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RESEARCH PAPER

CaWRKY6 transcriptionally activates *CaWRKY40*, regulates *Ralstonia solanacearum* resistance, and confers high-temperature and high-humidity tolerance in pepper

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Abstract

High temperature (HT), high humidity (HH), and pathogen infection often co-occur and negatively affect plant growth. However, these stress factors and plant responses are generally studied in isolation. The mechanisms of synergistic responses to combined stresses are poorly understood. We isolated the subgroup IIb WRKY family member *CaWRKY6* from *Capsicum annuum* and performed quantitative real-time PCR analysis. *CaWRKY6* expression was upregulated by individual or simultaneous treatment with HT, HH, combined HT and HH (HTHH), and *Ralstonia solanacearum* inoculation, and responded to exogenous application of jasmonic acid (JA), ethephon, and abscisic acid (ABA). Virus-induced gene silencing of *CaWRKY6* enhanced pepper plant susceptibility to *R. solanacearum* and HTHH, and downregulated the hypersensitive response (HR), JA-, ethylene (ET)-, and ABA-induced marker gene expression, and thermotolerance-associated expression of *CaHSP24*, ER-small *CaSHP*, and Chl-small *CaHSP*. *CaWRKY6* overexpression in pepper attenuated the HTHH-induced suppression of resistance to *R. solanacearum* infection. *CaWRKY6* bound to and activated the *CaWRKY40* promoter *in planta*, which is a pepper WRKY that regulates heat-stress tolerance and *R. solanacearum* resistance. *CaWRKY40* silencing significantly blocked HR-induced cell death and reduced transcriptional expression of *CaWRKY40*. These data suggest that *CaWRKY6* is a positive regulator of *R. solanacearum* resistance and heat-stress tolerance, which occurs in part by activating *CaWRKY40*.

Keywords: *CaWRKY6*, *Capsicum annuum*, high temperature and high humidity, *Ralstonia solanacearum*.

Introduction

Plants encounter numerous biotic and abiotic stresses in natural and agronomic habitats. A combination of high temperature (HT), high humidity (HH), and pathogen infection is one of the most harmful stress conditions for plants. HT, HH, or HT and HH (HTHH) promotes pathogen growth and compromises *R*-gene-mediated disease responses (Zhou *et al.*, 2004),

Abbreviations: ABA, abscisic acid; CaMV, cauliflower mosaic virus; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; ET, ethylene; ETH, ethephon; GFP, green fluorescent protein; GUS, β-glucuronidase; HA, haemagglutinin; HH, high humidity; hpi, h post-inoculation; hpt, h post-treatment; HT, high temperature; HTHH, high temperature and high humidity; JA, jasmonic acid; MeJA, methyl jasmonate; PAMP, pathogen-associated molecular pattern; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, salicylic acid; TF, transcription factor; TRV, tobacco rattle virus.

which leads to serious disease burdens and heavy crop losses, particularly in continuous cropping fields of Solanaceae such as pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabacum*). Numerous studies have investigated the responses of model plants and crops to single stresses. These studies identified and characterized the following two distinct types of plant immune responses to perceived pathogen attack. Pattern recognition receptors are ubiquitous membrane receptors that perceive conserved pathogen structures [denoted as pathogen-associated molecular patterns (PAMPs)] at the plasma membrane and trigger PAMP-mediated immunity. Intracellular resistance proteins (R-proteins) initiate effector-triggered immunity by recognizing effector proteins secreted by pathogens into the cytoplasm (Jones and Dangl, 2006, Hein *et al.*, 2009).

Plants sense heat stress differently from how they sense pathogen attack. At least four sensors are involved in detecting heat stress, including a plasma membrane channel that initiates an inward calcium flux, a histone sensor in the nucleus, and two unfolded protein sensors located in the endoplasmic reticulum and in the cytosol (Mittler *et al.*, 2012). There are stress-specific signalling pathways, but alternative signalling cascades also have been implicated in plant responses to pathogen infection and heat stress, including Ca^{2+} signalling, reactive oxygen species (ROS) burst, kinases, phytohormones [salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA)], and transcription factors (TFs) such as HSF and WRKY (Snyman and Cronje, 2008; Clarke *et al.*, 2009; Lee *et al.*, 2012; Dang *et al.*, 2013). This suggests that plant responses to different stresses are coordinated by complex and interconnected signalling pathways, and by extensive crosstalk between the plant responses to heat stress and pathogen infection. The crosstalk appears to be complex, because HT suppresses plant resistance to viruses, bacteria, fungi, and nematodes (Lee *et al.*, 2012), and promotes pathogen proliferation (Siebold and von Tiedemann, 2013). The opposite effects also have been reported (Bonde *et al.*, 2012). For example, HT enhanced the resistance of spring wheat to stripe rust (*Puccinia striiformis* f. sp. *tritici*) (Carter *et al.*, 2009). A subset of Toll interleukin-1 receptor nucleotide-binding site leucine-rich repeat genes were induced under heat stress (Prasch and Sonnewald, 2013), and the function of nucleotide-binding site leucine-rich repeat proteins was regulated by heat-shock proteins such as HSP90 (Belkhadir *et al.*, 2004). Therefore, deciphering the complex relationship between pathogen infection and HTHH responses is required to understand the molecular basis of plant immunity under HTHH and to facilitate breeding for durable plant resistance to diseases.

Transcriptional reprogramming has been identified for a vast array of genes in different plants in response to pathogen infection or heat shock (Walley and Dehesh, 2010; Gruner *et al.*, 2013). Accumulating evidence suggests the existence of common regulons in the plant response to multiple stress conditions (Li *et al.*, 2013). By contrast, plant responses to multiple stresses are different from responses to individual stresses. The majority of the transcriptome changes in plant responses to simultaneously applied stresses were not predicted based on the responses to single stress (Atkinson and Urwin, 2012; Prasch and Sonnewald, 2013; Rasmussen *et al.*, 2013). These results suggest that TFs may play crucial roles in coordinating plant responses to heat shock and pathogen infection. However, the role of different TFs in synergistic plant responses to combined HTHH and pathogen infection remains largely unknown.

Plant WRKY proteins contain one or two conserved WRKY domains with the sequence WRKYGQK, which binds conserved W-boxes [TTGAC(C/T)] present in the promoter regions of target genes across different plant species (Eulgem *et al.*, 2000; Rushton *et al.*, 2002). WRKY TFs are encoded by a large family with 72 members in *Arabidopsis*, 81 members in rice, and 55 members in cucumber (Dong *et al.*, 2003). These proteins can be grouped into three subfamilies on the basis of phylogeny and the basic of the WRKY domain(s) structure (Eulgem *et al.*, 2000). Members of the WRKY superfamily participate in biological processes such as plant growth, development, abiotic stress responses [e.g. drought, salt stress, heat stress (Li *et al.*, 2010; Dang *et al.*, 2013), HH (Zhou *et al.*, 2004), and cold stress] and biotic stress responses [e.g. pathogen infection, PAMP treatment, herbivores, and viruses (Eulgem *et al.*, 2000; Dang *et al.*, 2013)].

WRKY gene expression often responds to several stress factors, and a WRKY protein may participate in the regulation of several seemingly disparate processes (Dang *et al.*, 2013). WRKY proteins can function via protein–protein interaction, autoregulation, or cross-regulation to form complicated WRKY networks (Cheng *et al.*, 2012). The evidence indicates that complex molecular regulatory networks participate in WRKY-mediated biological processes. Previous studies show that WRKY TFs such as *AtWRKY25* (Li *et al.*, 2009), *AtWRKY26*, *AtWRKY33* (Li *et al.*, 2011), *AtWRKY39* (Li *et al.*, 2010), and *OsWRKY11* (Wu *et al.*, 2009) are involved in crosstalk between responses to heat stress and pathogen infection in *Arabidopsis*. *AtWRKY25*, *AtWRKY26*, and *AtWRKY33* functionally interact and play overlapping and synergetic roles in plant thermotolerance. *AtWRKY25* and *AtWRKY33* play partial roles in thermotolerance (Li *et al.*, 2009), and function as negative regulators of SA-mediated defence responses (Zheng *et al.*, 2007). *CaWRKY40*, a WRKY TF of *C. annuum*, acts as positive regulator in pepper responses to heat-shock treatment and *Ralstonia solanacearum* infection (Dang *et al.*, 2013). These results suggest that WRKY TFs play important roles in the plant response to heat stress and pathogen infection. However, functional characterization of these WRKY TFs was performed in plants that were treated with single stresses (heat stress or pathogen inoculation). Thus, the roles and molecular mechanism of WRKY TFs in synergistic plant responses to a combination of heat stress, HH, and pathogen attack are unknown.

