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ORIGINAL RESEARCH

A Novel Pepper (*Capsicum annuum* L.) WRKY Gene, *CaWRKY30*, Is Involved in Pathogen Stress Responses

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Abstract WRKY proteins are a superfamily of transcription factors involved in many plant processes including plant defense responses to biotic and abiotic stresses. We isolated a WRKY gene from pepper during the incompatible interaction between the pepper cultivar HDA149 and *Meloidogyne incognita*. The full-length gene, named as *CaWRKY30*, has a 1,533-bp cDNA sequence and contains an open reading frame of 1,095 bp, encodes a putative polypeptide of 364 amino acids with a theoretical protein size of 41.2 kDa, and contains one WRKY domain followed by a zinc-finger motif. The genomic sequence of *CaWRKY30* contains three exons and two introns. Southern blot analysis confirmed that *CaWRKY30* exists as a single copy in the pepper cultivar HDA149 genome. Quantitative RT-PCR showed that *CaWRKY30* is up-regulated by application of various pathogens including avirulent *M. incognita*, *Tobacco mosaic virus*, *Ralstonia solanacerum*, and *Phytophthora capsici* Leonian. Furthermore, the transcripts of *CaWRKY30* were rapidly induced after treatment with phytohormones salicylic acid (SA). However, the expression of *CaWRKY30* was down-regulated by virulent *M. incognita* and phytohormones methyl jasmonic acid (MeJA). In addition, the nuclear localization of CaWRKY30 was determined when a CaMV35s:: *CaWRKY30*-eGFP fusion construct was expressed in onion epidermal cells. These results suggested that *CaWRKY30* might be involved in plant defense mechanisms against the diverse pathogen infection.

Keywords WRKY transcription factor · Pepper · Root-knot nematode · Expression analysis

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Introduction

Pepper (*Capsicum annuum* L.) is an economically important vegetable in China and other temperate regions, and it is susceptible to the adverse effects plant-parasitic nematodes (PPNs). Among the PPNs, *Meloidogyne incognita* can stimulate the hyperplasia and hypertrophy of the surrounding cells, leading to the formation of the typical root gall. Plant nutrient and water uptake are substantially reduced because of the resulting damage to the root system; the infested plants are therefore weak and give low yields. Considerable efforts are underway to develop pepper varieties more tolerant to PPNs pathogens.

Plants have evolved a variety of defense mechanisms to protect themselves from microbial pathogen infections. A common feature of plant defense responses is the transcriptional control of expression of stress-responsive genes (Rushton and Somssich 1998). Key factors in the plant defense response include the timely recognition of the invading pathogens and the effective activation of the host defense mechanism through complex signal transduction pathways (Yang et al. 1997). The latter comprises a number of transcription factors (TFs) whose abundance is altered as a result of the pathogen attack. The TFs are presumably involved in regulating the expression of defense-related genes; examples of TFs include the ethylene response factor (ERF), basic-domain leucine zipper (bZIP), MYB,

WRKY protein, and other zinc-finger factors, all of which have been observed to increase in response to various pathogen infections (Singh et al. 2002).

The name of the WRKY family is derived from the most prominent feature of these proteins, namely the WRKY domain, a 60-amino-acid region that contains the amino acid sequence WRKYGQK; this domain is highly conserved among family members, and it is usually followed by a C_2–H_2 or C_2–HC zinc-finger motif (Eulgem et al. 2000). WRKY transcription factors contain one or two conserved WRKY domains, which can recognize and bind to the TTGAC(C/T) W-box elements found in the promoters of a large number of plant defense-related genes (Eulgem et al. 1999; Yang et al. 1999). WRKY TFs can be classified into three groups based on both the number of WRKY domains and the features of their zinc-finger motif. Group I WRKY TFs contains two WRKY domains, whereas most proteins with one WRKY domain belong to group II. The group II proteins can be further divided into five subgroups based on the structure of the WRKY domain. Proteins containing only one WRKY domain and the zinc-finger motif C_2–HC instead of C_2–H_2 have been assigned to the group III (Eulgem et al. 2000).

Previous studies have demonstrated that WRKY family members appear to be involved in the regulation of various physiological programs that are unique to plants, such as pathogen defense, senescence, embryogenesis, seed coat, trichome development, regulation of biosynthetic pathways, and hormone signaling (Rushton et al. 2010; Zhang et al. 2004). They have also been demonstrated to be involved in the defense against phytopathogens such as fungi, bacteria, and viruses (Fan et al. 2011; Park et al. 2006). These responses to pathogen attack require large-scale transcriptional reprogramming and require transcription factor families such as WRKY genes (Eulgem 2005; Naoumkina et al. 2008).

An increasing number of WRKY proteins have been identified in responses to pathogen infections and hormone signaling (Li et al. 2006; Marchive et al. 2007). However, few reports describe WRKY proteins with essential roles in mediating defense responses to root-knot nematode (RKN) attacks in pepper. In this study, we isolated the WRKY transcription factor *CaWRKY30* during the incompatible interaction between *C. annuum* (HDA149) and *M. incognita*. We analyzed the structure of *CaWRKY30* and studied the expression of the gene during the interaction of pepper plants with avirulent/virulent *M. incognita* and following infections with diverse pathogens and treatments with two phytohormones, SA and JA. The subcellular localization of CaWRKY30 protein was examined using green fluorescent protein (GFP). We suggest that *CaWRKY30* is induced by pathogen infection and might play an important role during the pathogen stresses.

