Capsicum annuum WRKY protein CaWRKY1 is a negative regulator of pathogen defense

Sang-Keun Oh[1], Kwang-Hyun Baek[2], Jeong Mee Park[1], So Young Yi[1], Seung Hun Yu[3], Sophien Kamoun[4] and Doil Choi[1,2]

[1]Plant Genome Research Center, KRIBB, P.O. Box 115, Yusung, Daejeon 305-600, Korea; [2]Department of Plant Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea; [3]Laboratory of Plant Pathology, Chungnam National University, Daejeon 305-764, Korea; [4]Department of Plant Pathology, OARDC, The Ohio State University, Wooster, OH 44691-4096, USA

Author for correspondence:
Doil Choi
Tel: +82 2 880 4568
Fax: +82 2 873 2056
Email: doil@snu.ac.kr

Received: *8 August 2007*
Accepted: *12 October 2007*

Summary

• Plants respond to pathogens by regulating a network of signaling pathways that fine-tune transcriptional activation of defense-related genes.

• The aim of this study was to determine the role of *Capsicum annuum* WRKY zinc finger-domain transcription factor 1 (CaWRKY1) in defense. In previous studies, *CaWRKY1* was found to be rapidly induced in *C. annuum* (chili pepper) leaves by incompatible and compatible pathogen inoculations, but the complexity of the network of the WRKY family prevented the function of CaWRKY1 in defense from being elucidated.

• Virus-induced gene silencing of *CaWRKY1* in chili pepper leaves resulted in decreased growth of *Xanthomonas axonopodis* pv. *vesicatoria* race 1. *CaWRKY1*-overexpressing transgenic plants showed accelerated hypersensitive cell death in response to infection with tobacco mosaic virus and *Pseudomonas syringe* pv. *tabaci*. Lower levels of pathogenesis-related gene induction were observed in *CaWRKY1*-overexpressing transgenic plants following salicylic acid (SA) treatments.

• This work suggests that the newly characterized CaWRKY1, which is strongly induced by pathogen infections and the signal molecule SA, acts as a regulator to turn off systemic acquired resistance once the pathogen challenge has diminished and to prevent spurious activation of defense responses at suboptimal concentrations of SA.

Key words: chili pepper (*Capsicum annuum*), disease resistance, hypersensitive response, plant defense, systemic acquired resistance, WRKY transcription factor.

New Phytologist (2007) **doi**: 10.1111/j.1469-8137.2007.02310.x

Introduction

Plants trigger a rapid resistance response, called the hypersensitive response (HR), following attack by pathogens, and activate a complex series of responses that lead to systemic acquired resistance (SAR) (Hammond-Kosack & Parker, 2003; Greenberg & Yao, 2004). A key factor in the plant defense response is timely recognition of the invading pathogens and effective activation of the host defense mechanism through complex signal transduction pathways (Yang *et al.*, 1997). Plant defense responses are therefore regulated by a network of signaling pathways that fine-tune transcriptional activation of defense-related genes (Rushton & Somssich, 1998). To date, the molecular events that follow pathogen recognition are not completely understood, but it is generally accepted that plants activate a large number of defense-related genes which are not usually expressed under normal growth conditions (Glazebrook, 2001). Transcriptional regulation of gene expression in response to pathogens is largely mediated by the expression of particular transcription factors and alteration of the target DNA-binding activities of these transcription factors (Rushton & Somssich, 1998; Chen &

Chen, 2002). Many *cis*-acting elements within the promoters of pathogen-regulated genes have been isolated (Rushton & Somssich, 1998).

The WRKY transcription factor family are *trans*-acting elements that have been extensively studied over the past 10 yr because of their involvement in transcriptional reprogramming during plant immune responses (Eulgem, 2006). WRKY transcription factors share a consensus core amino acid sequence WRKYGQK and they can be induced by pathogen infection, treatment with a pathogen-derived elicitor, or treatment with defense signaling molecules such as salicylic acid (SA) (Rushton *et al.*, 1996; Eulgem *et al.*, 1999; Yang *et al.*, 1999; Chen & Chen, 2002). WRKY transcription factors are encoded by a multigene family, with at least 72 and 100 members in Arabidopsis (Dong *et al.*, 2003) and rice (*Oryza sativa*) (Wu *et al.*, 2005), respectively. Although members of the WRKY gene family share different numbers of WRKY domains, they appear to have distinct functions in physiology through the regulation of different groups of genes (Eulgem *et al.*, 2006). The WRKY domain is a 60 amino acid region defined by the conserved amino acid sequence WRKYGQK at its N-terminal end together with a zinc-finger-like motif (Eulgem *et al.*, 2000). Interactions between WRKY proteins can result in both homocomplexes and heterocomplexes, which alter transcriptional regulation (Xu *et al.*, 2006). WRKY transcription factors regulate various physiological programs, including pathogen defense, senescence, trichome development, and the biosynthesis of secondary metabolites. WRKY proteins specifically recognize the W-box (sequence TTGAC) within the promoters of many defense-related genes, such as pathogenesis-related (*PR*) genes, and thus induce their expression (Rushton *et al.*, 1996; Eulgem *et al.*, 1999; Yang *et al.*, 1999; Yang & Chen, 2001). For example, WRKY proteins induced by pathogen attack recognize the elicitor response elements of the parsley (*Petroselinum crispum*) *PR-1* gene (Rushton *et al.*, 1996) and the promoter of the tobacco (*Nicotiana tabacum*) class I chitinase gene (*CHN50*; Yang *et al.*, 1999). The involvement of WRKY proteins in plant defense responses was further characterized by examining gene expression patterns in different SAR-inducing or -repressing conditions using gene chip analyses (Maleck *et al.*, 2000).

Many inducible defense responses are involved in both host and nonhost resistance responses and are elicited by both parasite-specific and nonspecific signals (Heath, 2000). Host resistance is usually restricted to particular pathogen races and is commonly controlled by resistance genes that are active against specific pathogen genotypes (Dangl, 1996). Unlike host resistance, nonhost resistance occurs in an entire plant species in response to any species of pathogen (Heath, 2000). Nonhost resistance has been proposed to be a response to nonspecific parasite factors and to reflect a common response in plant disease resistance (Heath, 2000). To attain our objective of understanding the molecular and cellular mechanisms involved in the plant–pathogen interaction, we have isolated transcription factors that are differentially expressed during incompatible pathogen infections in chili pepper (*Capsicum annuum*) plants. One of the differentially expressed genes was the putative *C. annuum* WRKY DNA-binding protein 1 (*CaWRKY1*; GenBank accession number EF468464), which was isolated from chili pepper plants (*C. annuum* cv. Bukang) infected with the nonhost pathogen *Xanthomonas axonopodis* pv. *glycines* 8ra.

Here, the characteristics and functions of CaWRKY1, a novel pathogen-responsive WRKY transcription factor, are reported. Studies on CaWRKY1 employing silencing and overexpression experiments indicate that the expression of CaWRKY1 seems to result in a reduction of basal resistance to pathogens. Previous studies on the *WRKY* gene family have been complicated by the functional redundancy that exists among structurally related members of the WRKY family and the limited *in vivo* roles of individual WRKYs (Eulgem, 2006). However, our detailed data on the function of CaWRKY1 allow us to conclude that CaWRKY1 acts as a negative regulator for plant defense against pathogens.

Materials and Methods

Plant cultivation and pathogen inoculation

Chili pepper plants (*Capsicum annuum* L. cv. Bukang) were mainly used in the experiments. All chili pepper plants were grown in a controlled environment room under a regime of 16 h light and 8 h dark at 27 ± 2°C. Eight-week-old chili pepper plants were used for the treatments with various pathogens or chemicals. The bacterial strain used in this study was *Xanthomonas axonopodis* pv. *glycines* 8ra (Hwang *et al.*, 1992), which causes pustule disease on soybean (*Glycine max* L.) *Xanthomonas axonopodis* pv. *glycines* 8ra was grown in yeast extract peptone (YEP) medium, resuspended in sterile 10 mM $MgCl_2$ solution at a concentration of 0.05 A_{600} (corresponding to 1×10^8 colony-forming units (CFU) ml^{-1}), and infiltrated into chili pepper leaves using a 1-ml needleless syringe. Control plants were inoculated with 10 mM $MgCl_2$ solution only. Two chili pepper cultivars, ECW-20 (*bs1/bs1*, *bs2/bs2*, *bs3/bs3*) and ECW-20R (*bs1/bs1*, *Bs2/Bs2*, *bs3/bs3*), were used to study the temporal expression of *CaWRKY1* after infiltration of the two chili pepper cultivars with *Xanthomonas axonopodis* pv. *vesicatoria* race 3 (*avrBs2*). HRs were characteristic of the *avrBs2*-induced HR in chili pepper plants with the *Bs2* gene (Tai *et al.*, 1999). The temporal expression of the *CaWRKY1* gene was also detected in *Capsicum chinense* L. PI257284, which carries the L^3 resistance gene against the $P_{1,2}$ pathotype of the pepper mild mottle virus (PMMOV) (Boukema, 1982; Berzal-Herranz *et al.*, 1995). Eight-week-old *C. chinense* PI257284 was inoculated with sap prepared from the leaves of *Nicotiana benthamiana* infected with PMMOV and incubated at 25°C. Leaves from the control plants were mechanically wounded using carborundum (Oh *et al.*, 2005a).