C. annuum originated in tropical regions of Central and South America. It is a vegetable with global agricultural importance and a typical Solanaceae that is sensitive to soil-borne diseases. The agricultural productivity of pepper is diminished by problem diseases such as *Phytophthora* blight and bacterial wilt (caused by *Phytophthora capsici* and *R. solanacearum*, respectively), which are more serious under conditions of HTHH. A better understanding of the

molecular mechanisms underlying plant responses to HTHH and pathogen infection could facilitate the development of pepper cultivars with high disease resistance under conditions of HTHH.

In the present study, we isolated a full-length cDNA of a member of the pepper WRKY TF family, designated *CaWRKY6*. We characterized its gene expression and function under conditions of 100% humidity and found that *CaWRKY6* was induced by *R. solanacearum* inoculation, heat-stress treatment, and exogenously applied JA, ethephon (ETH), and ABA. Overexpression of *CaWRKY6* in transgenic tobacco conferred resistance to *R. solanacearum* infection and tolerance to HTHH. *CaWRKY6*-silenced pepper plants were more susceptible to *R. solanacearum* infection and HTHH. These results suggest that *CaWRKY6* acts as a regulator of pepper disease resistance under HTHH.

Materials and methods

Plant materials and growth conditions

Pepper (*C. annuum*) cultivar GZ03 was provided by the pepper breeding group in Fujian Agriculture and Forestry University. The seeds of pepper GZ03 and *Nicotiana benthamiana* were sown in a soil mix [peat moss:perlite, 2:1 (v/v)] in plastic pots and placed in a greenhouse. Pepper plants were grown in the greenhouse at 25 °C, 60–70 µmol photons m^{-2} s^{-1}, a relative humidity of 70%, and a 16 h light/8 h dark photoperiod.

Pathogens and inoculation procedures

R. solanacearum strain FJC100301 was isolated previously in our laboratory and amplified according to the method of Dang *et al.* (2013). The bacterial cell solution was diluted to 10^8 colony-forming units (cfu) ml^{-1} (OD_{600}=0.8) with 10 mM $MgCl_2$. Pepper plants were inoculated by infiltrating 100 µl of the resulting *R. solanacearum* suspension into the third leaves from the apical meristem using a syringe with a needle. The leaves were harvested at the indicated time points for the preparation of RNA.

Plant treatment with exogenous hormones and abiotic/biotic stresses

Pepper plants at the four-leaf stage were sprayed with 1 mM SA, 100 µM methyl jasmonate (MeJA), 100 µM ABA, or 100 µM ETH. Mock plants were sprayed with the corresponding solvent or sterile ddH_2O. For heat-stress treatment, pepper plants at the eight-leaf stage were kept under high (39 °C) or normal (25 °C) temperature. To study the relative *CaWRKY6* transcript levels in response to *R. solanacearum* infection, pepper plants were inoculated at the eight-leaf stage by injection of 100 µl of bacterial suspension (10^8 cfu ml^{-1}) or distilled sterile 10 mM $MgCl_2$. For concurrent treatments of HTHH and *R. solanacearum* inoculation, pepper plants were inoculated at the eight-leaf stage by injection of 100 µl of *R. solanacearum* suspension, placed in a growth chamber at 39 °C and 100% humidity, and harvested at different time points.

Analysis of CaWRKY6 subcellular localization

The full-length cDNA of *CaWRKY6* was cloned into the plant expression vector pMDC45 downstream of the two copies of the cauliflower mosaic virus (CaMV) 35S promoters and in frame with green fluorescent protein (GFP) using the Gateway cloning technique (Invitrogen). The constructs *35S::CaWRKY6-GFP* and *35S::GFP* (used as a control) were transformed into *Agrobacterium tumefaciens* strain GV3101, which was cultured in induction medium (10 mM ethanesulfonic acid (pH 5.7), 10 mM $MgCl_2$, and 200 mM acetosyringone), harvested when the OD_{600} was approximately 1.0, and diluted to OD_{600}=0.8. Bacterial suspensions expressing *p35S::CaWRKY6-GFP* and *p35S::GFP* were injected into *Nicotiana benthamiana* leaves using a syringe without a needle. 4′,6-Diamidino-2-phenylindole (DAPI) staining was performed as described previously (Wang *et al.*, 2013). GFP and DAPI fluorescence was imaged using an Olympus fluorescence light microscope (Tokyo, Japan) with an excitation wavelength of 488 nm with a 505–530 nm band-pass emission filter and an excitation wavelength of 405 nm with a 435–480 nm band-pass emission filter. Photographs were overlaid and assembled using Image-Pro Plus (Cybernetics USA, Herndon, VA, USA).

Biolistic transformation of onion epidermal cells for transient expression

The effector vector pK7WG2-*CaWRKY6* and the reporter plasmid *2×W-p35S$_{core}$::GUS* and *2×Wm-p35S$_{core}$::GUS* were constructed and used in our previous study (Dang *et al.*, 2013). Inner epidermal peels from onion bulbs were placed on basic MS agar medium and subjected to biolistic bombardment (PDS-1000/He; Bio-Rad). Onion epidermal cells were co-transformed with plasmids of *2×W-p35S$_{core}$::GUS* and *p35S::CaWRKY6*, or individually transformed with *2×Wm-p35S$_{core}$::GUS* and *p35S::CaWRKY6* (1 mg of each plasmid). Transfected tissues were incubated for 24 h at 25 °C in the dark and stained for β-glucuronidase (GUS) activity (Jefferson *et al.*, 1987) before examining with an Olympus microscope.

HTHH treatment

To ensure that cell death under HTHH did not result from photo-oxidative stress, pepper plants silenced for *CaWRKY6* or transformed with empty vector control pepper plants were exposed to 37 °C heat stress under 100% humidity in 16 h light/8 h dark photoperiod for 36 h, and kept at 25 °C for 24 h before harvesting for further analysis.

Histochemical staining

Staining with trypan blue and 3,3′-diaminobenzidine (DAB) was done according to the previously published method of Choi *et al.* (2012). For trypan blue staining, pepper or tobacco leaves were boiled in trypan blue staining solution for 2 min, left at room temperature for 8 h, transferred into a chloral hydrate solution (2.5 g of chloral hydrate dissolved in 1 ml of distilled water), and boiled for 20 min to destain. After multiple changes of chloral hydrate solution to reduce the background, samples were mounted in 70% glycerol. For DAB staining, the leaves were stained overnight in 1 mg ml^{-1} of DAB. Stained leaves were cleared by boiling in lactic acid:glycerol:absolute ethanol [1:1:3 (v/v/v)] and then destained overnight in absolute ethanol (Korasick *et al.*, 2010). Representative images of DAB and trypan blue staining were photographed with a light microscope (Leica, Wetzlar, Germany).

Virus-induced gene silencing (VIGS) of CaWRKY6 *in pepper plants*

For VIGS silencing of *CaWRKY6*, the tobacco rattle virus (TRV)-based VIGS system was employed. The PYL192 and PYL279 VIGS vectors have been described previously (Liu *et al.*, 2002). A fragment of the transcribed region of *CaWRKY6* was amplified using gene-specific primers (5′-AA AAAGCAGGC TACGGTAGCTAGACAATTATGCTGC-3′ and 5′-AGAAAGC TGGGTCCAAAAAAAAATCTTATCAACTTG-3′), and cloned into the PYL279 VIGS vector using the Gateway cloning technique (Invitrogen). Vector construction and VIGS analysis were carried

out according to the method used in our precious study (Dang *et al.*, 2013).

Transient CaWRKY6 *expression assay*

GV3101 harbouring the *pK7WG2-CaWRKY6* vector was cultured to OD_{600}=1.0 in induction medium [10 mM ethanesulfonic acid (pH 5.7), 10 mM $MgCl_2$, 200 mM acetosyringone] and diluted to OD_{600}=0.8. This was injected into pepper or *N. benthamiana* leaves using a syringe without a needle. The plants were kept in a growth room for 2 d, and the injected leaves were then harvested for further use.

Quantitative real-time PCR (qPCR)

To determine the relative transcript levels of selected genes, qPCR was performed with specific primers (see Supplementary Table S1 at *JXB* online for gene-specific primers) according to the manufacturer's instructions for the Bio-Rad Realtime PCR system (Bio-Rad, Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa Perfect Real Time). Total RNA was isolated from pepper plants and wild-type and transgenic tobacco seedlings using TRIzol reagent (Invitrogen), and reverse-transcribed using the PrimeScript RT-PCR kit (TaKaRa, Dalian, China). Real-time PCR and corresponding data processing were performed as described previously (Dang *et al.*, 2013).