Material and Methods

Plant and Nematode Materials

The pepper (*C. annuum* L.) cultivar HDA149 contains a single dominant resistance gene (*Me3*, which confers resistance to pepper plants against to the RKN *M. incognita*) and was developed by Dr. Alain Palloix (INRA Montravet, France). The plants were grown in a greenhouse with a 16-h-light and 8-h-dark photoperiod at 25±2°C. The *Me3* avirulent culture of RKN, *M. incognita*, was maintained on the susceptible pepper cultivar Qiemen in a greenhouse, and the *Me3* virulent culture of RKN was maintained on pepper HDA149.

Isolation of *CaWRKY30*

The rhizosphere soil of 6-week-old pepper seedlings was inoculated with 800J2 of avirulent *M. incognita*. The plant root tips were harvested 12-, 24-, and 48-h post-inoculation and mixed for total RNA extraction. Nematode inocula were collected from a large number of juveniles freshly hatched from egg masses derived from a population of *M. incognita* that were reproduced in a greenhouse.

A 259-bp fragments of cDNAs, which putatively encodes a WRKY transcription factor, was screened from a transcriptome database by Solexa high-throughput deep sequencing during the incompatible interaction between pepper HDA149 and the avirulent *M. incognita* (data was not shown). Special primers (Table 1) used for RACE amplification were designed according to the fragments using the Primer Premier 5.0 software. For 3′ RACE amplification, first-strand cDNA was synthesized from 5 μg of total RNA using Superscript III (Invitrogen) according to the instruction manual (Invitrogen) and diluted fivefold. The reactions were performed in a final volume of 20 μl, and each contained 1 μl of diluted cDNA. PCR

Table 1 Primer names and sequences

Primers	Primer sequences (5′–3′)
5′RACE GS-w1	AAAGACGCTGAATAGCCGCTACCT
5′RACE GS-w2	ACATGCTGCTTGCTCATTCTTGTC
3′RACE GS-w3	GCGTTGATGAGTTTGAGAGC
CaWRKY30-F	GCGATGGATTGTGCAGTTAACTGG
CaWRKY30-R	CAGTCATGAGAAAAAATTTGGATT
CaWRKY30-GFPF	CTCGGGATGGATTGTGCAGTTAAC
CaWRKY30-GFPR	GGATCCTGAGAAAAAATTTGGATTG
CaWRKY30-qF	AATGTTGCGTTGGTGGCTCG
CaWRKY30-qR	AACTCCGTTTCTTGTCATCGC
β-actin qF	TGCAGGAATCCACGAGACTAC
β-actin qR	TACCACCACTGAGCACAATGTT

amplification was carried out at an initial denaturation of 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 10 min using the 3′ RACE primer (Table 1). For 5′ RACE amplification, first-strand cDNA was synthesized from 10 μg of total RNA using M-MLV Reverse Transcriptase according to FirstChoice® RLM-RACE Kit protocol (Ambion, Inc. USA). The 5′ RACE outer primer and 5′ RACE inner primer were synthesized to carry out nested PCR according to the 5′ RLM-RACE protocol (Ambion, Inc. USA). PCR products of both the 3′ RACE and 5′ RACE were purified, cloned into the pGEM-T vector (Tiangen Co. Ltd, Beijing) and sequenced. The sequences were analyzed and translated using DNAman and Primer 5.0. Based on the sequenced cDNA sequences from the 3′ RACE and 5′ RACE, a pair of primers (CaWRKY30-F and CaWRKY30-R; Table 1) was designed to clone the full-length cDNA of the *CaWRKY30* gene.

The genomic sequence of *CaWRKY30* was also cloned using the same primer that was used to clone the full-length cDNA. The exon–intron distribution was determined by comparing the genomic and cDNA sequences of *CaWRKY30* use the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). The molecular weight, structural analysis, and isoelectric point determination of the deduced CaWRKY30 protein were predicted on the ExPASy Proteomics Server (http://www.expasy.ch/tools/).

Southern Blot Analysis

Genomic DNA was isolated from pepper leaves as described by Lee et al. (Lee et al. 2002). Briefly, 20 μg of genomic DNA was digested with *Hind*III, *Eco*RV, and *Bam*HI, separated by electrophoresis on a 0.8% agarose gel, denatured, and blotted onto a Nylon membrane (Amersham, USA). The full-length cDNA of *CaWRKY30* was used as a probe and labeled with DIG-labeled. Hybridization and detection were performed with the DIG system under standard conditions according to the manufacturer's instructions (Roche, Gemany).

Hormone Treatments

For the hormone treatments, leaves of intact plants were sprayed with 0.1 mM MeJA or 5 mM SA prepared in water. MeJAwas first dissolved in absolute ethanol to prepare a 100-mM stock solution, which was then diluted with sterile water to a final concentration of 0.1 mM. Mock plants treated with water containing 0.1% (*v*/*v*) ethanol were placed in a separate chamber. Leaves from mock and hormone-treated plants were harvested at 2-, 6-, 9-, 12-, 16-, 24-, and 48-h post-inoculation (hpi), immediately frozen in liquid nitrogen and stored at −80°C until qRT-PCR was performed.

