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ORIGINAL PAPER

CanTF, a Novel Transcription Factor in Pepper, Is Involved in Resistance to *Phytophthora capsici* as well as Abiotic Stresses

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Abstract

Stress is usually considered an important factor resulting in plant injury. In this study, we identified a novel transcription factor gene, *CanTF* (*Capsicum annuum* transcription factor IIB), and characterized its role in the response to biotic and abiotic stresses. The full-length *CanTF* cDNA consists of 1488 bp, with a 1281-nucleotide open reading frame (ORF), and encodes a protein containing 426 amino acids with a theoretical molecular weight of 46.84 kDa. Real-time quantitative PCR revealed that *CanTF* is a stress-induced gene, with increased expression levels under both biotic and abiotic stresses. The expression of *CanTF* in pepper organs, especially roots, was highly induced by inoculation with an avirulent strain of *Phytophthora capsici*. Additionally, the differential expression of *CanTF* was observed under abiotic stresses, i.e., earlier expression was detected after cold, drought, and SA treatments than after salt and H_2O_2 treatment, suggesting its role in responses to various abiotic stresses. Furthermore, the silencing of *CanTF* by virus-induced gene silencing reduced the expression of defense-related genes (*CaPR1*, *CaDEF1*, and *CaSAR82*) under *P. capsici* inoculation. POD and root activity levels were lower after gene silencing than in controls, demonstrating the positive regulatory effect of *CanTF* against *P. capsici*. These results suggested that *CanTF* is a stress-induced gene involved in strengthening the pepper defense against biotic and abiotic stresses.

Keywords Pepper · Transcription factor (TF) · *Phytophthora capsici* · Abiotic stress · Virus-induced gene silencing

Introduction

Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops worldwide (Pimenta et al. 2016). Pepper is challenged by various biotic and abiotic stresses, such as cold stress (Sánchez-Bel et al. 2012), salinity (Penella et al. 2016), drought (Park et al. 2015), and pathogens (Lim et al. 2015). For example, chilling injury can cause lipid peroxidation and severely impair plant tolerance to low temperatures (Padma et al. 1997), while drought and salt stresses result in abnormal

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changes in physiology, such as photosynthesis, and low resistance to osmotic stress (Delfine et al. 2002; Park et al. 2016). Similarly, biotic stress can cause injury during pepper growth. For example, *Phytophthora capsici* can alleviate plant defense by secreting various pectin methyl-esterases during all stages of infection (Li et al. 2011; Zhang et al. 2016). Development is often disrupted by interactions between pepper plants and *P. capsici*. These biotic and abiotic stresses solely or jointly cause disorders of physiological metabolism during plant growth, resulting in plant injury. To combat these injuries, pepper has evolved several protective mechanisms, including the regulation by transcription factors (TFs) and hormones that enhance resistance to different stresses (Kim et al. 2009; Guo et al. 2015).

The importance of TFs in plant defense is evidenced by their ubiquity in plants (Jones and Dangl 2006). Regulation by TFs in plant development can activate the complex adaptive mechanisms and hence improve the resistance of plants to different stresses (Khong et al. 2015). For example, AP2/ERF TF binding to the promoter regions of pathogenesis-related genes can modulate their expression levels in response to pathogens (Ohme-Takagi and Shinshi 1995; Amorim et al. 2017). WRKY TFs are implicated in the regulation of plant defense mechanisms against pathogens by recognizing the W-box in promoter regions of some genes (Rushton et al. 2010). For example, SpWRKY3 can enhance resistance to *Phytophthora infestans* by inducing PR gene expression and reducing ROS accumulation (Cui et al. 2017). Most plant bZIP proteins binding to cis-elements can play important roles in plant immunity against pathogens (Alves et al. 2013; Noman et al. 2017). For example, StbZIP61, a potato transcription factor, regulates the dynamic biosynthesis of salicylic acid in defense against *P. infestans* infection (Zhou et al. 2018). Furthermore, GmPIB1, a bHLH TF, enhance resistance to *Phytophthora sojae* in soybean by repressing the expression of *GmSPOD1* (Cheng et al. 2018).

