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CaC3H14 encoding a tandem CCCH zinc finger protein is directly targeted by CaWRKY40 and positively regulates the response of pepper to inoculation by *Ralstonia solanacearum*

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SUMMARY

Tandem CCCH zinc finger (TZnF) proteins have been implicated in plant defence, but their role in pepper (*Capsicum annuum*) is unclear. In the present study, the role of CaC3H14, a pepper TZnF protein, in the immune response of pepper plants to *Ralstonia solanacearum* infection was characterized. When fused to the green fluorescent protein, CaC3H14 was localized exclusively to the nuclei in leaf cells of *Nicotiana benthamiana* plants transiently overexpressing CaC3H14. Transcript abundance of *CaC3H14* was up-regulated by inoculation with *R. solanacearum*. Virus-induced silencing of *CaC3H14* increased the susceptibility of the plants to *R. solanacearum* and down-regulated the genes associated with the hypersensitive response (HR), specifically *HIR1* and salicylic acid (SA)-dependent *PR1a*. By contrast, silencing resulted in the up-regulation of jasmonic acid (JA)-dependent *DEF1* and ethylene (ET) biosynthesis-associated *ACO1*. Transient overexpression of *CaC3H14* in pepper triggered an intensive HR, indicated by cell death and hydrogen peroxide (H_2O_2) accumulation, up-regulated *PR1a* and down-regulated *DEF1* and *ACO1*. Ectopic overexpression of CaC3H14 in tobacco plants significantly decreased the susceptibility of tobacco plants to *R. solanacearum*. It also up-regulated HR-associated *HSR515*, immunity-associated *GST1* and the SA-dependent marker genes *NPR1* and *PR2*, but down-regulated JA-dependent *PR1b* and ET-dependent *EFE26*. The *CaC3H14* promoter and was bound and its transcription was up-regulated by CaWRKY40. Collectively, these results indicate that CaC3H14 is transcriptionally targeted by CaWRKY40, is a modulator of the antagonistic interaction between SA and JA/ET signalling, and enhances the defence response of pepper plants to infection by *R. solanacearum*.

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Keywords: *Capsicum annuum*, CCCH-type zinc finger, immunity, *Ralstonia solanacearum*.

INTRODUCTION

Through the co-evolution of plants and their potential pathogens, plants have developed efficient defences against micro-organisms, enabling them to effectively limit pathogen infection. Many of the genes and proteins associated with plant defence modify the expression patterns of the plant's transcriptomes and proteomes, thereby conferring resistance to specific pathogens (Attard *et al.*, 2014; Dörmann *et al.*, 2014; Grewal *et al.*, 2012; Laura *et al.*, 2015; Rudd *et al.*, 2015). A zag–zig–zag model has been established that consists of two layers of immunity: effector-triggered immunity (ETI) and pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Hein *et al.*, 2009; Jones and Dangl, 2006). The difference between PTI and ETI is quantitative, and they share overlapping signalling mechanisms, including Ca^{2+}, reactive oxygen species, hormones [e.g. salicylic acid (SA), jasmonic acid (JA) and ethylene (ET)], mitogen-activated protein kinase (MAPK) cascades and various transcription factors (TFs). However, the mechanism by which plants perceive stress and translate the signals into an appropriate defence response is unknown. This is particularly true in plants that do not respond in a manner similar to well-studied model systems.

Plant defences appear to be primarily regulated transcriptionally through the action of various TFs (Riechmann and Ratcliffe, 2000; Wray, 2003). The action of these TFs may interconnect, creating transcriptional networks (Garner *et al.*, 2016; Moore *et al.*, 2011; Tsuda and Somssich, 2015; Wang *et al.*, 2006). Zinc finger (Znf) proteins are a large and diverse family of TFs, characterized by the Znf motif. The protein motif consists of cysteine (C) or histidine (H) residues coordinated with several zinc ions (Hall, 2005). Znf proteins have been classified into several different types based

on the number and order of the C and H residues binding the zinc ions. These types include C2H2, C2C2, C2HC, C2C2C2C2, C2HCC2C2 and C3H1 (also called CCCH) (Berg and Shi, 1996; Mackay and Crossley, 1998; Moore and Ullman, 2003; Schumann *et al.*, 2007). The CCCH Znf family is unique because it regulates gene expression by direct binding of mRNA or DNA (Li *et al.*, 2001; Pomeranz *et al.*, 2010; Wang D *et al.*, 2008) and possesses nuclease activity (Addepalli and Hunt, 2008). Other Znf families regulate gene expression with the aid of DNA-binding or protein-binding proteins. Genome-wide annotation analyses identified 67 genes coding for CCCH Znf proteins in rice (*Oryza sativa*), 68 in Arabidopsis (Wang D *et al.*, 2008), 34 in *Medicago truncatula* (Dang *et al.*, 2013), 68 in maize (Peng *et al.*, 2012) and 91 in *Populus* (Chai *et al.*, 2012). The CCCH family can be divided into seven groups based on phylogenetic analysis (Peng *et al.*, 2012). Members of the CCCH family appear to be involved in many aspects of plant regulation, including growth (von Saint Paul *et al.*, 2011), development (Grabowska *et al.*, 2009), plant architecture (Wang L *et al.*, 2008) and plant immunity (Deng *et al.*, 2012; Guo *et al.*, 2009; Lee *et al.*, 2013). The proteins involved in these aspects of plant physiology include PEI1, AtSZF1/AtSZF2 (Sun *et al.*, 2007), SOMNUS (Wang L *et al.*, 2008), AtTZF1, AtOZF1 (Huang *et al.*, 2011) and AtOZF2 in Arabidopsis; OsDOS (Kong *et al.*, 2006), OsTZF1, Ehd4, OsGZF1 and OsLIC (Wang L *et al.*, 2008) in rice; GhZFP1 in cotton (Guo *et al.*, 2009); and CsSEF1 in cucumber (Grabowska *et al.*, 2009). However, the majority of the members in this family remain functionally unidentified, especially in non-model plants.

Another important group of plant TFs is the WRKY family. Its members have one or two highly conserved WRKY domain(s) and a Znf-like motif (Ulker and Somssich, 2004). These conserved WRKY domains recognize and bind the highly conserved W-box (TTGACC/T) (Eulgem *et al.*, 2000); however, this binding can be affected by the flanking sequences of the W-box (Agarwal *et al.*, 2011; Ciolkowski *et al.*, 2008). DNA binding allows these TFs to act as regulators of plant immunity (Buscaill and Rivas, 2014; Garner *et al.*, 2016; Pandey and Somssich, 2009). The W-box is highly enriched in promoters of immunity-associated genes, including genes that encode various TFs, indicating that WRKY TFs may fulfil their function by complexing with various TFs to form different transcriptional pathways or networks (Birkenbihl *et al.*, 2017; Liu S *et al.*, 2015). However, the precise position of most of the WRKY TFs in the transcriptional networks is unknown.

Pepper (*Capsicum annuum*) is an important vegetable worldwide and a prototypical member of the family Solanaceae. It is susceptible to several soil-borne pathogens, specifically *Ralstonia solanacearum* and *Phytophthora capsici*, the causal agents of bacterial wilt and Phytophthora blight, respectively. In pepper production, these diseases frequently cause heavy yield losses. Plant breeding is a powerful tool for the development of crop varieties with enhanced performance and superior traits. A better understanding of the molecular mechanisms underlying plant immunity could lead to genetic strategies to improve plant disease resistance. However, few genes have been functionally characterized in pepper, and the molecular mechanisms of pepper immunity have not been characterized completely. In a previous study, we concluded that, in pepper, CaWRKY40 promotes an immune response to *R. solanacearum* infection, but we did not identify its mechanism of action. Here, we have obtained a full-length cDNA of a CCCH-type gene, designated *CaC3H14*, through random sequencing of a normalized cDNA library from pepper. Our data are consistent with the hypothesis that *CaC3H14* is directly targeted and transcriptionally regulated by CaWRKY40, and that CaC3H14 enhances pepper resistance to *R. solanacearum* infection.

RESULTS

Cloning and sequence analysis of *CaC3H14* cDNA

Tandem CCCH zinc finger (TZnF) proteins have been implicated in plant growth and development, as well as plant defence responses. To date, no TZnF protein has been functionally characterized in pepper. Using cDNA-amplified fragment length polymorphism, a transcript-derived fragment (TDF), which was transcriptionally up-regulated by inoculation with *R. solanacearum* and contained two conserved CCCH domains (data not shown), was identified, and its corresponding full-length cDNA was cloned from a cDNA library. The cDNA was 1860 bp in length and harboured an open reading frame (ORF) that encoded a protein with three Znf CCCH domains. We designated it CaC3H14, because its amino acid sequence had the highest similarity (68%–83%) to the protein encoded by the *C3H14* gene when compared with other members in *C. annuum* and several known CCCH subfamily members from *Solanum lycopersicum* and *Nicotiana attenuata* (Fig. S1, see Supporting Information).