Infections Using Different Pathogens

Six-week-old pepper plants (*C. annuum* L. HDA149) were used for the inoculations with different pathogens. HDA149 is susceptible to the *Tobacco mosaic virus* (TMV); in fact, we used some TMV strains in this study. The TMV inoculation, which consisted of a suspension in phosphate buffer (50 mM) containing 1 mg of tobacco TMV leaf per 5 ml phosphate buffer, was applied to the surface of fully expanded leaves of pepper plants and rubbed with carborundum. Mock-inoculated plants were rubbed with phosphate buffer and carborundum only. The bacterial strain used in this study was *Ralstonia solanaearum* TM60, which causes wilt disease on pepper plants, was cultured in TTC medium (10 g l^{-1} peptone, 3 g l^{-1} beef extract, 5 g l^{-1} NaCl and 10 mg l^{-1} TTC, pH 7.4), and the *R. solanaearum* inocula (10^6 CFU ml^{-1}) were infiltrated into leaves; mock plants were treated with TTC medium. *Phytophtora capsici* Leonian Phyc-CS, which causes pepper phytophthora blight, was grown on oatmeal agar at 25°C, and then placed at 28°C with fluorescent illumination to induce sporangium production. Spores were collected with sterilized water and the suspension was passed through miracloth to separate the fungal material from pieces of agar. To infect plants, pathogen inoculations were conducted by spraying the seedlings with *P. capsici* spore suspension (10^5 spores ml^{-1}) on the leaves. Plants infected with *P. capsici* were incubated under 100% relative humidity. Control plants were treated in the same way but with sterile distilled water instead. Leaves from pathogen- and control-treated plants were harvested at 3-, 6-, 9-, 12-, 16-, 24-, and 48-h post-inoculation (hpi), immediately frozen in liquid nitrogen, and stored at −80°C until further processing.

For avirulent *M. incognita* infection, the inoculation of J2 nematodes was performed as described previously. Root tips were collected at 3-, 6-, 12-, 24-, 48-, 72-h, 7-d and 10-d post-inoculation (hpi/dpi). For virulent *M. incognita* treatment, root tips were collected as detailed for avirulent *M. incognita*. Mock plants were inoculated with water. All of the samples were immediately frozen in liquid nitrogen and stored at −80°C until qRT-PCR was performed.

Subcellular Localization of CaWRKY30

The coding region (CDS) of *CaWRKY30* without the termination codon was amplified by RT-PCR from pepper cDNA using the primers *CaWRKY30*-GFPF and *CaWRKY30*-GFPR (Table 1). The PCR amplification products containing the *Ava*I and *Bam*HI sites were digested with *Ava*I and *Bam*HI. After purification, the PCR products were cloned into the pCaMV35S-eGFP vector (Zhang et al. 2008) that had been digested with *Ava*I and *Bam*HI. The

pCaMV35S::eGFP empty vector was used as the control. In transient expression analysis, inner epidermal peels from onion (*Allium cepa*) bulbs were placed on basic MS media and cultured for 24 h in a dark environment. Then, the plasmid DNA of the appropriate fusion construction (2.5 μg of pCaMV35S::*CaWRKY30*-eGFP or pCaMV35S::eGFP) was transformed into the onion cells using a particle gun (PDS-1000/He; Bio-Rad) according to the manufacturer's instructions. After bombardment, the epidermises were cultured for 24 h at 25°C in the dark. Finally, the peels were mounted on slides for GFP observation under a confocal fluorescence microscope (Olympus, Japan).

Real-Time RT-PCR Analysis

Total RNA was extracted from hormone- and pathogen-treated leaf tissue and nematode-inoculated root tip tissue using the TRIzol method (Invitrogen). The integrity of the RNA was assessed on a 1% agarose gel. Total RNA (2 μg) was reverse transcribed with SuperScript® III First-Strand Synthesis System (Invitrogen, USA) using an oligo(dT) primer according to the manufacturer's instructions. The cDNA synthesis reactions were treated with RNAse H, diluted 40-fold in sterile double-distilled water and 1.6 μl of the diluted cDNA served as the template for PCR. For quantitative PCR, 2× SYBR® Premix Ex Taq TM II (Takara) was used according to the manufacturer's recommendations on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Specific primer pairs (CaWRKY30-qF and CaWRKY30-qR, Table 1) targeting a 123-bp amplified fragment were designed using the Oligo 5.0 software packages. The expression level of *CaWRKY30* was measured in leaf samples treated with hormones and root samples inoculated with nematodes. Quantitative PCR was carried out in a final volume of 20 μl containing 0.4 μM of each primer and 1× SYBR® Premix Ex Taq TM II (Takara). The conditions of amplification were as follows: 95°C for 3 min, then 40 cycles of 95°C for 10 s, 57°C for 30 s, and 72°C for 10 s. All samples were tested in quadruplicate and three independent biological replicates; β-actin transcripts were used as internal controls. Differences were considered significant when the P values were <0.05 (Student's t test).