The transcription factor IIB (TFIIB) family is well characterized, since it acts as a bridge between RNA polymerase II and the pre-initiation complex (PIC) and serves as the interaction target for multiple classes of trans-activator proteins that regulate gene expression in a specific manner (Lagrange et al. 2003). TFB-like proteins are characterized by an N-terminal zinc ribbon, a variable linker segment, and a cyclin fold domain, and there are a number of van der Waals contacts, salt bridges, and hydrogen bonds in the DNA–TBP–TFIIB interaction (Nikolov et al. 1995). Although the TFIIB protein does not exhibit sequence-specific DNA binding, a close association with DNA was discovered based on interactions with the phosphodiester backbone of DNA at the TATA element and its extension of the DNase footprint after joining a TBP–DNA complex (Malik et al. 1993; Nikolov et al. 1995). In *Arabidopsis thaliana*, 14 different TFIIB-like proteins encoding proteins in three major TFIIB subfamilies have been discovered (Knutson 2013). AtTFIIB1 has positive effects on pollen tube growth and endosperm development (Zhou et al. 2013), demonstrating its key role in the regulation of plant development. TFIIB provides protection against oxidative stress imposed by the extracellular environment or generated by cellular respiratory activity (de Faria and Fernandes 2006). However, very little information is available regarding the role of the TFIIB1 family in pepper defense against different stresses.

In the current study, a novel gene belonging to the TFIIB family, *CanTF*, was isolated from the pepper cultivar A3, and its characteristics under different stresses were evaluated. The objective of our study was to reveal the biological role of *CanTF* in the responses to biotic and abiotic stresses. The results provide insights in the mechanisms by which *CanTF* regulates pepper development under environmental stress and provides a basis for resistance breeding.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of the pepper (*Capsicum annuum* L.) cultivar A3 (susceptible to HX-9 and resistant to PC of *P. capsici*) were obtained from the *Capsicum* Research Group, College of Horticulture, Northwest A&F University, China. Seeds were germinated, and seedlings were grown under the conditions described by Wang et al. (2013a).

Pathogen Preparation and Inoculation Procedures

P. capsici zoospore suspension was prepared according to the protocol used by Wang et al. (2013a) as follows: compatible (HX-9) and incompatible (PC) *P. capsici* strains were grown on PDA medium for 7 days, cut into pieces, and placed into sterile distilled water, followed by incubation in the light for 4 days, incubation for 1 h at 4 °C, and shaking for 1 h at 28 °C to release zoospores. The zoospores were collected by filtering through four layers of cheesecloth and were adjusted to 1×10^4 per millimeter. Pepper seedlings with six true leaves were inoculated by adding 2.5 mL of virulent/avirulent strains of *P. capsici* zoospore suspensions to each pot using the root drench method.

Stress Treatments

Various abiotic stresses, including cold, salt, and drought, along with hormone treatments were applied. Pepper seedlings were exposed to 4 °C for cold stress. For salt and

drought stress treatments, the seedlings were immersed in 0.4 M sodium chloride (NaCl) and 0.4 M mannitol solutions, while water was used for the control-treated plants. Leaf samples from the treated and control seedlings were collected at 0, 2, 4, 8, 12, and 24 h post-stress treatment. For pathogen treatment, pepper seedlings with six-true leaves were inoculated by adding 2.5 mL of the virulent/avirulent *P. capsici* zoospore suspension to each pot using the root drench method. Leaf and root samples from treated and control (mock) plants were collected at 0, 2, 4, 8, 12, 24, 36, 48, and 72 h after inoculation. For hormonal treatments, each hormone (5 mM salicylic acid (SA), 50 μM methyl jasmonate (MeJA), and 10 μM H_2O_2) was supplemented with 0.5% Tween-20 and sprayed on pepper seedlings. Leaves from mock and treated plants were collected at 0, 2, 4, 8, 12, 24, and 48 h after treatment. All collected samples were instantly frozen in liquid nitrogen and stored at − 80 °C. All treatments were performed and analyzed thrice in separate experiments.

Sequence Analysis of the *CanTF* Gene

A multiple sequence alignment of CanTF amino acid sequences was obtained using DNAMAN (Version 5.0). The amino acid sequence of CanTF and homologs in other plant species were aligned using CLUSTALW as described by Guo et al. (2016), and MEGA6.0 was used to construct a phylogenetic tree by the neighbor-joining method (Benson et al. 2000).

Tissue-Specific Expression of *CanTF* in Pepper

To evaluate the tissue-specific expression of *CanTF*, samples were collected from roots, stems, leaves, flowers, green fruits, and mature fruits of the pepper cultivar A3, immediately frozen in liquid nitrogen, and stored at − 80 °C until RNA extraction.

RNA Isolation and Real-Time RT-PCR Analysis

RNA was extracted from samples at different time points, as mentioned previously, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For reverse transcription, 0.5 μg of total RNA was used for first-strand cDNA synthesis using a PrimeScript™ Kit (TaKaRa Bio Inc., Dalian, China). Real-time quantitative PCR (qRT-PCR) was performed as described by Guo et al. (2016). *Ubi3* (AY486137.1) was used as internal control (reference gene) (Wan et al. 2011). The primer sequences used in the study are shown in Table 1. Relative *CanTF* expression levels were determined by the comparative $2^{-\Delta\Delta CT}$ method (Litvak and Schmittgen 2001).