Expression pattern of *CaC3H14* in pepper plants

To confirm *CaC3H14* was induced following *R. solanacearum* infection, plants were inoculated with the highly pathogenic *R. solanacearum* strain FJC100301. Infected leaves were harvested to isolate total RNA. *CaC3H14* transcript levels were assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR). Figure 1 shows that *CaC3H14* transcription is significantly enhanced following inoculation with the pathogen compared with control plants. Transcription of *CaPR1a*, an SA-dependent pathogenesis-related (PR) gene in plants, is also induced. Expression of a homologous gene is up-regulated during infection of *Nicotiana benthamiana* by *R. solanacearum* (Zhang *et al.*, 2012).

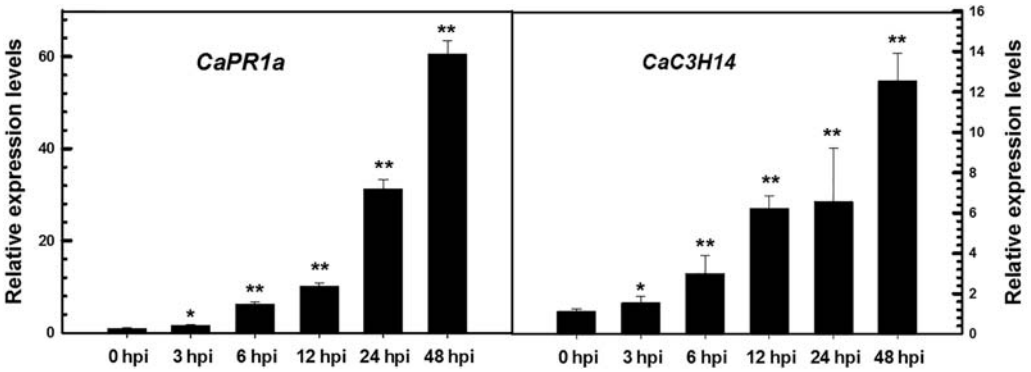

Fig. 1 Relative expression levels of *CaC3H14* and *CaPR1a* in pepper plants infected with *Ralstonia solanacearum* at various hours post-inoculation (hpi) from quantitative real-time polymerase chain reaction (PCR) analysis. The relative transcript levels of *CaC3H14* were compared with those in control plants, which were assigned as unity. *CaPR1a* was used as a positive control. Data represent the mean ± standard deviation (SD) of two independent experiments, each with three replicates ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ (Student–Newman–Keuls test).

Subcellular localization of CaC3H14

The subcellular localization of a protein usually provides a clue to its function, and so we identified the subcellular localization of CaC3H14. We transiently overexpressed (OE) a CaC3H14-green fluorescence protein (GFP) fusion protein in epidermal cells of *N. benthamiana* leaves. GFP images were visualized using a confocal microscope. CaC3H14-GFP was exclusively observed in the nuclei based on co-localization with 4,6-diamidino-2-phenylindole (DAPI) staining (Fig. 2). Thus, we concluded that CaC3H14 was localized to the nucleus.

Silencing of *CaC3H14* enhances susceptibility of pepper to *R. solanacearum*

To further study the role of CaC3H14 in the response to infection by *R. solanacearum* strain FJC100301, we performed a loss-of-function experiment in pepper seedlings using virus-induced gene silencing (VIGS) of *CaC3H14*. Quantitative PCR analysis showed that *CaC3H14* was effectively silenced in *TRV::CaC3H14* pepper plants. The number of *CaC3H14* transcripts in *TRV::CaC3H14* (*CaC3H14*-silenced) plants was reduced to approximately 30% of that in *TRV::00* (control) plants, and *CaC3H14* transcript levels in *R. solanacearum*-infected *CaC3H14*-silenced plants were approximately 5%–15% of those in *R. solanacearum*-infected control plants at 24 and 48 h post-inoculation (hpi; Fig. 3a). *Ralstonia solanacearum* replication was significantly enhanced in *CaC3H14*-silenced pepper plants compared with that in control plants (Fig. 3b). When roots were inoculated with the pathogen, *CaC3H14*-silenced plants consistently showed more severe disease symptoms than control plants (Fig. 3c). A dynamic disease index of inoculated control and *CaC3H14*-silenced plants, performed from 6 to 12 days post-inoculation (dpi), confirmed the increased bacterial growth in *CaC3H14*-silenced plants relative to that in control plants (Fig. 3d). Quantitative RT-PCR was performed to evaluate the expression levels of defence-related genes in inoculated, *CaC3H14*-silenced plants. *CaC3H14* silencing significantly down-regulated the expression of hypersensitive cell death marker genes, such as *CaHIR1*, and the immunity-associated gene *CaPR1a*, whereas it up-regulated the expression of the defence-related genes *CaDEF1* and *CaACO1* (Fig. 3e). We concluded that *CaC3H14* silencing enhanced the susceptibility of pepper to *R. solanacearum*.

Transient overexpression of *CaC3H14* induces cell death and defence-related gene expression in pepper plants

As transient overexpression of Myc- or HA-tagged immunity-associated proteins has been frequently employed to define the

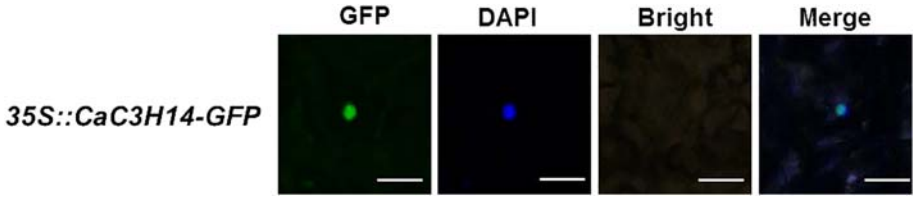

Fig. 2 Subcellular localization of CaC3H14. *Nicotiana benthamiana* leaves were infiltrated with cells of *Agrobacterium tumefaciens* strain GV3101 containing the *35S::CaC3H14-GFP* construct. Fluorescence of the green fluorescent protein (GFP) was detected 48 h after inoculation. Bar, 50 μm. DAPI, 4,6-diamidino-2-phenylindole.