Results and Discussion

Cloning and Sequence Analysis of the *CaWRKY30* Gene

ESTs corresponding to 17 putative transcription factors were selected from a pepper transcriptome sequence database by Solexa high-throughput sequencing after the pepper plants were infected with avirulent *M. incognita* (data not shown). Genes that were either induced or repressed by avirulent *M. incognita* were chosen, and their expression levels analyzed (data not shown). Sequences encoding several putative transcription factors that were induced by avirulent *M. incognita* inoculation were screened. A 259-bp fragment representing the partial cDNA sequence of *CaWRKY30* gene can act as a putative transcription factor in response to an avirulent *M. incognita* attack. Based on the 259-bp EST sequence, specific oligonucleotide primers were designed used for 5′RACE and 3′RACE cloning experiments. The N′- and C′-ends of the cDNAs encoding putative WRKY proteins were obtained, and the full-length WRKY cDNAs were then amplified by PCR using primers designed to anneal to either end of the gene sequence. Then, a WRKY transcription we named *CaWRKY30* (FJ360844) was isolated. The nucleotide sequence of *CaWRKY30* cDNA is 1,533 bp long and contains an open reading frame encoding a polypeptide of 364 amino acids. The molecular mass of the predicted protein is 41.2 kDa, and the isoelectric point was calculated to be 6.57. Sequence analysis showed that *CaWRKY30* has one WRKY domain, which is highly conserved among all WRKY proteins, and one C_2HC zinc-finger-like motif consisting of three cysteines and one histidine in the central and C-terminal regions (Fig. 1). The *Arabidopsis* WRKY TFs *AtWRKY41* (NP_192845) and *AtWRKY30* (NP_568439), which contains one WRKY domain belonging to group III like *CaWRKY30*, share relatively high homology with *CaWRKY30* and have about 39% and 33.3% identity to the deduced amino acid of *CaWRKY30*, respectively. *AtWRKY41* is known to be a key regulator in the cross talk of the salicylic acid and jasmonic acid pathways (Higashi et al. 2008). *CaWRKY30*, an *Arabidopsis* WRKY transcription factor ortholog, and *AtWRKY41* each have a similar putative zinc-finger motif ($C–X_7–C–X_{23}–H–X_1–C$) and a nuclear localization signals (Fig. 1). These data indicate that *CaWRKY30* is a group III member of the WRKY superfamily.

When pepper HDA149 genomic DNA was digested with three different restriction enzymes and hybridized with a cDNA fragment of *CaWRKY30*, only one hybrid band were detected in three different lanes which contains the DNA digested with *Eco*RV, *Bam*HI, and *Hind*III, respectively (Fig. 2a). This result confirmed that the *CaWRKY30* gene is present as a single copy in the genome of pepper HDA149. Interestingly, all *Arabidopsis* WRKY genes might be present as single copy (Eulgem et al. 2000), suggesting that WRKY genes may be have a common feature exist in plant genome.

The *CaWRKY30* genomic sequences were obtained from *C. annuum* DNA, and *CaWRKY30* consisted of two introns and three exons (Fig. 2b). The WRKY domain-encoding region in *CaWRKY30* was interrupted by an intron.

