Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2013) **14**(2), 131–144 DOI: 10.1111/j.1364-3703.2012.00836.x

CaWRKY58, encoding a group I WRKY transcription factor of *Capsicum annuum*, negatively regulates resistance to *Ralstonia solanacearum* infection

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SUMMARY

WRKY transcription factors are encoded by large gene families across the plant kingdom. So far, their biological and molecular functions in nonmodel plants, including pepper (*Capsicum annuum*) and other Solanaceae, remain poorly understood. Here, we report on the functional characterization of a new group I WRKY protein from pepper, termed CaWRKY58. Our data indicate that CaWRKY58 can be localized to the nucleus and can activate the transcription of the reporter *β-glucuronidase* (*GUS*) gene driven by the *35S* core promoter with two copies of the W-box in its proximal upstream region. In pepper plants infected with the bacterial pathogen *Ralstonia solanacearum*, *CaWRKY58* transcript levels showed a biphasic response, manifested in an early/transient down-regulation and late up-regulation. *CaWRKY58* transcripts were suppressed by treatment with methyl jasmonate and abscisic acid. Tobacco plants overexpressing *CaWRKY58* did not show any obvious morphological phenotypes, but exhibited disease symptoms of greater severity than did wild-type plants. The enhanced susceptibility of *CaWRKY58*-overexpressing tobacco plants correlated with the decreased expression of hypersensitive response marker genes, as well as various defence-associated genes. Consistently, *CaWRKY58* pepper plants silenced by virus-induced gene silencing (VIGS) displayed enhanced resistance to the highly virulent *R. solanacearum* strain FJC100301, and this was correlated with enhanced transcripts of defence-related pepper genes. Our results suggest that *CaWRKY58* acts as a transcriptional activator of negative regulators in the resistance of pepper to *R. solanacearum* infection.

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INTRODUCTION

Plants are frequently exposed to attack by a wide variety of microbial pathogens. In addition to constitutive defences, such as a waxy cuticle or preformed antimicrobial compounds (Grennan, 2006), plants employ inducible innate immunity, which consists of two interconnected branches, known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones and Dangl, 2006). PTI is activated by PAMPs or microbe-associated molecular patterns (MAMPs) through plant cell surface pattern recognition receptors (PRRs), resulting in nonhost resistance. Adapted pathogens secrete effector molecules which suppress PTI. As a countermeasure, plants evolve resistance (R) proteins that recognize effectors and activate ETI in a gene-for-gene mode (Pieterse *et al*., 2009). PTI and ETI are interconnected with each other via various regulatory components, such as PRRs and R proteins (Qi *et al*., 2011), ADP ribosylation factor and guanine nucleotide exchange factor (Nomura *et al*., 2011), and activate local as well as systemic defence responses (Fu *et al*., 2009). Extensive gene expression and metabolism reprogramming has been associated with PTI and ETI, and appears to be controlled by complex signalling networks. Previous studies have shown that various phytohormones (Lenz *et al*., 2011), reactive oxygen intermediates (De Vleesschauwer *et al*., 2009), mitogen-activated protein (MAP) kinases (Ren *et al*., 2010) and transcription factors (TFs), such as WRKY TFs, have roles in both PTI and ETI (Pandey and Somssich, 2009; Tsuda and Katagiri, 2010). Differences in transcriptional responses between ETI and PTI appear to be quantitative rather than qualitative (Maleck *et al*., 2000). The activation of immune responses during ETI is faster, more prolonged and more robust than those observed during PTI (Jones and Dangl, 2006). Phytohormones, especially salicylic acid (SA) and jasmonic acid (JA), play crucial roles in PTI and ETI (Koornneef and Pieterse, 2008). SA-dependent plant defences are often triggered by biotrophic pathogens (Glazebrook,

2005), whereas JA-dependent plant defences are generally activated by necrotrophic pathogens (Spoel *et al*., 2007) and herbivores (insects; Zhang *et al*., 2011). The balance between these defence hormones is dependent on the recognized pathogens, and SA and JA signalling can act antagonistically or synergistically, dependent on their concentration, allowing for the fine tuning of the spectrum of defence reactions (Kunkel and Brooks, 2002).

WRKY proteins are a class of zinc-finger-containing TFs encoded in all plants by large gene families, and are characterized by the presence of one or two 60-amino-acid WRKY domains (Eulgem *et al*., 2000; Rushton *et al*., 2010). In the genome of Arabidopsis and rice, 72 and 109 members of WRKY proteins have been found, respectively (Eulgem and Somssich, 2007; Zhang and Wang, 2005). WRKY members have been classified into three groups (I, II and III) and various subgroups (e.g. IIa, IIb, etc.) based on their primary structure (Eulgem *et al*., 2000). It is generally assumed that WRKY TFs act as major regulatory proteins by binding to the W-box [TTGAC(C/T)], a common *cis*-element in the promoters of their target genes. Many WRKY TFs have been found to participate in the regulation of defence responses to biotic and abiotic stresses (Rushton *et al*., 2010). In *Arabidopsis*, WRKY58 acts downstream of *NPR1*, a negative regulator of systemic acquired resistance (SAR; Wang *et al*., 2006), whereas WRKY39 is positively co-regulated by the SA and JA signalling pathways, enhancing thermotolerance (Li *et al*., 2010). Previous studies have suggested that WRKY proteins not only participate in the regulation of ETI, but also in the regulation of PTI, and that specific WRKY proteins can function as positive or negative regulators in the basal defence responses of plants (Eulgem and Somssich, 2007). Thus, WRKY proteins appear to participate in the tight regulation and fine tuning of the complex signalling and transcriptional networks of plant defence.

Pepper (*Capsicum annuum*) is a crop of agricultural importance worldwide, and a continuous cropping-sensitive crop because of diseases, such as phytophthora blight and pepper bacteria wilt (Hayward, 1991; Ristaino and Johnston, 1999). *Ralstonia solanacearum* is a devastating soil-borne bacterium causing wilting disease in over 200 economically important plant species, including pepper and tobacco (Boucher and Genin, 2004). Understanding the genetic mechanisms of plant disease resistance against *R. solanacearum* will benefit enormously the development of improved varieties of various crops. The manipulation of regulatory processes at the level of transcriptional regulation appears to be particularly promising, as this may allow a change in the expression characteristics of key components relevant for a specific trait, with minimal disturbance of other cellular processes. To date, only a small number of WRKY TFs from pepper have been cloned and functionally characterized, including CaWRKYb (Lim *et al*., 2011), CaWRKY2 (Oh *et al*., 2006), CaWRKY-a (Oh *et al*., 2006) and CaWRKY1 (Oh *et al*., 2008), which have been shown to play defence-related roles. In the present study, we report a novel WRKY protein from pepper, designated *CaWRKY58*, which acts as a negative regulator of immunity against *R. solanacearum*.

RESULTS

Cloning and sequence analysis of *CaWRKY58*

By searching the expressed sequence tags (ESTs) of *C. annuum* by TBLASTN (http://www.ncbi.nlm.nih.gov), we identified nine ESTs with similarity to AtWRKY58 TF. One of these ESTs was found to harbour two WRKY domains in its deduced amino acid sequence and was chosen to design and synthesize the primer. With the primer pairs, we performed a polymerase chain reaction (PCR)-based screen of a cDNA library constructed from the RNA of UVB-treated pepper leaves. A positive clone isolated was found to be 1493 bp in length, comprising 41 bp of 5′ untranslated region (UTR), a 1137-bp open reading frame (ORF) and a 315-bp 3′ UTR. Its ORF was predicted to encode a protein of 378 amino acid residues, harbouring two 60-amino-acid-containing WRKY domains (Eulgem *et al*., 2000). The relative molecular mass and theoretical p*I* of the predicted protein were 42.0 kDa and 8.21, respectively. Homology searches further confirmed the high similarity of the superfamily conserved domains to known WRKY TFs, and suggested that it represented a member of WRKY subgroup I (Eulgem *et al*., 2000). Alignment analysis of this WRKY sequence with related sequences revealed that it shared 100% amino acid identity with the protein encoded by a previously identified pepper WRKY gene (Q5DJV3) (http://www.uniprot.org). Furthermore, we found that it shared 46.0%, 44.0% and 51.0% identity with AtWRKY3 (Q9ZQ70), AtWRKY4 (Q9XI90) and AtWRKY58 (Q93WU7), respectively. As AtWRKY58 is the most closely related *Arabidopsis* WRKY protein, we termed the cDNA clone CaWRKY58, which exhibited 77.0% and 48.0% amino acid sequence homology with *Nicotiana tabacum* (common tobacco) WRKY-7 (Q94IB5) and *Vitis vinifera* (grape) WRKY2 (Q5IY47), respectively (Fig. 1). To date, there is no functional analysis of *CaWRKY58* or any related TF from Solanaceae available, and therefore we continued its characterization.