Chemical and wounding treatments

For chemical treatments, chili pepper plants were sprayed with 5 mM SA in water, 100 μM methyl jasmonate (MeJA) in 10% acetone, or 5 mM ethephone in 10% acetone. Wounding treatments were carried out by crushing the apical lamina of all the rosette leaves of chili pepper plants several times with a specula, resulting in ~50% wounding in the leaf area (Lee *et al.*, 2002). Leaves were harvested at 0–24 h after the treatments, frozen immediately in liquid nitrogen, and stored at −80°C before being used for RNA extraction.

DNA and RNA gel blot analyses

Genomic DNA was isolated from chili pepper leaves as described by Lee *et al.* (2002). Genomic DNA (20 μg) was digested with *Hin*dIII, *Xba*I, and *Dra*I, respectively, separated by electrophoresis in a 0.7% agarose gel, denatured, and blotted onto a Nytran membrane (Amersham Pharmacia, Sunnyvale, CA, USA). DNA blot hybridization was performed using the full-length *CaWRKY1* cDNA as the probe, which was labeled with ^{32}[P]-dCTP. For RNA gel blot analysis, total RNA (20 μg) were separated in a formaldehyde-containing agarose gel and transferred onto a Nytran membrane (Amersham Pharmacia). Each cDNA probe was labeled with ^{32}[P]-dCTP using the Prime-a-Gene System (Promega, Madison WI, USA).

Subcellular localization of CaWRKY1

The subcellular localization of CaWRKY1 was determined using the 35S-*CaWRKY1*-smGFP fusion protein (where smGFP is the soluble modified form of the green fluorescent protein). The full-length *CaWRKY1* open reading frame (ORF) without the termination codon was prepared by PCR using the *CaWRKY1* cDNA as a template and two primers (forward, 5′-GGATCCATGCTTGATGGGAG-3′ and reverse, 5′-GGATCCAGGATGGGGATAGACC-3′). The C-terminal region of the PCR-amplified *CaWRKY1* cDNA fragment was fused to the N-terminal region of the smGFP expression vector (David & Vierstra, 1996). The 35S-smGFP vector without *CaWRKY1* was used as a control. In-frame construction of the smGFP-*CaWRKY1* fusion was confirmed by sequencing the plasmid. For transient expression analysis, inner epidermal peels from onion bulbs were placed on basic Murashige–Skoog (MS) agar media (Murashige and Skoog, 1962), and plasmid DNA of the appropriate fusion construct (4 μg each of p35S-*CaWRKY1*-smGFP or p35S-smGFP) was introduced into the onion cells using a particle gun (PDS-1000 He^{-1}; Bio-Rad, Hercules, CA, USA). Bombardment was performed at 10 cm from the targets using 0.7-μm diameter gold particles under 28-inch Hg and 1100 psi helium pressure. The gold particles used were coated with supercoiled plasmid DNA using the method of Takeuchi *et al.* (1992). The MS plates with the bombarded epidermal peels were incubated for 12 h at 25°C in the dark. Fluorescence images were captured using a UV light incident fluorescence microscope (Zeiss, Axioskop, Germany) fitted with fluorescein isothiocyanate filters (an excitation filter of 520 nm and an emission filter of 488 nm).

Construction of *CaWRKY1* deletion mutants and yeast transactivation assay

All yeast manipulations were performed using the procedures recommended by the manufacturer (Clontech, Palo Alto, CA, USA). The *Eco*RI-*Xho*I inserts of *CaWRKY1* derivatives were inserted into the corresponding restriction site of *pLexA* vector (Clontech) to generate an in-frame fusion with the LexA (DNA-binding protein) domain. The newly made constructs included *pLexA*-CaWRKY1-F (1–157 amino acids), *pLexA*-CaWRKY1-N (1–95 amino acids), and *pLexA*-CaWRKY1-C (93–157 amino acids), as well as *pLexA* and *pLexA*-G4, which were used as controls. The constructs were co-transformed into the yeast strain EGY48 (*MATα*, *his3*, *trp1*, *ura3*, *lexA operator-LEU2* marker genes) with p8op-lacZ encoding a *lacZ* reporter gene under the control of the *LexA* operator. Transformed clones were selected on synthetic dextrose plates lacking leucine for 3 d at 30°C. β-galactosidase enzymatic assays for the yeast clones containing *LexA* fusions were performed using *o*-nitrophenyl β-D-galactopyran as a substrate, as previously described by Lee *et al.* (2002).

Bacterial expression of *CaWRKY1* and electrophoretic mobility shift assays

Whole CaWRKY1-coding regions were amplified using *CaWRKY1* cDNA as the template and a primer set (forward primer, 5′-GGATCCATGCTTGATGGGAG-3′ and reverse primer, 5′-AGTAACTTTCAAACTCGAGCTC-3′). The prepared full-length *CaWRKY1* was cloned into the *Bam*HI-*Xho*I site of the pGEX-4T vector (Amersham Pharmacia). The resulting in-frame fusion plasmid was transformed into *Escherichia coli* BL21 (DE3) to express the total 156 amino acids of CaWRKY1. Overexpression of glutathione S-transferase (GST)-*CaWRKY1* was induced by adding 0.4 mM isoprophyl-β-D-thiogalactoside and incubating at 30°C for 2 h. The purified recombinant GST-*CaWRKY1* protein, as described by Amersham Pharmacia, was used for the electrophoretic mobility shift assays (EMSAs) as described by Wang *et al.* (1998). The two synthetic oligonucleotides used in EMSAs were the W-box (5′-GGAACTTGAC CATTTGACCATTGACCACCAGGAAATCG-3′), which contained three TTGAC sequences, as found in the elicitor response element of the tobacco chitinase gene (Yang *et al.*, 1999), and the mW-box (5′-GGAACTTGAACATTT GAACATTGAACACCAGGAAATCG-3′), which contained three mutated TTGAA sequences instead of the three TTGAC sequences. Double-stranded synthetic oligonucleotides were labeled to give specific activities of approx. 5×10^4 cpm

ng^{-1} ^{32}[P]-γATP using T4 polynucleotide kinase (Promega). The reaction solution contained 12 µl of nuclear extraction buffer (25 mM HEPES-KOH, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 5 µg ml^{-1} antipain, 5 µg ml^{-1} leupeptin and 5 µg poly (dI-dC)), 1 µg of the purified recombinant *CaWRKY1* protein, and 1 ng of the labeled double-stranded synthetic oligo DNA. Formation of the DNA–protein complexes was achieved by incubation at room temperature for 20 min, and the DNA–protein complexes were resolved on a 10% polyacrylamide gel in 0.5 × TBE (Tris-HCL, boric acid, EDTA) at 4°C. Subsequently, the gel was dried and the locations of the DNA–protein complexes were recorded on an X-ray film.

Virus-induced gene silencing (VIGS) for *CaWRKY1*

The 285-bp N-terminal fragment of *CaWRKY1* amplified by PCR was cloned into the *Eco*RI-*Xho*I sites of the TRV2 vector (Liu *et al.*, 2002; Chung *et al.*, 2004). The primers for the PCR were 5′-GAATTCATGCTTGATGGGAG-3′ and 5′-CTCGAGGCCATCGTCTAGTATTTC-3′. The TRV2 derivative vectors *pTRV2-GFP* and *pTRV2-CaWRKY1*-N were transformed into *Agrobacterium tumefaciens* strain GV3101 and the transformed cells were selected on YEP media with 50 mg l^{-1} kanamycin, 5 mg l^{-1} tetracycline, and 100 mg l^{-1} rifampicin. The transformants were grown at 28°C, centrifuged, re-suspended to an A_{600} of 0.5 in a buffer (10 mM $MgCl_2$, 10 mM MES, and 200 µM acetosyringone), and kept with shaking at 22–25°C for 5 h. Cultures of the transformants were mixed at a 1 : 1 ratio with *A. tumifaciens* containing the TRV1 vector (OD_{600} nm = 0.5) and the mixture was infiltrated into cotyledons of germinating 'Bukang' chili pepper plants. The inoculated plants were transferred to a growth chamber maintained at 16°C for 1 d with 60% relative humidity, and then placed in a growth room at 25°C with a light intensity of approx. 400 µmol m^{-2} s^{-1} in a cycle of 16 h light and 8 h dark. For each experiment, 30 chili pepper plants were inoculated with the pTRV2-*GFP* vector and the pTRV2-*CaWRKY1*-N vector, respectively. Twenty-five days after this inoculation, *CaWRKY1*-silenced plants were inoculated with *X. axonopodis* pv. *vesicatoria* (race 1), and the numbers of bacterial cells were counted 7 d later. Half of the leaves were used for RNA extraction, and the other half were used for measurement of the bacterial population. The expression data for transcript copy numbers were analysed by Duncan's means separation, available in PROC ANOVA of the software SAS (SAS, Cary, NC, USA).

Overexpression of *CaWRKY1*

Transgenic plants were generated by transformation of tobacco leaves with the *CaWRKY1* full-length cDNA cloned into the plant expression vector pMBP1 under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter. Leaf discs of *N. tabacum* cv. Xanthi-nc (genotype NN) were transformed with the pMBP1-derived vector containing *A. tumefaciens* strain LBA4404 as described by Oh *et al.* (2005b). The primary transgenic tobacco lines (T_0) were selected by kanamycin resistance, and four transgenic lines were further selected by monitoring the constitutive expression levels of *CaWRKY1* transcripts in the absence of any treatment (data not shown). The selected transgenic lines were allowed to self-pollinate, and the T_1 seeds were used for further experiments.