Chromatin immunoprecipitation (ChIP) analysis

The full-length *CaWRKY6* cDNA was cloned into pEarleyGate 201 using Gateway cloning techniques (Invitrogen). ChIP was performed according to standard protocols. Briefly, approximately 2 g of pepper leaves transiently overexpressing *CaWRKY6:HA* were treated with either 10 mM bithionol sulfoxide or dimethylsulfoxide (solvent control) for 16 h and then fixed with 1.0% formaldehyde for 5 min. Antibody against haemagglutinin (HA) (Santa Cruz Biotechnology) were used for immunoprecipitation. Protein A–agarose beads were blocked with salmon sperm DNA and used to pull down the protein–DNA complex. Equal amounts of starting plant material and ChIP products were used for PCR with specific primers (see Supplementary Table S2 at *JXB* online for gene-specific primers) of the *CaWRKY40* or $2\times W\text{-}p35S_{core}::GUS$ promoters.

Results

Cloning and sequence analysis of the full-length CaWRKY6 *cDNA*

A normalized cDNA library of pepper was constructed previously from a native pepper inbred line. This library was used to isolate full-length *C. annuum* cDNAs. More than 1000 clones were randomly picked and sequenced. One of the positive cDNA clones was identified as a member of the WRKY superfamily of TFs. This cDNA was 2235 bp, and contained 83 bp of 5′-untranslated region, 1911 bp of open reading frame, and 241 bp of 3′-untranslated region. The open reading frame was predicted to encode a protein of 636 aa with a conserved WRKY domain (Eulgem *et al.*, 2000). The relative molecular mass was 69.2 kDa and the theoretical isoelectric point (pI) was 6.32. The sequence has been deposited in GenBank under accession no. KF736800. The isolated cDNA clone shared deduced amino acid sequence identity with the following genes: 86% identity with *SlWRKY6* (NCBI protein no. XP_004243486.1), 68% identity with *VvWRKY6* (XP_002269696.2), 60% identity with *GmWRKY6* (XP_003541953.1), 51% identity with *AtWRKY6* (NM_104910.2), 50% identity with *AtWRKY31* (XP002867796.1), and 50% identity with *AtWRKY42* (XP002872692.1) (Supplementary Fig. S1 at *JXB* online). Therefore, this cDNA clone was designated *CaWRKY6*. *CaWRKY6* was categorized into subgroup IIb based on the WRKY domain and the zinc-finger-like motif. So far, *CaWRKY6* has not been functionally characterized.

CaWRKY6 is localized to the nucleus

Sequence analysis using WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html) indicated that the predicted CaWRKY6 protein contains a putative nuclear localization signal ($_{397}$PVRKQVQ$_{403}$; Supplementary Fig. S1). To confirm nuclear localization, we generated a CaWRKY6–GFP fusion construct driven by the constitutive CaMV 35S promoter, and expressed the construct in *N. benthamiana* leaves. The subcellular location of the *p35S::CaWRKY6-GFP* fusion protein was determined using a fluorescence microscope and *p35S::GFP* as a control. Typical results showed the exclusive localization of CaWRKY6–GFP in the nucleus, whereas the GFP control was observed in multiple subcellular compartments including the cytoplasm and nucleus (Supplementary Fig. S2 at *JXB* online). These results suggested that CaWRKY6 is localized to the nucleus.

CaWRKY6 binds to the W-box and activates transcription in a W-box-dependent manner

Numerous studies have demonstrated the sequence-specific binding of WRKY proteins to typical W-boxes [TTGAC(C/T)] located in the promoters of defence-associated genes, which serve as pathogen-responsive regulatory elements. To test if CaWRKY6 could bind to W-boxes, we performed ChIP analysis with *p35S:HA-CaWRKY6* and a reporter vector containing *GUS* controlled by the CaMV 35S core promoter (−46 to +8 bp) with two copies of the W-box (TTGACC) or two copies of a mutated W-box (TTAACC) ($2\times W\text{-}p35S_{core}::GUS$ or $2\times Wm\text{-}p35S_{core}::GUS$) in the proximal upstream region (Fig. 1A, B).The results showed that CaWRKY6 could bind the W-box but could not bind the mutated W-box. To further assay if CaWRKY6 could activate transcription in a W-box-dependent manner, we performed transient co-expression experiments using the effector vector *p35S::CaWRKY6* and the reporter vector $2\times W\text{-}p35S_{core}::GUS$ with $2\times Wm\text{-}p35S_{core}::GUS$ as a negative control. Reporter vectors were either transformed individually or co-transformed with the effector construct into onion epidermal cells by particle bombardment followed by GUS histochemical assays for reporter gene expression. Onion epidermal cells co-transformed with the $2\times W\text{-}p35S_{core}::GUS$ and the effector plasmid were often stained dark blue. By contrast, epidermal cells transformed with either reporter vector alone, co-transformed with $p35S_{core}::GUS$ and *p35S::CaWRKY6*, or co-transformed with $2\times Wm\text{-}p35S_{core}::GUS$ and *p35S::CaWRKY6* were never stained blue (Fig. 1C). Tobacco