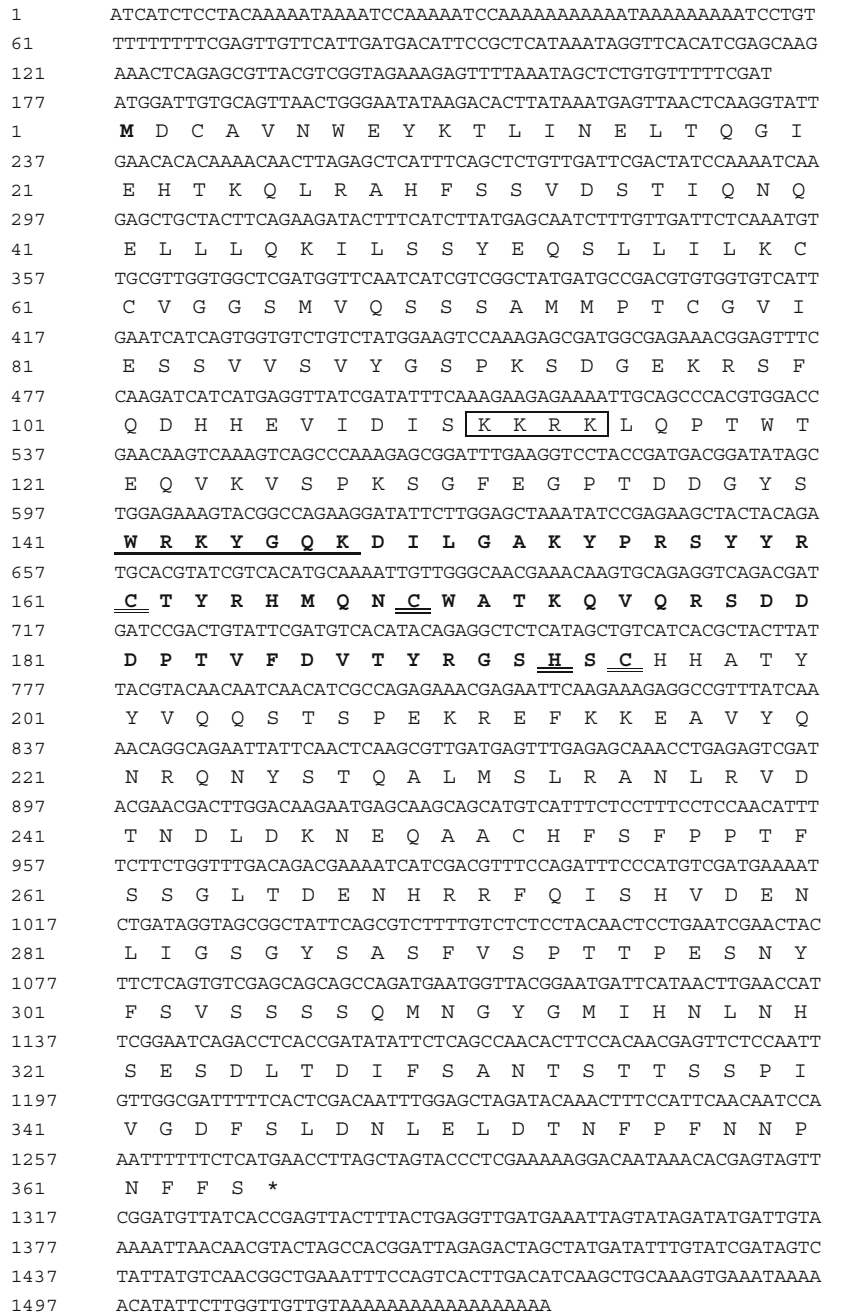

Fig. 1 Nucleotide and deduced amino acid sequences of the pepper *CaWRKY30* cDNA clone. The nucleic acids are presented in the *top line* and the deduced amino acid sequence is shown *below* it. The stop codon is indicated by the *asterisk*. The WRKY domain is shown in *bold* and WRKY sequences are *underlined*. The cysteines and histidines of the zinc-finger-like motif are *double underlined*. The putative nucleic localization signal sites are *boxed*

Interestingly, in *Arabidopsis*, the existence of an intron within the region encoding the C-terminal WRKY domain of group I members or the single WRKY domain of group II and III members is a common feature (Eulgem et al. 2000).

CaWRKY30 Localizes to the Plant Nucleus

CaWRKY30 is likely to be localized in the nucleus since it is a putative transcription factor. Our sequence analysis results showed that *CaWRKY30* contains a putative NLS sequence (^{111}KKRK^{114}), which is identical to consensus sequences found in spinach *SoWRKY1* (Huang et al. 2010). In addition, the presence of putative nuclear localization signals predicted by the BaCelLo predictor program (http://gpcr.biocomp.unibo.it/bacello/pred.htm) is consistent with this possibility. To confirm that the CaWRKY30 is indeed targeted to the nucleus, we constructed a green fluorescent protein (GFP) fusion of this WRKY protein. Then, we demonstrated that the transiently expressed WRKY::GFP fusion protein was localized exclusively to the nuclei of onion epidermal cells. With the control vector alone, the GFP signal was distributed in both the nucleus and cytoplasm (Fig. 3). The nuclear localization of CaWRKY30 protein supports its role as a transcriptional regulator.

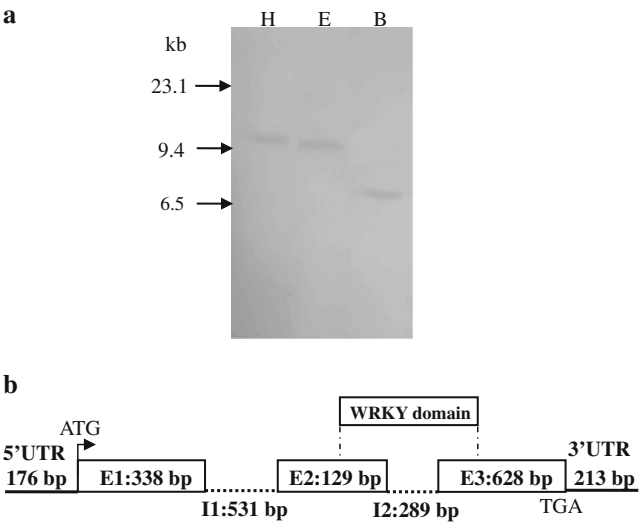

Fig. 2 **a** Southern blot analysis of *CaWRKY30* in the leaves of *C. annuum* (HDA149) (*H, Hind*III; *E, Eco*RV; *B*, *Bam*HI). Pepper genomic DNA (20 μg) was digested with *Eco*RV, *Bam*HI and *Hind*III and hybridized with the full-length *CaWRKY30* cDNA labeled with DIG. The sizes of the molecular weight *markers* are indicated on the *left*. **b** Characterization of the *C. annuum CaWRKY30* genomic structure. Exons are indicated by the *boxes* and introns are shown as *dotted lines*. *Arrows* indicate the transcription initiation site. The *bold lines* show the 5′UTR and 3′UTR, respectively. Interestingly, the WRKY domain is interrupted by an intron