CaWRKY58 proteins are predominantly localized to the nucleus

Sequence analysis using WoLF PSORT (http://wolfpsort.org/) indicated that the predicted CaWRKY58 protein contains three putative nuclear localization signals (^{165}PAKKKVE^{171}, ^{192}PTKQRKD^{198} and ^{266}PDAKRTK^{272}; Fig. 1). To confirm its nuclear localization, we generated the CaWRKY58–green fluorescent protein (GFP) fusion driven by the constitutive *CaMV35S* promoter. The subcellular locations of the *p35S::CaWRKY58-GFP* gene and *p35S::GFP* gene (negative control; Fig. 2A) in *Nicotiana benthamiana* leaves were visualized using a fluorescence microscope. Typical results indi-

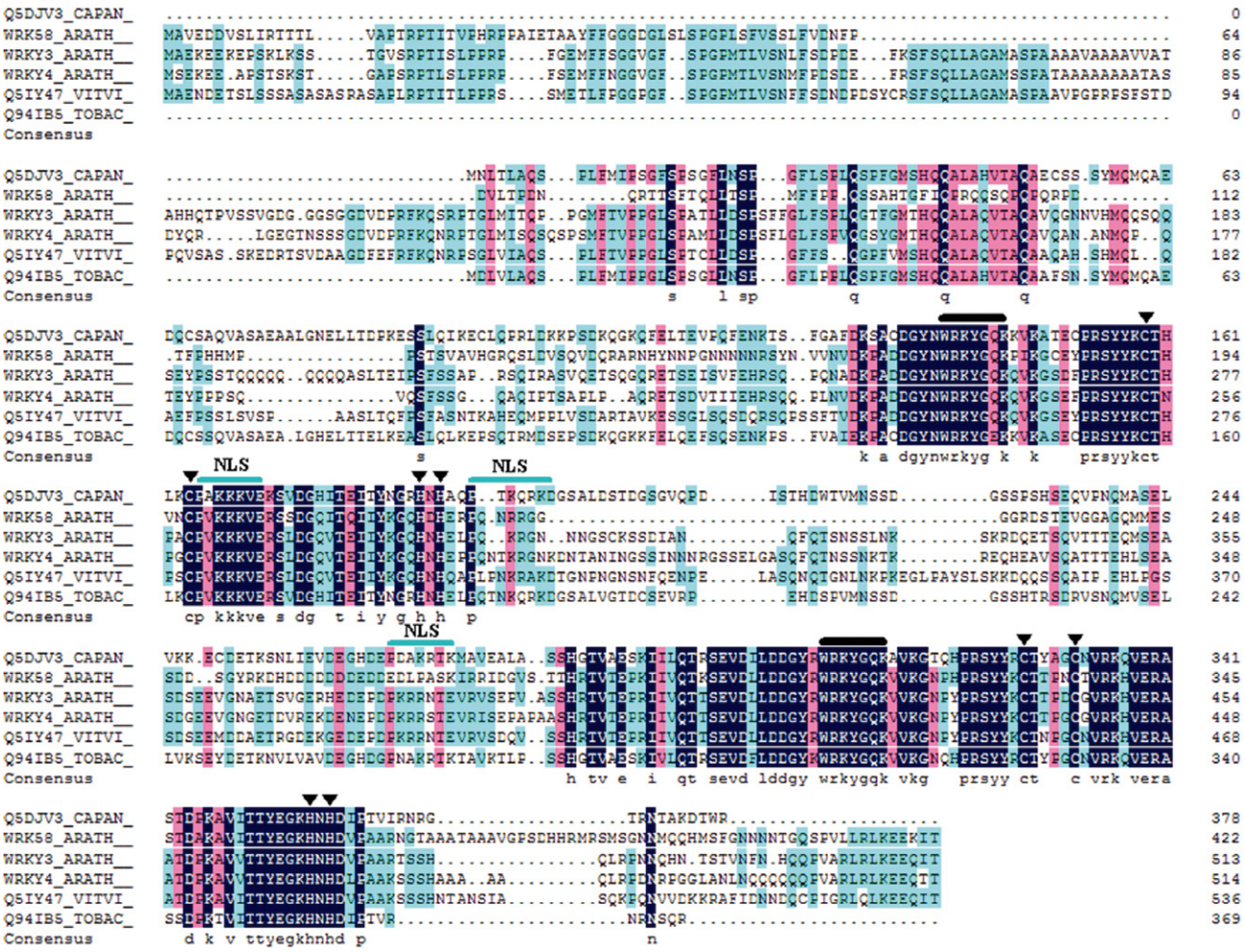

Fig. 1 Comparison of amino acid sequences of CaWRKY58 with representative related proteins *Arabidopsis thaliana* WRKY4 (Q9XI90), AtWRKY3 (Q9ZQ70), AtWRKY58 (Q93WU7), *Nicotiana tabacum* (common tobacco) WRKY DNA-binding protein (Q94IB5) and *Vitis vinifera* (grape) DNA-binding protein WRKY2 (Q5IY47). Green shading, 50%–75% identity; red shading, 75%–100% identity, black shading, 100% identity. Alignment was carried out by DNAMAN5.

cated exclusive localization of CaWRKY58–GFP in nuclei, whereas GFP alone occurred in multiple subcellular compartments, including the cytoplasm and nuclei (Fig. 2B).

CaWRKY58 can activate transcription

Numerous studies have demonstrated sequence-specific binding of WRKY proteins to the W-box [TTGAC(C/T)], which often serve as pathogen-responsive regulatory elements in the promoters of defence-associated genes, including the SA-responsive *pathogenesis-related* genes (Yamamoto *et al*., 2004), as well as many *WRKY* genes (Eulgem *et al*., 1999). To test whether this also applies to CaWRKY58, we performed transient co-expression experiments with an effector vector bearing the full-length *CaWRKY58* cDNA controlled by the *CaMV35S* promoter (*p35S::CaWRKY58*) and a reporter vector containing *GUS* controlled by the *CaMV35S* core promoter (–46 to +8 bp) with (*2* × *W-p35S*$_{core}$*::GUS*) or without (*p35S*$_{core}$*::GUS*) two copies of the W-box in its proximal upstream region (Fig. 3A). Reporter vectors were either transformed individually or co-transformed with the effector construct into onion epidermal cells by particle bombardment, followed by histochemical GUS assays for reporter gene expression. Onion epidermal cells co-transformed with the *2* × *W-p35S*$_{core}$*::GUS* and effector plasmids often stained dark blue; we found dark blue cells in around 25% of the fields examined (Fig. 3B). In contrast, cells transformed with either the reporter vector or co-transformed with *p35S*$_{core}$*::GUS* and *p35S::CaWRKY58* never stained blue.

Response of *CaWRKY58* transcripts to *R. solanacearum* infection

To test whether *CaWRKY58* is involved in immune responses, the transcriptional expression pattern of *CaWRKY58* in the leaves of

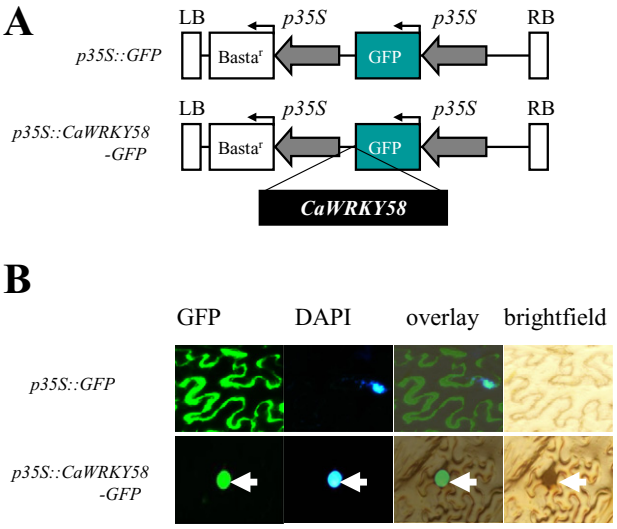

Fig. 2 Subcellular localization of CaWRKY58. (A) Schematic representation of *p35S::GFP* and *p35S::CaWRKY58-GFP* constructs. (B) CaWRKY58-GFP exclusively localized in the nucleus of *Nicotiana benthamiana* leaves; green fluorescent protein (GFP) alone localized throughout the whole cells; 4′,6-diamidino-2-phenylindole (DAPI) images indicate nuclear staining. All images were taken by fluorescence microscopy 24 h after agroinfiltration.

C. annuum in response to infection with *R. solanacearum*, the causal agent of pepper bacterial wilt, was determined by quantitative real-time PCR (qPCR; Fig. 4A). Compared with mock-treated control plants, *CaWRKY58* transcripts were reduced in leaves at 3–12 h post-inoculation (hpi) with the highly virulent *R. solanacearum* strain FJC100301. This decrease was detectable as early as 3 hpi, with the minimal level observable at 6 hpi. The *CaWRKY58* transcript levels returned to their ground state at 24 hpi and continued to increase further, reaching the highest level (1.6-fold induction) at 48 hpi. Induction of *CaPR1* transcript accumulation confirmed that infection with *R. solanacearum* was successful. These results suggest a role of *CaWRKY58* in the regulation of defence reactions of pepper against *R. solanacearum*.