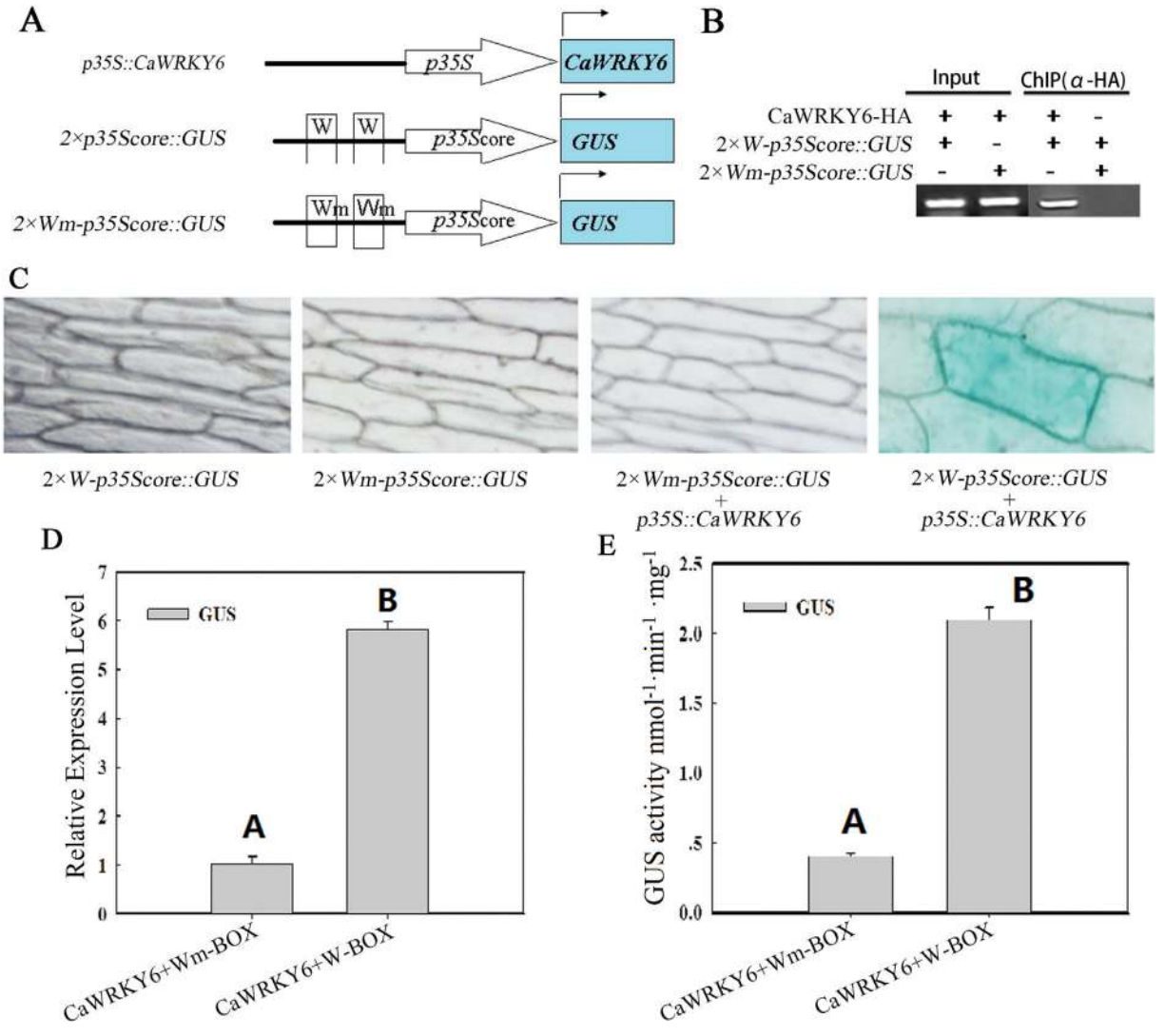

Fig. 1. *CaWRKY6 trans*-activation experiments performed by particle bombardment of onion epidermal cells. (A) Schematic diagram of the reporter and overexpression constructs used for co-transfection of onion epidermal cells. W, W-box; Wm, mutated W-box. (B) ChIP assay showing that CaWRKY6 binds to the W-box in *2×W-p35S$_{core}$::GUS in vivo*. Tobacco leaves were infiltrated with *Agrobacterium* GV3101 carrying *2×W-p35S$_{core}$::GUS or 2×w-p35S$_{core}$::GUS* and *35S:HA-CaWRKY6* (*CaWRKY6* constructed in the pK7WG2 vector). The transiently expressed leaves were harvested and fixed with 1% formaldehyde. The protein input was immunoprecipitated with anti-HA antibodies. The acquired DNAs were adjusted to the same concentration and PCRs were performed using *2×W-p35S$_{core}$::GUS*-specific primers. Lanes 1 and 2, input (total DNA–protein complex); lanes 3 and 4, DNA–protein complex immunoprecipitated with anti-HA antibody. (C) Onion epidermal cells co-transfected with the indicated reporter and effector plasmids. Dark blue staining was only observed after co-transfection of *2×W-p35S$_{core}$::GUS* with *p35S::CaWRKY6*. (D, E) qRT-PCR and GUS protein analysis indicating that CaWRKY6 binds to the W-box in *2×W-p35S$_{core}$::GUS in vivo*. Tobacco leaves were infiltrated with *Agrobacterium* GV3101 carrying *2×W-p35S$_{core}$::GUS or 2×w-p35S$_{core}$::GUS* and *35S:HA-CaWRKY6* (*CaWRKY6* constructed in the pK7WG2 vector).

leaves were infiltrated with *Agrobacterium* GV3101 carrying *2×W-p35S$_{core}$::GUS or 2×w-p35S$_{core}$::GUS* and *35S:HA-CaWRKY6* (*CaWRKY6* constructed in pK7WG2 vector). qPCR and GUS protein analysis indicating CaWRKY6 binds to W-box in *2×W-p35S$_{core}$::GUS in vivo* (Fig. 1D, E).

CaWRKY6 *expression is upregulated by* R. solanacearum *infection and HTHH conditions*

Cell death and hydrogen peroxide (H_2O_2) production in primary leaves of pepper inoculated with the highly virulent *R. solanacearum* strain FJC100301 were visualized by staining with trypan blue and DAB, respectively. No staining or only weak staining was observed in leaves adjacent to the primary leaves inoculated with *R. solanacearum* (Fig. 2A). To determine if *CaWRKY6* expression was modulated by *R. solanacearum* inoculation, transcriptional expression of *CaWRKY6* was measured by qPCR analysis during the compatible interaction with *R. solanacearum*. Compared with control plants, *CaWRKY6* transcripts were upregulated in leaves inoculated with *R. solanacearum* but not in systemic leaves or in leaves treated with $MgCl_2$ (Fig. 2B). The increased *CaWRKY6* transcript levels were maintained between 12 and 48 h post-inoculation (hpi) with maximal levels observed at 48 hpi (Fig. 3A). This clear transcriptional activation suggested that *CaWRKY6* participates in the response of pepper plants to *R. solanacearum* inoculation.

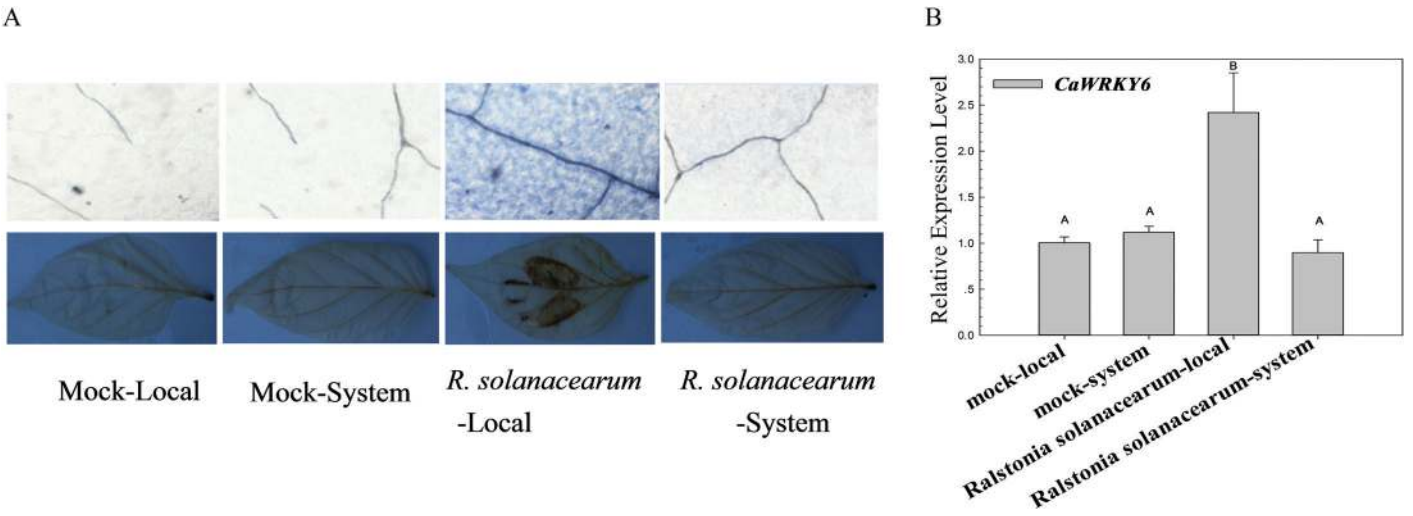

Fig. 2. Effect of *R. solanacearum* inoculation on pepper cell death and expression of *CaWRKY6*. (A) DAB and trypan blue staining of the primary and secondary leaves 24 h after inoculation of primary leaves with *R. solanacearum*. (B) qPCR analysis of relative transcript levels of *CaWRKY6* in primary and secondary leaves 24 h after inoculation of primary leaves with *R. solanacearum*. *R. solanacearum*-local, leaves inoculated with *R. solanacearum*; *R. solanacearum*-system, the second leaves next to the leaves inoculated with *R. solanacearum*; mock-local, mock in the pathogen-inoculated leaves; mock-system, mock in the leaves next to the inoculated leaves. Error bars indicate the standard error. Different upper-case letters indicate significant differences from three independent experiments based on the LSD test (P<0.01).

The relative abundance of *CaWRKY6* transcripts under conditions of heat shock and/or 100% humidity was determined by qPCR of four pepper plants at the four-leaf stage. *CaWRKY6* transcript levels increased in heat-shock-treated pepper plants from 24 to 48 h post-treatment (hpt) (Fig. 3A), and also increased under conditions of 100% humidity at 48 h hpt (Fig. 3A). Synergistic HTHH effects on CaWRKY6 transcripts levels were observed in pepper plants from 12 to 48 hpt (Fig. 3A). Transcriptional upregulation of *CaWRKY6* by *R. solanacearum* inoculation was found to be enhanced by environments with HT or HH, and the environment with HTHH, and the highest expression levels occurred in *R. solanacearum* inoculated pepper plants under HTHH.

Response of CaWRKY6 *transcript levels to exogenously applied Sam MeJA, ETH, and ABA*

The phytohormones SA, JA, and ET play crucial roles in the responses to biotic and abiotic stress and control the expression of defence genes. We assessed the potential role of these phytohormones in regulating *CaWRKYK6* expression. The relative abundance of *CaWRKY6* transcripts was determined by qPCR analysis of pepper plants at the four-leaf stage that were treated with exogenous SA, MeJA, and ETH (which is converted to ET by the plant). *CaWRKY6* transcript levels did not change after treatment with 1 mM SA at all tested time points (Fig. 3B). After treatment with 100 µm MeJA, *CaWRKY6* transcript levels increased from 6 to 12 hpt and peaked at 12 hpt (Fig. 3C). After treatment with 100 µM ETH, *CaWRKY6* transcript levels increased significantly from 6 to 48 hpt (Fig. 3D). The application of exogenous ABA (a phytohormone involved in plant responses to abiotic stress) significantly increased *CaWRKY6* expression from 24 to 48 hpt (Fig. 3E). These results suggested that *CaWRKY6* may function in plant defence and stress responses.