Cloning and Sequence Analysis of *CanTF*

The rapid amplification of cDNA ends (RACE) technique was used to obtain the full-length cDNA designated *CanTF*. According to the partial cDNA fragment of the reported sequence (accession number GD094649.1), a set of gene-specific primers (*CanTF*F, *CanTF*R) for 5′ and 3’ RACE was designed. Contig Express software and BLAST were used to assemble the full-length cDNA sequence. The full-length cDNA sequence was obtained from the leaves of the pepper cultivar A3 using the primer pair (*CanTF*QF and *CanTF*QR).

The pI and MW of CanTF were analyzed using the online tool (https://www.expasy.org/), and conserved domains were identified using an NCBI tool (available online: http://www.ncbi.nlm.nih.gov/cdd/). The secondary structure was predicted using ScratchProtein Predictor (https://www.ics.uci.edu/~baldig/scratch/). The transmembrane and signal peptides were predicted using the CBS prediction server (http://www.cbs.dtu.dk/services/).

VIGS of *CanTF* in Pepper

For gene silencing in pepper plants, the virus-induced gene silencing (VIGS) system was used (Choi et al. 2007). *CaPDS* was used for the phenotypic effectiveness of VIGS. A 256-bp fragment harboring a conserved and a non-conserved region of CanTF with primers containing restriction sites for EcoRI and BamHI enzymes was cloned into the TRV2 vector. Then, the freeze-thaw method was used to transform the TRV1, TRV2, TRV2-*CaPDS*, and TRV2-*CanTF* vectors into the *Agrobacterium tumefaciens* strain GV3101. The *A. tumefaciens* strain (GV3101) harboring TRV1 was mixed at a 1:1 ratio with TRV2, TRV2-*CaPDS*, and TRV2-*CanTF*. Then, a sterilized syringe without a needle was used to inoculate the *Agrobacterium* suspensions to the cotyledons of pepper plants. Plants were placed in a growth chamber with the same growth conditions described by Wang et al. (2013b).

After 5 weeks of infiltration, the upper 4th leaves from TRV2:00 (control) and TRV2: *CanTF* (silenced) plants were injected with the *P. capsici* suspension. In addition, control (TRV2:00) and silenced (TRV2: *CanTF*) plants were subjected to 4 °C for cold stress treatment, 0.4 M sodium chloride (NaCl) and mannitol for salt and drought stress treatments. Leaf samples were collected for the measurement of peroxidase activity assay and conductivity.

Measurements of Peroxidase Activity Assay and Conductivity

Peroxidase (POD) enzyme activity was quantified using the technique of Beffa et al. (1990). To evaluate the permeability

Table 1 Primer sequences used in the experiment

Code	Sequence (5′–3′)
*CanTF*F	CGTGTTTGAGGAATCGGAGTGTTGAAGC
*CanTF*R	TCAGCCTGGGTCTTCCGCTTGTCTTCT
CanTFQF	ATGCGGTGTCCGTACTGTTC
*CanTF*QR	ACATAAGGCTTTCTTTATCAGTTCA
*CaPDS*VF(VIGS)	GGGGAATTCTGTTGTCAAAACTCCAA GGTCTGTA
*CaPDS*VR	GGGGGATCCTTTCTCCCACTTGGTTC ACTCTTGT
*CanTF*VF	GGGGAATTCTAGAAGACAAGCGGAAG ACCCAGG
*CanTF*VR	GGGGGATCCTGGTTTAACAGGCTTGT CCCTCTCA
CaUbi-F	TGTCCATCTGTCCTCTGTTG
CaUbi-R	CACCCCAAGCACAATAAGAC
*CanTF*DF(qRT-PCR)	ATACTTATGAGCAACCTACTCCAAAT
*CanTF*DR	GTATGAAAAAGATGGTGGGATTG
*CaPR1*F(qRT-PCR)	GCCGTGAAGATGTGGGTCAATGA
*CaPR1*R	TGAGTTACGCCAGACTACCTGAGTA
*CaDEF1*F	GTGAGGAAGAAGTTTGAAAGAAAGTAC
*CaDEF1*R	TGCACAGCACTATCATTGCATACAATTC
*CaSAR82*F	GTTGTGACTATTGTTGTGCCTA
*CaSAR82*R	TAATCATAAACAAATCAATCTAAATC

of the membrane, conductivity was determined as described by Dionisio-Sese and Tobita (1998).