Transcriptional response of *CaWRKY58* to exogenous SA, methyl jasmonate (MeJA), abscisic acid (ABA) and ethylene (ET) application

Phytohormones, such as SA, JA, ABA and ET, serve as important signalling molecules in the regulation of plant defence reactions against biotic and abiotic stresses and play crucial roles in the mediation of the expression of downstream defence genes (Fujita *et al*., 2006). To evaluate the possible involvement of *CaWRKY58* in the signalling cascades utilized by these hormones, *CaWRKY58* transcript levels were determined by qPCR in four-leaf pepper plants exogenously treated with SA, MeJA, ABA and ET. In response to 5 mM SA, the transcript levels of *CaWRKY58* increased within 1 h post-treatment (hpt) to 12 hpt, reaching maximal levels at 6 hpt (2.1-fold relative to mock-treated control plants; Fig. 4B). After 24 h, the *CaWRKY58* transcript levels declined. *CaPR1* transcripts accumulated strongly after application of SA. *CaWRKY58* transcript levels were reduced between 1 and 48 hpt with 100 μM MeJA, whereas the level of the known JA-responsive gene *CaPIN2* was strongly induced (Fig. 4C). Between 1 and 48 hpt with ABA, *CaWRKY58* transcripts declined (Fig. 4D). By contrast, the transcripts of the known ABA-responsive *CaDepro* gene accumulated strongly during the same time interval. Application of 10 mM ET enhanced the *CaWRKY58* transcript levels between 1 and 48 hpt, reaching a maximum around 3 hpt (Fig. 4E). Similarly, transcripts of the known ET-responsive *CaACC oxidase* gene were strongly induced.

Overexpression of *CaWRKY58* increases the susceptibility to *R. solanacearum* infection in tobacco

The differential expression of *CaWRKY58* in response to *R. solanacearum* inoculation and exogenous SA and MeJA application suggested a role for this gene in plant immunity. To test this possibility, we generated transgenic tobacco T_2 lines constitutively expressing *CaWRKY58* driven by the *CaMV35S* promoter (Fig. 5A). We did not observe any phenotypic differences between *CaWRKY58-OE* transgenic T_2 lines and the wild-type parental tobacco line (WT-K326). The highly virulent FJC100301 strain of *R. solanacearum* was used to infect the tobacco plants of *CaWRKY58-OE* and the wild-type. All three tested transgenic lines exhibited enhanced disease symptoms in response to *R. solanacearum* inoculation (not shown). The line *CaWRKY58-OE-1*, which showed the greatest *CaWRKY58* relative transcript levels (not shown) of all the tested lines, was chosen for detailed disease resistance assays. At 4 days post-inoculation (dpi), we observed clear wilting symptoms on *CaWRKY58-OE* line plants, whereas the wild-type exhibited only slight wilting symptoms (Fig. 5B, photographs were taken of plants of a typical transgenic line, *CaWRKY58-OE-1*). At 21 dpi, extremely severe wilting symptoms developed strongly in both the wild-type and *CaWRKY58-OE-1* plants (Fig. 5C). *CaWRKY58-OE* lines expressed *CaWRKY58*, whereas *CaWRKY58* transcripts were not detected in wild-type tobacco plants (Fig. 5D). At 4, 6, 9 and 12 dpi with *R. solanacearum*, clear wilting symptoms were detected in tobacco leaves. At these time points, wild-type tobacco plants exhibited approximately 22%, 61%, 68% and 69%, respectively, of the intensity of wilting symptoms observed in *CaWRKY58-OE-1* plants (Fig. 5E). Similar results were observed in wild-type and *CaWRKY58-OE-1* plant leaves injected with 10 μL of 10^8 colony-forming units (cfu) of *R. solanacearum* using a syringe needle. At 5 dpi, severe wilting symptoms were observed in the leaves of bacterium-injected *CaWRKY58-OE-1* plants, whereas only limited wilting symptoms were present in leaves of wild-type tobacco plants. At 12 dpi, the wilting symptoms were clearly more pronounced in *CaWRKY58-OE-1* than in wild-type plants (Fig. 5F).

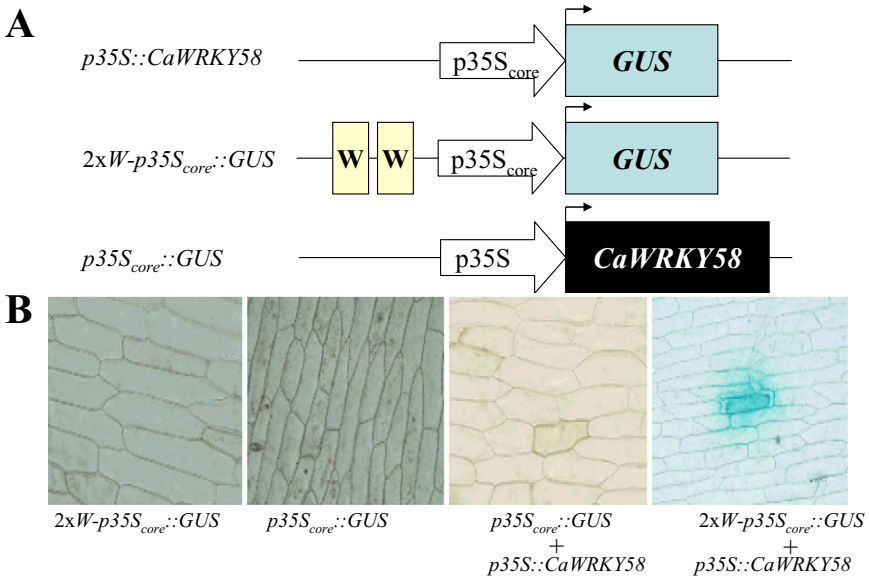

Fig. 3 CaWRKY58 *trans*-activation experiments performed by particle bombardment with onion epidermal cells. (A) Schematic diagram of the reporter and overexpression constructs used for co-transfection in particle-bombarded onion epidermal cells. (B) Onion epidermal cells co-transfected with the indicated reporter and effector plasmids. Dark blue staining was only observed after co-transfection of *2 × W-p35Score::GUS* with *p35S::CaWRKY58*.

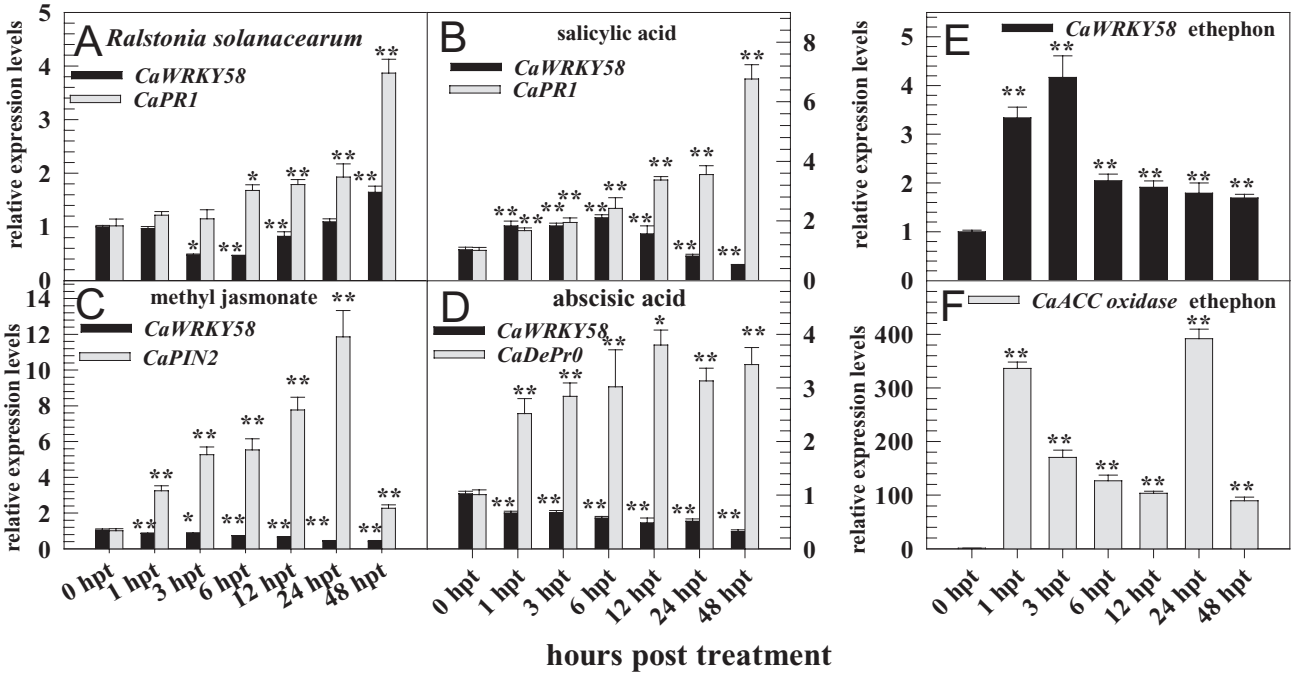

Fig. 4 Quantitative real-time polymerase chain reaction (qPCR) analysis of relative *CaWRKY58* transcript levels in biotic and abiotic stresses. (A) *CaWRKY58* transcript levels measured at different time points in the upper (systematic) pepper leaves after inoculation with the highly virulent *Ralstonia solanacearum* FJC100301 in the roots. The *CaPR1* gene was used as positive control. (B–D) Relative *CaWRKY58* transcript levels in pepper leaves at various time periods after treatment with salicylic acid (SA, 5 mM), methyl jasmonate (MeJA, 100 μM) and abscisic acid (ABA, 100 μM). The *CaPR1*, *CaPIN2* and *CaDePro* genes were used as positive controls, respectively. (E, F) Relative *CaWRKY58* transcript levels in pepper leaves at various time periods after treatment with ethephon (10 mM) with the *CaACC oxidase* gene used as a positive control. (A–F) *CaWRKY58* or marker gene transcript levels in stress- or hormone-treated pepper plants were compared with those in mock-treated control plants, which were set to a relative expression level of '1'. Error bars indicate the standard error; the experiments were repeated three times, together with at least three independent repetitions of the biological experiments. Asterisks indicate significant differences (Student–Newman–Keuls test, $*P < 0.05$ or $**P < 0.01$).