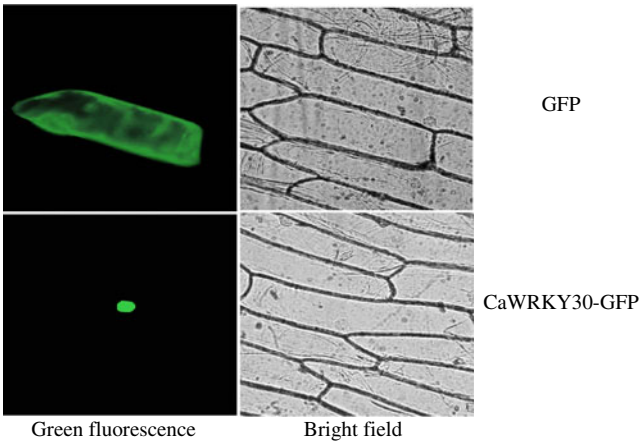

Fig. 3 Subcellular localization of the CaWRKY30 GFP fusion protein in onion epidermal cells. The images in the *top row* show the control plasmid expressing only the GFP. The *second row* shows the WRKY30-GFP fusion protein expressed in onion epidermal cells. The cells were examined under bright field (*right*) and fluorescence (*left*) exposures. The fluorescence photographs of onion epidermal cells were taken using a confocal fluorescence microscope (Olympus, Japan) fitted with fluorescein isothiocyanate filters (excitation 480 nm, emission 530 nm, dichroic beamsplitter 510 nm)

Expression Analysis of *CaWRKY30* Genes in Response to the RKN *M. incognita*

To investigate whether *CaWRKY30* transcripts are induced after inoculation with *M. incognita*, we used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to monitor the *CaWRKY30* expression pattern in pepper HDA149. The *CaWRKY30* transcripts were suppressed during the early stages upon infection with the compatible virulent *M. incognita* pathogen, lower transcript levels were detected at all of the investigated time points, i.e., at 3, 6 and 12 h, compared to the control, and the effect was more obvious after 48 h of inoculation (Fig. 4a). However, the transcripts of *CaWRKY30* was induced by inoculation with the incompatible avirulent *M. incognita* pathogen at 3 h after inoculation, the transcript levels were maintained for 48 h and reached its highest levels at 24 h after inoculation (Fig. 4b). By 72 h and 7 d after inoculation, the level of expression had decreased, implying that *CaWRKY30* may play a role at the early stage during the incompatible interaction of nematode and pepper plants. A number of previous studies have demonstrated that WRKY genes are induced by various pathogens. The expression profiles of all 13 WRKY group III transcription factors in *Arabidopsis* have been investigated, and the results indicated that the expression of almost all 13 genes was attributable to pathogen infection and SA production (Kalde et al. 2003). During the compatible interaction of the root-knot nematode and *Arabidopsis*, global analysis has shown that the successful establishment of RKN infestation is associatied with the suppression of plant defense responses (Jammes et al. 2005). Thus, most of the nematode-regulated genes involved in defense pathways were repressed, and 17 of the 21 WRKY genes identified are down-regulated in nematode-infected root tissues (Jammes et al. 2005), whereas the accumulation of WRKY transcripts appears to be a general characteristic of plant defenses in response to pathogens (Eulgem 2005). *AtWRKY23* is up-regulated almost immediately upon nematode infection and knockdown lines show increased resistance to the cyst nematode *Heterodera schachtii* (Grunewald et al. 2008). Along with previous studies, our results indicate that expression of *CaWRKY30* is induced upon infection with the avirulent nematode *M. incognita* but repressed by the virulent *M. incognita*; this suggests that *CaWRKY30* associated with the *Me3* gene-mediated root-knot nematode resistance in pepper plants.

Moreover, to investigate the expression levels of the *CaWRKY30* gene in different organs of the pepper plants under normal conditions, total RNA was extracted from roots, stems, leaves, flowers, and young fruits. Quantitative PCR showed that a high level of *CaWRKY30* transcripts was detected in roots; the lowest was in young fruits, and similar transcripts were detected in stems, leaves, and flowers (Fig. 4c). Previous studies have shown that the organ-specific expression of WRKY genes from pepper plants is induced by various biotic and abiotic stresses (Oh et al. 2008; Park et al. 2006). The relative high expression of *CaWRKY30* in roots suggests that *CaWRKY30* may be associated with defense responses in roots.