Determination of Root Activity

The triphenyltetrazolium chloride (TTC) was used to measure root activity according to the methods of Wang et al. (2013a). *P. capsici* post-inoculation root samples of about 0.5 g were collected from control and *CanTF*-silenced plants. A modified TTC method was used to measure root activity as described by Jin et al. (2016). The treatments were evaluated in three biological replications, and measurements were repeated thrice.

Statistical Analysis

Data were evaluated by an analysis of variance (ANOVA) using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± standard deviation (SD) of three replicates for all parameters. A least significant difference ($P \leq 0.05$) test was used to identify significant differences among the treatments.

Results

Sequence Analysis of *CanTF*

The full-length cDNA of *CanTF* was obtained by RACE. *CanTF* consisted of 1488 nucleotides, including a 5′-untranslated region (UTR) of 100 bp, an open reading frame (ORF) of 1281 bp and a 3′-UTR of 107 bp with a poly (A+) tail of 26 bp (GenBank accession number FJ617518). The ORF encoded a predicted protein of 426 amino acids with a theoretical molecular weight (MW) of 46.84 kDa and isoelectric point (pI) of 5.39. A structural analysis revealed that *CanTF* belongs to the transcription factor IIB (TFIIB) superfamily, containing a zinc-binding region (C3-X2-C6-X18-C25-X2-C28) and two binding sites (130-174, 226-312), as shown in Fig. 1, while TMpred showed that CanTF does not contain a transmembrane helix. Therefore, CanTF probably lacks a transmembrane domain. Further sequence analysis indicated that the deduced CanTF protein does not contain a signal peptide. In addition, high amino acid sequence homology was observed between CanTF and other TFIIB proteins based on multiple alignments obtained using ClustalW. The percent identities of *CanTF* relative to *Solanum tuberosum* (XP_006340594.1), *Solanum lycopersicum* (NP_001308007.1), *Nicotiana tomentosiformis* (XP_009601399.1), *Nicotiana sylvestris* (XP_009791322.1), *Prunus persica* (XP_007203626.1), *Vigna radiata* var. *radiate* (XP_014519199.1), *Glycine soja* (KHN36117.1), *Morus notabilis* (XP_010095267.1), *Gossypium arboretum* (KHG10594.1), *Theobroma cacao* (XP_007046979.1), and *Prunus mume* (XP_008241653.1) were 94%, 93%, 93%, 93%, 75%, 73%, 71%, 73%, 73%, 74%, and 68%, respectively (Fig. 1). The phylogenetic analysis was carried out using MEGA6.0. Two clusters were observed using the amino acid sequences of 14 TFIIB genes from *Solanum lycopersicum* (NP_001308007.1), *Solanum pennellii* (XP_015064013.1), *Solanum tuberosum* (XP_006340594.1), *Capsicum annuum* (NP_001311673.1), *Nicotiana sylvestris* (XP_009791324.1), *Theobroma cacao* (XP_007046979.2), *Brassica oleracea* var. *oleracea* (XP_013597636.1), *Arabidopsis thaliana* (NP_195383.1 and NP_001078502.4), *Arabidopsis lyrata* subsp. *lyrata* (XP_002867001.1), *Vigna radiata* var. radiate (XP_014519199.1), *Cicer arietinum* (XP_004512022.1), *Morus notabilis* (XP_010095267.1), and *Prunus mume* (XP_008241653.1) (Fig. 2). *CanTF* (FJ617518) of pepper and other Solanaceae plants contained smaller subunit TFIIB sequences.

Tissue-Specific Expression of *CanTF* in Pepper

To investigate the tissue-specific expression level of *CanTF*, RNA was extracted from leaves, stems, roots, flowers, and both green and red fruits (Fig. 3). The results indicated that