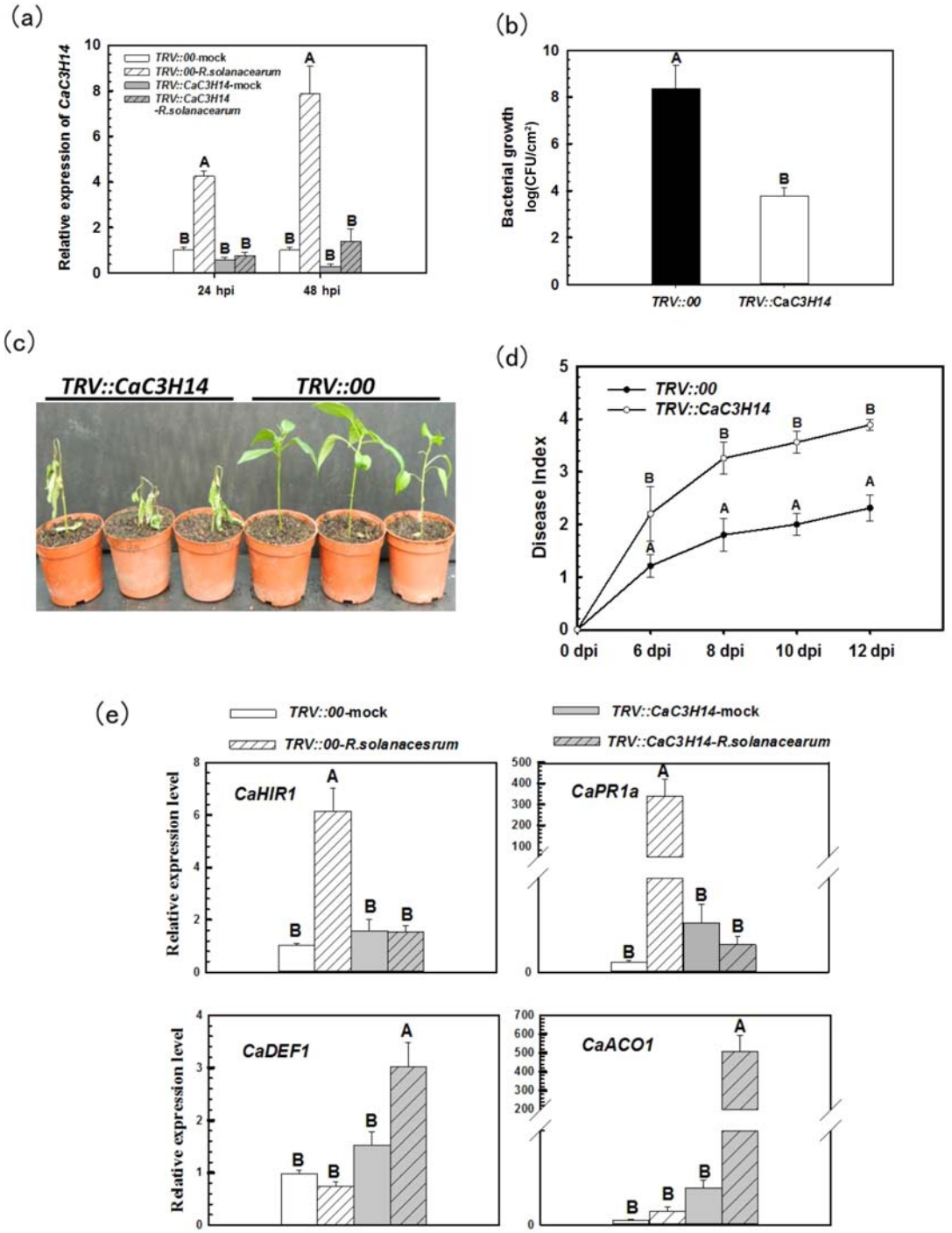

Fig. 3 Virus-induced gene silencing (VIGS) of *CaC3H14* enhanced the susceptibility of pepper plants to *Ralstonia solanacearum*. (a) The silencing efficiency of CaC3H14 in *TRV::CaC3H14* plants, with or without inoculation, detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) at 24 and 48 h post-inoculation (hpi). The relative transcription level of *CaC3H14* in *TRV::CaC3H14* plants was compared with that in mock-treated *TRV::00* plants, which was set as unity. (b) *Ralstonia solanacearum* growth in inoculated *CaC3H14*-silenced or control plants at 48 hpi. CFU, colony-forming unit. (c) Disease symptoms of *TRV::00* and *TRV::CaC3H14* plants at 6 days post-inoculation (dpi) by root irrigation. (d) Dynamic disease index of *TRV::00* and *TRV::CaC3H14* pepper plants from 6 to 12 dpi; data represent the mean ± standard deviation (SD) of three biological replicates each comprising five plants. (e) The effect of *CaC3H14* silencing on the relative expression of defence-related genes in inoculated plants at 24 hpi. The relative transcription of immunity-associated marker genes in *R. solanacearum*-inoculated *TRV::00* and *TRV::CaC3H14* and mock-treated *TRV::CaC3H14* plants was compared with that in mock-treated *TRV::00* plants, which was set as unity. In (a), (b), (d) and (e), data represent the mean ± SD of two independent experiments, each with three replicates ($n = 6$). Different letters indicate significant difference as determined by Student–Newman–Keuls test ($P < 0.01$).

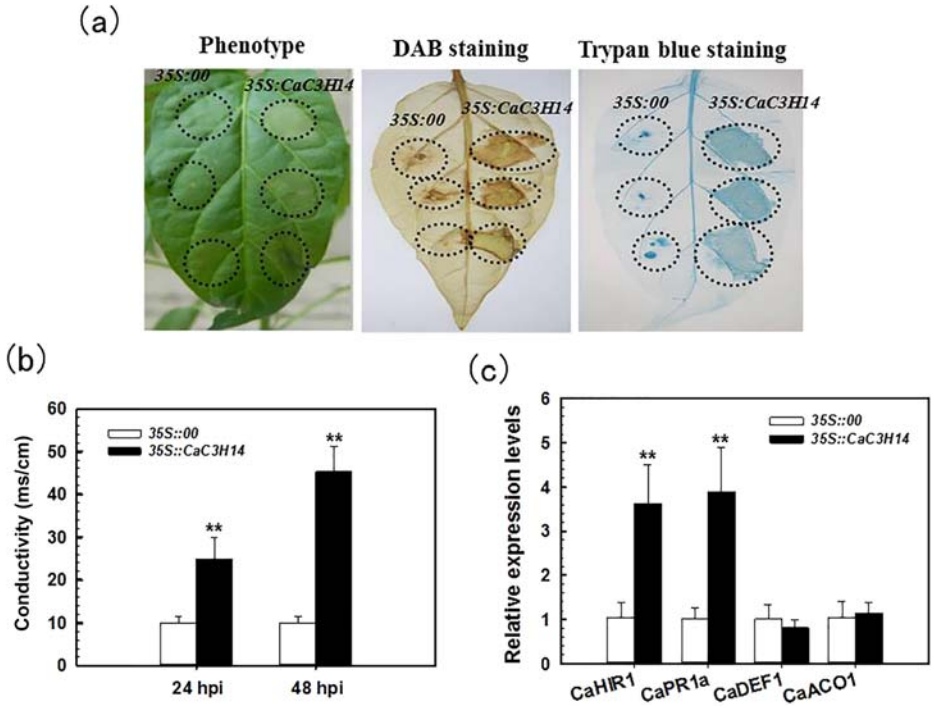

Fig. 4 Transient overexpression of *CaC3H14* induced cell death and defence-related gene expression in pepper plants. (a) Photographs of pepper leaves which had been infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying the 35S::*00* (empty vector) or 35S::*CaC3H14* construct. Left panel: infiltrated pepper leaves at 4 days post-inoculation (dpi); middle panel: after staining with 3,3′-diaminobenzidine (DAB) to detect hydrogen peroxide; right panel: after staining with trypan blue to assess cell death. (b) Electrolyte leakage of pepper leaves measured by conductivity after agro-infiltration with *A. tumefaciens* strain GV3101 cells containing the 35S::*00* or 35S::*CaC3H14* construct. (c) Relative expression levels of defence-related genes, assessed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, in leaves transiently overexpressing *35S::CaC3H14* or *35S::00* at 24 h post-inoculation (hpi). The transcript levels of defence-related genes in *CaC3H14* transient overexpressing leaves were compared with those in control plants, which were assigned as unity. In (b) and (c), data represent the mean ± standard deviation (SD) of two independent experiments, each with three replicates ($n = 6$). $^{**}P < 0.01$ (Student–Newman–Keuls test).

roles of these proteins in plant immunity(Choi and Hwang, 2011; Kim *et al.*, 2014), we used an HA-tagged transient overexpression approach to define the role of CaC3H14 in pepper immunity. GV3101 cells containing 35S::*CaC3H14-HA* or *35S::00* were infiltrated into pepper leaves. Transient *CaC3H14* overexpression was detected by immunoblotting against human influenza haemagglutinin antibody (HA; data not shown) (Fig. S3, see Supporting Information). Overexpression of *CaC3H14-HA* visibly triggered cell death after 4 dpi, whereas controls did not (Fig. 4a, left panel). Hypersensitive response (HR)-associated cell death was assessed by staining with trypan blue to identify necrotic cells. Control leaves produced a weak to non-existent HR-mediated necrotic response, whereas leaves transiently overexpressing *CaC3H14-HA* clearly induced a necrotic response (Fig. 4a, right panel). Hydrogen peroxide (H_2O_2) production was detected using 3,3′-diaminobenzidine (DAB) staining. There was visible DAB staining in the leaves transiently expressing *CaC3H14-HA*, but not in control leaves (Fig. 4a, middle panel). We also performed an ion leakage test to analyse cell necrosis severity caused by plasma membrane damage in leaves transiently overexpressing *CaC3H14-HA* (Fig. 4b). There was more ion leakage in leaves transiently overexpressing *CaC3H14-HA* compared with that in control leaves at 48 and 72 h after agro-infiltration with GV3101 cells.

To test whether transient overexpression of *CaC3H14* could alter the expression of defence-related genes in pepper plants, we examined the transcript levels of *CaHIR1*, *CaPR1a*, *CaDEF1* and *CaACO1*. The relative transcript levels of *CaHIR1* and *CaPR1a* were significantly higher in *CaC3H14* transiently overexpressing leaves than in control leaves, whereas the relative transcript levels of *CaDEF1* and *CaACO1* were not significantly changed (Fig. 4c).

Ectopic overexpression of *CaC3H14* enhances resistance of tobacco to *R. solanacearum* attack

To confirm the role of CaC3H14 in plant immunity, transgenic tobacco plants overexpressing CaC3H14 were generated, and the effect of ectopic *CaC3H14* overexpression on tobacco immunity was assayed. Nineteen independent T_0 transgenic tobacco lines constitutively expressing *CaC3H14* driven by the CaMV35S promoter were acquired, and their corresponding T1 and T2 lines