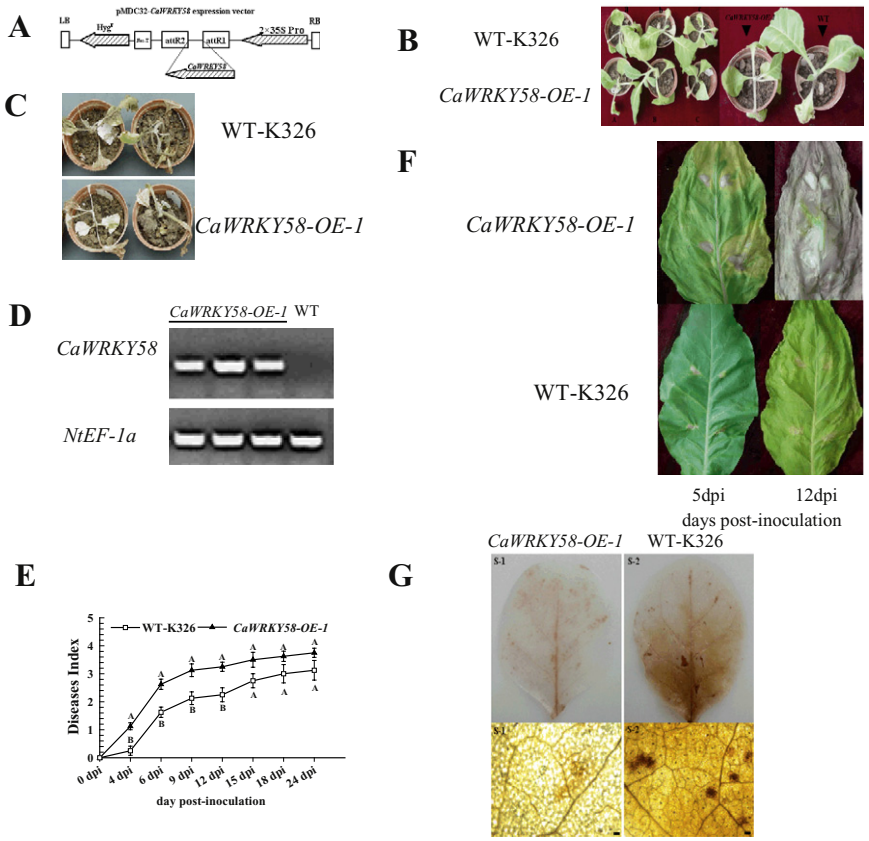

Fig. 5 Transgenic T_2 tobacco plants overexpressing *CaWRKY58* exhibit enhanced susceptibility to *Ralstonia solanacearum*. (A) Schematic representation of the pMDC32-*CaWRKY58* constructs. LB and RB, left and right borders of the T-DNA; attR1 and attR2, recombination sites for Gateway cloning; 2 × 35SPro, two copies of the *Cauliflower Mosaic Virus* 35S promoter; Nos-T, nos-terminator; Hyg^r, hygromycin resistance gene. (B) Seven-week-old plants of the representative transgenic *CaWRKY58-OE-1* line and its parental wild-type line (WT-K326) 4 days post-inoculation (dpi) of their third leaves with 10 μL of a suspension of 10^8 colony forming units (cfu)/mL of the highly virulent *R. solanacearum* strain FJC100301. (C) This photograph was taken at 21 dpi. (D) Semi-quantitative PCR analysis of *CaWRKY58* transcript levels in transgenic and wild-type tobacco plants; *NtEF*-1-α served as endogenous control. (E) *Ralstonia solanacearum*-inoculated leaves scored at internal time points as a disease index ranging from 0 to 4: 0, no wilting; 1, 1%–25% wilted; 2, 26%–50% wilted; 3, 51%–75% wilted; 4, 76%–100% wilted or dead. Averages presented are based on three biological replicates, each comprising five plants, Error bars indicate the standard error. Different letters indicate significant differences as determined by Student–Newman–Keuls test (uppercase difference $P < 0.01$). (F) Parallel experiments were performed in wild-type line (WT-K326) and *CaWRKY58-OE-1* plants inoculated with 10 μL of 10^8 cfu/mL *R. solanacearum* through the third leaves employing needle syringes. The photographs were taken at 5 and 12 dpi. (G) Decreased H_2O_2 production in *CaWRKY58-OE-1* leaves compared with wild-type leaves infected with the highly virulent strain of *R. solanacearum* FJC100301. FJC100301-noninfected leaves were harvested and stained with 3,3′-diaminobenzidine (DAB) at 48 hours post-inoculation. Bars, 0.2 mm.

We also stained *R. solanacearum*-infected *CaWRKY58-OE-1* and wild-type leaves with 3,3′-diaminobenzidine (DAB), which polymerizes instantly and locally on contact with H_2O_2 to form reddish-brown polymers. H_2O_2 release is associated with the development of hypersensitive response (HR) cell death (Van Breusegem and Dat, 2006). Consistent with the lack of HR-like lesions, *CaWRKY58-OE-1* plants exhibited weaker DAB-stained spots relative to the wild-type after inoculation with *R. solanacearum*, indicating lower levels of H_2O_2 accumulation (Fig. 5G).

Consistent with the lack of HR-like lesions and the enhanced susceptibility of *CaWRKY58-OE-1* plants, they exhibited reduced transcript levels of the HR marker genes *NtHSR201* and *NtHSR515* (reduced by 1.85-fold and 2.29-fold, respectively, relative to wild-type plants). Similarly, transcript levels of the SA-regulated *NtPR1a/c*, *NtPR2* and *NtPR4* (Sohn *et al*., 2007) were decreased by 1.64-fold, 2.21-fold and 2.33-fold, respectively, ET signalling-associated marker genes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) *NtACS1* and *NtACS6* (Penrose and Glick, 1997) were reduced 1.33-fold and 1.60-fold, respectively, JA-regulated *NtPR1b* (Sohn *et al*., 2007) was reduced 3.86-fold, and transcripts of *Ntosmotin* (Singh *et al*., 1989), a protein regulated by ABA and implicated in the adaptation to low water potential, declined 2.66-fold in *CaWRKY58-OE-1* plants relative to the wild-type. However, transcript levels of other stress-related genes, such as *NtHIN1*, *NtPR3*, *NtEFE26*, *NtCAT1*and *NtGST1*

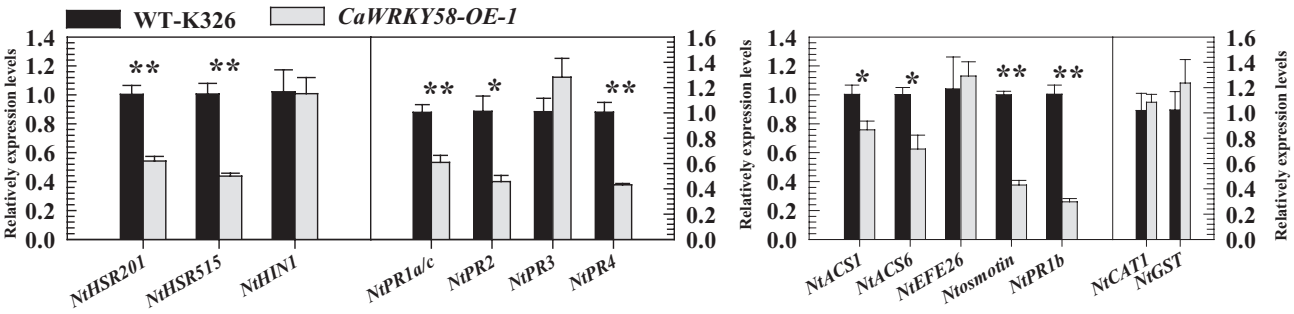

Fig. 6 Quantitative real-time polymerase chain reaction (qPCR) analysis of the relative transcript levels of defence marker genes in *CaWRKY58-OE-1* compared with wild-type tobacco plants. WT, untransformed wild-type tobacco K326. Defence-related gene transcript levels of wild-type tobacco were used as reference, and were set to a relative expression level of '1'. Error bars indicate the standard error; the experiments were repeated three times, together with at least three independent repetitions of the biological experiments. Asterisks indicate significant differences (Student–Newman–Keuls test, $*P < 0.05$ or $**P < 0.01$).

(Takahashi and Nagata, 1992; Takahashi *et al*., 1997), did not show any statistically significant difference ($P < 0.05$) relative to those of wild-type plants (Fig. 6).

NtHSR201, *NtHSR515*, *NtPR1b*, *NtPR1a/c*, *NtPR2* and *NtACS6*, all of which exhibited decreased constitutive transcript levels in *CaWRKY58-OE-1* plants in at least one of the two tested time points, and their transcripts accumulated to significantly lower levels in *CaWRKY58-OE-1* plants relative to the wild-type (Fig. 7). Thus, the constitutive expression and *R. solanacearum* responsiveness of this set of six genes is clearly (directly or indirectly) repressed by the *CaWRKY58* transgene.