Healthy, fully expanded leaves of *pMBP1*-transgenic and *CaWRKY1*-transgenic T_1 tobacco plants were challenged with tobacco mosaic virus and *Pseudomonas syringe* pv. *tabaci* strain 11528. *Pseudomonas syringe* pv. *tabaci* 11528 was grown overnight in liquid Luria-Bertani medium containing 100 µg ml^{-1} rifampicin. *Pseudomonas syringe* pv. *tabaci* 11528 cultures (OD_{600} = 0.002, approx. 7 × 10^3 CFU ml^{-1}) were re-suspended in 10 mM $MgCl_2$, and then infiltrated into transgenic tobacco leaves using a 1-ml needleless syringe. *In planta* growth of bacterial cells was measured on three replicate plants. Three 1.0-cm^2 leaf discs per plant were collected from the pathogen-infiltrated region of each *CaWRKY1*- and *pMBP1*-transgenic tobacco leaf. The bacterial populations were measured by grinding the leaf discs in 10 mM $MgCl_2$ and plating serial dilutions on LB agar plates containing 100 µg ml^{-1} rifampicin on a daily basis for 5–8 d after the challenge inoculation.

Results

Silencing of *CaWRKY1* in chili pepper plants increases the growth rates of bacteria

To characterize the loss-of-function phenotype of *CaWRKY1* in chili pepper plants, pTRV2-*CaWRKY1*-N, which contained 285 bp of the N-terminal region of *CaWRKY1* (Fig. 1a), was constructed to silence *CaWRKY1*. *CaWRKY1*-silenced chili pepper plants did not show any significant difference in morphology compared with the control plants, which were silenced with the pTRV2-*GFP* vector. Because the transcript levels of *CaWRKY1* in chili pepper were too low to be detected under normal growth conditions, expression levels of *CaWRKY1* were determined in chili pepper plants treated with 5 mM SA, which induced *CaWRKY1*. The expression levels of *CaWRKY1* were significantly reduced in *CaWRKY1*-N-silenced chili peppers compared with the control plants (Fig. 1b); however, the *PR-1* gene, a positive marker for SA treatment, was detected in all SA-treated chili pepper plants (Fig. 1b).

CaWRKY1-N-silenced chili pepper plants (*C. annuum* cv. Bukang) were tested for levels of resistance to *X. axonopodis* pv. *vesicatoria* race 1, which is a compatible pathogen to *C. annuum* cv. Bukang. Symptoms of the disease and the numbers of *X. axonopodis* pv. *vesicatoria* race 1 in the leaves of chili pepper plants were monitored for 7 d after inoculation (DAI) (Fig. 1c,d). Suppression of *CaWRKY1* resulted in reductions in symptoms of disease (Fig. 1c) and also in the

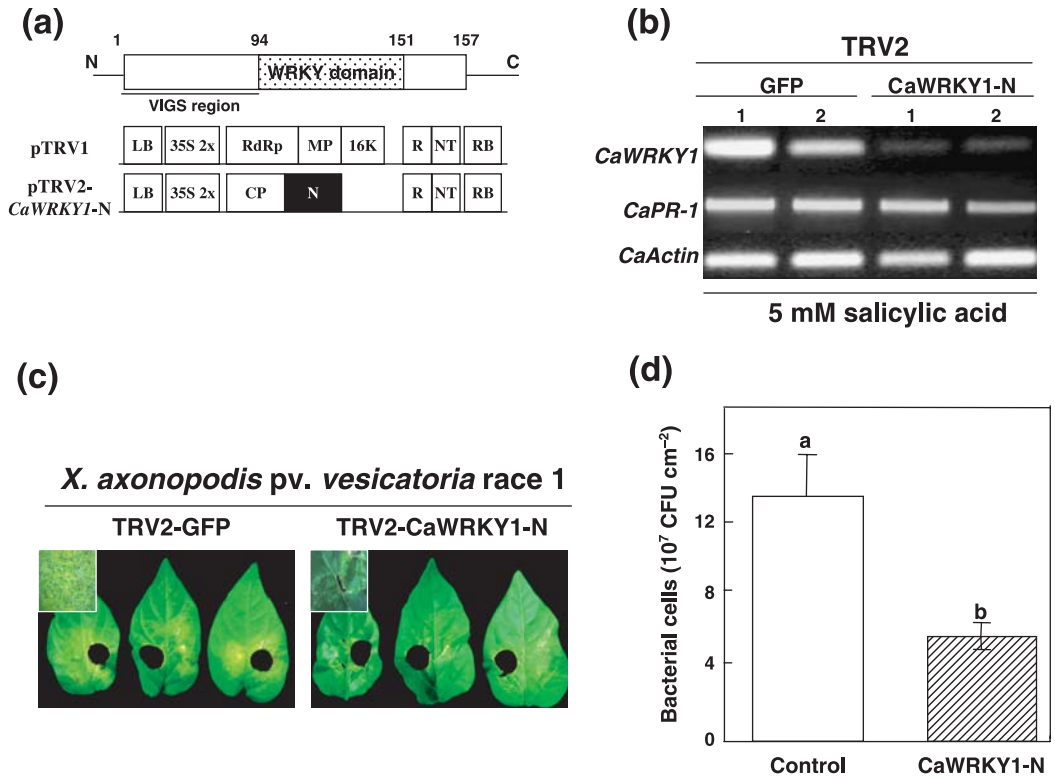

Fig. 1 Silencing of the *CaWRKY1* gene in chili pepper (*Capsicum annuum*) plants. Four-week-old chili pepper seedlings were inoculated with the pTRV1 and pTRV2-*CaWRKY1*-N vectors by agroinfiltration and, approx. 25 d later, upper leaves were challenged with inoculation with *Xanthomonas axonopodis* pv. *vesicatoria* race 1. (a) Schematic representation of the pTRV1 and pTRV2-*CaWRKY1*-N constructs. 35S, cauliflower mosaic virus (CaMV) 35S promoter; RdRp, RNA-dependent RNA polymerase; MP, movement protein; *PR-1*, pathogenesis-related 1; 16K, 16K protein; CP, coat protein; NT, nos terminator; LB and RB, the left and right borders of the T-DNA. The underlined *CaWRKY1* cDNA N-terminal region used for the virus-induced gene silencing (VIGS) is marked with a black box in pTRV2-*CaWRKY1*-N. (b) Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of expression levels of *CaWRKY1* in the *CaWRKY1*-silenced chili pepper plants treated with 5 mM salicylic acid (SA). Two leaves were tested in the green fluorescent protein (*GFP*)- or *CaWRKY1*-silenced chili peppers, respectively. (c) Photographs showing the leaves of pTRV2-*GFP* and pTRV2-*CaWRKY1*-N 7 d after inoculation with *X. axonopodis* pv. *vesicatoria* race 1. (d) Numbers of *X. axonopodis* pv. *vesicatoria* race 1 in the leaves of pTRV2-*GFP* and pTRV2-*CaWRKY1*-N silenced plants. The leaves of pTRV2-*GFP* and pTRV2-*CaWRKY1*-N silenced plants were inoculated with *X. axonopodis* pv. *vesicatoria* race1 (3×10^4 CFU ml^{-1} in $MgCl_2$), and the colony-forming units (CFU) per cm^2 of plant extract were counted 7 d after inoculation (DAI). Each bar represents the mean + SE derived from six leaf discs from three independently silenced plants.

numbers of bacteria (Fig. 1d). The numbers of bacteria present in the *CaWRKY1*-silenced plants were approximately fourfold less than the numbers in the control plants (Fig. 1c).

Transgenic tobacco plants overexpressing *CaWRKY1* have decreased basal levels of resistance

To address the role of *CaWRKY1* in disease resistance in plants, we generated transgenic tobacco plants constitutively expressing *CaWRKY1* under the control of the 35S promoter in the pMBP1 vector (Fig. 2a). Twenty-one T_0 transgenic tobacco plants were generated, and nine transgenic lines showing the highest expression levels were selected (data not shown). Among the nine transgenic lines, the four T_1 transgenic lines that stably expressed *CaWRKY1* (lines 1, 3, 7, and 16) (Fig. 2b) were selected for further analysis. The *CaWRKY1*-overexpressing transgenic lines did not exhibit any apparent phenotypic abnormalities compared with wild-type plants (data not shown). Upon tobacco mosaic virus inoculation, in nontransgenic or pMBP1 vector-only transformed tobacco plants, the typical HR of tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) possessing the *N* gene was observed. However, despite the presence of the *N* gene, the four *CaWRKY1*-expressing transgenic lines inoculated with tobacco mosaic virus appeared to fail to restrict the virus in the necrotic lesions (Fig. 2c,d). When the T_2 *CaWRKY1*-transgenic tobacco plants were inoculated with tobacco mosaic virus, approximately equal numbers of local necrotic lesions (40–60 necrotic lesions per leaf), which are indicative of HR, appeared at 3–5 DAI in the control and *CaWRKY1*-transgenic lines (Fig. 2c). Interestingly, in three independent transgenic lines (*pMBP1-CaWRKY1* lines 1, 3, and 7; not line 16), the size of the local necrotic lesions was significantly increased compared with the control plants (Fig. 2c,d). *nahG*