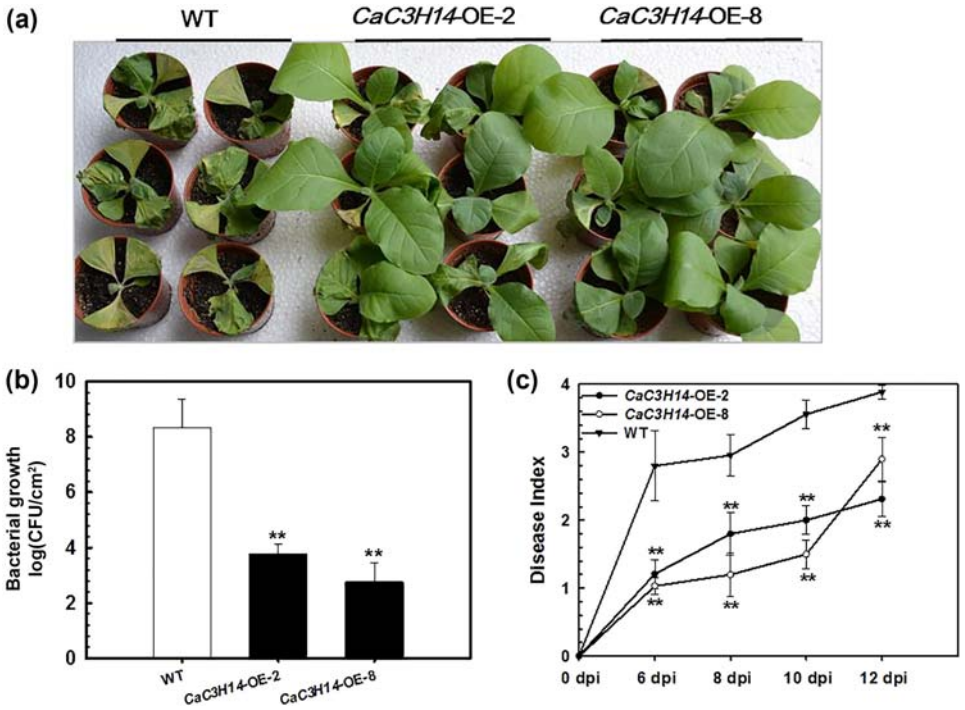

Fig. 5 Ectopic overexpression of *CaC3H14* decreased susceptibility of the transgenic tobacco plants to *Ralstonia solanacearum* strain FJC100301 compared with wild-type (WT) plants. (a) Disease symptoms of *R. solanacearum*-inoculated tobacco plants at 7 days post-inoculation (dpi) by root irrigation. (b) Growth of *R. solanacearum* in the third leaves of *R. solanacearum*-inoculated plants of transgenic lines (*CaC3H14-OE-2* and *CaC3H14-OE-8*) and WT control at 36 h post-inoculation (hpi). (c) Dynamic disease index of plants inoculated with *R. solanacearum* using root irrigation, from 6 to 12 dpi. Data represent the mean ± standard deviation (SD) of three biological replicates, each comprising five plants. $^{**}P < 0.01$ (Student–Newman–Keuls test).

were generated. No significant altered growth and development were found between the plants of the acquired T_2 lines and wild-type (WT) plants. Two T_2 transgenic lines, *CaC3H14-OE-2* and *CaC3H14-OE-8*, with average numbers of *CaC3H14* transcripts and normal growth and development were selected for further analysis. The two transgenic plant lines and WT plants were inoculated with *R. solanacearum* strain FJC100301 using root irrigation. At 7 dpi, we observed wilting symptoms in all three types of plant, but these symptoms were more severe in WT plants than in the two transgenic lines (Fig. 5a). Bacterial growth in the inoculated leaves of the plants was assessed by measuring the number of colony-forming units (CFUs) at 36 hpi. At this time point, there were significantly fewer CFUs in transgenic plants than in WT plants (Fig. 5b). The disease index was assessed from 6 to 12 dpi and was significantly higher in WT plants than in either transgenic line (Fig. 5c).

To confirm the effects of CaC3H14 overexpression observed phenotypically in infected plants and to investigate the possible modes of action, we measured the transcription of defence-related genes in *CaC3H14-OE* transgenic and WT tobacco plants using quantitative RT-PCR. We examined the transcript levels of HR-associated genes *NtHSR515* (Czernic *et al.*, 1996) and *NtGST1* (Peng *et al.*, 2004), the SA-responsive genes *NtNPR1* (Ghanta *et al.*, 2011) and *NtPR2*, the JA-responsive gene *PR1b* (Sohn *et al.*, 2007) and the ET-associated gene *NtEFE26* (Sohn *et al.*, 2007). There were significantly more transcripts of the HR-associated genes *NtHSR515* and *NtGST1* in *CaC3H14-OE* transgenic plants than in WT plants, whereas the numbers of *NtNPR1*, *NtPR2*, *NtPR1b* and *NtEFE26* transcripts were not significantly different between transgenic and WT plants. By contrast, there were fewer *NtPR1b* transcripts in transgenic plants than WT plants (Fig. 6).

We also evaluated the transcript levels of these defence-related genes in tobacco plants 48 h after inoculation with *R. solanacearum*. Transcription of *NtHSR515*, *NtGST1*, *NtNPR1* and *NtPR2* in WT plants was up-regulated to different degrees, and up-regulation was potentiated by *CaC3H14* overexpression in transgenic plants. Transcription of *NtPR1b* and *NtEFE26* was significantly up-regulated in inoculated WT plants, but *CaC3H14* overexpression significantly reduced the up-regulation in inoculated transgenic plants (Fig. 6).

Chromatin immunoprecipitation (ChIP) analysis of CaWRKY40 binding to the *CaC3H14* promoter

As both *CaC3H14* and *CaWRKY40* are transcriptionally up-regulated following *R. solanacearum* infection, and three typical W-boxes were identified in the promoter region of *CaC3H14* (Fig. 7a), we hypothesized that CaWRKY40 acts as a regulator of *CaC3H14*. We performed ChIP to determine if CaWRKY40 binds the *CaC3H14* promoter. Cells of *Agrobacterium tumefaciens* strain GV3101 containing the *35S::CaWRKY40-HA* or *35S::00* construct were infiltrated into pepper leaves, and the leaves were harvested 48 h later for chromatin isolation. The isolated chromatin was randomly sheared into fragments of 300 − 500 bp, and the

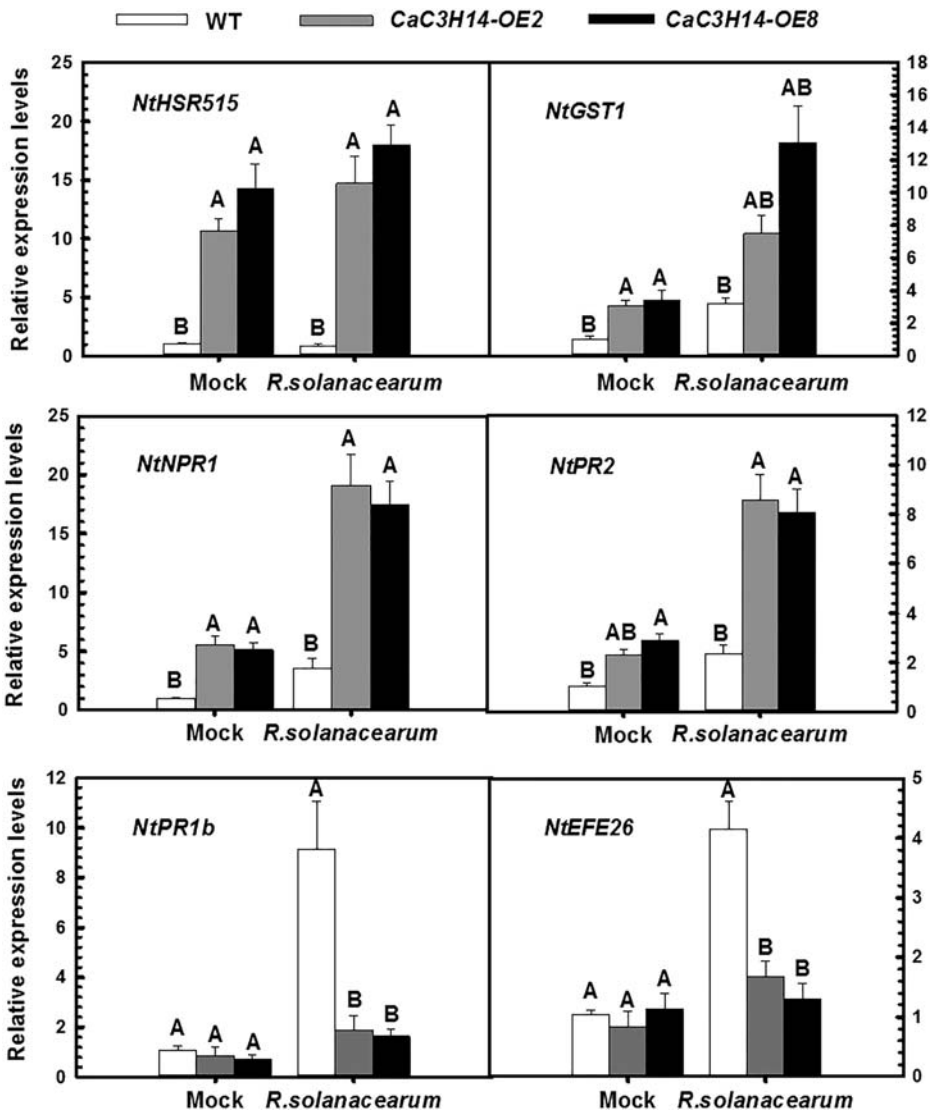

Fig. 6 Relative expression of defence-related genes in transgenic tobacco lines overexpressing *CaC3H14* or wild-type (WT) tobacco plants, assessed using quantitative reverse transcription-polymerase chain reaction (RT-PCR). Plants were inoculated with *Ralstonia solanacearum* using root irrigation and assessed 48 h later. Controls were mock-treated WT plants and transgenic plants. Expression in the former was set to unity. Data represent the mean ± standard deviation (SD) of two independent experiments, each with three replicates ($n = 6$). Different letters indicate significant difference as determined by Student–Newman–Keuls test ($P < 0.01$).