A second set of tobacco genes influenced by *CaWRKY58* includes *NtCAT1*, *NtGST1* and *NtEFE26*. At least in one of the two tested time points after *R. solanacearum* infection, the transcripts of each of these three genes accumulated in *CaWRKY58-OE-1* plants to lower levels than in wild-type tobacco. However, *CaWRKY58* does not appear to significantly enhance their constitutive expression.

In a third set of tobacco genes, *NtNPR1* and *Ntacc deaminase*, in at least one of the two tested time points after *R. solanacearum* infection, transcripts of each of these genes accumulated in *CaWRKY58-OE-1* plants to lower levels than in wild-type tobacco.

Taken together, our transcript data were consistent with the observed defence phenotype of *CaWRKY58-OE* plants. Overexpression of *CaWRKY58* probably enhances the susceptibility of tobacco to *R. solanacearum* by reducing the transcript levels of key HR-associated genes and other defence genes.

Effect of virus-induced gene silencing (VIGS) of *CaWRKY58* on *R. solanacearum* resistance in pepper

We also performed loss-of-function experiments with *CaWRKY58*-silenced pepper seedlings. A 310-bp fragment of *CaWRKY58* was used to construct the VIGS vector PYL-279-*CaWRKY58* (Fig. 8A). The efficiency of *CaWRKY58* silencing was evaluated in PYL-279 and PYL-279-*CaWRKY58* pepper plants, and *CaWRKY58* transcript levels were found to be reduced by approximately 90% in VIGS-*CaWRKY58* pepper plants relative to those of PYL-279 plants (Fig. 8B). PYL-279-*CaPDS* (*phytoene desaturase* gene) photobleaching plants were used as a control to identify leaves successfully silenced for *CaWRKY58* expression (Fig. 8C). *CaWRKY58*-silenced leaves exhibited a higher level of *R. solanacearum* resistance relative to nonsilenced wild-type plants, indicating that the silencing of *CaWRKY58* attenuated the *R. solanacearum* susceptibility (Fig. 8D).

Consistent with the decline in *R. solanacearum* susceptibility in PYL-279-*CaWRKY58* relative to PYL-279 pepper plants, the transcript levels of the defence-related pepper genes *CaPR1*, *Caβ-1,3-glucanase*, *CaChitinase*, *CaPR4*, *CaPR10*, *CaNPR1*, *CaACC oxidase* and *CaSIG4* were enhanced in PYL-279-*CaWRKY58* relative to PYL-279 pepper plants in at least one of the two tested time points (6 or 24 h after infection with *R. solanacearum*, Fig. 9). Both the results of *CaWRKY58* overexpression in tobacco and *CaWRKY58* silencing in pepper consistently support a role of this TF as a negative regulator of immunity against *R. solanacearum*.

DISCUSSION

Frequently confronted with a large variety of stresses in their natural ecosystems, plants have evolved elaborate and complicated defence mechanisms, including physiological and metabolic reprogramming, to protect themselves against stresses and to survive. These processes are largely transcriptionally regulated by different TFs and a complicated signalling network. A large body of evidence suggests that WRKY TFs regulate plant host genes in response to pathogen infection, either as positive or negative regulators (Oh *et al*., 2008), including as a positive regulator in response to some pathogens and a negative regulator in response to others (Lai *et al*., 2008).

The results in this study showed that pepper *CaWRKY58* is a *WRKY* gene that encodes a nuclear localized WRKY transcriptional activator that can bind to DNA molecules with W-box sequences. The overexpression of *CaWRKY58* decreased the resistance of

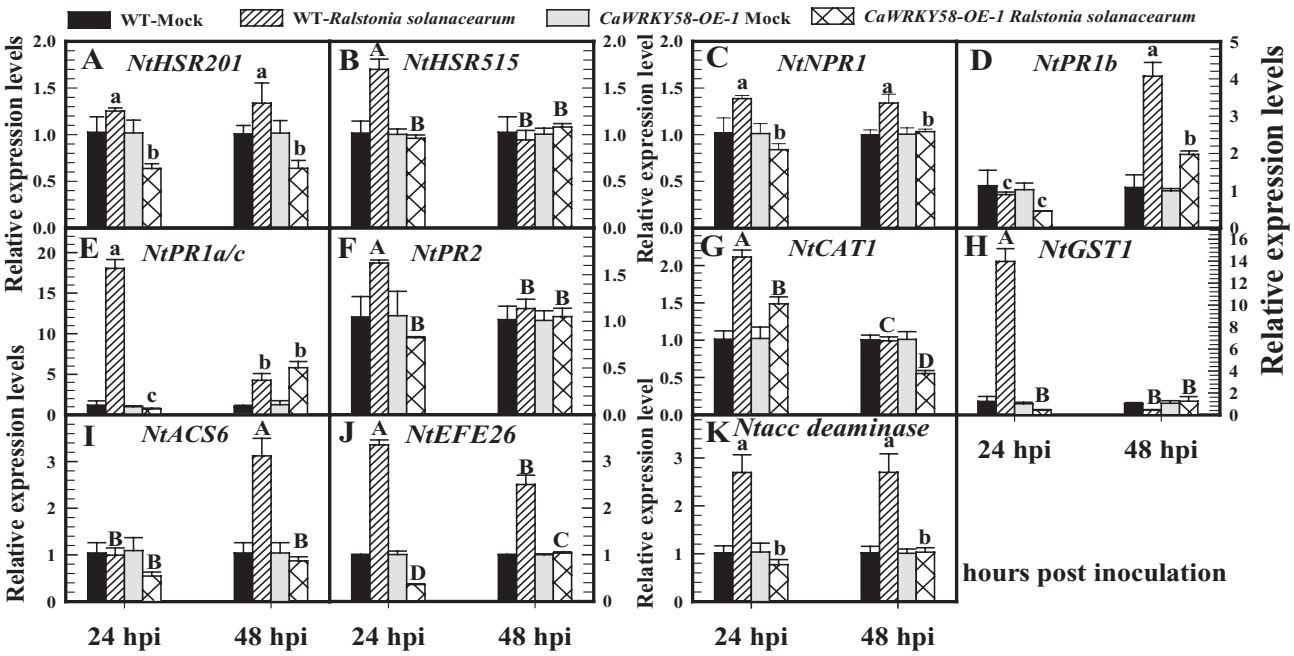

Fig. 7 Relative expression levels of hypersensitive response (HR)-associated and stress-related genes in wild-type (WT) and *CaWRKY58-OE-1* tobacco plants at 24 or 48 h post-inoculation by quantitative real-time polymerase chain reaction (qPCR). (A, B) Relative transcript levels of HR marker genes *NtHSR201* and *NtHSR515*. (C) Relative expression levels of *NtNPR1*. (D) Relative expression levels of the jasmonic acid (JA)-responsive gene *NtPR1b*. (E, F) Relative transcript levels of the salicylic acid (SA)-responsive genes *NtPR1a/c* and *NtPR2*. (G, H) Relative expression levels of *NtCAT1* and *NtGST1*. (I–K) Relative expression levels of ethylene production-associated genes *NtACS6*, *NtEFE26* and *Ntacc deaminase*. Absolute transcript levels of the respective genes in mock-treated wild-type or *CaWRKY58-OE-1* were used as reference, which were set to a value of '1'. Error bars indicate the standard error; the experiments were repeated three times, together with at least three independent repetitions of the biological experiments. Different letters indicate significant differences as determined by Student–Newman–Keuls test (lowercase difference $P < 0.05$; uppercase difference $P < 0.01$).

transgenic tobacco to *R. solanacearum* infection, as well as to *NtHSR201*, *NtHSR515*, SA and JA *PR* gene transcripts. In contrast, *CaWRKY58*-silenced pepper plants displayed enhanced resistance to *R. solanacearum* attack. These results strongly suggest that *CaWRKY58* functions as a negative regulator of pepper resistance to *R. solanacearum* infection. Previous studies have reported that other WRKY TFs, such as *AtWRKY4* (Lai *et al*., 2008), *AtWRKY11*, *AtWRKY17* (Journot-Catalino *et al*., 2006), *AtWRKY27* (Mukhtar *et al*., 2008), *AtWRKY48* (Xing *et al*., 2008), *AtWRKY58* (Wang *et al*., 2006) in *Arabidopsis*, *AtWRKY62* in rice (Peng *et al*., 2008) and *CaWRKY1* in pepper (Oh *et al*., 2008), also act as negative regulators of defence reactions. For example, in rice, *OsWRKY62* has been reported to act as a negative regulator of basal and *Xa21*-mediated defence against *Xanthomonas oryzae* pv. *oryzae*. In pepper, *CaWRKY1*-overexpressing transgenic plants show increased HR cell death in response to infection with tobacco mosaic virus and *Pseudomonas syringae* pv. *tabaci*. In contrast, *CaWRKY1*-silenced pepper plants exhibit decreased growth of *Xanthomonas axonopodis* pv. *vesicatoria* race 1 (Oh *et al*., 2008). Other types of defence-associated TFs, such as OsERF922 (Liu *et al*., 2012), NAC (Wang *et al*., 2009) and ERF (McGrath *et al*., 2005), have also been found to act as negative regulators of disease resistance. Among these, the WRKY proteins mentioned above, CaWRKY1 and CaWRKY58, are WRKY proteins of *C. annuum* that belong to subgroup IIc and group I, respectively. The silencing of *CaWRKY1* (Oh *et al*., 2008) and *CaWRKY58* by VIGS results in enhanced resistance to pathogen infection, respectively, suggesting that there is no functional redundancy of *CaWRKY1* and *CaWRKY58* as negative regulators in the genome of pepper in the resistance to pathogen attack. It will be interesting in the future to reveal whether CaWRKY1 and CaWRKY58 are involved in the same pathways during biotic stress responses.