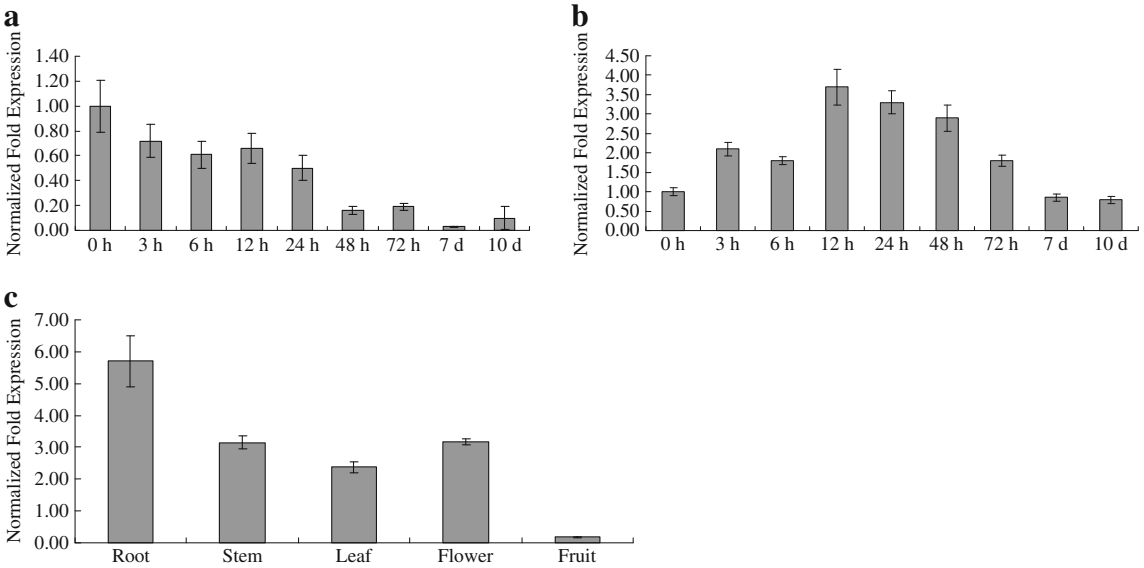

Fig. 4 Expression analyses of *CaWRKY30* in response to *M. incognita*. The expression level of *CaWRKY30* was calculated relative to that of noninoculated (ck) pepper plants. The β-actin gene was used as an internal reference. The normalized fold expression of the *CaWRKY30* gene 0 h was set equal to 1.0. Each *bar graph* represents the mean±SD of three independent biological replicates **a** virulent *M. incognita* inoculation **b** avirulent *M. incognita* inoculation. **c** Tissue-specific expression of *CaWRKY30* in different tissues of pepper plants under normal conditions

Expression of *CaWRKY30* upon Salicylic Acid and Methyl Jasmonic Acid Inoculation

As indicated earlier, JA and SA are important signaling molecules that are implicated in plant defense responses against pathogen attacks (Bostock 2005; Reymond and Farmer 1998). When pepper plants were sprayed with 5 mM SA, the expression of *CaWRKY30* expression was induced dramatically. The *CaWRKY30* transcripts accumulated as early as 2 h after treatment, and the transcripts levels increased to 19-fold that of the control in 9 h; then we observed a gradual decrease to about normal levels at 48 h after the inoculation, which was the last observed time point (Fig. 5a), indicated that *CaWRKY30* expression was considerably induced or up-regulated by the signal molecule SA. To investigate whether *CaWRKY30* gene expression is induced in response to methyl jasmonate (MeJA) treatment, 0.1 mM MeJA was applied by the same method used for the SA treatment. The RT-PCR analysis showed that the *CaWRKY30* transcripts were suppressed as early as 2 h after treatment, but they decreased to the lowest levels 16 and 48 h after treatment (Fig. 5b), suggested that *CaWRKY30* expression was suppressed or down-regulated after treatment with MeJA. In *Arabidopsis*, *AtWRKY70* belongs to group III and is a common component in the SA- and JA-mediated signal pathways; the expression of *AtWRKY70* is activated by SA and repressed by JA, and *AtWRKY70* seems to act as an activator of SA-induced genes and a repressor of JA-response genes (Li et al. 2004). Recent research showed that *AtWRKY70* not only acts as a convergence point that determines the balance between the SA- and JA-dependent defense pathways, but it is also required for R-gene-mediated resistance (Knoth et al. 2007; Li et al. 2006). Thus, *CaWRKY30* may play roles that are similar to those

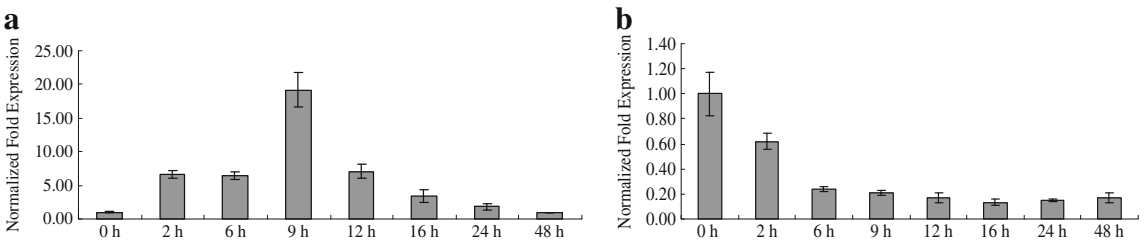

Fig. 5 Expression of the *CaWRKY30* gene after **a** salicylic acid (SA) and **b** methyl jasmonate (MeJA) treatments. The expression level of *CaWRKY30* was calculated relative to that of noninoculated (ck) pepper plants. The β-actin gene was used as an internal reference. The normalized fold expression of the *CaWRKY30* gene 0 h was set equal to 1.0. Each *bar graph* represents the mean±SD of three independent biological replicates

AtWRKY70, but these need to be further elucidated. In sunflower, MeJA did not induce *HaWRKY* expression, but most of the *HaWRKY* genes were induced by SA, among of them, *HaWRKY76* was up-regulated by SA, but repressed by MeJA (Giacomelli et al. 2010). In cotton, *GhWRKY3* is up-regulated by SA, and might be play an important role in plant defense responses (Guo et al. 2011). In pepper plants, the expression of *CaWRKY1* transcripts was detected at 1.5 h after SA treatment, but the MeJA did not induce the expression of *CaWRKY1* (Oh et al. 2008). These results indicate that *CaWRKY30* may participate in the early stages of the SA responses.