fragments bound to the target proteins were immunoprecipitated with HA. The resulting DNA fragments were used as templates for PCR analysis with specific primer pairs. The results showed that only PW3 produced amplified product from immunoprecipitated DNA template derived from CaWRKY40-HA transiently overexpressing pepper leaves; however, PW1–2 and the control primer pair (CP), which was designed as a negative control based on a promoter region without a W-box, did not amplify any product from the immunoprecipitated DNA template derived from CaWRKY40-HA (Fig. 7a,b), indicating that the W3-box might be responsible for CaWRKY40 targeting. This result was further confirmed by microscale thermophoresis (MST) in solution; the K_d value of binding of the DNA fragment to CaWRKY40 was 8.8142E-07, whereas that of the GFP control was 2.8E-08 (Fig. 7c); all of these data suggest the direct targeting of CaWRKY40 to *CaC3H14*, probably via the W3-box. To test the effect of *R. solanacearum* inoculation on the binding of CaWRKY40 to the *CaC3H14* promoter, ChIP analysis and real-time PCR were performed following infection. The results showed that the *CaC3H14* promoter of infected pepper leaves was more enriched with CaWKRY40 than that of mock-treated control plants (Fig. 7d). To test the specificity or selectivity of the targeting of CaWRKY40 to CaC3H14, the binding of CaWRKY27 and CaWRKY58, the two other WRKY TFs implicated in pepper immunity by our previous studies (Dang *et al.*, 2014; Wang *et al.*, 2013), to the promoter of CaC3H14 was tested by ChIP analysis. The results showed that the primer pair PW3 amplified clear product from immunoprecipitated DNA templates derived from CaWRKY40-HA, but not from CaWRKY27-HA or CaWRKY58-HA transiently overexpressing pepper leaves, indicating the selectivity of targeting of *CaC3H14* by CaWRKY40 (Fig. 7e).

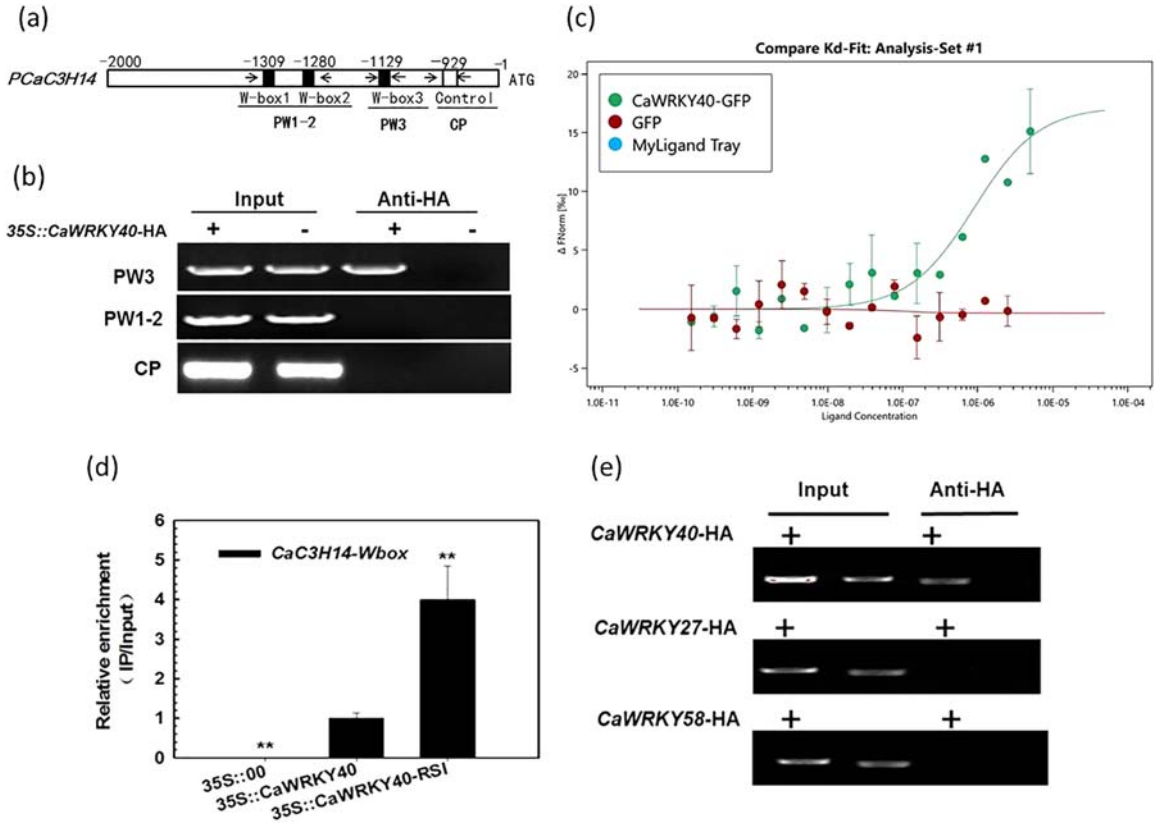

Fig. 7 *CaC3H14* is directly targeted and transcriptionally regulated by CaWRKY40. (a) The distribution of W-boxes in the *CaC3H14* promoter and the primer pairs used in chromatin immunoprecipitation (ChIP) analyses. PW1–2, PW3, primer pairs of the fragment containing W-box 1 and 2, which are close to each other, and W-box 3, respectively. CP, control primer pair (negative control), which was designed based on a region without a W-box. (b) Assay of binding of CaWRKY40 to the *CaC3H14* promoter determined by ChIP-polymerase chain reaction (PCR) with different specific primer pairs. Pepper leaves were inoculated with *Agrobacterium tumefaciens* strain GV3101 cells containing the construct of *35S::CaWRKY40-HA* and harvested 48 h after inoculation. The immunoprecipitated DNA was used as a template for PCR. (c) Interaction of CaWRKY40 with the promoter fragment of CaC3H14 by microscale thermophoresis in solution. Data represent the mean ± standard deviation (SD) of three replicates. (d) The binding of CaWRKY40 to the *CaC3H14* promoter was enhanced by *Ralstonia solanacearum* inoculation. Pepper leaves were inoculated with *Agrobacterium tumefaciens* strain GV3101 cells containing a construct of *35S::CaWRKY40-HA*; at 24 h post-inoculation, they were inoculated with *R. solanacearum*, and 24 h later they were harvested. The PW3 primer pair was used for real-time PCR; the data represent the mean ± SD of two independent experiments, each with three replicates ($n = 6$). $^{**}P < 0.01$ (Student–Newman–Keuls test). (e) Assay of binding of CaWRKY27 and CaWRKY58 to the *CaC3H14* promoter determined by ChIP-PCR with different specific primer pairs using CaWRKY40 as positive control; the Anti-HA+ derived from CaWRKY27 or CaWRKY58 transient overexpressing leaves was used as template with PW3 as the primer pair for PCR. Input, total DNA–protein complex; HA, human influenza haemagglutinin; Anti-HA+, DNA–protein complex immunoprecipitated with anti-HA antibody (α-HA).

Effect of transient CaWRKY40 expression on *CaC3H14* transcriptional expression

The data from the ChIP analysis were consistent with the hypothesis that *CaC3H14* is a direct target gene of CaWKRY40. Thus, we explored the effect of CaWRKY40 on *CaC3H14* transcription. We first assessed the transcript levels of *CaC3H14* in *CaWRKY40*-overexpressing, *CaWRKY40*-silenced and control pepper leaves using real-time RT-PCR analysis. Following inoculation, transient overexpression of *CaWRKY40* significantly activated *CaC3H14* expression in leaves at both 24 and 48 hpi (Fig. 8a). The silencing of *CaWRKY40* was performed by VIGS; the success of *CaWRKY40* silencing was measured by detection of the transcript level of *CaWRKY40* in *R. solanacearum*-inoculated *TRV::CaWRKY40* plants. The specificity of *CaWRKKY40* silencing was tested by detecting the transcription of *CaWRKY40b* in mock-treated *TRV::CaWRKY40* pepper plants. *CaWRKY40b* is a negative regulator down-regulated by *R. solanacearum* inoculation and shares the highest deduced amino acid sequence identity (64.27%) to that of CaWRKY40 amongst all of the pepper *WRKY*s; *CaWRKY40b* is not transcriptionally regulated by CaWRKY40 under room temperature (our unpublished data). The results showed that the transcript levels of *CaWRKY40* in *R. solanacearum*-inoculated *TRV::CaWRKY40* plants were significantly decreased compared with those in control plants (Fig. S2a,