Defence reactions against disease-causing agents are energy-consuming processes (Brown, 2002). Previous studies have shown that AtWRKY4, AtWRKY11, AtWRKY17, AtWRKY25, CaWRKY1 and OsWRKY62 act as negative regulators of disease resistance, and are transcriptionally up-regulated in response to pathogen infection, as well as the exogenous application of SA or JA. These negative regulators are thought to prevent the inappropriate activation of defence responses at suboptimal concentrations of signal molecules, or to turn off the activated defence reaction once the invasion of pathogens has been dealt with (Wang *et al*., 2006). Another possibility is that these genes might be activated by pathogens to suppress host defence as a counter-defence mechanism of the pathogen. For example, *AvrPto* of *P. syringae* functions as a kinase inhibitor, which interacts directly with receptor kinases,

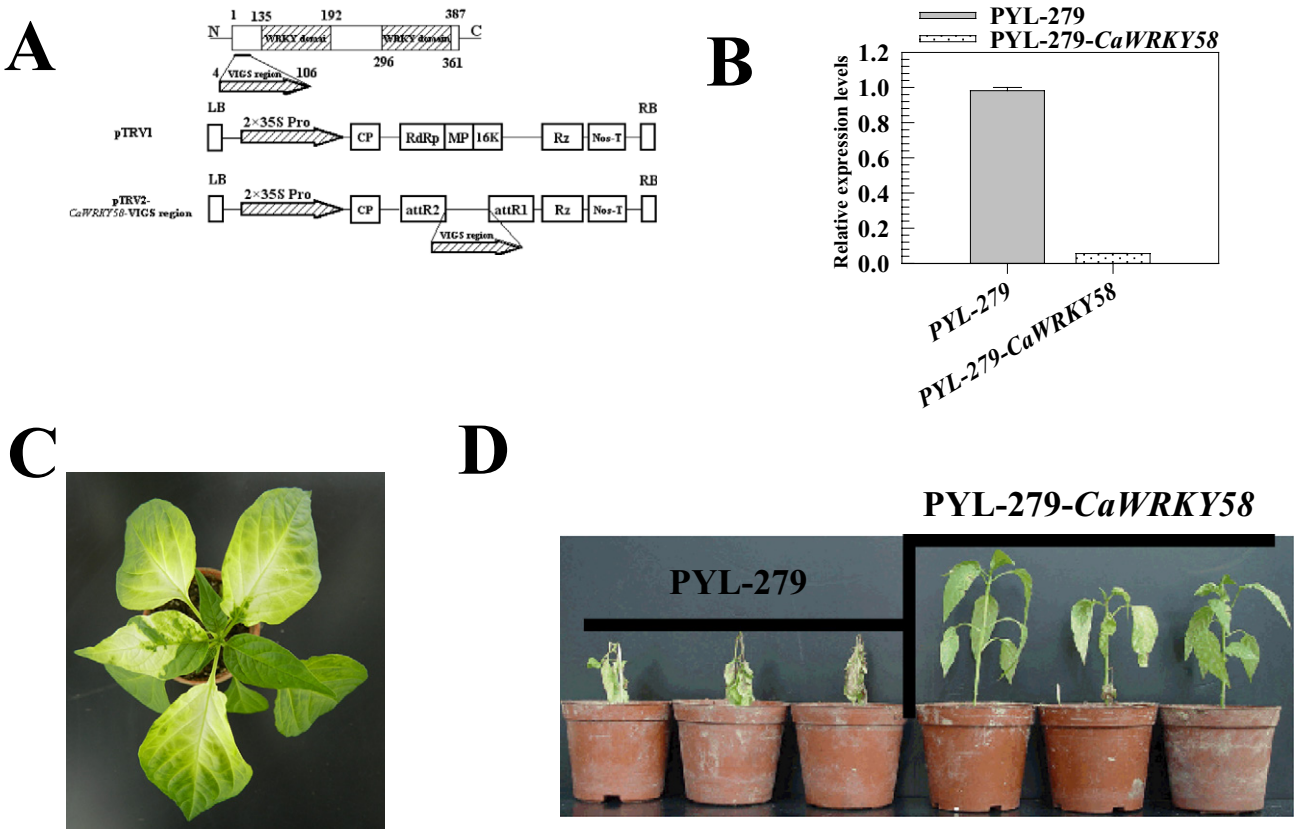

Fig. 8 Loss-of-function analysis of *CaWRKY58* in pepper plants. One-month-old pepper seedlings, 21 days after agroinfiltration with pTRV1 (PYL-192) and pTRV2-*CaWRKY58* (PYL-279-*CaWRKY58*) virus-induced gene silencing (VIGS) vectors. The upper leaves were used to examine *CaWRKY58* transcript levels in PYL-279 and PYL-279-*CaWRKY58* pepper plants. (A) Schematic representation of the pTRV1 and pTRV2-*CaWRKY58*-VIGS constructs. LB and RB, left and right borders of the T-DNA; 2 × 35SPro, two copies of the *Cauliflower Mosaic Virus* 35S promoter; CP, viral coat protein; RdRp, RNA-dependent RNA polymerase; MP, movement protein; 16K, 16-kDa protein; Rz, ribozyme; attR1 and attR2, Gateway recombination sites; Nos-T, nos-terminator; VIGS region. (B) Relative *CaWRKY58* transcript levels in PLY-279 and PYL-279-*CaWRKY58* pepper plants measured by quantitative real-time polymerase chain reaction (qPCR; error bars indicate the standard error; the experiments were repeated three times, together with at least three independent repetitions of the biological experiments. (C) The silencing effect of the pepper *phytoene desaturase* (*PDS*) gene; photobleaching plants were visible 21 days after agroinfiltration. (D) Effect of *Ralstonia solanacearum* in PYL-279 and PYL-279-*CaWRKY58* pepper upper leaves; *R. solanacearum* was taken from stem exudates or the vascular portion of wilted pepper plants; the levels of resistance to *R. solanacearum* were tested in PYL-279 and PYL-279-*CaWRKY58* pepper plants, and the photographs were taken at 15 days post-inoculation.

such as Arabidopsis and tomato FLS2, to block PTI, and leads to host susceptibility (Xiang *et al*., 2008), or modifies the host ABA signalling pathway to suppress defence responses (de Torres-Zabala *et al*., 2007). HvWRKY1/2, TFs of barley, have been shown to act as repressors by direct association with MLA10, a barley resistance protein against the powdery mildew fungus, in the MLA10-mediated blockade of PAMP-activated basal defence (Shen *et al*., 2007). However, we found that the transcriptional expression of *CaWRKY58* was down-regulated in response to *R. solanacearum* infection, as well as exogenously applied SA or MeJA. We speculate that *CaWRKY58* may act as a constitutive negative regulator, tightly suppressing PTI- and ETI-related responses in the absence of microbes to avoid unnecessary energy expenditure (Schwessinger and Zipfel, 2008). In response to the recognition of pathogens, such as *R. solanacearum*, the expression of *CaWRKY58* may be down-regulated through SA- and JA-mediated signalling to derepress PTI. Thus, the suppression of *CaWRKY58* expression may serve as a switch to activate defence responses. Similar observations have also been reported for the Arabidopsis NAC TF ATAF1, which acts as a negative regulator of defence responses against necrotrophic fungal and bacterial pathogens, and is also rapidly suppressed by infection with *Pseudomonas syringae* pv. *tomato* DC3000 or after treatment with SA and JA (Wang *et al*., 2009).