Expression of the *CaWRKY30* Gene in Response to Various Pathogens

To investigate whether *CaWRKY30* was induced by other stimuli, pepper leaves treated with various pathogens were harvested for RNA extraction at the time points indicated. After plants were challenged with the bacterial pathogen *Ralstonia solanacerum*, the *CaWRKY30* transcript abundance was significantly increased and reached its maximal level 3 h after treatment (Fig. 6).

We then examined the expression patterns of *CaWRKY30* in the leaves of pepper (HDA149) in response to infection with the fungal pathogen *Phytophthora capsici*. As shown in Fig. 6, the *CaWRKY30* transcripts were significantly affected by *P. capsici* and were significantly up-regulated at all of the time points (Fig. 6).

The levels of *CaWRKY30* gene expression in response to a viral pathogen attack were also monitored. *T. mosaic virus* (TMV) is an important pathogen, which often causes pepper virus disease and then affects the yield and quality. Highest levels of the *CaWRKY30* transcript were detected in pepper leaves 3 h after inoculation with TMV, and the expression level was highest at 48 h and then decreased to lower levels at 72 h, which was the last time point we monitored (Fig. 6). In summary, our results indicate that *CaWRKY30* was induced by *R. solanacerum*, *P. capsici*, and TMV. Furthermore, a similar temporal pattern in the transcript abundance of *CaWRKY30* was observed upon inoculation with *R. solanacerum* and *P. capsici*.

A large number of WRKY genes are known to be induced by various pathogens, such as viruses (Chen and Chen 2000; Park et al. 2006), fungi (Chujo et al. 2007), bacteria (Chen and Chen 2002) and nematodes (Jammes et al. 2005). WRKY factors appear to play a major role in transcriptional reprogramming during a variety of immune responses (Eulgem 2005). In *Arabidopsis*, 49 of the 72 WRKY genes investigated were differentially regulated in plants after infection with an avirulent strain of *P.syringae* or with SA treatment (Dong et al. 2003). *AtWRKY33* is required for resistance to necrotrophic fungal pathogens, and its transcripts were induced in response to pathogen infection (Zheng et al. 2006). In canola, a set of 10 *BnWRKY* genes are induced by the fungal pathognes *Sclerotinia sclerotiorum* and *Alternaria brassicae* (Yang et al. 2009). The cotton WRKY gene, *GnWRKY3* is up-regulated after infection with *Rhizoctonia solani*, *Colletotrichum gossypii*, and *Fusarium oxysporum* f. sp. *Vasinfectum* (Guo et al. 2011). The pepper WRKY protein, *CaWRKY1* is a negative regulator of pathogen defense, and the expression of *CaWRKY1* is rapidly induced by incompatible and compatible pathogen inoculations (Oh et al. 2008). When considering these previous studies, our results indicate that expression of *CaWRKY30* is very rapidly induced upon pathogen infection, and suggesting that *CaWRKY30* might be play an important role in the plant defense response to pathogens.

The plant defense mechanisms against pathogen infections are complex. What has become unequivocal, however, is that plants undergo extensive transcriptomic reprogramming in a highly dynamic and temporal manner. This process involves the induction and up-regulation as well as the down-regulation of a large number of genes in plants. This regulated response is represented by a network of various transcription factors. WRKY transcription factors are the main regulatory proteins forming such a network (Eulgem and Somssich 2007). In this study, a WRKY TF named *CaWRKY30*, which

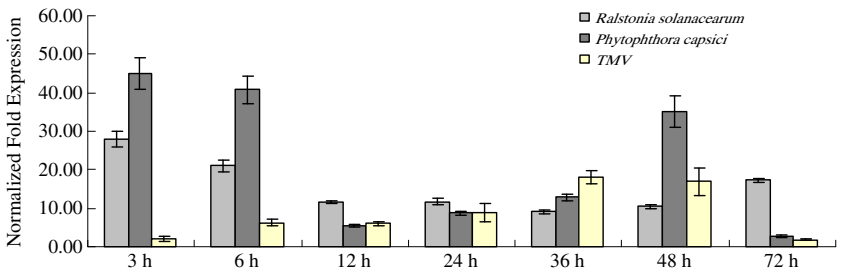

Fig. 6 Relative expression level of the *CaWRKY30* gene in the leaves of pepper plants after inoculation with *R. solanacerum*, *P. capsici*, and *T. mosaic virus* (TMV). The expression level of *CaWRKY30* was calculated relative to that of noninoculated (ck) pepper plants. The β-actin gene was used as an internal reference. The normalized fold expression of the *CaWRKY30* gene 0 h was set equal to 1.0. Each *bar graph* represents the mean±SD of three independent biological replicates

was rapidly induced upon inoculation with avirulent *M. incognita* and several pathogens, was isolated from pepper plants. Transgenic pepper plants or heterologous plants that overexpress the *CaWRKY30* gene, and/or loss-of-function studies by RNAi or virus-induced gene silencing of *CaWRKY30*, as well as the identification of the target gene, might be used to elucidate the role(s) of *CaWRKY30* in the defense against pathogen infections.

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