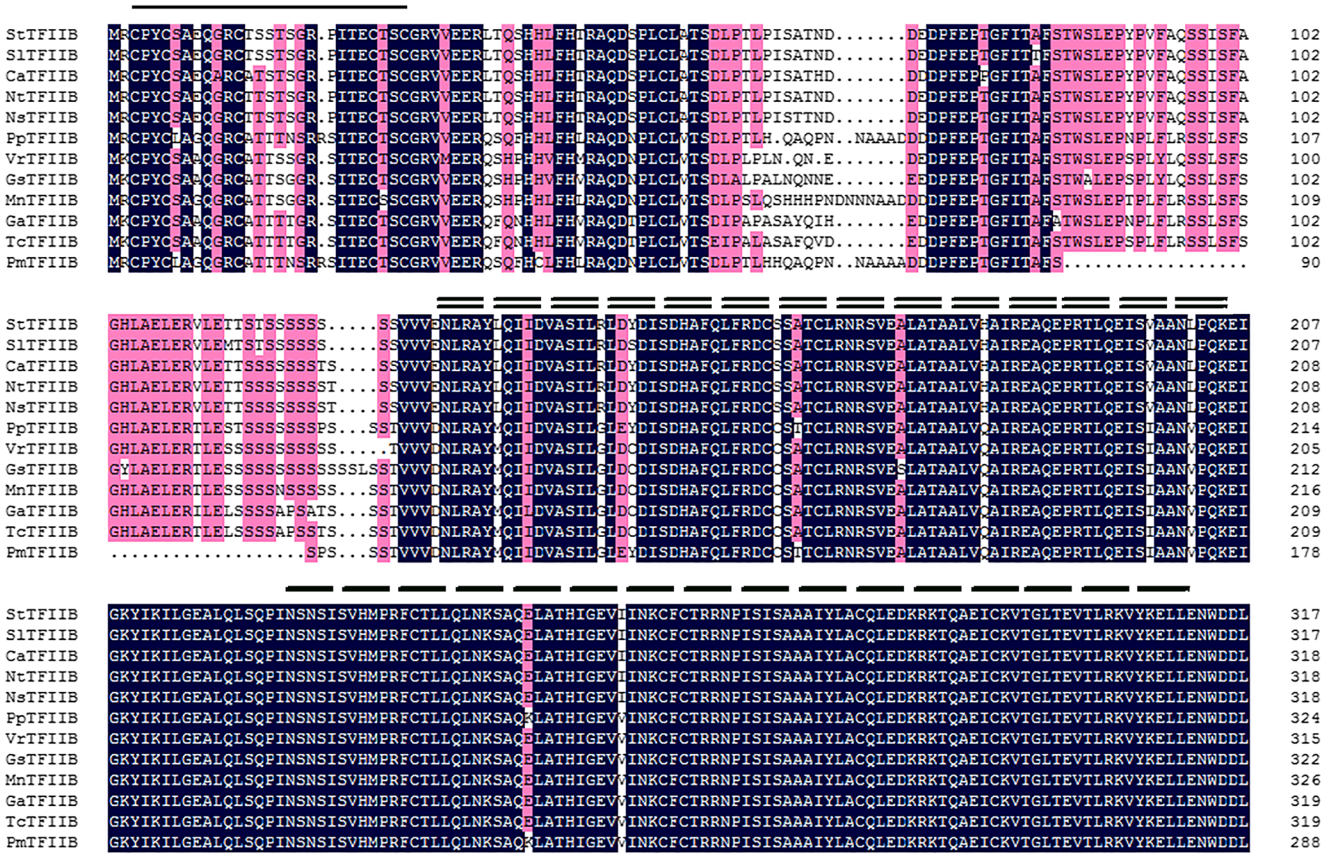

Fig. 1 Multiple sequence alignment of the amino acid sequence of CanTF with amino acid sequences of StTFIIB (*Solanum tuberosum*, XP_006340594.1), SlTFIIB (*Solanum lycopersicum*, NP_001308007.1), NtTFIIB (*Nicotiana tomentosiformis*, XP_009601399.1), NsTFIIB (*Nicotiana sylvestris*, XP_009791322.1), PpTFIIB (*Prunus persica*, XP_007203626.1), VrTFIIB (*Vigna radiata var. radiata*, XP_014519199.1), GsTFIIB (*Glycine soja*, KHN36117.1), MnTFIIB (*Morus notabilis*, XP_010095267.1), GaTFIIB (*Gossypium arboretum*, KHG10594.1), TcTFIIB (*Theobroma cacao*, XP_007046979.1), and PmTFIIB (*Prunus mume*, XP_008241653.1). Dark-blue shading and pink shading reflect 100% and 75% conservation, respectively. Gaps (−) were introduced to maximize the alignment. The zinc-binding region is indicated by a solid line and the two-binding sites are indicated by the dashed and the single short lines

the expression levels of *CanTF* were significantly higher in both green and red fruits than in other tissues, with no significant difference between these fruits. Among other tissues, the expression levels were highest in leaves, followed by stems, flowers, and roots, but the differences were not significant.

Expression of *CanTF* in Response to *P. capsici* Infection

CanTF expression levels were quantified in the roots and leaves of post-inoculation *P. capsici* strains (HX-9 and PC). Based on qRT-PCR, *CanTF* was strongly up-regulated by the incompatible strain of *P. capsici* (Fig. 4). In roots, the expression level of *CanTF* was significantly increased by inoculation with the PC strain at 72 h post-inoculation, followed by 48 h and 12 h. For the HX-9 strain, the expression level was slightly increased at 2, 48, and 72 h after inoculation, without any significant differences among these and other time points (Fig. 4a). In leaves, *CanTF* expression levels exhibited almost the same trend as those in roots for both strains (HX-9 and PC), as shown in Fig. 4b. Generally, the transcript level of *CanTF* in leaf was greater for the PC strain than the HX-9 strain. The inoculation of plants with the HX-9 strain increased the expression in leaves to peak levels (3.325 ± 1.19) at 2 h post-inoculation, followed by 72 h (2.41 ± 1.499), while the expression level at 24 h ranked third. For inoculation with the PC strain, the expression level increased at three different time points and reached the highest levels at 72, 24, and 48 h after inoculation.