In addition to responding to exogenously applied SA and MeJA, we also found that transcript levels of *CaWRKY58* were down-regulated by exogenously applied ABA, but up-regulated by ET. Recently, it has been reported that SA, JA and ET, as well as auxin, ABA, cytokines and brassinosteroids, are involved in extensive crosstalk between various defence and developmental pathways

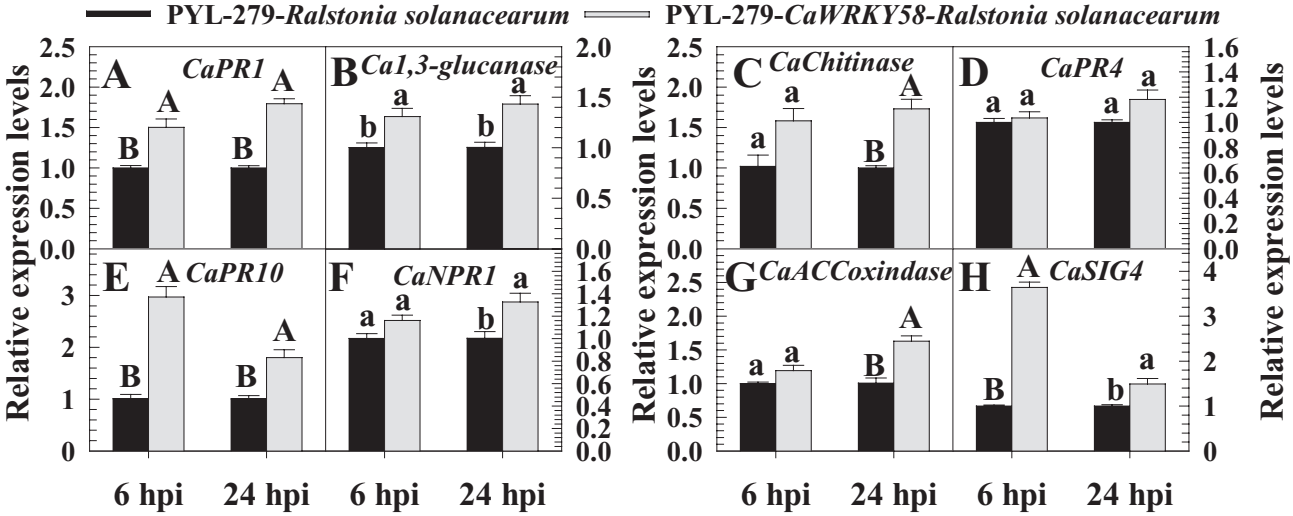

Fig. 9 Relative transcript levels of defence-related genes in PYL-279 and PYL-279-*CaWRKY58* pepper plants at 6 or 24 h post-inoculation by quantitative real-time polymerase chain reaction (qPCR). (A–E) Transcript levels of *CaPR1*, *Caβ-1,3-glucanase*, *CaChitinase*, *CaPR4* and *CaPR10*. (F) Relative expression levels of *CaNPR1*. (G) Relative expression levels of the ethylene production-associated gene *CaACC oxidase*. (H) Relative transcript levels of *CaSIG4*. (A–H) Absolute transcript levels of the respective genes in *Ralstonia solanacearum*-infected PYL-279 pepper plants were used as reference, which were set to a value of '1'. Error bars indicate the standard error and the experiments were repeated three times, together with at least three independent repetitions of the biological experiments. Different letters indicate significant differences as determined by Student–Newman–Keuls test (lowercase difference $P < 0.05$; uppercase difference $P < 0.01$).

(Robert-Seilaniantz *et al*., 2007). Further analyses on phytohormone signalling crosstalk mechanisms will provide new insights into how *CaWRKY58* negatively regulates defence responses to *R. solanacearum* and, possibly, other types of pathogen.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Pepper (*C. annuum*, 8#, a cultivar provided by the pepper breeding group at Fujian Agriculture and Forestry University, which is moderately resistant to *R. solanacearum* strain FJC100301) was used. Seeds were sown in a soil mix (peat moss : perlite; 2/1, v/v) in plastic pots. *CaWRKY58-OE* lines and wild-type (WT-K326) tobacco (cultivar K326 provided by the tobacco breeding group at Fujian Agriculture and Forestry University, which is highly susceptible to *R. solanacearum* strain FJC100301) were grown in a growth room. T_2 or T_3 seeds of transgenic tobacco lines were surface sterilized with 75% alcohol for 30 s and 10% H_2O_2 for 10 min, washed five times with sterile double-distilled H_2O and placed on Murashige and Skoog medium supplemented with 50 mg/L hygromycin for 2–3 weeks. Surviving individuals were transferred to soil mix (peat moss : perlite; 2/1, v/v) and grown in a growth room for another 2–3 weeks. *CaWRKY58-OE* lines and wild-type tobacco of the same size were transferred into soil mix (peat moss : general soil; 1/1, v/v) in plastic pots for another 3–4 weeks. Pepper and tobacco plants were grown in a growth room at 25 ± 2 °C, 60–70 μmol photons/m^2/s and a relative humidity of 70%, under a 16-h light/8-h dark cycle.

Isolation and sequencing of cDNA of *CaWRKY58*

By searching ESTs of *C. annuum* by TBLASTN (http://www.ncbi.nlm.nih.gov), we identified nine ESTs with similarity to AtWRKY58 TF. One of these ESTs was found to harbour two WRKY domains in its deduced amino acid sequence and was chosen to design and synthesize a pair of specific primers. With the primers (5′TCCAGCGAAGAAGAAGGT3′, 5′TGAGAAG GAGAAGACCCA3′), the corresponding full-length cDNA was isolated from the cDNA library by a PCR-based 96-well screening method described by Munroe *et al*. (1995), and the positive clones (λTriplEx2) were converted to pTriplEx2 by *in vivo* excision following the user manual. All of the sequencing was performed by TaKaRa (Dalian, China).

Pathogens and inoculation procedures

Ralstonia solanacearum FJC100301 was isolated from the wilted samples of pepper from Fujian province (China). Exudates of the stem and the stem vascular portion from these plants were purified on tetrazolium chloride medium (Kelman, 1954). *Ralstonia solanacearum* strains were cultured at 200 rpm, 28 °C in PSA medium (200 g potato, 20 g sucrose, 3 g beef extract, 5 g tryptone and 1 L of double-distilled H_2O) and homogenized in sterile 10 mM $MgCl_2$ for 36 h, and the cell density was calculated to be 10^8 cfu/mL [optical density at 600 nm (OD_{600}) = 0.8].

Tobacco plants were inoculated by infiltrating 10 μL of the resulting *R. solanacearum* suspension into the third leaves from the top using a syringe with a needle. The respective fourth leaves were harvested at the indicated time points for the preparation of RNA and histochemical staining.

To study the *CaWRKY58* silencing phenotype in pepper plants under *R. solanacearum* inoculation, two incisions, 3 mm apart, were made in the upper leaves of pepper plants by cutting with scissors which had been dipped into *R. solanacearum* suspension (10^8 cfu/mL) for at least 30 min.

Application of plant hormones, abiotic and biotic stresses

Pepper plants at the four-leaf stage were sprayed with 5 mM SA and 100 μM MeJA (both in 10% distilled ethanol). Mock treatment was performed by spraying control plants with 10% ethanol. One-month-old pepper plants were sprayed with 100 μM ABA and 10 mM ethephon in sterile double-distilled H_2O. Control plants were sprayed with sterile double-distilled H_2O. To study the relative *CaWRKY58* transcript levels in response to *R. solanacearum* infections, pepper plants were inoculated at the eight-leaf stage by the addition of 50 mL of bacterial suspension (10^8 cfu/mL).

Construction of transgenic *CaWRKY58*-overexpressing plants

Transgenic plants were generated by transformation of tobacco leaves with the *CaWRKY58* full-length cDNA cloned into the *CaMV*35S promoter-containing plant expression vector pMDC32 by the Gateway cloning technique (Invitrogen, Carlsbad, CA, USA) to yield pMDC32-*CaWRKY58*. Leaf discs of *N. tabacum* cv. K326 were transformed with the pMDC32-*CaWRKY58*-containing *Agrobacterium tumefaciens* strain LBA105, as described by Oh *et al*. (2005). The initial transgenic tobacco lines (T_0) were selected by hygromycin^r, and 15 transgenic lines were further confirmed by *CaWRKY58*-specific primer PCR and semi-quantitative PCR, respectively. T_1 seeds collected from regenerated T_0 plants, and T_2 or T_3 seeds, were employed for future analysis.

Subcellular localization

Onion epidermal cells were bombarded with plasmids containing *CaMV*35S promoter-driven *CaWRKY58-GFP*. A full-length *CaWRKY58* ORF without the termination codon was prepared by PCR using the *CaWRKY58* cDNA in pTriplEx2 as template with specific primers (5′CGGAATTCAT GAATTTGACGTTGGCTCA3′, 5′CGGGATCCTTCTCAACGCCAAGTATCTTT3′) harbouring *Eco*RI and *Sma*I sites. The PCR amplification products and the vector pEGAD (Cutler *et al*., 2000) were both digested with *Eco*RI and *Sma*I, and the corresponding bands were recovered and ligated into pEGAD to yield the expression vector *p35S::CaWRKY58-GFP*. The vector *p35S::GFP* was used as a control (Tong *et al*., 2007). *p35S::CaWRKY58-GFP* and *p35S::GFP* were transformed into *Agrobacterium tumefaciens* strain GV3101. The cells of GV3101 harbouring *p35S::CaWRKY58-GFP* and *p35S::GFP* were co-infiltrated into *N. benthamiana* leaves. One day after agroinfiltration, the cell layers were mounted in staining buffer containing 0.1% 4′,6-diamidino-2-phenylindole (DAPI) in 5% dimethylsulphoxide (DMSO) and 1% Tween-20. GFP fluorescence was imaged in a fluorescence microscope, with an excitation wavelength of 488 nm and a 505–530-nm bandpass emission filter. DAPI fluorescence was also imaged in the fluorescence microscope using an excitation wavelength of 405 nm and a 435–480-nm bandpass emission filter. The photograph was further overlaid by Imagepro Plus (Cybernetics USA, Herndon, VA, USA).