Effect of CaWRKY6 *silencing on resistance of pepper to* R. solanacearum *and thermotolerance*

To test the role of *CaWRKY6* in immunity and thermotolerance, we performed loss-of-function experiments in pepper seedlings that had a *CaWRKY6* gene silenced by VIGS. We generated TRV::CaPDS, which silences the phytoene desaturase gene (PDS) and induces a photobleaching phenotype, as an additional control to determine the success of gene silencing (Fig. 4A). The two vectors TRV1 (PYL192) and TRV2 (PYL279) were transformed separately into *A. tumefaciens* strain GV3101. The two resulting GV3101 cells were mixed and co-injected into leaves of pepper seedlings, and the seedlings were incubated at 16 °C for 56 h, after which they were kept at 25 °C. Three independent experiments were performed, and we obtained approximately 100 plants of *TRV::00* and 100 plants of *TRV::CaWRKY6*. Five plants were randomly selected to check the efficiency of gene silencing. In *TRV::CaWRKY6* pepper plants, *CaWRKY6* transcript levels were reduced to ~30% of those in *TRV::00* plants (Fig. 4B). We also checked the most homologous gene of *CaWRKY6* in pepper genome, another WRKY (*CA02g17910*), and no significant change in its expression was detected in *CaWRKY6*-silenced (*TRV::CaWRKY6*) and the control (*TRV::00*) plants, indicating that the silence of the *CaWKRY6* gene was specific.

A highly virulent strain of *R. solanacearum* FJC100301 was used to inoculate five individual plants for each of the *TRV::CaWRKY6* and *TRV::00* plants. At 4 dpi, we observed definite wilting symptoms on *TRV::CaWRKY6* pepper leaves, but the *TRV::00* empty vector control plants exhibited only faint wilting symptoms (Fig. 4C). We also stained *R. solanacearum*-infected *CaWRKY6*-silenced and control leaves with DAB (an indicator of H_2O_2 accumulation) and trypan blue (an indicator of cell death or necrosis). Strongly

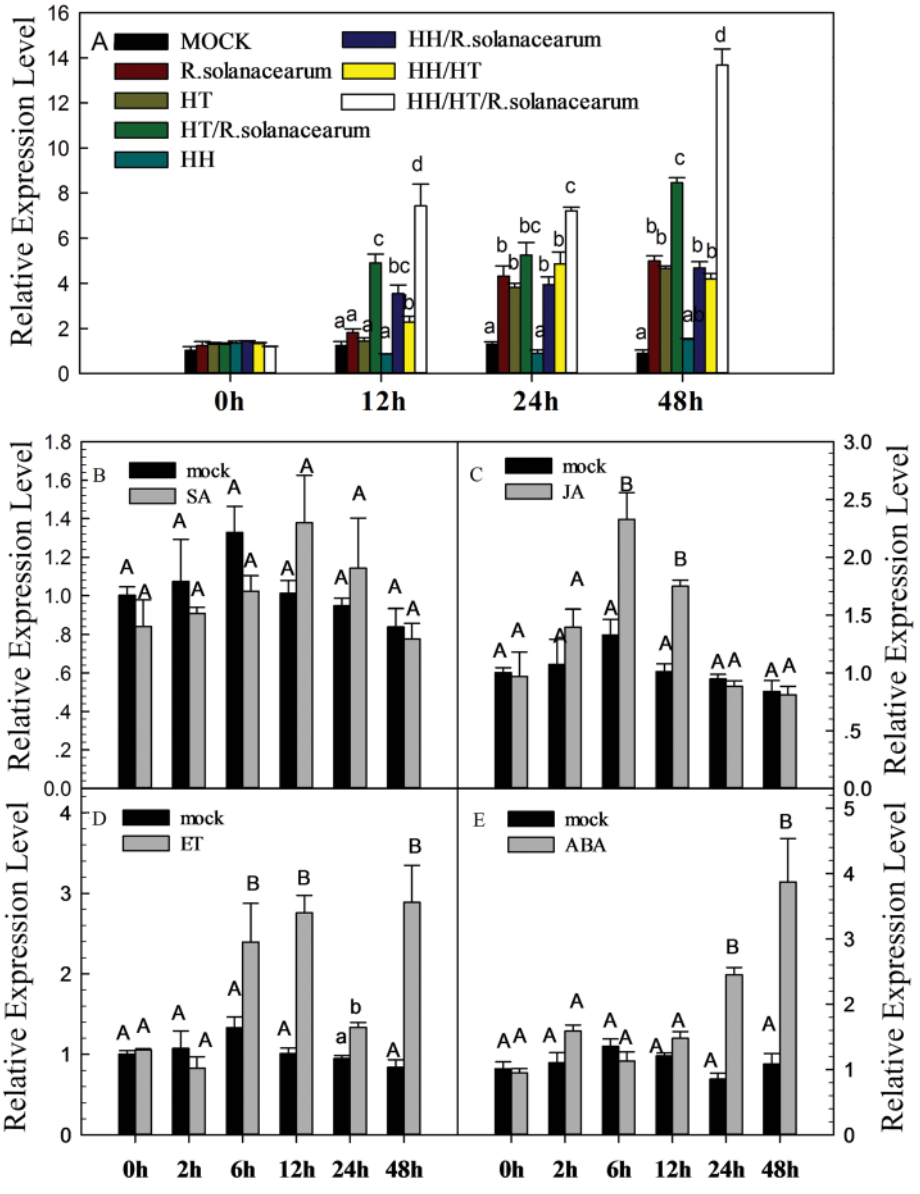

Fig. 3. qPCR analyses of relative *CaWRKY6* transcript levels in leaves of pepper plants exposed to biotic and abiotic stresses. (A) Leaves at different time points after inoculation with the highly virulent *R. solanacearum* strain FJC100301, with 37 °C heat shock under 100% humidity. (B–E) Leaves at various times after treatment with 1 mm SA (B), 100 μm MeJA (C), 100 μm ETH (D), or 100 μm ABA (E). The transcript levels in stress- or hormone-treated pepper leaves were compared with those in mock-treated control plants (normalized to a relative expression level of 1). Error bars indicated the standard error. Different capital letters indicate significant differences from three independent experiments based on the LSD test ($P<0.01$). Different lower-case letters indicate significant differences from three independent experiments based on the LSD test ($P<0.05$).

polymerized DAB (dark brown) and hypersensitive response (HR)-mimicking cell death were detected in the control leaves at 24 hpi, whereas the intensities of DAB and trypan blue staining were distinctly reduced in *CaWRKY6*-silenced leaves (Fig. 4D).

The expression of known pepper defence genes involved in the response to pathogen infection was analysed by qPCR. The transcript levels of the defence-related pepper genes *CaHIR1*, *CaPO2*, *CaNPR1*, *CaDEF1*, *CaACO1*, and *CaABR1* were reduced in *CaWRKY6*-silenced leaves compared with controls in at least one of the two tested time points (0 or 24 h after infection with *R. solanacearum* FJC100301; Fig. 4E). When challenged with HT under 100% humidity, *CaWRKY6*-silenced leaves exhibited significantly increased thermosensitivity compared with that of the wild-type control plants (Fig. 4C). The expression of known pepper defence genes involved in the response to HT was analysed by qPCR. The transcript levels of the HT-related pepper genes *CaHSP24*, ER-small *CaHSP*, and Chl-small *CaHSP* were decreased in *CaWRKY6*-silenced leaves relative to control pepper plants in at least one of the two tested time points (Fig. 4F).