Expression of *CanTF* in Response to Abiotic Stresses and Hormonal Treatments

To evaluate the role of *CanTF* in protection against abiotic stresses, pepper plants were exposed to cold, salt, and drought stresses (Fig. 4c–e). Compared to the control, the expression

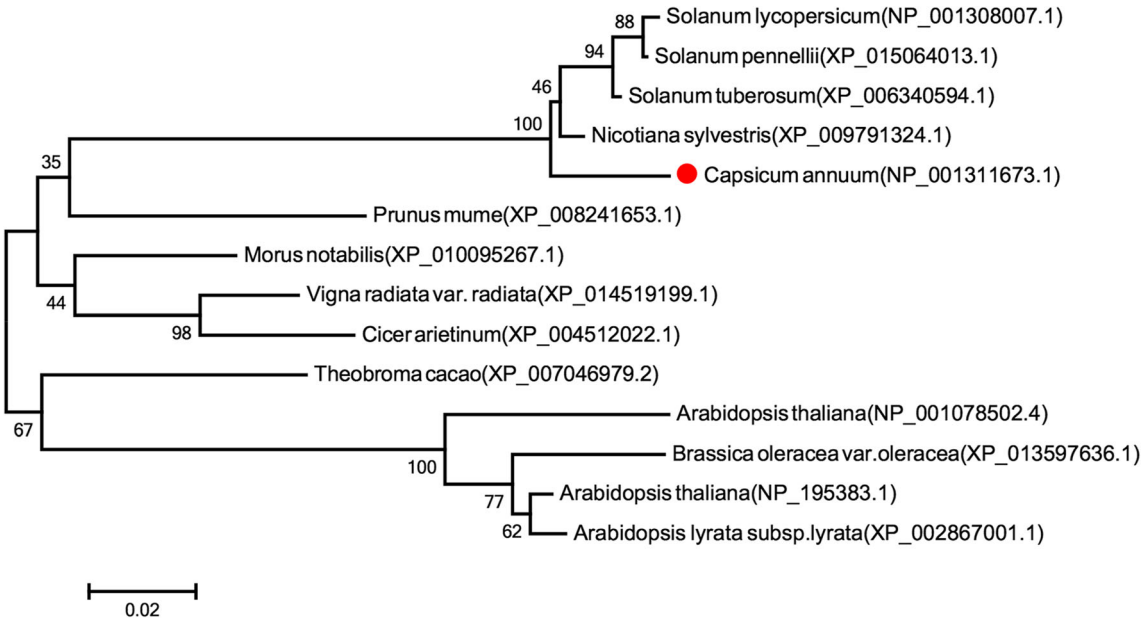

Fig. 2 Phylogenetic tree of proteins homologous to CanTF and TFIIB proteins from other species. The rooted gene tree (majority-rule consensus from 1000 bootstrap replicates) was obtained by a heuristic search using MEGA6.0. Bootstrap values are indicated at each branch node. GenBank accession numbers are in parentheses after each species and gene name. The red point represents CanTF (NP_001311673.1). Scale bar indicates the similarity coefficient

level of *CanTF* was about 3-fold higher (3.82) at 4 h post-cold stress (4 °C) (Fig. 4c), while exposure to salt stress significantly increased the expression level by 3-fold or more at 12 h (3.08) and 24 h (3.32) (Fig. 4d). *CanTF* also respond to drought stress; its expression increased 9-fold (9.44) at 2 h post-treatment (Fig. 4e).

While evaluating the expression levels of *CanTF* in response to signaling molecules, leaf samples from plants treated with SA, MeJA, and H_2O_2 were collected, and their cDNA was used for qRT-PCR to analyze expression levels. Plants treated with H_2O_2 showed a slight increase in the expression of *CanTF* at 2 h (10.79) post-treatment, followed by a slight decrease and an increase to the maximum level at 48 h post-treatment, which was almost 56-fold increase compared with the expression level in the control (Fig. 5a). In response to SA treatment, the expression level of *CanTF* increased dramatically by 77-fold (77.36) at 2 h post-treatment as compared to the control, but showed no significant effect at other time points (Fig. 5b). Plants treated with MeJA showed initially downregulated expression, reaching the lowest level at 4 h post-treatment, followed by an upregulation, reaching a maximum at 12 h, and downregulation after 12 h, but the differences in the expression level of *CanTF* were not significant (Fig. 5c).