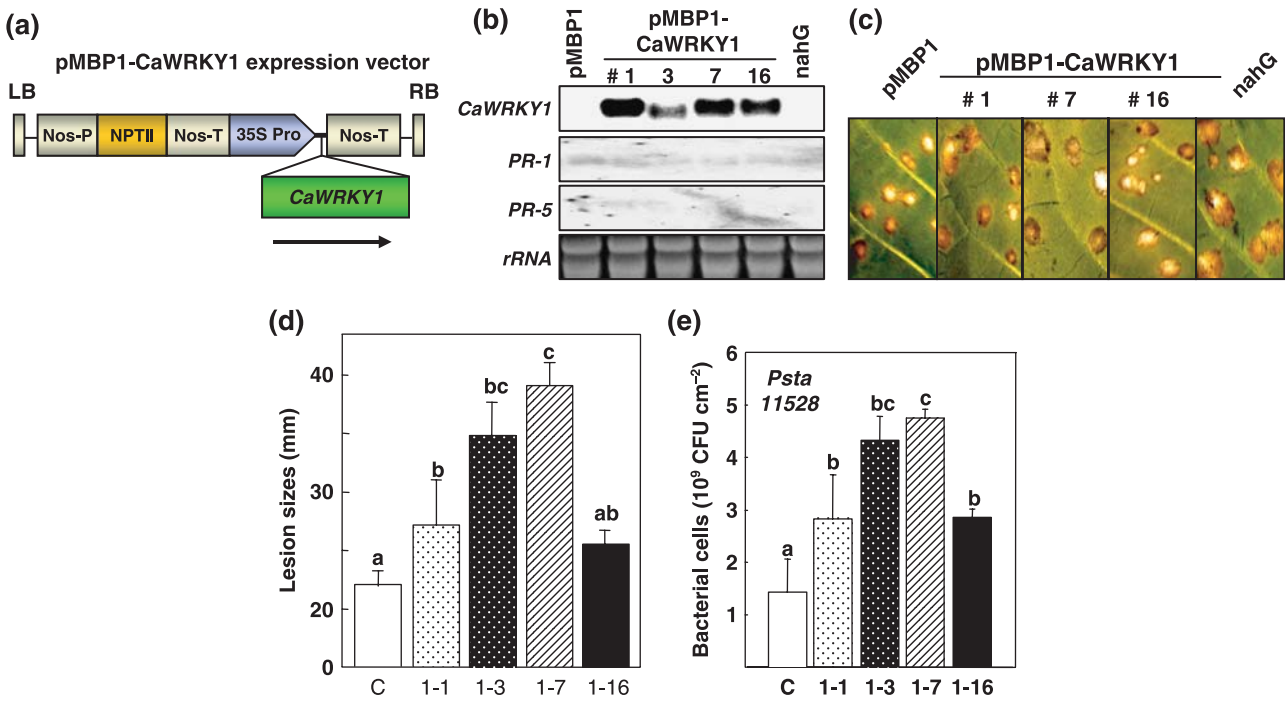

Fig. 2 Constitutive expression of *Capsicum annuum CaWRKY1* in tobacco (*Nicotiana tabacum*). (a) Schematic representation of the pMBP1-*CaWRKY1* construct. LB and RB, the left and right borders of the T-DNA. Nos-P, nos promoter; NPTII, neomycin phosphotransferase II; Nos-T, nos-terminater; 35S Pro, cauliflower mosaic virus 35S promoter. (b) Northern blot analyses of the expression of *CaWRKY1* in the *CaWRKY1*-transgenic tobacco plants. The expression levels of *CaWRKY1* were analysed in 5-wk-old transgenic tobacco plants with pMBP1, pMBP-*CaWRKY1* (lines 1, 3, 7, and 16), and pMBP-*nahG* (salicylate hydroxylase) by northern blotting. The expression levels of pathogenesis-related (*PR*) genes *PR-1* and *PR-5* were also determined. (c) Photographs of hypersensitive response (HR)-induced cell death in the pMBP1, pMBP1-*CaWRKY1* transgenic lines (lines 1, 7, and 16), and pMBP1-*nahG*-transgenic tobacco plants infected with tobacco mosaic virus for 5 d. (d) The lesion sizes induced by tobacco mosaic virus infection in the control and the *CaWRKY1*-transgenic tobacco lines were assessed at 7 d after inoculation (DAI). C, control; 1-1, 1-3, 1-7 and 1-16, lines 1, 3, 6 and 16, respectively. (e) Transgenic tobacco plants were inoculated with *Pseudomonas syringae* pv. *tabaci* (*Psta*) 11528 at OD_{600} nm = 0.001 in 10 mM $MgCl_2$, and the colony-forming units (CFU) per cm^2 of plant extract were counted at 5 DAI. Each value represents the mean + SE from four independent experiments.

(salicylate hydroxylase)-transgenic tobacco plants, which contain low amounts of SA as a result of the degradation of SA to catechol by *nahG* (van Wees & Glazebrook, 2003), also had larger local necrotic lesions than the control plants (Fig. 2c). Furthermore, constitutive expression of *CaWRKY1* also accelerated the growth of the bacterial pathogen *P. syringe* pv. *tabaci* 11528, the fire blight pathogen of tobacco, in the transgenic tobacco plants. At 3 DAI, the leaves of *CaWRKY1*-transgenic plants contained more severe chlorotic lesions than the control plants (data not shown). The disease symptoms could be correlated with the numbers of bacteria in the leaves of the *CaWRKY1*-transgenic lines, in which bacteria had grown two- to fourfold faster than in the control plants at 5 DAI (Fig. 2e).

The expression levels of defense-related genes, such as *PR* genes (*PR-1*, *2*, *4*, and *5*), *SAR8.2* (Lee & Hwang, 2006), *GST*, and ascorbate peroxidase (*APX*), were investigated in the *CaWRKY1*-transgenic lines treated with 2 mM SA. All *CaWRKY1*-transgenic lines had down-regulated expression of *PR-1*, *PR-2*, and *PR-4* relative to control plants at all the examined time-points (Fig. S1). Transgenic lines 3, 7, and 16, but not line 1, had lower expression of *PR-5* than the control plants. All *CaWRKY1*-transgenic lines showed significantly lower expression of *SAR8.2* and *GST* than the control plants. Although there was a smear indicating *APX* expression in the control plants treated with 2 mM SA, none of the *CaWRKY1*-transgenic lines showed any sign of *APX* expression.

Molecular characterization of CaWRKY1

The cDNA encoding the whole *CaWRKY1* gene contains a single open reading frame encoding a deduced protein of 157 amino acids with a calculated molecular mass of 18.18 kDa. The deduced CaWRKY1 protein has structural similarities with Arabidopsis WRKY 50, 51, and 59, which belong to group II(C) of the *WRKY* gene family (Eulgem *et al.*, 2000) (Fig. 3). The members of group II(C) have a single WRKY

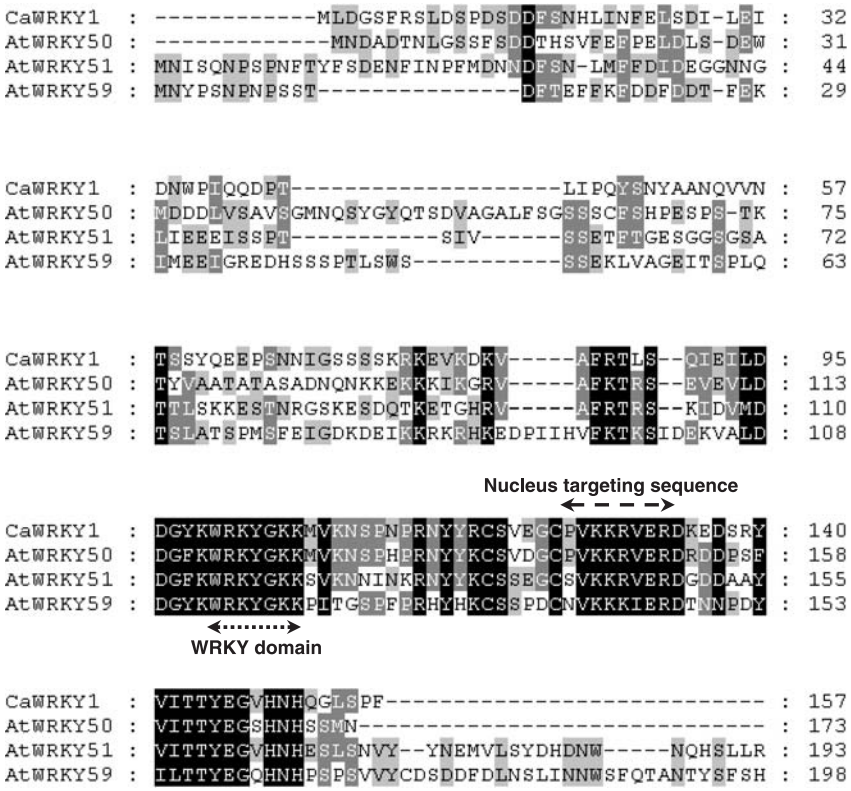

Fig. 3 Comparison of the amino acid sequences of *Capsicum annuum* CaWRKY1 and Arabidopsis WRKY proteins. The amino acid sequence of CaWRKY1 was aligned with those of Arabidopsis WRKY50, 51, and 59 (GenBank accession numbers AC005965, AB019236, and AC007019, respectively). CaWRKY1 and Arabidopsis WRKY50, 51, and 59 share the same WRKY core domain as WRKYGKK. The WRKY domain sequence and the nucleus-targeting sequence (PVKKRVER) are underlined in the figure. Alignment was performed using the CLUSTALW multiple alignment program (http://www.ebi.ac.uk/clustalw/) (Chenna *et al.* 2003).

domain next to a [K/R]EPRVAV[Q/K]T[K/V]SEVD[I/V]L sequence. CaWRKY1 has a unique 'WRKYGKK' core sequence instead of the major WRKY core sequence WRKYGQK (Eulgem *et al.*, 2000). The conserved 60-amino acid WRKY domain containing the WRKYGKK motif has been shown to interact with the *cis*-acting W-box which contains an invariant TTGAC-core element (Eulgem *et al.*, 2000).