Co-transfection experiments

Two complementary single strands of oligonucleotide (5′GATCCTTAT TCAGCCATCAAAAG*TTGACC*AATAATTTATTCAGCCATCAAAAGTTGACCAA TAATT3′; 5′CTAGAATTATTGGTCAACTTTTGATGGCTGAATAAATTATT*GGTC AA*CTTTTGATGGCTGAATAAG3′) were annealed in 50 mM NaCl at a naturally descending temperature from 70 °C to room temperature to form a double-strand DNA bait harbouring two directly repeated W1 (*TTGACC*) box units from the parsley *PR1* genes (Rushton *et al*., 1996). The resulting double-stranded DNA stretch was inserted into the *Bam*HI and *Xba*I sites of pBT10-GUS (Rushton *et al*., 2002) to form the reporter plasmid *2* × *W-p35S$_{core}$::GUS*. The effector plasmid was constructed by inserting the full length of *CaWRKY58* cDNA into the Gateway destination vector pMDC32 downstream from the *CaMV35S* promoter. Inner epidermal peels from onion bulbs were placed on MS medium and subjected to biolistic bombardment (Bio-Rad PDS-1000/He, Hercules, CA, USA) with various combinations of the effector plasmid and reporter plasmid (1 μg of each plasmid). Transfected onion tissue was incubated for 24 h at 25 °C in the dark and stained (Jefferson, 1987) prior to examination with an Olympus microscope (Tokyo, Japan).

VIGS of *CaWRKY58* in pepper

The Tobacco Rattle Virus-based VIGS system was employed for *CaWRKY58* silencing. The PYL192 and PYL279 VIGS vectors have been studied previously (Liu *et al*., 2002). The *CaWRKY58* fragments were amplified using gene-specific primers (5′AAAAAGCAGGCTTACGTTGGCTCAATCTC CTTT3′, 5′AGAAAGCTGGGTCTGTCTGATGGTTTCTTATCC3′), and were cloned into the pTRV2 VIGS vector by the Gateway cloning technique (Invitrogen). The PYL192 vector, PYL279 vector with or without *CaWRKY58*, respectively, and PYL279 vector with a 250-bp or 500-bp *PDS* fragment were then transformed into the *Agrobacterium tumefaciens* strain GV3101, respectively. Briefly, *Agrobacterium* strains with the PYL192 vector and with PYL279, PYL279-*CaWRKY58* and PYL279-*PDS* (OD_{600} = 1.0) were mixed in a 1:1 ratio, respectively, and the mixture was infiltrated into cotyledons of 2-week-old pepper plants using a 1-mL sterile syringe without a needle. The *Agrobacterium*-inoculated pepper plants were grown in a growth chamber at 16 °C in the dark for 56 h with 45% relative humidity, and then transferred into a growth room at 25 ± 2 °C, 60–70 μmol photons/m^2/s and a relative humidity of 70%, under a 16-h light/8-h dark cycle.

Histochemical staining

Infection and development of pathogens were assessed by staining infected plants with 1 mg/mL DAB. After overnight treatment with DAB, the stained leaves were cleared by boiling in lactic acid : glycerol : absolute ethanol (1:1:3, v/v/v), and destained overnight in absolute ethanol (Korasick *et al*., 2010). Representative phenotypes were photographed with a light microscope (Leica, Solms, Germany).

QPCR

QPCR for the relative expression levels of target genes was performed with specific primers (see Table 1 for gene-specific primers) according to

Table 1 Main primers for polymerase chain reaction (PCR) used in this study.

Gene	Accession no.	Left primer (5′ to3′)	Right primer (5′ to3′)	Size (bp)
*CaWRKY58**	AY740531.1	ATCAGTGTTCCGCTCAGGTG	GCTCCGAGGGCATTCAGTA	275
CaActin	AY572427	AGGGATGGGTCAAAAGGATGC	GAGACAACACCGCCTGAATAGC	290
CaPR1	AF348141.1	GCCGTGAAGATGTGGGTCAATGA	TGAGTTACGCCAGACTACCTGAGTA	108
Ca1,3-glucanase	AF227953.1	AGAGATTGTTGTGTCCGAGAGTG	TCCAGTTCAGGGTTCTTGTTATTCT	189
CaChitinase	AY775335.1	ATCACCAGTGGCATTGTCTTGTCTC	AGCAACTTCTTTCTTACGGGCAGTA	235
CaPR4	AF244122.1	CAACCCGCAGAACATCAACTGG	CCTCAAGCATCTACCGCAAGCA	160
CaPR10	AF244121.1	CAAGTCCACAGCCTCAGTTGCC	TCCAGCACCACCATCACCTTCA	130
CaNPR1	X61679.1	ACTTCTTCGCCGACGCCAAG	GCCAACACATTCACCAGAGCATC	190
CaPIN2	AF242734.1	GCAACTATTACAGCGTCATCGG	GGGTCAGACTCTCCTTCACAAA	226
CaDePro	AY225438.1	GACACTTCAGTTCCAGTTGAGA	CCTCCTCCGTCTTCTTCTGT	109
CaACC oxidase	AB434925.1	CCATTGTGGTCAACCTTGGC	GCATCGCTTCCTGGATTGTAA	136
CaSIG4	AF354454.1	CCTTCATACATCAGGCACCACT	AACAACGGCAACACAATCACAG	138
NtHIN1	Y07563	CGACCTAACAAAGTCAAGTTCTACG	CTCTATCTCCCAATAAAACCAAGC	283
NtHSR201	X95343	CAGCAGTCCTTTGGCGTTGTC	GCTCAGTTTAGCCGCAGTTGTG	173
NtHSR515	X95342	TTGGGCAGAATAGATGGGTA	TTTGGTGAAAGTCTTGGCTC	499
NtPR1a/c	X05959	AACCTTTGACCTGGGACGAC	GCACATCCAACACGAACCGA	271
NtPR2	M60460	TGATGCCCTTTTGGATTCTATG	AGTTCCTGCCCCGCTTT	175
NtPR3	X51425	CAGGAGGGTATTGCTTTGTTAGG	CGTGGGAAGATGGCTTGTTGTC	222
NtPR4	AF154635.1	GGAAAACGGAAAGGTAAGAAGAGG	GGACACGAGGTAGGTATCACAACAA	222
Ntosmotin	X61679.1	TTCAATGCTGCTGGTAGGGG	GGTTAGTCGGGGCGAAAGTC	187
NtPR1b	X66942	AACCCATCCATACTATTCCTTG	GCCGCTAACCTATTGTCCC	202
NtNPR1	U76707	GGCGAGGAGTCCGTTCTTTAA	TCAACCAGGAATGCCACAGC	234
NtACS1	X65982	CATTAGCGAGGATTCGGAGTT	GTGGTGAATGAGGGATAGGAGA	180
NtACS6	X51599	ATGCCAAGGAAAGGGATTCTACA	TGGGAGGTTTGGGCGAAGA	132
Ntacc deaminase	Z46349.1	TCTGAGGTTACTGATTTGGATTGG	TGGACATGGTGGATAGTTGCT	264
NtEFE26	Z29529	CGGACGCTGGTGGCATAAT	CAACAAGAGCTGGTGCTGGATA	267
NtCAT1	AY128694.1	CAACTTCCTGCTAATGCTCCAA	TGCCTGTCTGGTGTGAATGA	245
NtGST1	D10524	AGCACCCTTACCTTTCCCTC	GCTTTCCTTCACAGCAGCATCA	284
NtEF-1α	D63396	TGCTGCTGTAACAAGATGGATGC	GAGATGGGGACAAAGGGGATT	134

*Primer for relative transcript levels of *CaWRKY58* detection.

the instructions provided for an Applied Biosystems 7500 RT-PCR system (Carlsbad, CA, USA) and SYBR® Premix Ex Taq™ II (TaKaRa Perfect Real Time). Total RNA was isolated from pepper wild-type and transgenic tobacco plants using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The RNA sample was then reverse transcribed with a PrimeScript™ RT-PCR kit (Oh *et al*., 2005) in a 10-μL volume. The resulting cDNA was distilled 10-fold, and amplified with SYBR® Premix Ex Taq™ II (TaKaRa Perfect Real Time) using an Applied Biosystems 7500 RT-PCR system in a 10-μL volume with the following programme: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 34 s, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 15 s. The amplification of the target genes was monitored every cycle by SYBR-Green fluorescence. The Ct (threshold cycle), defined as the RT-PCR cycle at which a statistically significant increase in reporter fluorescence was first detected, was used as a measure for the starting copy numbers of the target gene. Three replicates of each experiment were performed, and normalized transcript levels data of target genes were analyzed by qPCR and Livak method ($2^{(-\Delta\Delta CT)}$, Livak and Schmittgen, 2001).

The relative expression levels of the target genes of pepper, and wild-type and transgenic tobacco, were normalized to the expression of *CaActin* and *NtEF-1α*, respectively.

ACKNOWLEDGEMENTS

We thank Mark D. Curtis for kindly providing the Gateway destination vectors, Weisshaar for pBT10-GUS and Dr S. P. Dinesh-Kumar (Yale University) for the pTRV1 and pTRV2 vectors. This work was supported by grants from the National Natural Science Foundation of China (30971718), the Research Fund for the Doctoral Program of Higher Education of China (20093535110004), the Transgenic Major Program (2009ZX08001-015B) and the Natural Science Foundation of Fujian Province, China (2008J049).

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