Transient expression of CaWRKY6 *induces the HR, cell death, and accumulation of* H_2O_2 *in pepper leaves*

HR is a mechanism used by plants to prevent pathogen spreading from infected cells to uninfected cells, which

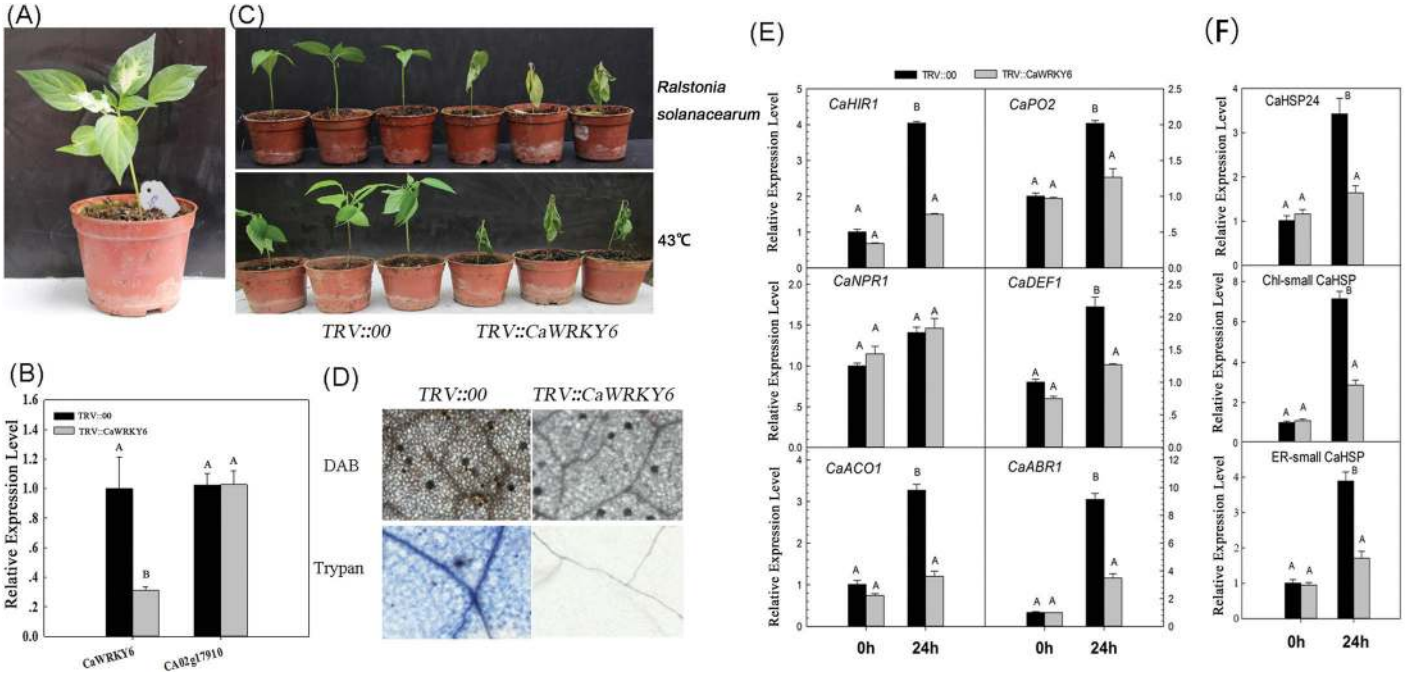

Fig. 4. *CaWRKY6*-silenced pepper plants display a greater susceptibility to *R. solanacearum* FJC100301 infection and heat stress. (A) Silencing of pepper PDS was phenotypically visible at 30 d after agroinfiltration (dag). (B) qPCR analysis of expression of *CaWRKY6* and its orthologue *CA02g17910* in *CaWRKY6*-silenced (*TRV::CaWRKY6*) and control (*TRV::00*) plants. (C) Effect of *R. solanacearum* FJC100301 infection on *CaWRKY6*-silenced (*TRV::CaWRKY6*) and control (*TRV::00*) plants. (D) Effect of HT treatment on *CaWRKY6*-silenced and control leaves. The pepper plants were treated at 43 °C for 24 h, and then kept under normal temperature conditions (25 °C) for 24 h before checking the phenotype. (E) qPCR analyses of defence or thermotolerance-related gene transcript levels in *CaWRKY6*-silenced (*TRV::CaWRKY6*) and control (*TRV::00*) plants after inoculation with or without *R. solanacearum* or heat-stress treatment.

involves the production of ROS and induction of cell death in infected cells. We tested whether *CaWRKY6* was involved in the regulation of HR. *CaWRKY6* was transiently expressed in pepper leaves by infiltration with *A. tumefaciens* GV3101 carrying *35S::00* (empty vector) or *35S::CaWRKY6*, and expression was assessed by staining with trypan blue to identify necrotic cells. The empty vector control did not induce HR-mediated cell death or a necrotic response, whereas the *35S::CaWRKY6* construct distinctly induced a necrotic response in pepper leaves (Fig. 5A).

H_2O_2 production in pepper leaves transiently expressing *CaWRKY6* was visualized by staining with DAB. DAB staining was weak or absent in leaves transiently expressing the empty vector (Fig. 5A). We used an ion leakage test to analyse the severity of cell necrosis caused by plasma membrane damage in cells expressing *CaWRKY6*. Pepper leaves transiently expressing *CaWRKY6* exhibited more ion leakage at 24 and 48 h after agroinfiltration than that in leaves expressing the empty vector control (Fig. 5B). qPCR analysis of *CaWRKY6* transcripts during transient expression of *35S::00* or *35S::CaWRKY6* showed that transcript levels were higher in leaves expressing *35S::CaWRKY6* than in control leaves (Fig. 5C). We also examined changes in the expression of defence-related genes including the SA-responsive *CaNPR1* and *CaSAR8.2*, JA-responsive *CaDEF1*, ET biosynthesis-associated *CaACO1* and *CaPR4*, ABA-responsive *CaABR1*, HR marker *CaHIR1*, ROS detoxification-associated *CaPO2*, and heat-shock response genes *CaHSP24*, ER-small *CaSHP*, and Chl-small *CaHSP* (Fig. 5D, E). The results showed that the relative transcript levels of *CaDEF1*, *CaACO1*, *CaPR4*, *CaHIR1*, *CaABR1*, *CaPO2*, *CaHSP24*, ER-small *HSP*, Chl-small *HSP* increased continuously during transient expression of *CaWRKY6*. This indicated that transient *CaWRKY6* overexpression could induce HR and thermotolerance in pepper plants.

The inter-relationship between CaWRKY6 *and* CaWRKY40

Our data showed similarities between the results for *CaWRKY6* in the present study and those for *CaWRKY40* from a previous study (Dang *et al.*, 2013). *CaWRKY40* was upregulated by heat stress with 100% humidity and by *R. solanacearum* FJC100301 inoculation, and acted as a regulator of resistance to FJC100301 inoculation and tolerance to HTHH. Therefore, we used qPCR to test the expression of *CaWRKY6* in pepper leaves that transiently expressed *CaWRKY40*, and the expression of *CaWRKY40* in pepper leaves transiently expressing *CaWRKY6* or silenced for *CaWRKY6*. The results showed that the expression of *CaWRKY6* was enhanced in *CaWRKY40*-expressing leaves (Fig. 6A), and the expression of *CaWRKY40* increased in *CaWRKY6*-expressing leaves (Fig. 6B). This indicated a possible functional interconnection between *CaWRKY6* and *CaWRKY40*, which was further supported by gene silencing. The results showed that, when challenged with *R. solanacearum* FJC100301 inoculation or heat shock, *CaWRKY6* silencing attenuated the transcriptional expression of *CaWRKY40* (Fig. 6C, D),