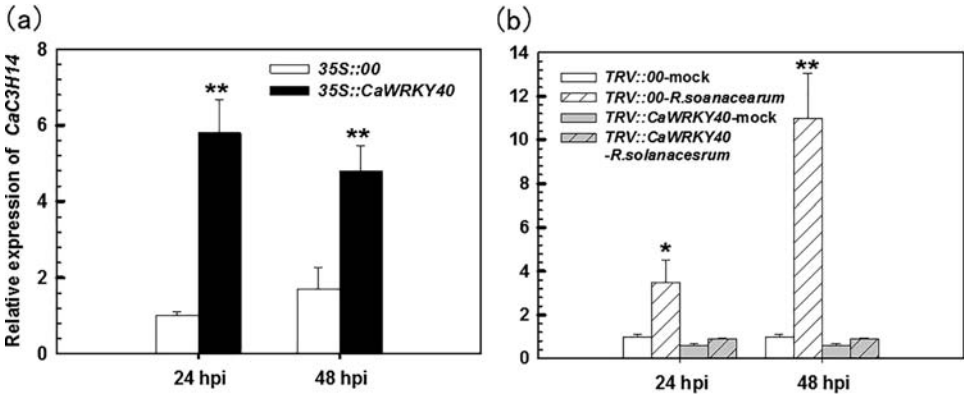

Fig. 8 The transcription of *CaC3H14* in pepper was up-regulated by transient overexpression of *CaWRKY40* and down-regulated by *CaWRKY40*-silencing using virus-induced gene silencing (VIGS). (a) Relative expression levels of *CaC3H14* in plants in which *CaWRKY40* was transiently overexpressed. Expression in mock-treated controls (*35S::00*) was set to 1. (b) Relative expression levels of *CaC3H14* in *CaWRKY40*-silenced plants. Expression in mock-treated control plants (*35S::00 or TRV::00*) was set to 1. dpi; days post inoculation with *R. solanacearum;* data represent mean ± SD of two independent experiments, each with three replicates (n = 6); *, $p < 0.05$; **, $p < 0.01$ (Student–Newman–Keuls test).

see Supporting Information), whereas the transcript levels of *CaWRKY40b* did not decrease in *TRV::CaWRKY40*; instead, the transcript levels of *CaWRKY40b* were enhanced in *TRV::CaWRKY40* plants compared with *TRV::00* plants, indicating the specific silencing of *CaWRKY40* by VIGS (Fig. S2b). From our unpublished data, CaWRKY40b is a negative regulator and is down-regulated by *Ralstonia* infection, and this gene is also negatively regulated by CaWRKY40; therefore, the specific silencing of *CaWRKY40* de-repressed the transcription of *CaWRKY40b*. The transcription of *CaC3H14* was detected in *TRV::CaWRKY40* plants. The results showed that *CaC3H14* transcript levels in *CaWRKY40*-silenced pepper leaves were significantly lower than those in control plant leaves (Fig. 8b). These data indicate that CaWRKY40 directly targets *CaC3H14* and positively regulates its transcription in *R. solanacearum*-infected pepper plants.

DISCUSSION

CCCH genes are important for plant growth, development and environmental responses (Bogamuwa and Jang, 2013; Higuera *et al.*, 2014; Huang *et al.*, 2012; Jan *et al.*, 2013; Kim *et al.*, 2008; Kong *et al.*, 2006; Lin *et al.*, 2011; Lu *et al.*, 2014; Yang *et al.*, 2013; Zhou *et al.*, 2014). Studies of these genes have mainly focused on model plants, such as rice and Arabidopsis, and the determination of their role in members of the Solanaceae family, such as pepper, is limited. Here, we characterized the role of one of these genes, *CaC3H14*, in the immune response of pepper to infection by *R. solanacearum*. We concluded that *CaC3H14* positively regulates the immune response in pepper to *R. solanacearum* and is targeted by CaWRKY40.

We isolated *CaC3H14* from a pepper cDNA library. Its deduced amino acid sequence contains three typical CCCH Znf motifs: Cx8Cx5Cx3H, Cx8Cx5Cx3H and Cx7Cx5Cx3H. When this protein was fused with GFP, the fusion products were present exclusively in the nucleus, similar to the CCCH Znf proteins OsGZF1 and GhZFP1 in their respective plants (Guo *et al.*, 2009; Yang *et al.*, 2013). However, other CCCH proteins, including OsTZF1 and AtTZF1, have dynamic subcellular localization patterns in the cytoplasm and the nucleus (Jan *et al.*, 2013; Pomeranz *et al.*, 2010, 2011), indicating that CCCH Znf proteins have diverse subcellular localization, probably related to their functions.

When plants are infected by pathogens, they must mount a large-scale transcriptional reprogramming of defence-associated genes (Bartsch *et al.*, 2006; Ramonell *et al.*, 2005; Rowland *et al.*, 2005). In this study, *CaC3H14* was significantly up-regulated in response to *R. solanacearum* inoculation, and we hypothesized that this protein is important for the pepper immune response. Several lines of evidence supported this hypothesis. First, we performed several loss- and gain-of-function analyses. Silencing of *CaC3H14* using VIGS significantly enhanced the susceptibility of pepper plants to *R. solanacearum*, whereas its transient overexpression triggered an intensive HR. Evidence for the HR was twofold: transient overexpression of *CaC3H14* was accompanied by enhanced trypan blue staining, indicative of cell death, a common event in ETI which may also occur in PTI (Tsuda and Katagiri, 2010). Second, there was an accumulation of H_2O_2 in overexpressing plants. Potentiated H_2O_2 production is believed to confer cell death (Dang *et al.*, 2013). We also observed that ectopic overexpression of *CaC3H14* consistently enhanced the resistance of tobacco plants to *R. solanacearum* inoculation. Our hypothesis was also supported by the results of an expression assay of some immunity-associated marker genes. Specifically, in pepper plants, the HR-associated gene *HIR1* and the SA-dependent PR gene *PR1a* were significantly down-regulated by silencing of *CaC3H14*, but up-regulated by its transient overexpression. We also tested

the HR-associated genes *NtHSR515* (Czernic *et al.*, 1996; Takahashi *et al.*, 2004; Tronchet *et al.*, 2001) and *NtGST1* (Peng *et al.*, 2004), as well as the SA-dependent genes *NtPR2* (Li *et al.*, 2015; Naoumkina *et al.*, 2008) and *NtNPR1* (Ghanta *et al.*, 2011). These genes were consistently up-regulated by ectopic overexpression of *CaC3H14* in transgenic tobacco plants. All of these data suggest that CaC3H14 acts as a positive regulator of pepper immunity against *R. solanacearum* attack.

We next reasoned that *R. solanacearum*-induced *CaC3H14* transcription would require regulation. We have observed previously that *CaWRKY40* is induced by inoculation with *R. solanacearum* and enhances the immune response in pepper (Dang *et al.*, 2013). Because there are three W-boxes in the promoter of *CaC3H14*, we hypothesized that CaWRKY40 regulates the transcription of *CaC3H14* by binding one of these W-boxes. By ChIP assay, it was found that, amongst the three typical W-boxes, only the W3-box appeared to be bound by CaWRKY40, which was further supported by the data from MST analysis. However, the W3-box was not found to be bound by CaWRKY27 and CaWRKY58, the two other pepper WRKY TFs implicated in pepper immunity against *R. solanacearum* by our previous studies (Dang *et al.*, 2014; Wang *et al.*, 2013); this suggests that the binding of CaWRKY40 to the W3-box containing the *CaC3H14* promoter is selective; however, its possible binding by other WRKY TFs cannot be ruled out at present. We also observed that, on *R. solanacearum* infection, pepper plants transiently overexpressing *CaWRKY40* exhibited significantly enhanced *CaC3H14* transcription, whereas TRV::*CaWRKY40* plants exhibited significantly down-regulated *CaC3H14* transcript abundance, compared with control plants. These data provide solid evidence that *CaC3H14* is a direct target of CaWRKY40 during the response of pepper to *R. solanacearum* infection.

Notably, the binding of CaWRKY40 to the *CaC3H14* promoter was enhanced by *R. solanacearum* infection, and the transcription of immunity-associated genes, which were modulated by CaC3H14, was similarly affected by *R. solanacearum* infection. It is possible that both CaWRKY40 and CaC3H14 are modulated by some unidentified components activated by *R. solanacearum* infection. WRKY proteins have been found to interact with many other proteins, such as MAPK, VQ proteins, calmodulin, 14-3-3, chromatin remodelling proteins and other TFs (e.g. WRKY, NAC), and modify the target specificity or transcriptional activity of these proteins (Alves *et al.*, 2014; Chi *et al.*, 2013; Giri *et al.*, 2013; Shan *et al.*, 2016). Other CCCH proteins include AtTZF5, which interacts with MARD1 and RD21A (Bogamuwa and Jang, 2016), and GhZFP1, which enhances salt stress tolerance and fungal disease resistance in transgenic tobacco by interacting with GZIRD21A and GZIPR5 (Guo *et al.*, 2009). Further characterization of the proteins that interact with CaWRKY40 and CaC3H14 during the response of pepper to *R. solanacearum* infection should clarify the underlying mechanism of the pepper immune response to *R. solanacearum*.