Silencing of *CanTF* Weakened the Defense Response of Pepper Against *P. capsici*

Five weeks after infiltration, the visible symptoms of photobleaching due to a loss of chlorophyll were observed in *CaPDS*-silenced plant leaves (Fig. 6a), confirming that VIGS was successful. Furthermore, a qRT-PCR analysis of RNA extracted from the leaves of *CanTF*-silenced plants (TRV2:*CanTF*) and empty vector control plants (TRV2:00) was performed to clarify the efficiency of *CanTF* silencing by VIGS. As shown in Fig. 6b, the levels of *CanTF* transcripts were significantly reduced to different extents in *CanTF*-silenced plants compared to those in the empty vector control. These results indicated that *CanTF* was partially silenced. Thus, to verify the role of *CanTF* in defense response, the fourth to fifth leaves from the top of the silenced (TRV2:*CanTF*) and control (TRV2:00) pepper plants were removed and inoculated with HX-9 strain of *P. capsici*. On the 4th day of inoculation, more lesions were detected on the

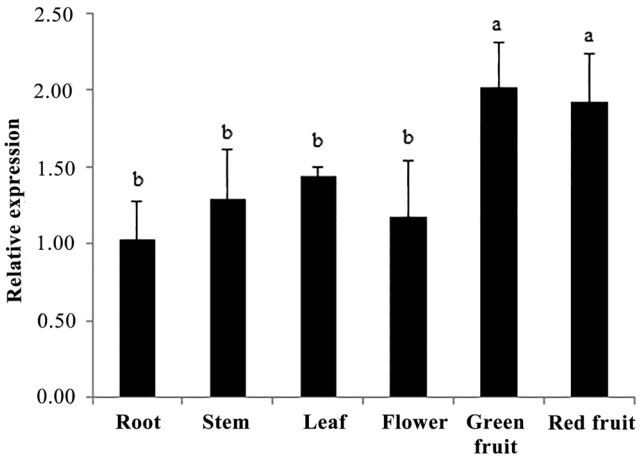

Fig. 3 Tissue-specific expression of *CanTF* in different organs of pepper plants. Results are presented as means ± SD of three independent biological replicates