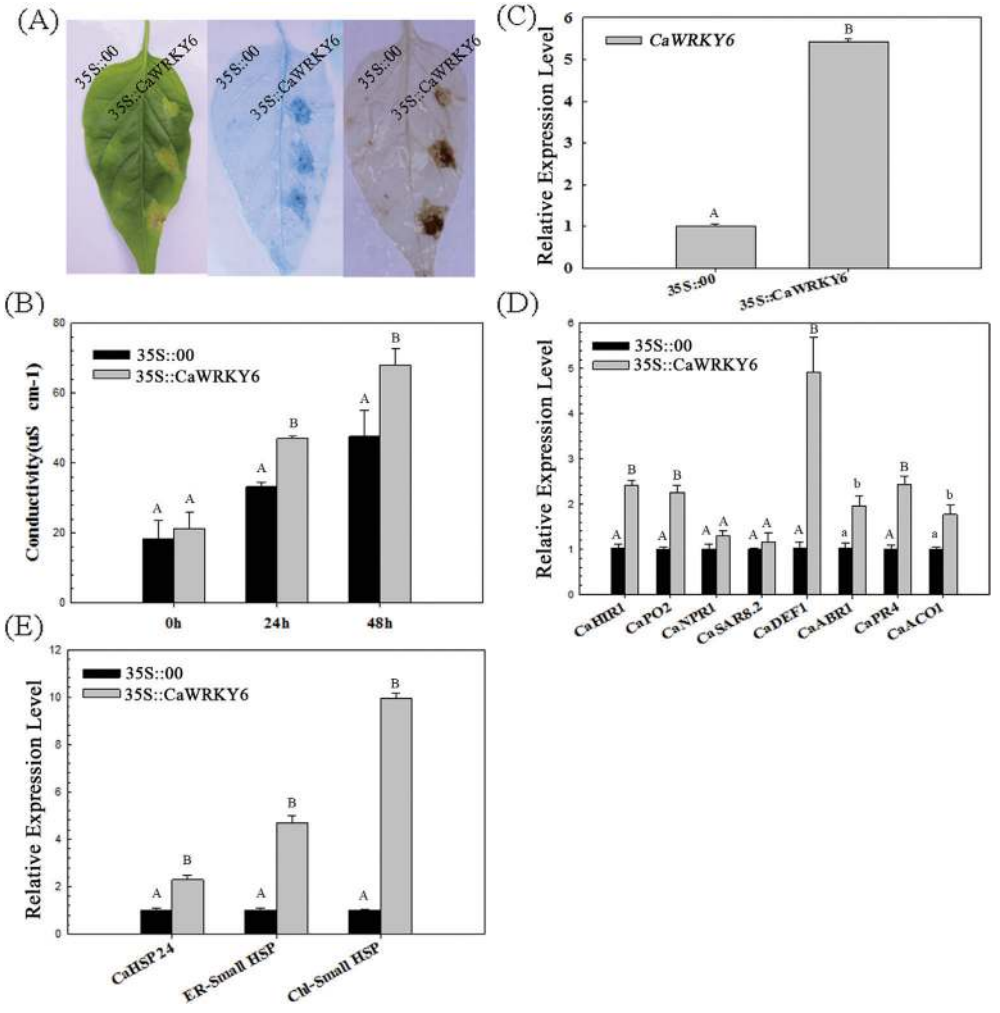

Fig. 5. Cell death response in pepper leaves infiltrated with *A. tumefaciens* GV3101 carrying the *35S:00* (pK_7WG_2 empty vector) or *35S:CaWRKY6* construct. (A) Phenotypes of *CaWRKY6*-expressing leaves at 4 dag (left), trypan blue staining of leaf tissues at 1 dag (middle), and DAB staining of leaf tissues at 1 dag (right). (B) Electrolyte leakage assay of *CaWRKY6*-expressing leaves at different time points after agroinfiltration. (C) qPCR analysis of *CaWRKY6* expression in control and *CaWRKY6*-expressing leaves. (D, E) Real-time qPCR analysis of defence- or thermotolerance-related gene expression in *CaWRKY6*-expressing leaves. Transcript levels were normalized to the expression of pepper *CaActin* measured in the same samples. Error bars indicated the standard error. Significant differences from three independent experiments based on the LSD test are denoted by upper-case ($P<0.01$) and lower-case ($P<0.05$) letters.

whereas, *CaWRKY40* silencing did not affect *CaWRKY6* expression (Fig. 6E, F). Consistently, *CaWRKY40* silencing significantly reduced the HR-mimicking cell death triggered by *CaWRKY6*, whereas *CaWRKY6* silencing did not affect HR-mediated cell death induced by *CaWRKY40* (Fig. 6G). These results suggested that *CaWRKY6* may function upstream of *CaWRKY40*.

The ChIP study showed that CaWRKY6 bound to the *CaWRKY40* promoter, which contains four classic W-boxes (TTGACCY) (Fig. 6H), and this supports our proposal that *CaWRKY6* regulates the pepper response to HTHH and *R. solanacearum* FJC100301 inoculation by acting upstream of *CaWRKY40*. Since *CaHIR1*, *CaPO2*, *CaDEF1*, *CaABR1*, *CaPR4*, and *CaACO1* were previously found to be transcriptionally modulated by CaWRKY40, to test whether these marker genes are common targets of CaWRKY6 and CaWRKY40, a ChIP analysis was carried out with CaWRKY6 and CaWRKY40 against the promoters of *CaHIR1*, *CaPO2*, *CaDEF1*, *CaHSP24*, ER-small *HSP*. The results showed that both CaWRKY6 and CaWRKY40 bound to the promoters of these marker genes (Fig. 7).

Discussion

Our results demonstrated that CaWRKY6 is a subgroup IIb member of the WRKY family in *C. annuum*, and activates *CaWRKY40* by binding to the promoter. CaWRKY6 acts as positive regulator of pepper thermotolerance to HT, HTHH, and pepper resistance to *R. solanacearum* infection combined with HTHH.

There is evidence that pepper CaWRKY6 participates synergistically in pepper immunity to *R. solanacearum* and thermotolerance. First, *CaWRKY6* was induced by *R. solanacearum* infection, HT, HH, and HTHH, and by exogenous application of MeJA, ETH, and ABA [the phytohormones involved in plant immunity (Zimmerli *et al.*, 2004) and the heat-stress response (Larkindale and Knight, 2002)]. Close association between transcriptional upregulation by biotic

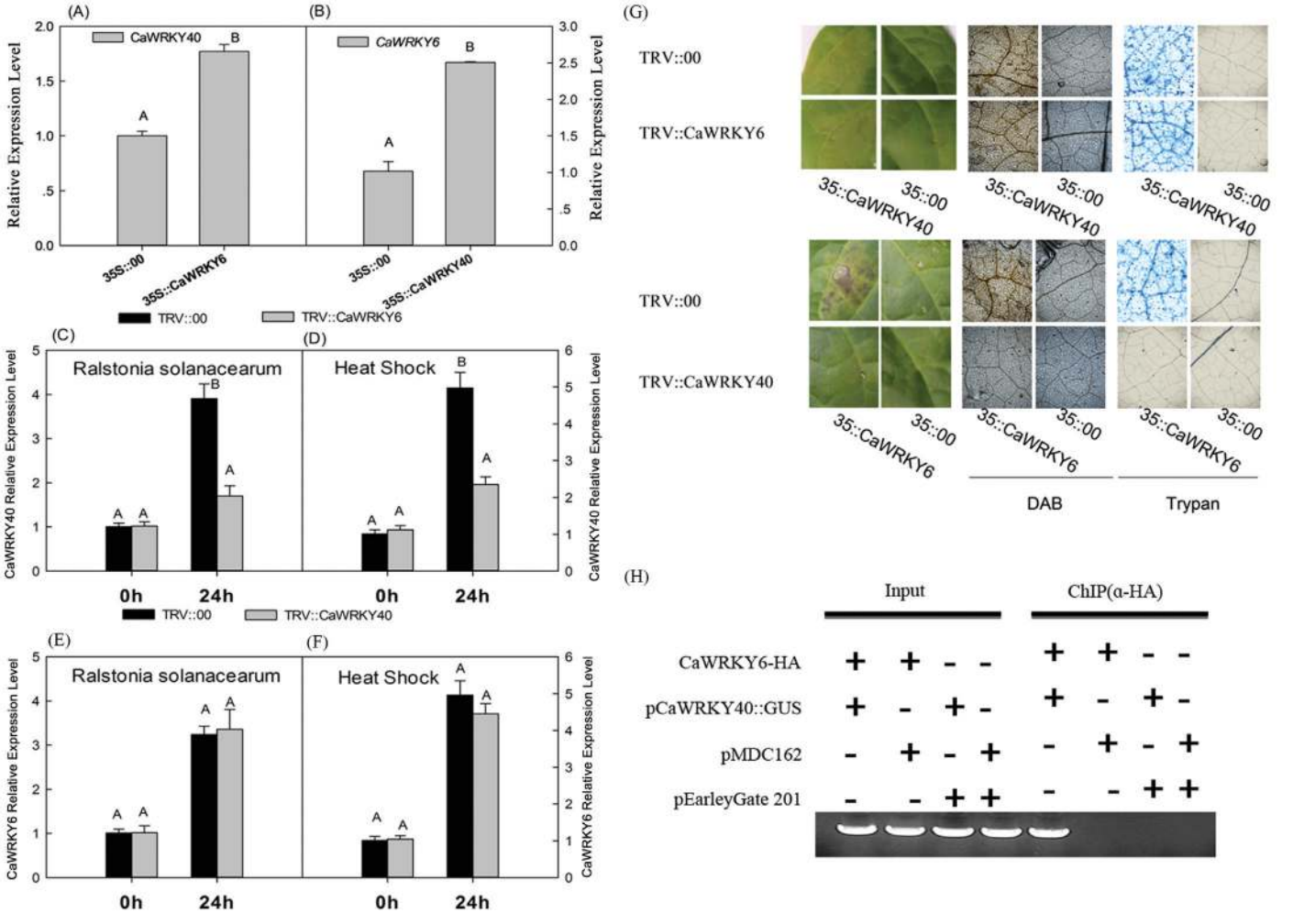

Fig. 6. Analysis of the inter-relationship between *CaWRKY6* and *CaWRKY40*. (A) Transcriptional expression of *CaWRKY40* in pepper leaves transiently overexpressing *CaWRKY6*. (B) Transcriptional expression of *CaWRKY6* in *CaWRKY40* transiently overexpressing pepper leaves. (C, D) qPCR analyses of *CaWRKY40* in *CaWRKY6*-silenced and control pepper plants after inoculation with *R. solanacearum* FJC100301 (C) or heat-stress treatment (43 ° for 24 h) (D). (E, F) qPCR analysis of *CaWRKY6* in *CaWRKY40*-silenced and control pepper plants after *R. solanacearum* FJC100301 infection (E) or heat-stress treatment (43 °C for 24 h) (F). (G) Cell death in pepper leaves infiltrated with *A. tumefaciens* GV3101 harbouring the *35S:00*, *35S:CaWRKY6*, or *35S:CaWRKY40* construct in *TRV::00* or *CaWRKY6*- or *CaWRKY40*-silenced pepper plants. (H) CaWRKY6 binds to the *CaWRKY40* promoter *in vivo* as detected by ChIP. DNAs were immunoprecipitated using anti-HA antibodies, adjusted to the same concentration, and PCRs were performed using *2×W-p35Score::GUS*-specific primers. Lanes 1–3, input (total DNA–protein complex); lanes 4–6, DNA–protein complex immunoprecipitated with anti-HA antibody.