The plant hormones SA, JA and ET are important for plant defence (Kunkel and Brooks, 2002; Robert-Seilaniantz *et al.*, 2011) and are produced in response to infection by a variety of pathogens. The responses to pathogen infection differ depending on the nature of the pathogen and its infection strategy (De Vos *et al.*, 2005). SA was originally shown to be involved in defence against biotrophic pathogens, and JA was shown to be involved in defence responses to necrotrophic pathogens (Glazebrook, 2005). However, there is intensive crosstalk between SA and JA/ET signalling pathways, and this crosstalk is an important regulatory mechanism in itself. The crosstalk is believed to provide regulation so that the immune response is tailored to the type of pathogen encountered (Pieterse *et al.*, 2012). Complex antagonistic and synergistic regulatory relationships between the SA and JA signalling sectors of the plant immune signalling network have been found in Arabidopsis in response to different pathogens (De Vos *et al.*, 2005; Glazebrook *et al.*, 2003; Sato *et al.*, 2010). An additive or synergistic interaction between JA and SA signalling sectors was revealed during PTI, and they are partially antagonistic to each other during ETI (Tsuda *et al.*, 2009). The latter phenomenon seems to allow for compensatory effects if a defined sector is disabled as a result of interference with pathogen effectors. In our study, overexpression of *CaC3H14* in either pepper or tobacco triggered the expression of SA-dependent immunity-associated genes, but not JA- or ET-dependent immunity-associated marker genes, whereas silencing of *CaC3H14* down-regulated SA-dependent PR genes, but enhanced JA- and ET-dependent PR genes in pepper plants. We interpret this as evidence that CaC3H14 regulates the antagonistic interaction between SA and JA/ET signalling, similar to the role of *AtWRKY70*. The overexpression of *AtWRKY70* increases resistance to virulent pathogens and results in the constitutive expression of SA-induced PR genes, whereas its suppression activates JA-responsive/coronatine insensitive 1 (COI1)-dependent genes (Li *et al.*, 2004, 2006). We speculate that the up-regulation of *CaC3H14* with a biotrophic lifestyle in the early stages of infection by *R. solanacearum* might initiate the HR and activate SA-dependent PR genes, whilst blocking unnecessary JA/ET-dependent defence genes. Notably, although *CaC3H14* was directly and transcriptionally regulated by CaWRKY40 during the response of pepper to *R. solanacearum* infection, the apparent relationship of *CaC3H14* to SA, JA and ET signalling is different from that of CaWRKY40. The latter appears to regulate the synergistic interaction between SA and JA/ET signalling (Dang *et al.*, 2013). Although the underlying mechanism is not clear, we hypothesize that unknown proteins might contribute to this difference, either at the transcriptional or post-transcriptional level. Further investigation may reveal the mechanism.

It is worth noting that *CaC3H14*-overexpressing transgenic tobacco plants exhibited significantly enhanced resistance to *R. solanacearum* inoculation with little apparent effect on growth or development, consistent with other reports (Dang *et al.*, 2011; Hong *et al.*, 2017; Shen *et al.*, 2015; Vaid *et al.*, 2015). For example, overexpression of *MoSM1* by the rice blast fungus *Magnaporthe oryzae* induces broad-spectrum resistance against fungal and bacterial diseases in rice plants without reducing abiotic stress tolerance or grain yield (Hong *et al.*, 2017). Also, overexpression of the receptor-like kinase ERECTA improves thermotolerance in rice and tomato without affecting growth (Shen *et al.*, 2015). A possible explanation is that the random insertion of the expression cassette of foreign genes results in many transgenic clones that carry genes with adverse effects on growth or development, but only the clones with low or moderate expression levels that exhibit no significant growth penalty can generate transgenic plants. For example, transgenic plants expressing high levels of *AtWRKY18*, a pathogen- and SA-induced Arabidopsis TF, were stunted in growth, but *AtWRKY18* expressed at moderate levels did not cause substantial negative effects on plant growth (Chen and Chen, 2002). Another possibility is that the adverse effects of the activation of SA-dependent genes following *CaC3H14* overexpression could be counteracted to some degree by depression of JA- and ET-dependent defence pathways. In addition, there was no difference in the transcript levels of marker genes of plant immunity, such as *NtPR1a* and *HSR515*, in WT tobacco plants and *CaC3H14-OE* lines; however, when the plants were inoculated with *R. solanacearum*, these marker genes were significantly up-regulated. It seems likely that CaC3H14 mediates signalling components that are activated by infection with *R. solanacearum*.

Based on the collective results of this study, we propose that *CaC3H14* is transcriptionally targeted by CaWRKY40. It appears to modulate the antagonistic interaction between SA and JA/ET signalling and to enhance the defence response of pepper plants to infection by *R. solanacearum*.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds of pepper (*C. annuum* cultivar GZ03) and tobacco (*Nicotiana tabacum* cultivar Honghuadajinyuan and *N. benthamiana)* were sown in a soil mix [peat moss : perlite, 2 : 1 (v/v)] in plastic pots and placed in a growth room at 25 °C, 70% relative humidity, with 60–70 mmol photons/m^2/s for 16 h, followed by 8 h in the dark.

Inoculation with bacteria

Bacteria of *R. solanacearum* strain FJC100301, which is highly virulent to pepper, were isolated from wilted samples of pepper grown in Fujian Province (China). Bacteria were cultured as described previously by Dang *et al.* (2013). The bacteria were incubated at 28 °C in potato sucrose agar (PSA) medium (200 g/L of potato extract, 20 g/L of sucrose, 3 g/L of beef extract, 5 g/L of tryptone) for 36 h with shaking at 200 rpm, and were pelleted by spinning for 10 min at 10 000 ***g*** and resuspended in 10 mM $MgCl_2$ solution. The bacterial suspension was diluted to 10^8 CFU/mL [equivalent to an optical density at 600 nm (OD_{600}) of 0.8] and used for inoculation.

Bacterial inoculations were performed when the pepper or tobacco plants had eight leaves, unless otherwise indicated. Roots were inoculated by irrigating the soil with 1 mL of suspension. For leaf inoculation, the third leaf from the plant apex was inoculated with 10 μL of suspension by infiltrating it on the leaf using a needle-less syringe once the leaf was fully expanded. Control plants were inoculated with 10 mM $MgCl_2$. Samples were collected at various times after inoculation for further analysis.

Disease index measurement

Ralstonia solanacearum-inoculated pepper or tobacco plants were scored every 3 days following the method of Dang *et al.* (2013).

Isolation and sequence analysis of cDNA of *CaC3H14*

A full-length cDNA with high sequence similarity to *C3H14* of other plant species was acquired by random sequencing of a normalized cDNA library of *C. annuum* constructed in our laboratory. The deduced amino acid sequence served as a query to search for homologues from other plant species at the website: http://www.ncbi.nlm.nih.gov/. The alignment analysis of homologous sequences was performed using DNAMAN5 software.

Construction of the vectors

All of the vectors in the present study were constructed by Gateway cloning technology (Invitrogen, Carlsbad, CA, USA).To construct vectors for overexpression and subcellular localization analysis, the full-length ORF of CaC3H14 was cloned into the entry vector pDONR207 using a BP reaction. It was then cloned into the destination vectors pK7WG2 for overexpression and pMDC103 for subcellular localization by LR reactions. To construct the vector of *35S::CaWRKY27-HA*, *35S::CaWRKY40-HA* or *35S::CaWRKY58-HA*, the full-length ORFs of *CaWRKY27*, *CaWRKY40* and *CaWRKY58* without the stop codon were cloned into entry vector pDONR207 by BP reaction, also by Gateway cloning technology, and then into destination vector pEarleyGate 201 by LR reaction. To construct a vector for VIGS, a specific 351-bp fragment of the ORF of *CaC3H14* and a 300-bp fragment of the 3′-untranslated region (3′-UTR) of *CaWKRY40* were employed for vector construction. The specificities of these two fragments were confirmed by homology sequence searching by BLAST against the database genome sequences of CM334 (http://peppergenome.snu.ac.kr/) and Zunla-1 (http://peppersequence.genomics.cn/page/species/blast.jsp). The specific fragments were cloned into the entry vector pDONR207, and then cloned into the pYL279 vector. All of the primers used in vector construction are listed in Table S1 (see Supporting Information).

Subcellular localization

The *A. tumefaciens* strain GV3101 containing *35S::CaC3H14-GFP* was grown overnight and resuspended in an induction medium (10 mM MES,

10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6). Bacterial suspensions of OD_{600} = 0.8 were used to inoculate *N. benthamiana* leaves. At 48 hpi, the fluorescence of GFP was assessed by imaging with a laser scanning confocal microscope (TCS SP8, Leica, Solms, Germany), using an excitation wavelength of 488 nm and a 505–530-nm bandpass emission filter.