The number of copies of the *CaWRKY1* gene in the chili pepper genome was estimated by Southern blot analyses. The *CaWRKY1* gene is estimated to be present as one copy in the genome of chili pepper cv. Bukang. When chili pepper genomic DNA was digested with three different restriction enzymes and hybridized with the full-length *CaWRKY1* cDNA, an approx. 5.5-kb *Dra*I fragment and a restriction site for *Hin*dIII or *Xba*I within the *CaWRKY1* gene were detected (Fig. 4a). The expression levels of the *CaWRKY1* gene were investigated in different parts of chili pepper plants under nonstressful conditions. Expression of *CaWRKY1* was not detectable in roots, stems, petals, or stigmas plus petals, but was detectable as a smear in leaves, young fruits, and seeds (Fig. 4b).

CaWRKY1 is induced by pathogen infection

The expression patterns of *CaWRKY1* in the leaves of chili pepper in response to infection with different pathogens, namely *X. axonopodis* pv. *glycines* 8ra, *Pseudomonas syringe* pv. *syringae* 61 (Huang *et al.*, 1988), PMMOV, and *X. axonopodis* pv. *vesicatoria* race 3, were studied. The *CaPR-1* gene was used as a positive marker identifying successful infection with the various pathogens. Although *X. axonopodis* pv. *glycines* 8ra and *P. syringe* pv. *syringae* 61 are not pathogens of chili pepper, they elicit an HR on infected leaves. *CaWRKY1* transcripts were detected at only 0.5 h after *X. axonopodis* pv. *glycines* 8ra infiltration, and expression levels remained high for 24 h, whereas no induction or very small amounts of *CaWRKY1* transcripts were detected in leaf tissue treated with 10 mM $MgCl_2$ (Fig. 5a). *CaPR-1* expression was detected 1.5 h after infiltration, and increased until 24 h after *X. axonopodis* pv. *glycines* 8ra infiltration. In response to an incompatible pathogen, *P. syringe* pv. *syringae* 61, *CaWRKY1* transcripts were detected at high levels at 0.5 h after the *P. syringe* pv. *syringae* 61 infiltration; however, the levels of *CaWRKY1* expression were reduced 3 h after infiltration. High expression levels of *CaWRKY1* were detected 9 and 15 h after infiltration (Fig. 5b). *CaPR-1* transcripts were detected 6 h after infiltration, and expression levels increased until 24 h after *P. syringe* pv. *syringae* 61 infiltration.

The levels of *CaWRKY1* gene expression in response to a viral pathogen attack were also monitored. Pepper leaves (*Capsicum chinense*) PI257284 carrying the L^3 resistance locus is resistant to PMMOV (pathotype $P_{1,2}$). Inoculation of pepper leaves with PMMOV elicited an HR at 3 DAI. *CaWRKY1* transcripts were detected at 24 h after the PMMOV inoculation,

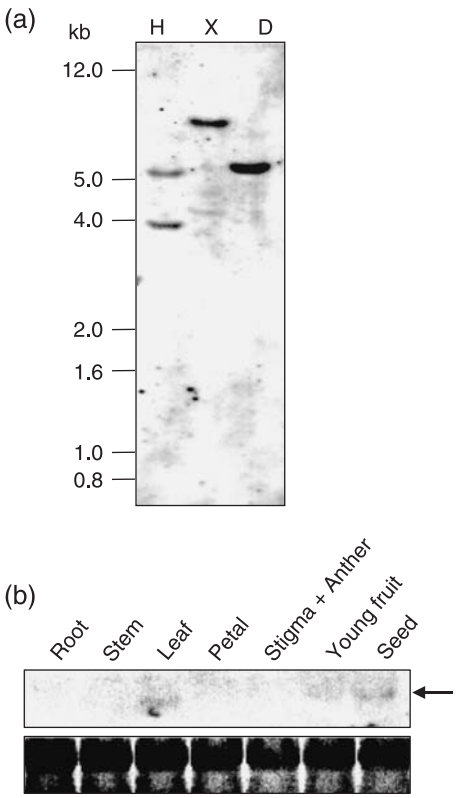

Fig. 4 Characterization of *Capsicum annuum CaWRKY1* genomic structure and expression. (a) Southern blot analysis of CaWRKY1 in the leaves of *C. annuum* (chili pepper). Chili pepper genomic DNA (20 μg) was digested with *Hin*dIII (H), *Xba*I (X), or *Dra*I (D) and hybridized with the full-length *CaWRKY1* cDNA labeled with ^{32}[P]-dCTP. The sizes of the molecular weight markers are indicated in kilobases on the left. (b) Tissue-specific expression of *CaWRKY1* in various tissues of chili pepper plants. Total RNAs extracted from various tissues were hybridized with the full-length *CaWRKY1* cDNA labeled with ^{32}[P]-dCTP. Ribosomal DNA labeled with ^{32}[P]-dCTP was also used as a probe to identify the equal loading of total RNA.

and transcripts were detected at the highest levels at 48 h after the inoculation. Interestingly, *CaPR-1* was detected 24 h after the PMMOV inoculation, and expression levels increased until 96 h after the inoculation, which was the end-point for the observation (Fig. 5c).

We examined the expression levels of *CaWRKY1* to elucidate the behavior of *CaWRKY1* in *R* gene-mediated disease resistance. Expression of *CaWRKY1* was detected in Early Calwonder (ECW) and Early Calwonder-20R (ECW-20R) inoculated with *X. axonopodis* pv. *vesicatoria* race 3, which causes a pepper bacterial spot disease. ECW (*bs2/bs2*) is susceptible to *X. axonopodis* pv. *vesicatoria* race 3 (*avrBs2*), but ECW-20R is resistant to this pathogen. When ECW and ECW-20R were infiltrated with *X. axonopodis* pv. *vesicatoria* race 3, ECW did not exhibit any visible response to the infection within 36 h post-infiltration, whereas ECW-20R developed symptoms of HR on the infiltrated leaf tissue within 20 h post-infiltration (Tai *et al.*, 1999). *CaWRKY1* transcripts in ECW-20R were detected within 1.5 h after *X. axonopodis* pv. *vesicatoria* race 3 infiltration and remained at high levels at 48 h post-inoculation (Fig. 5d). The expression patterns of *CaWRKY1* in ECW-20R were very different from those in ECW-20R, which expressed *CaWRKY1* only at 72 h post-inoculation. The incompatible interaction of ECW-20R with *X. axonopodis* pv. *vesicatoria* race 3 induced high levels of expression of *CaPR-1* within 6 h post-inoculation and increased expression of *CaPR-1*, whereas the compatible interaction of ECW with *X. axonopodis* pv. *vesicatoria* race 3 induced high levels of expression of *CaPR-1* 24 h after inoculation.

SA induces the expression of *CaWRKY1*

To evaluate the effects of plant defense signaling molecules on *CaWRKY1* expression, we examined the expression of *CaWRKY1* transcripts in chili pepper leaves treated with SA, ethephon, or MeJA, respectively. Expression levels of the *CaPR-1*, *CaPINII* (*CaProteinase inhibitor II*) and *CaACO* (*CaAcc oxidase*) genes were also monitored as positive markers for treatment with SA, MeJA, and ethephon, respectively (Reymond & Farmer, 1998). There was no detectable accumulation of *CaWRKY1* and *CaPR-1* transcripts in control plants sprayed with 10% acetone (Fig. 6a). When 5 mM SA in 10% acetone was sprayed onto chili pepper leaves, however, the expression of *CaWRKY1* transcripts was detected within 1.5 h after SA treatment, reached its highest levels between 3 and 6 h after the treatment, and gradually decreased 6 h after SA treatment (Fig. 6b). Other defense-related signal molecules such as MeJA and ethephon did not induce the expression of *CaWRKY1* (Fig. 6c,d). The effect of wounding on the expression of *CaWRKY1* was also evaluated, but wounding was not found to induce any expression of *CaWRKY1* (Fig. 6e).

CaWRKY1 is localized in the plant nucleus

The C-terminal region of the CaWRKY1 protein contains a stretch of basic amino acids, PDSKRWK, which was predicted to be a nuclear localization signal (NLS) by the PSORT program (http://psort.nibb.ac.jp). To confirm the location of the CaWRKY1 protein in the nucleus, we performed *in vivo* targeting experiments using a vector composed of *CaWRKY1* fused to the soluble modified form of the green fluorescent protein (smGFP), which serves as a fluorescent marker (David & Vierstra, 1996). The fusion construct (Fig. S2a) was transfected into onion epidermal cells by biolistic bombardment as described by Takeuchi *et al.* (1992). Onion epidermal cells transfected with the 35S-*CaWRKY1*-smGFP construct emitted green fluorescence exclusively in nuclei, whereas the epidermal cells transfected with the 35S-smGFP construct emitted fluorescence from entire cells (Fig. S2b). These results indicate that CaWRKY1 is located in the nucleus.

