKY40 into *E. coli* strain BL21 (DE3). Expression of the fusion proteins was induced by the addition of 1 mM (final concentration) isopropyl- β -D-thiogalactoside (IPTG) at 20°C for 12 h. We confirmed the presence of the soluble proteins in the supernatant of the *E. coli* strain BL21 (DE3) cell lysate by SDS-PAGE.

Pull-down assays.

We undertook a pull-down approach to identify CaWRKY40 interactors: *E. coli* BL21 (DE3) cells containing pDEST-15-CaWRKY40 were cultivated on LB media to OD_{600} = 0.5 and then a solution of IPTG (50 mg/ml) was added to induce the biosynthesis of CaWRKY40-GST overnight. Next, the total protein was isolated and mixed with that isolated from *R. solanacearum*-inoculated pepper leaves (with uninfected pepper leaves as the control). Leaves were lysed in extraction buffer (50 mM phosphate buffer [pH 7.0], 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β -mercaptoethanol) by freezing with liquid nitrogen, and ground using a pestle and mortar. We immobilized the soluble fusion protein CaWRKY40-GST onto Beaver-Beads GSH (Smart-Lifesciences) and incubated the mixture with rotation for 3 h at 4°C. Proteins bound to the beads were subsequently washed with buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄; pH 7.4) and eluted with elution buffer B (50 mM Tris-HCl and 10 mM reduced glutathione). Eluted proteins were resolved by SDS-PAGE; one swimming lane for RSI treatment and one lane for the control on the gel were cut off for LC-MS/MS analysis.

LC-MS/MS analysis.

Isolated proteins and potential CaWRKY40 interactors were analyzed on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific), as previously described (Wang et al. 2018; Zuo et al. 2001). The samples were dissolved in 10 μ l of a 10% formic acid solution, then analyzed using LC-MS/MS with an online sodium spray ion source. Peptide samples (5 μ l) were loaded into the trap column (Acclaim PepMapC18, 100 μ m by 2 cm; Thermo Fisher Scientific) at a flow rate of 10 μ l/min, and subsequently separated on a 60-min gradient on the analytical column (Acclaim PepMapC18, 75 μ m by 15 cm). The column flow was controlled at 300 nL/min, and the electrospray voltage was 2 kV. Full scan spectra (m/z 350 to 1,550) were obtained at a mass resolution of 60 K, and higher energy collisional dissociation MS/MS scans were subsequently performed at a resolution of 30 K with a dynamic exclusion for 30 s.

The original MS collection files were imported into Proteome Discover 2.1 for retrieval. We searched peptide fragments against the Zunla pepper database, and used BLAST searches at the NCBI database to annotate the functions of the corresponding proteins.

ChIP analysis.

We carried out ChIP assays as previously described (Qiu et al. 2018; Sun et al. 2015). Briefly, pepper leaves infiltrated with *A. tumefaciens* strain GV3101 cells harboring 35S:00, 35S:CaWRKY28-GFP, or 35S:CaWRKY40-GFP constructs were collected at 48 hpi for chromatin isolation. Extracted chromatin were sonicated to generate DNA fragments between 200 and 500 bp in length, which we incubated with magnetic beads (Thermo Fisher Scientific) linked to anti-GFP antibody for 2 h at 4°C, following the manufacturer's instructions. The immunoprecipitated DNA fragments were used as template for semi-quantitative PCR or RT-qPCR with the specific primers listed in Supplementary Table S1.

MST assay.

We assessed the interaction between CaWRKY28(m) and CaWRKY40 by MST assay, as previously described (Harazi

et al. 2017). Briefly, GFP (as a control) or CaWRKY28-GFP was used as a fluorescent label, while CaWRKY40-GST purified from *E. coli* strain BL21 (DE3) was used as a nonfluorescent label. Protein interaction between CaWRKY28-GFP or GFP and CaWRKY40-GST was measured at various CaWRKY40-GST concentrations with 20 nM CaWRKY28(m)-GFP or GFP. We diluted the CaWRKY40-GST solution to a range of concentrations from 1.0×10^{-10} to 1.0×10^{-3} nM, then incubated the CaWRKY40-GST fusion protein with 20 nM labeled protein for 10 min in interaction buffer. The samples were then loaded into Monolith NT.115 Capillaries (catalog MO-K002; NanoTemper Technologies) using 50% IR laser power and an LED excitation source, with $\lambda = 470$ nm at ambient temperature. We calculated the Kd values for CaWRKY28-GFP and CaWRKY40-GST or GFP and CaWRKY40-GST interactions using the NanoTemper Analysis 1.2.20 software (Papageorgiou et al. 2016; Zillner et al. 2012).

RT-qPCR assay.

For the RT-qPCR assay, we used a Bio-Rad Real-Time PCR system (Bio-Rad Laboratories) and the SYBR Premix Ex Taq II system (TaKaRa Bio) with specific primers listed in Supplementary Table S1. We normalized transcript levels to the reference gene *CaACTIN* (GQ339766). The Livak method was used to analyze the data (Livak and Schmittgen 2001).

EMSA analysis.

EMSA assays were carried out as described previously (Shen et al. 2020). To test binding against recombinant CaWRKY28 and CaWRKY28m protein, we synthesized wild-type and mutated probes by PCR, where one primer was labeled with the fluorescent dye Cy5. The recombinant proteins were incubated with Cy5-labeled wild-type or mutated probes, in 5× binding buffer. The mixture was separated by PAGE and scanned on an Odyssey CLX Infrared imager (LI-COR).

Measurement of conductivity.

We measured leaf ion leakage by determining conductivity following a previously described method (Choi et al. 2012; Hwang and Hwang 2011; Shen et al. 2016a). Leaf disks (6 mm in diameter) were taken from pepper leaves infiltrated with *A. tumefaciens* strain cells harboring an empty vector, 35S:CaWRKY28-GFP, 35S:CaWRKY40-GFP, or the 1:1 mixture of *A. tumefaciens* strain cells containing 35S:CaWRKY28-GFP and 35S:CaWRKY40-GFP. The disks were harvested at the indicated time points, then incubated in 5 ml of double-distilled water for 1 h at 28°C. Ion conductivity was measured using a Mettler Toledo 326 ion meter (Mettler Toledo).

Statistical analyses.

Statistical analysis in the present study was performed with the DPS software package. Data represented the means $\pm$ standard deviation obtained from three or four replicates; different uppercase letters above the bars indicated significant differences among means ($P < 0.01$), as calculated with Fisher's protected least significant difference test.

AUTHOR-RECOMMENDED INTERNET RESOURCES

China National GeneBank: <https://db.cngb.org/search/?q=CNP0001104>
Pepper genome platform (PGP): <http://peppergenome.snu.ac.kr>

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