VIGS of *CaC3H14 and CaWRKY40* in pepper plants

For VIGS of *CaC3H14* or *CaWRKY40* in pepper plants, *A. tumefaciens* strain GV3101 harbouring *pYL192* with *pYL279-CaC3H14*, *pYL279-CaWRKY40* or *pYL279* (negative control) was resuspended in induction medium at a 1 : 1 ratio (OD_{600} = 0.6) and co-infiltrated into cotyledons of 2-week-old pepper plants. Details of the procedure have been published previously (Cai *et al.*, 2015; Cheng *et al.*, 2016; Dang *et al.*, 2013; Shen *et al.*, 2016a, 2016b). The specificity of *CaWRKY40* silencing was assayed by the detection of transcript levels of *CaWRKY40b* in *CaWRKY40*-silenced pepper plants.

Transient expression of *CaC3H14* in pepper leaves

For transient expression analysis, *A. tumefaciens* strain GV3101 harbouring either *35S::CaC3H14* or the empty vector *35S::00* was grown overnight and resuspended in induction medium. Bacterial suspension (OD_{600} = 0.8) was infiltrated into leaves of pepper plants.

Generation of transgenic *CaC3H14*-overexpressing tobacco plants

Tobacco cultivar Honghuadajinyuan was used to generate *CaC3H14*-overexpressing tobacco plants by transforming leaf discs with *A. tumefaciens* strain GV3101 harbouring the *35S:CaC3H14* vector. Twenty independent T_0 transgenic tobacco lines were selected by kanamycin, and further confirmed by PCR and quantitative PCR. Two T_2 transgenic lines that exhibited mid-range numbers of *CaC3H14* transcripts were selected for analyses in this study.

Histochemical staining

Leaves were stained for histological analysis by trypan blue or DAB as described by Choi *et al.* (2012) and our previous studies (Cai *et al.*, 2015; Dang *et al.*, 2014; Liu Z-Q *et al.*, 2015; Zhang *et al.*, 2015).

Measurement of electrolyte leakage

Electrolyte leakage of the inoculated sites was measured to evaluate cell death. Leaf discs of 6 mm in diameter were taken at 24 and 48 hpi, placed into sterilized distilled water and slowly shaken at room temperature. Electrolyte leakage was measured using a Mettler Toledo 326 (Mettler, Zurich, Switzerland).

Overproduction and purification of the recombinant CaWRKY40-GFP

For the overproduction of CaWRKY40-GFP, the pET-11a-CaWRKY40-GFP plasmid was used to transform the *Escherichia coli* expression strain BL21(DE3). The protein expression of the two proteins and their purification were carried out following the method of Papageorgiou *et al.* (2016).

Interaction of CaWRKY40 with promoter fragment of *CaC3H14* by MST in solution

The interaction of CaWRKY40 with the promoter fragment of *CaC3H14* was performed by MST (Zillner *et al.*, 2012). In this experiment, GFP in the fused protein CaWRKY40-GFP was used as fluorescent label, and a fragment containing a W-box within the promoter of *CaC3H14*, which was amplified by PCR with a specific primer pair (PW3) and further purified, was used as the non-fluorescent molecule. The protein–DNA interactions were measured at various DNA concentrations keeping the protein concentration constant at 20 μM. The DNA used in 16 different concentrations was prepared using serial dilutions. The initial concentration of the DNA was 0.08 nM. The interaction buffer was 20 mM sodium phosphate, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA) and 100 mM NaCl. The protein and DNA were mixed and balanced for 10 min, after which the samples were loaded into a hydrophilic capillary for DNA–protein interaction measurement in a monolith NT.115 5 instrument (NanoTemper Technologies GmbH, Munich, Germany) using 50% IR laser power and an LED excitation source with λ = 470 nm at ambient temperature. The NanoTemperAnalysis 1.2.20 software was used to fit the data and determine the apparent K_d values (Papageorgiou *et al.*, 2016; Zillner *et al.*, 2012).

ChIP analysis

ChIP assays were performed as described previously (Sun *et al.*, 2015) with slight modification. Leaves were inoculated with GV3101 cells containing *35S::CaWRKY27-HA*, *35S::CaWRKY40-HA* or *35S::CaWRKY58-HA* and *35S::HA*, and collected at 24 hpi. For each sample, about 4 g of leaves were treated with 1.0% formaldehyde for 8 min. Glycine (3 M) was added to a final concentration of 0.125 M glycine. The sample was then vacuum infiltrated for an additional 5 min to stop crosslinking. Nuclear extracts were isolated and resuspended sequentially with extraction buffer I (0.4 M sucrose, 10 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 U protease inhibitors), buffer II (0.25 M sucrose, 10 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 U protease inhibitors) and buffer III (1.7 M sucrose, 10 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 1 U protease inhibitors). After this series, they were digested with micrococcal nuclease (Takara, Dalian, China) according to the manufacturer's instructions. Magnetic beads (Invitrogen) linked with antibody against HA (anti-HA tag, rabbit polyclonal antibody, Sigma, St Louis, Missouri, The United States) or anti-FLAG (negative control) were added to digested samples and eluted following the manufacturer's instructions. Two millilitres of 10 mg/mL proteinase K were added to each sample, incubated at 45 °C for 1 h, extracted twice with the same volume of Tris-saturated phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v), centrifuged at 4 °C and 20 000 ***g*** for 15 min and the aqueous phase was moved to another tube. DNA was then precipitated by adding twice the volume of 100% ethanol, 1/10 volume of 3 M sodium acetate and 1 mL of 2 M glycogen, and incubated at −20 °C overnight. DNA was pelleted by spinning for 20 min at 16 700 ***g***, and the pellets were washed with 70% ethanol, dried at room temperature, resuspended in 50 μL of TE buffer and stored at −20 °C for further use. The immunoprecipitated DNA was analysed for the enrichment of CaWRKY40 at the promoter region of the target genes using semi-quantitative PCR or quantitative

real-time PCR. For quantitative real-time PCR, fold increases in immunoprecipitated DNA were calculated relative to the input DNA (relative enrichment) and the internal control, *CaActin*. The primers used for real-time PCR analysis in ChIP assays are listed in Table S1.

Immunoblot analysis

Protein extraction buffer was used to extract the total protein of pepper samples. At 4 °C, total extracted protein was incubated together with anti-HA agarose (Thermo Fisher Scientific, Waltham, MA, USA) overnight. Beads were collected using a magnetic rack and washed three times with Tris-buffered saline and Tween-20 (0.05%). Eluted protein was examined by immunoblotting using anti-HA–peroxidase antibodies (Abcam, Cambridge, UK).

Quantitative real-time RT-PCR

To determine the relative transcription levels of selected genes, real-time PCR was performed with specific primers (Table S1) according to the manufacturer's instructions for the BIO-RAD Real-time PCR system (Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa, Dalian China). Total RNA preparation and real-time RT-PCR were carried out as described previously (Cai *et al.*, 2015; Dang *et al.*, 2014; Zhang *et al.*, 2015). Two independent biological experiments were performed in triplicate. Data were analysed using the Livak method (Livak and Schmittgen, 2001) and expressed as a normalized relative expression level ($2^{-\Delta\Delta CT}$) of the respective genes (mock-treated or control plants). The relative transcript level of each sample was normalized to *CaActin* or *NtEF1a*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 The primers used in vector construction and gene expression analyses by polymerase chain reaction (PCR) in the present study

Fig. S1 The structural domains of CaC3H14 and comparison of its amino acid sequence with that of homologues in other plant species. The accession number of CaC3H14 is XP_016543877, highlighted by a red ellipse. The three ZnF_C3H1 domains, CX8CX5CX3H, CX8CX5CX3H and CX7CX5CX3H, are indicated with black lines. The homologues of CaC3H14 in other plant species include: XP_016543677 (*Capsicum annuum*), XP_019245694 (*Nicotiana attenuata*), XP_019266162 (*N. attenuata*), XP_010312058 (*Solanum lycopersicum*) and XP_004249283 (*S. lycopersicum*). Blue shading, 50%–75% identity; red shading, 75% identity; black shading, 100% identity. The blue ellipse indicates the specific sequence of CaC3H14. The alignment analysis was made using DNAMAN5 software.

Fig. S2 The success and specificity of *CaWRKY40* silencing by virus-induced gene silencing (VIGS). (a) The transcript levels of *CaWRKY40* in *TRV::CaWRKY40* and control pepper plants with or without *Ralstonia solanacearum* inoculation; the transcript level in mock-treated control plants (*TRV::00*) was set to unity. (b) The relative transcript levels of *CaWRKY40b* in *TRV::CaWRKY40* and control pepper plants; the transcript level of TRV::00 was set to unity. dpi, days post-inoculation with *R. solanacearum*; data represent mean ± standard deviation (SD) of six replicates ($n = 6$). Different letters indicate significant difference as determined by Student–Newman–Keuls test ($P < 0.01$).

Fig. S3 The detection of the success of CaC3H14-HA transient overexpression in pepper leaves by western blot with haemagglutinin antibody.