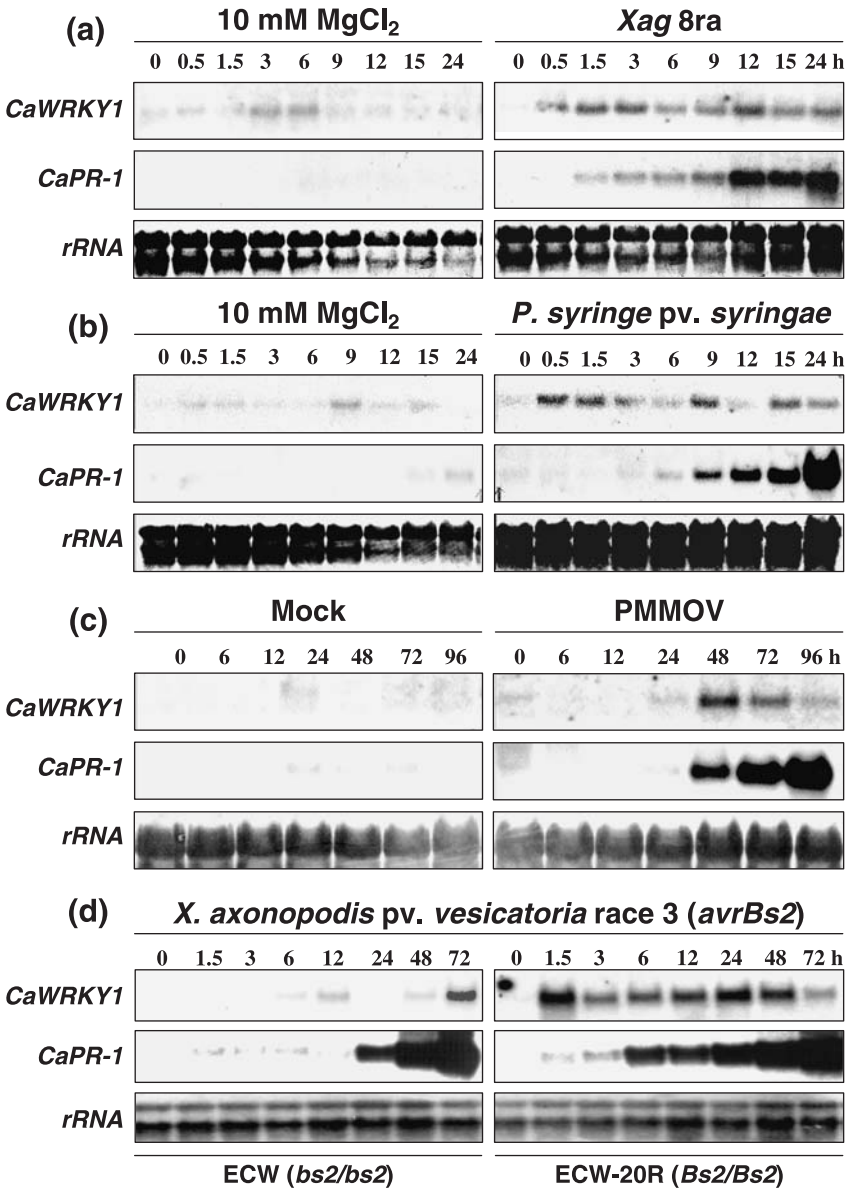

Fig. 5 Characterization of the expression of *Capsicum annuum CaWRKY1* in response to various pathogens. Total RNA was extracted from the leaves at the indicated time-points, and hybridized with ^{32}[P]-dCTP-labeled *CaWRKY1* cDNA and chili pepper pathogenesis-related 1 (*PR-1*) cDNA. The equal loading of total RNA was identified by hybridizing with a ^{32}[P]-dCTP-labeled ribosomal DNA probe. (a) Expression levels of *CaWRKY1* in the leaves of chili pepper (*C. annuum* cv. Bukang) inoculated with the bean pustule pathogen *Xanthomonas axonopodis* pv. *glycines* (*Xag*) 8ra at a concentration of 1×10^8 CFU ml^{-1} in 10 mM $MgCl_2$. (b) Expression levels of *CaWRKY1* in the leaves of chili pepper plants (cv. Bukang) inoculated with the bean pathogen *Pseudomonas syringe* pv. *syringae* 61 in 10 mM $MgCl_2$. (c) Expression levels of *CaWRKY1* in the leaves of 8-wk-old *Capsicum chinense* PI257284 inoculated with pepper mild mottle virus (PMMOV). *Capsicum chinense* PI257284 contains the L^3 resistance gene conferring resistance to the $P_{1,2}$ pathotype of PMMOV. Leaves of *C. chinense* PI257284 were mechanically wounded using carborundum for PMMOV infection. (d) Expression levels of *CaWRKY1* in the leaves of cultivars ECW and ECW-20R inoculated with the pepper pathogen *X. axonopodis* pv. *vesicatoria* race 3 at 1×10^8 CFU ml^{-1}. The ECW (*bs2/bs2*) cultivar is susceptible to *X. axonopodis* pv. *vesicatoria* race 3, whereas the ECW-20R (*Bs2/Bs2*) cultivar is resistant to this pathogen.

CaWRKY1 protein activates transcription in yeast and specifically binds to the W-box

Yeast transactivation activity assays were performed to determine whether CaWRKY1 displays transactivation activity. The LexA DNA-binding domain was fused to the full-length *CaWRKY1* (amino acids 1 to 156; pI = 5.95), the N-terminal *CaWRKY1* (amino acids 1 to 95; pI = 4.27) and the C-terminal *CaWRKY1* (amino acids 96 to 156; pI = 9.30), which were named pLexA-F, pLexA-N, and pLexA-C, respectively. We used the LexA DNA-binding domain (pLexA) or the LexA DNA-binding domain fused to the GAL4 (activator) activation domain (pLexA-G4) as negative and positive controls, respectively (Fig. 7a). These various constructs were used to transform the yeast strain EGY48, and the transcriptional activation of these constructs was identified by measuring the activation of the *LEU2* and *LacZ* (β-*galactosidase*), dual reporter genes under the control of the *LexA* operator in yeast (Fig. 7b). When the clones containing the various constructs were grown on synthetic dextrose plates lacking leucine for 3 d at 30°C, only the pLexA-G4 positive control and pLexA-N grew on the plates (Fig. 7b). Expression of the LexA DNA-binding domain alone, or expression of the LexA DNA-binding domain fused to the full-length or C-terminal *CaWRKY1*, did not activate the *LEU2* reporter gene. The pLexA-G4 positive control and pLexA-N activated transcription of the reporter gene by 25.4-fold and 20.8-fold compared with pLexA (negative control), respectively (Fig. 7c). Therefore, the acidic N-terminal region of CaWRKY1 (pI = 4.27) seems to contain a potential transcriptional activation domain.

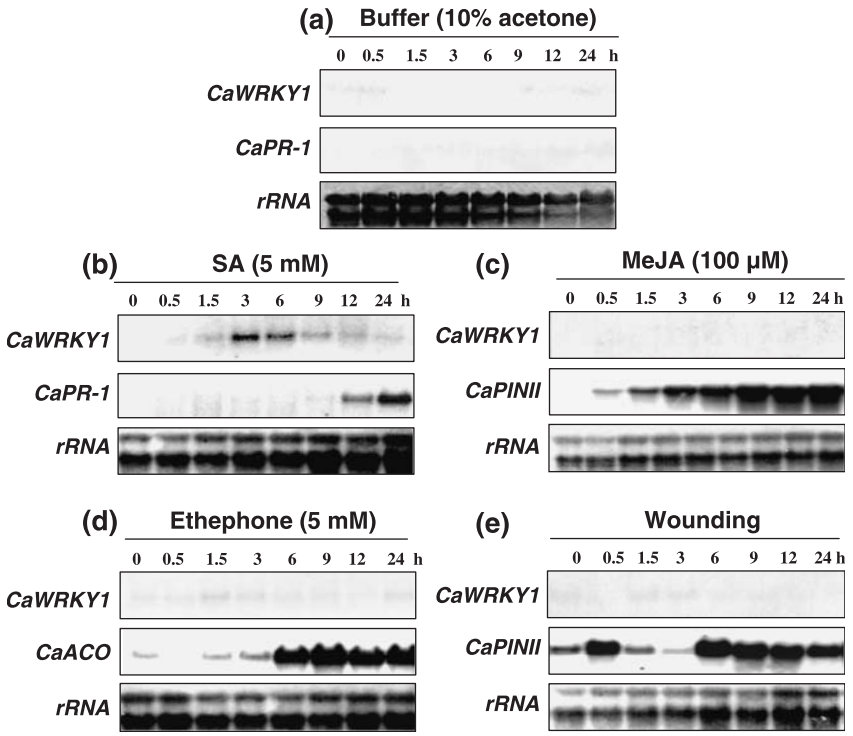

Fig. 6 Expression analysis for the induction of *Capsicum annuum CaWRKY1* by defense signaling molecules and wounding. Transcripts of CaWRKY1 were detected in chili pepper plants (*Capsicum annuum* cv. Bukang) treated with (a) 10% acetone alone, (b) salicylic acid (SA) in 10% acetone, (c) methyl jasmonate (MeJA) in 10% acetone, (d) ethephon in 10% acetone, or (e) wounding at various time-points. Wounding was applied to the rosette leaves of chili pepper plants by crushing the apical lamina several times with a specula, resulting in effective wounding of ~50% of the leaf area. Transcripts of the genes pathogenesis-related 1 (*CaPR-1*), *CaACO* (*CaAcc oxidase*) and *CaPINII* (*Caproteinase inhibitor II*) were also monitored for the positive markers identifying SA, ethephon, MeJA, or wounding treatment, respectively. Ribosomal DNA labeled with ^{32}[P]-dCTP was used to identify the equal loading of total RNA.