and/or abiotic stresses and roles in plant responses to these stresses have frequently been reported in a large number of WRKY genes previously (Dong *et al.*, 2003; Chen *et al.*, 2012; Wei *et al.*, 2013). Secondly, *CaWRKY6*-silenced pepper plants displayed higher susceptibility to *R. solanacearum* infection and attenuated basal thermotolerance under 100% humidity, and downregulated the expression of HR marker gene *CaHIR1*, JA-dependent *CaDEF1*, ET biosynthesis-associated *CaACO1*, and ABA-dependent *CaABR1*. When *CaWRKY6*-silenced pepper plants were inoculated with *R. solanacearum*, the silencing of *CaWRKY6* also downregulated expression of thermotolerance-associated *HSP24*, ER-small *HSP*, and Chl-small *HSP* (Abbaszadegan *et al.*, 1997) when the plants were challenged by heat stress. Thirdly, overexpression of *CaWRKY6* significantly activated the expression of HR-associated, JA-dependent, and ET-dependent *PR* genes (*CaHIR1*, *CaPO2*, *CaDEF1*, and *CaACO1*), thermotolerance-associated *HSP24*, ER-small *HSP* and Chl-small *HSP*, and ABA-dependent *ABR1*, which was strongly induced by infection of avirulent *Xanthomonas campestris* pv. *vesicatoria* and by ABA application, and is essential for induced cell death associated with ABA–SA antagonism (Choi and Hwang, 2011). Similar synergistic regulation of heat-stress and pathogen infection responses were reported for *AtWRKY39* (Dong *et al.*, 2003; Li *et al.*,, 2010) and *CaWRKY40* (Dang *et al.*, 2013).

Phytohormones are important signalling molecules involved in the plant response to pathogen infection and heat stress, and in the crosstalk between the plant response to biotic and abiotic stress (Jefferson *et al.*, 1987). As mentioned above, *CaWRKY6* was upregulated by exogenously applied JA, ET, and ABA. Consistently, transient overexpression of *CaWRKY6* in pepper plants induced JA-, ET-, and ABA-associated marker genes, whereas silence of *CaWRKY6* in

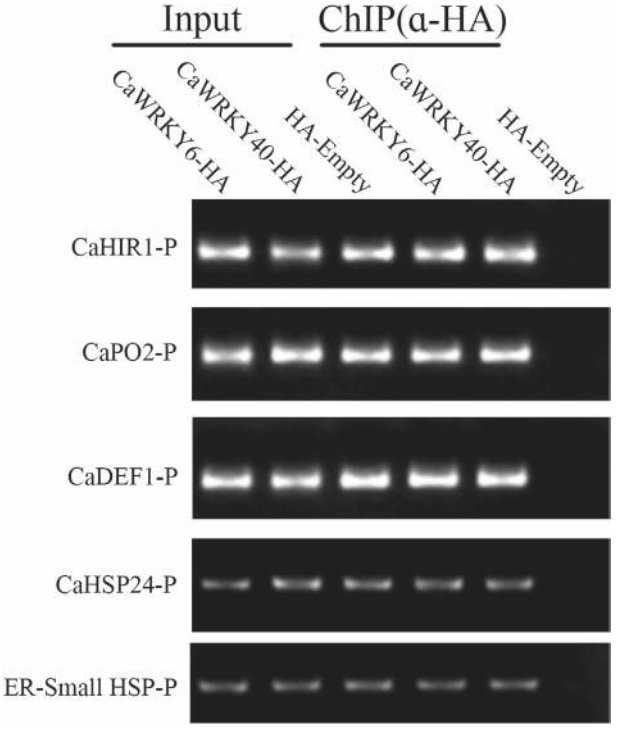

Fig. 7. Both CaWRKY6 and CaWRKY40 bind to the promoters of defence and thermotolerance-associated marker genes *in vivo* by ChIP. DNAs immunoprecipitated using anti-HA antibodies were adjusted to the same concentration and PCRs were performed using specific primers of the promoters of the tested defence and thermotolerance-associated marker genes. Lanes 1–3, input (total DNA–protein complex); lanes 4–6, DNA–protein complex immunoprecipitated using anti-HA antibody.

pepper plants downregulated JA-, ET-, and ABA-associated marker genes. All these data strongly suggested that *CaWRKY6* is potentially associated with signalling pathways mediated by JA, ET, and ABA. ABA-, JA-, and ET-mediated signalling was previously linked not only to plant immunity (Adie *et al.*, 2007; Garcia-Andrade *et al.*, 2011; Chen *et al.*, 2013) but also to the crosstalk between biotic and abiotic stress-signalling networks (Zhang *et al.*, 2009; Liu *et al.*, 2012; Chen *et al.*, 2013), suggesting that the synergistic response of pepper to *R. solanacearum* and heat stress mediated by *CaWRKY6* is by JA, ET, and ABA pathways; however, other signalling pathways cannot be excluded.

Members of the WRKY TF superfamily may be functionally connected and form a transcriptional network composed of positive and negative feedback loops and feed-forward modules (Eulgem and Somssich, 2007; Cheng *et al.*, 2012). The expression and function of *CaWKRY6* exhibited similarities to that of *CaWRKY40* (Dang *et al.*, 2013) in that both genes are upregulated by *R. solanacearum* and HTHH, and their overexpression in pepper both conferred increased resistance to *R. solanacearum* and tolerance to HTHH. In the present study, the gain-of-function and loss-of-function analyses suggested an inter-relationship and expression of *CaWKRY6* and *CaWRKY40*. *CaWRKY6* might act upstream of *CaWRKY40* in pepper in response to pathogen infection or/and HTHH, suggesting that *CaWRKY6* and *CaWRKY40* may act in co-regulatory networks. This hypothesis was confirmed by ChIP analyses, which showed that CaWRKY6 bound to the *CaWRKY40* promoter. Additionally, our data for ChIP analysis showed that both CaWRKY6 and CaWRKY40 can bind to the promoter of *CaHIR1*, *CaPO2*, *CaDEF1*, *CaHSP24* and ER-small HSP, suggesting that these marker genes are at least a part of the common targets of CaWRKY6 and CaWRKY40, and CaWRKY6 not only modulates the expression of these genes by activating CaWRKY40 but also activates these genes directly. However, these two functionally related WRKY genes might be regulated by different signalling pathways. Besides JA, ET, and ABA signalling, *CaWRKY40* is also regulated by SA signalling (Dang *et al.*, 2013), whereas *CaWRKY6* is regulated by JA-, ET-, and ABA-mediated but not by SA-mediated signalling. *CaWRKY6* regulates induced immunity in pathogen-infected leaves but not in systemic leaves. We proposed that transcriptional expression of *CaWRKY40* might be partially regulated by *CaWRKY6* associating with JA-, ET-, and ABA-mediated signalling, and that expression of *CaWRKY40* may be regulated by other factors in an SA-dependent manner. Further investigation is required to determine the role of *CaWRKY6–CaWRKY40* interaction, how pepper resistance to disease resistance under HTHH is fine-tuned, and how the *CaWRKY6–CaWRKY40* interaction is modulated by different signal transduction pathways in different environments.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Pepper primers used for qPCR in this study.

Supplementary Table S2. Pepper primers used for ChIP PCR in this study.

Supplementary Fig. S1. Comparison of amino acid sequences of *CaWRKY6* with representative related proteins.

Supplementary Fig. S2. Subcellular localization of *CaWRKY6*.

Acknowledgements

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