WRKY proteins can bind to W-box (TTGAC) elements using the WRKY DNA-binding motif (Yang *et al.*, 1999). To determine the DNA-binding capacity of CaWRKY1 for binding to the W-box elements, we isolated a recombinant CaWRKY1 protein fused to glutathione S-transferase (GST) expressed in *E. coli*. Two WRKY probes were prepared; one had a repeat of the wild-type W-box (W-box; TTGAC) and the other had a repeat of the mutated W-box (mW-box; TTGAA) (Fig. 7d). EMSAs were performed to determine the binding capability of the recombinant CaWRKY1-GST protein for binding to the W-box or mW-box. The CaWRKY1-GST protein bound only to the W-box (TTGAC), not to the mW-box (TTGAA) (Fig. 7e). The DNA-binding specificity of CaWRKY1-GST for binding to the W-box was confirmed again by competition assays (data not shown).

Discussion

WRKY proteins are a group of DNA-binding transcription factors involved in many plant processes, including plant responses to biotic and abiotic stresses (Eulgem *et al.*, 2000). The expression of a number of *WRKY* genes is known to be induced by infection by various pathogens, including viruses (Yang *et al.*, 1999; Chen & Chen, 2000), bacteria (Chen & Chen, 2002), and oomycetes (Dellagi *et al.*, 2000). Among the 72 Arabidopsis *WRKY* genes, 49 were found to be differentially regulated following treatment with an avirulent strain of a bacterial pathogen or SA (Dong *et al.*, 2003). Of these 49 *WRKY* genes, 43 were up-regulated, suggesting that defense-related expression of *WRKY* genes involves extensive transcriptional activation and repression of genes associated with plant defense responses.

We have isolated *CaWRKY1*, a gene encoding a group II(C) WRKY transcription factor.

CaWRKY1 proteins contain a putative NLS sequence (^{126}PVKKRVER^{133}), which is identical to consensus sequences found in simian virus 40 that are similar to an NLS (Garcia-Bustos *et al.*, 1991), and, indeed, the targeting experiment shows that CaWRKY1 can be localized to the nucleus (Supplementary Material). Plant WRKY proteins have been shown to bind a WRKY core sequence [TTGAC(C/T)] known as a W-box element (de Pater *et al.*, 1996; Rushton *et al.*, 1996; Yang *et al.*, 1999). The CaWRKY1 protein also binds sequence-specifically to the W-box (TTGAC) (Fig. 7e), but not to the mutated W-box (TTGAA), which many WRKY proteins cannot bind to (Yu *et al.*, 2001; Chen & Chen, 2002). These results re-emphasize the importance of the last nucleotide 'C' in the sequence of W-box elements for determining the binding of WRKY proteins to the W-box.

CaWRKY1 contains one WRKY domain with a WRKYGKK core sequence instead of having the major WRKY core sequence WRKYGQK (Eulgem *et al.*, 2000). In Arabidopsis, only WRKY 50, 51, and 59 have been found to contain the WRKY core domain sequence WRKYGKK (Wu *et al.*, 2005). In particular, Arabidopsis WRKY 50 and 51 share structural similarities with CaWRKY1, and can be classified into group II(C) (Eulgem *et al.*, 2000). Interestingly, the

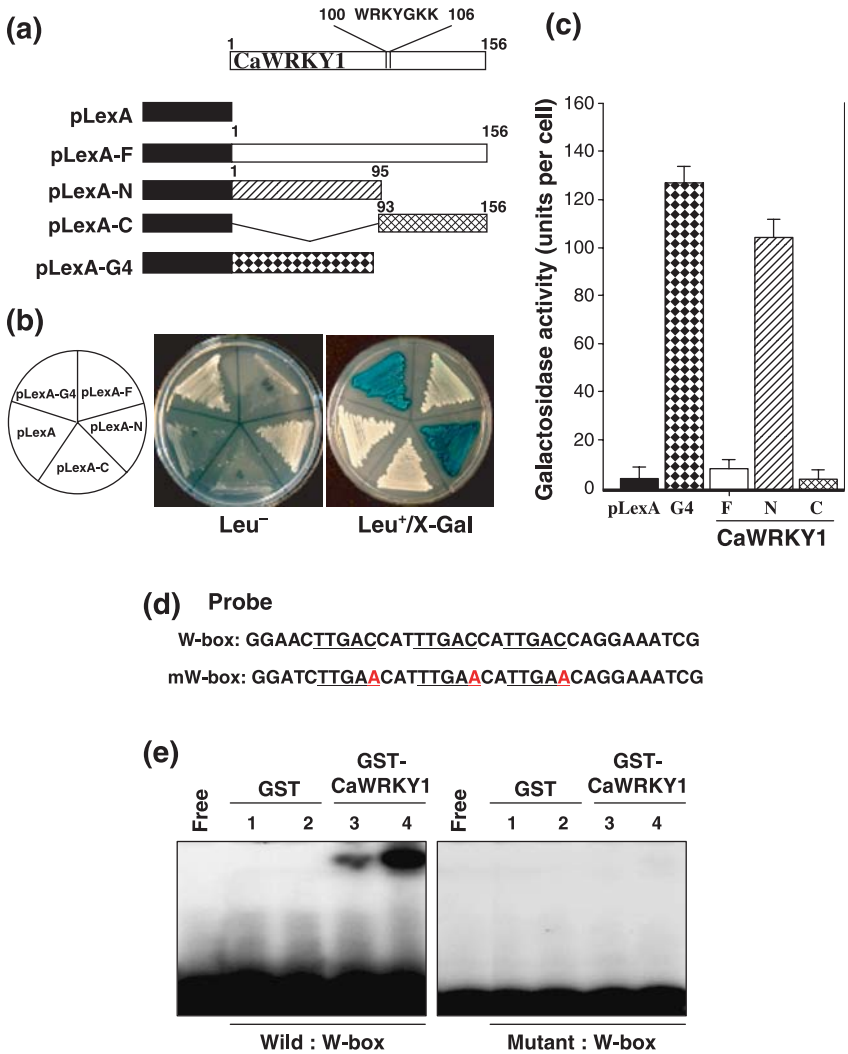

Fig. 7 *Capsicum annuum* CaWRKY1 transcription activation and binding to the W-box element. (a) Diagram showing all the reporter constructs for analysis of LexA (DNA-binding protein)-dependent transactivation of CaWRKY1 in the yeast strain EGY48. The vectors were constructed with pLexA (pLexA vector only), G4 pLexA-G4 (GAL4 activation domain), pLexA-CaWRKY1-F (amino acids 1–156), pLexA-CaWRKY1-N (amino acids 1–95), and pLexA-CaWRKY1-C (amino acids 93–156). (b) Clones transformed with the different vectors were grown on synthetic dextrose (SD) plates lacking leucine for 3 d at 30°C. Transcription activation was monitored by the detection of yeast growth without leucine and β-galactosidase activity. (c) β-galactosidase activities for all the reporter constructs. Enzymatic assays for *LacZ* (β-*galactosidase*) expression of transformants were performed using ONPG (O-nitrophenyl β-O-galactopyramoide) as the substrate. One unit of β-galactosidase is defined as the amount that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minute per cell. Data points are the average + SE from three independent replications. (d) Sequences of the wild-type W-box probe (W-box: repetition of TTGAC sequence) and the mutated W-box probe (mW-box: repetition of TTGAA sequence). W-boxes are underlined, and the mutated bases in the mW-box are shown in red. (e) Electrophoretic mobility shift assay for assessing the binding of glutathione S-transferase (*GST*)-*CaWRKY1* to the W-box. The assays were performed by incubating the W-box probe or the mW-box probe with the GST protein or with the GST-CaWRKY1 recombinant protein. Lanes marked as 'Free' contained only 1 ng of the free probes; GST lanes 1 and 2 contained 1 μg of the GST protein; GST-CaWRKY1 lanes 3 and 4 contained 1 μg of the recombinant GST::CaWRKY1 protein. GST lane 1 and GST-CaWRKY1 lane 3 contained 1 ng of the labeled double-stranded oligo DNA, and GST lane 2 and GST-CaWRKY1 lane 4 contained 2 ng of the labeled double-stranded oligo DNA.

CaWRKY1 gene and the Arabidopsis genes *WRKY50* and *WRKY51* seem to share very distinct patterns of regulation. In response to avirulent pathogens and SA, Arabidopsis *WRKY50* and *WRKY51* were strongly induced (Dong *et al.*, 2003), and these factors also induced high levels of expression of *CaWRKY1* (Fig. 5). Furthermore, the lack of induction of *WRKY50* and *WRKY51* in *nahG*-transgenic Arabidopsis (Dong *et al.*, 2003) and of *CaWRKY1* in *nahG*-transgenic tobacco (data not shown) suggests that SA is a signal molecule regulating the expression of *WRKY50*, *WRKY51* and *CaWRKY1*. The similar molecular structures and regulation mechanisms of *CaWRKY1* and Arabidopsis *WRKY50* and *WRKY51* suggest that these *WRKY* genes might play particular roles in plant physiology.

The CaWRKY1 protein seems to act as a negative regulator in defense mechanisms. Evidence for such a function includes the reduced levels of basal resistance to pathogens in *CaWRKY1*-overexpressing transgenic tobacco plants and the increased defense capacities of *CaWRKY1*-silenced chili pepper plants. When *CaWRKY1*-overexpressing transgenic tobacco lines were infected with tobacco mosaic virus, they showed broader necrotic lesions than wild-type plants or control plants transformed with the pMBP1 empty vector (Fig. 2c,d). Furthermore, constitutive expression of *CaWRKY1* resulted in enhanced growth of *P. syringe* pv. *tabaci* 11528, the fire blight pathogen, in tobacco (Fig. 2e). In contrast to the reduced levels of resistance to pathogens in plants overexpressing *CaWRKY1*, tolerance to *X. axonopodis* pv. *vesicatoria* race 1 was increased in *CaWRKY1*-silenced chili pepper plants (Fig. 1d). All data indicate that the expression of the CaWRKY1 protein decreases tolerance to pathogens; therefore, the up-regulation of *CaWRKY1* in response to pathogens or SA may function as a negative regulator in defense. *CaWRKY1*-overexpressing transgenic tobacco plants down-regulated defense-related genes, such as *PR* genes, *SAR8.2*, *GST*, and *APX*, in response to SA treatment. These results indicate that CaWRKY1 seems to act as a repressor down-regulating defense-related genes. There have been several reports of genes acting as negative regulators in pathogen defense (Zeng *et al.*, 2004; Wang *et al.*, 2006). In rice, *spotted leaf11*, which encodes a U-box/armadillo repeat protein, acted as a negative regulator in plant cell death and defense (Zeng *et al.*, 2004). In Arabidopsis, WRKY58 has been shown to function as a negative regulator in defense (Wang *et al.*, 2006). A *WRKY58* mutant had the phenotype of a mutant with constitutive resistance, and treatment of this mutant with suboptimal concentrations of benzothiadizole (BTH) resulted in increased resistance to a pathogen (Wang *et al.*, 2006). These negative regulators are thought to prevent inappropriate activation of defense responses at suboptimal concentrations of signal molecules or to turn off SAR once the invasion of pathogens has been dealt with (Wang *et al.*, 2006).

Plant defense involves adjustment of metabolism and growth, and thus involves not only induction and up-regulation but also down-regulation of many genes in plants. Moreover, plant defense requires feedback repression to maintain optimal protein levels of pathogenesis-induced transcriptomes. The CaWRKY1 protein characterized here, which was strongly induced by treatments with pathogens and SA, may serve as a negative regulator acting as a safety device to block stimulated activation of defense responses at suboptimal concentrations of SA. Furthermore, the existence of CaWRKY1 as a negative regulator for defense, turning off SAR whenever the threat of pathogen challenge diminishes, may be a prerequisite for the survival of plants. Further studies are needed to elucidate the mechanisms of the repression of *PR* and defense-related genes by CaWRKY1.

Acknowledgements

This research was supported by grants from the Crop Functional Genomics Center (CFGC), one of the 21st Century Frontier Research Programs of the Ministry of Science and Technology, to DC.

References

Berzal-Herranz A, de la Cruz A, Tenllado F, Díaz-Ruíz JR, López L, Sanz AI, Vaquero C, Serra MT, García-Luque I. 1995. The *Capsicum* L^3 gene-mediated resistance against the tobamoviruses is elicited by the coat protein. *Virology* **209**: 498–505.

Boukema IW. 1982. Resistance to TMV in *Capsicum chacoense* Hunz. is governed by an allele of the L-locus. *Capsicum Newsletter* **3**: 47–48.

Chen C, Chen Z. 2000. Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY-DNA binding proteins from tobacco. *Plant Molecular Biology* **42**: 387–396.

Chen C, Chen Z. 2002. Potentiation of developmentally regulated plant defense by *AtWRKY18*, a pathogen-induced *Arabidopsis* transcription factor. *Plant Physiology* **129**: 706–716.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* **31**: 3497–3500.

Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D. 2004. A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Molecules and Cells* **17**: 377–380.

Dangl JL, Dietrich RA, Richberg MH. 1996. Death don't have no mercy: cell death programs in plant–microbe interactions. *Plant Cell* **8**:1793–1807.

David SJ, Vierstra RD. 1996. Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*. *Weeds World* **3**: 43–48.

De Pater S, Greco V, Pham K, Memelink J, Kijne J. 1996. Characterization of a zinc-dependent transcriptional activator from Arabidopsis. Nucleic Acids Research 24: 4624–4631.

Dellagi A, Heilbronn J, Avrova HO, Montesano M, Palva ET, Stewart HE, Toth IK, Cooke DEL, Lyon GD, Birch PRJ. 2000. A potato gene encoding a WRKY-like transcription factor is induced in interactions with *Erwinia carotovora* subsp. *atroseptica* and *Phytophthora infestans* and is coregulated with class I endochitinase expression. *Molecular Plant–Microbe Interactions* **13**: 1092–1101.

Dong J, Chen C, Chen, Z. 2003. Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Molecular Biology* **51**: 21–37.

Eulgem T. 2006. Dissecting the WRKY web of plant defense regulators. *PLoS Pathogens* **2**: 1028–1030.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE. 2000. The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5**: 199–206.

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE. 1999. Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *EMBO Journal* **18**: 4689–4699.

Garcia-Bustos J, Hertman J, Hall MN. 1991. Nuclear protein location. *Biochimica et Biophysica Acta* **1071**: 83–101.

Glazebrook J. 2001. Genes controlling expression of defense responses in Arabidopsis – 2001 status. *Current Opinion in Plant Biology* **4**: 301–308.

Greenberg JT, Yao N. 2004. The role and regulation of programmed cell death in plant–pathogen interactions. *Cellular Microbiology* **6**: 201–211.

Hammond-Kosack KE, Parker JE. 2003. Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. *Current Opinion in Biotechnology* **14**: 177–193.

Heath MC. 2000. Nonhost resistance and nonspecific plant defenses. *Current Opinion in Plant Biology* **3**: 315–319.

Huang HC, Schuurink R, Denny TP, Atkinson MM, Baker CJ, Yucel I, Hutcheson SW, Collmer A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *Journal of Bacteriology* **170**: 4748–4756.

Hwang I, Lim SM, Shaw PD. 1992. Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glycines*. *Journal of Bacteriology* **174**: 1923–1931.

Lee S-C, Hwang B-K. 2006. Identification and deletion analysis of the promoter of the pepper SAR8.2 gene activated by bacterial infection and abiotic stresses. *Planta* **224**: 255–267.

Lee SJ, Lee MY, Yi SY, Oh SK, Choi SH, Her NH, Choi D, Min BW, Yang SG, Harn CH. 2002. PPI1: a novel pathogen-induced basic region-leucin zipper (bZIP) transcription factor from pepper. *Molecular Plant–Microbe Interactions* **15**: 540–548.

Liu Y, Schiff M, Dinesh-Kumar SP. 2002. Virus-induced gene silencing in tomato. *Plant Journal* **31**: 777–786.

Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, Dangl JL, Dietrich RA. 2000. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nature Genetics* **26**: 403–410.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**: 473–497.

Oh S-K, Lee S, Yu SH, Choi D. 2005a. Expression of a novel NAC domain-containing transcription factor (CaNAC1) is preferentially associated with incompatible interactions between chili pepper and pathogens. *Planta* **220**: 876–887.

Oh S-K, Park JM, Joung YH, Lee S, Chung E, Kim S-Y, Yu SH, Choi D. 2005b. A plant EPF-type zinc-finger protein, *CaPIF1*, involved in defense against pathogens. *Molecular Plant Pathology* **6**: 269–285.

Reymond P, Farmer EE. 1998. Jasmonate and salicylate as global signals for defense gene expression. *Current Opinion in Plant Biology* **1**: 404–411.

Rushton PJ, Somssich IE. 1998. Transcriptional control of plant genes responsive to pathogens. *Current Opinion in Plant Biology* **1**: 311–315.

Rushton PJ, Torres TJ, Parniske M, Wernert P, Hahlbrock K, Somssich IE. 1996. Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO Journal* **15**: 5690–5700.

Tai TH, Dahlbeck D, Clark ET, Gajiwala P, Pasion R, Whalen MC, Stall RE, Staskawicz BJ. 1999. Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. *Proceedings of the National Academy of Sciences, USA* **96**: 14153–14158.

Takeuchi Y, Dotson M, Keen NT. 1992. Plant transformation: a simple particle bombardment device based on flowing helium. *Plant Molecular Biology* **18**: 835–839.

Van Wees SCM, Glazebrook J. 2003. Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. *Plant Journal* **33**: 733–742.

Wang D, Amornsiripanitch N, Dong Z. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathogens* **2**: 1042–1050.

Wang Z, Yang P, Fan B, Chen Z. 1998. An oligo selection procedure for identification of sequence-specific DNA-binding activities associated with the plant defense response. *Plant Journal* **16**: 515–522.

Wu K, Guo Z-J, Wang H-H, Li J. 2005. The WRKY family of transcription factors in rice and Arabidopsis and their origins. *DNA Research* **12**: 9–26.

Xu X, Chen C, Fan B, Chen Z. 2006. Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* **18**: 1310–1326.

Yang P, Chen Z. 2001. A family of dispersed repetitive DNA sequences in tobacco contain clusters of W-box elements recognized by pathogen-induced WRKY DNA-binding proteins. *Plant Science* **161**: 655–664.

Yang P, Wang Z, Fan B, Chen C, Chen Z. 1999. A pathogen- and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of the tobacco class I chitinase gene promoter. *Plant Journal* **18**: 141–149.

Yang Y, Shah J, Klessig DF. 1997. Signal perception and transduction in plant defense response. *Genes and Development* **11**: 1621–1639.

Yu D, Chen C, Chen Z. 2001. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* **13**: 1527–1540.

Zeng LR, Qu S, Bordeos A, Yang C, Baraoidan M, Yan H, Xie Q, Nahm BH, Leung H, Wang GL.2004. Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-Box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *Plant Cell* **16**: 2795–2808.

Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Effect of constitutive overexpression of *CaWRKY1* on the expression of defense-related genes.

Fig. S2 Nuclear localization of the CaWRKY1-GFP fusion protein.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02310.x
(This link will take you to the article abstract.)

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than about missing material) should be directed to the journal at *New Phytologist* Central Office.