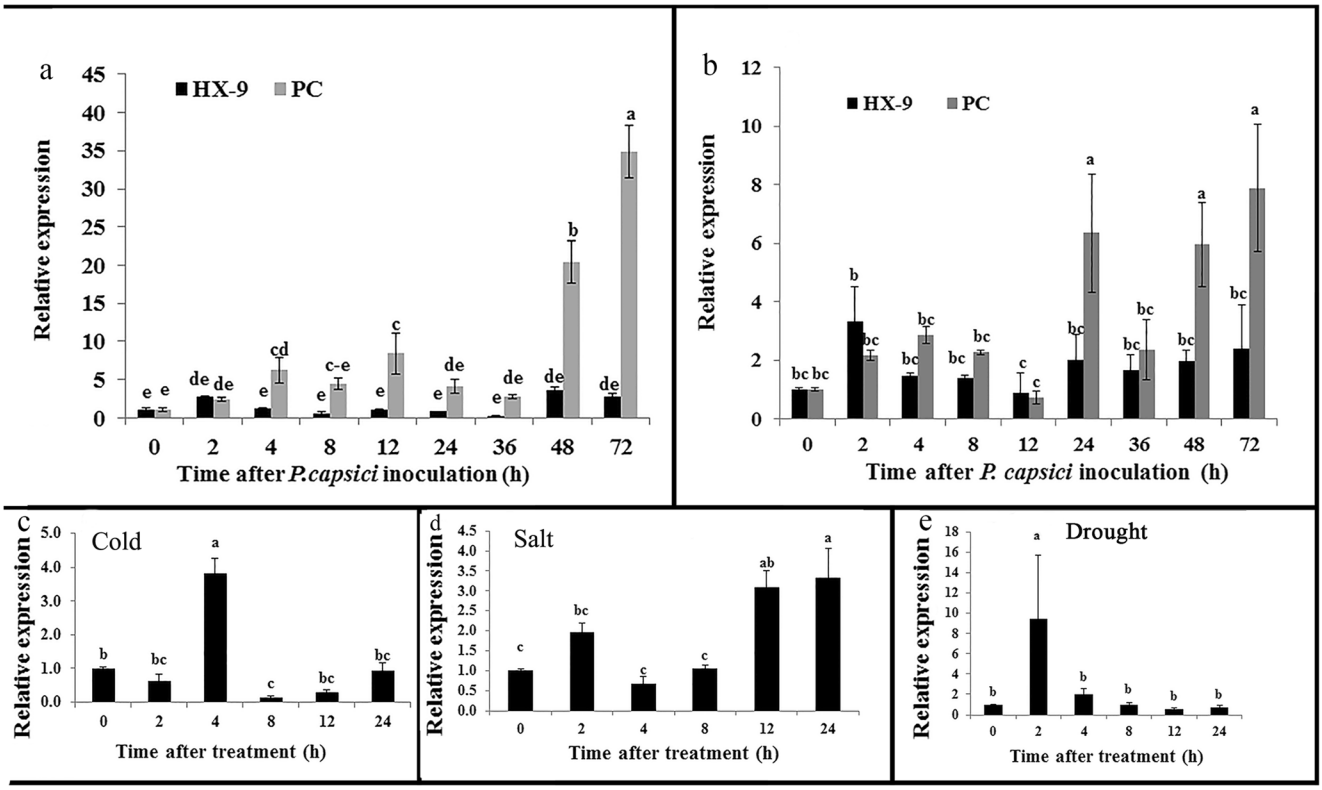

Fig. 4 Real-Time RT-PCR analysis of relative *CanTF* expression levels in pepper plants after *Phytophthora capsici* stress and abiotic stress. **a** *Phytophthora capsici* infection in roots. **b** *P. capsici* infection in leaves. **c** Cold stress. **d** Salt stress. **e** Drought stress

leaves of the *CanTF*-silenced plants (Fig. 7b) than on the control (TRV2:00) plant leaves (Fig. 7a). In addition, POD activity was measured in the leaves of control (TRV2:00) and silenced plants after HX-9 strain infection. The results indicated that POD activity was increased in both silenced and control plants, but the silenced plants had the lowest POD activity as compared to that of the control (TRV2:00).

After inoculate with the PC strain, the expression levels of *CanTF*, *CaPR1*, *CaDEF1*, and *CaSAR82* were elevated, but the increases in TRV2:00 plants were greater than those in *CanTF*-silenced plants. Significant increases in the expression of *CanTF*, *CaPR1*, and *CaDEF1* were detected at 24 h post-inoculation in the non-silenced plants, while no significant increases in expression were observed in silenced plants (Fig. 8a–d). Similarly, after the inoculation of the HX-9 strain, the expression levels of *CanTF* and defense-related genes (*CaPR1*, *CaPR1*, *CaDEF1*, and *CaSAR8*.2) were upregulated at 24 h post-inoculation (Fig. 8e–h). The upregulation was

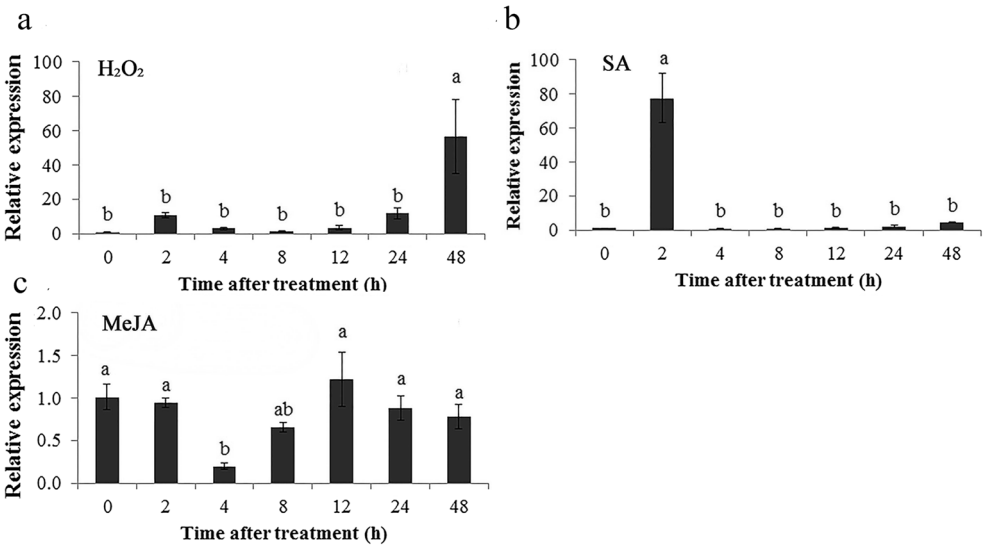

Fig. 5 Real-time RT-PCR analysis of relative *CanTF* expression levels in leaves of pepper plants after hormonal treatments. **a** H_2O_2 treatment. **b** SA treatment. **c** MeJA treatment. Results are presented as means ± SD of three independent biological replicates

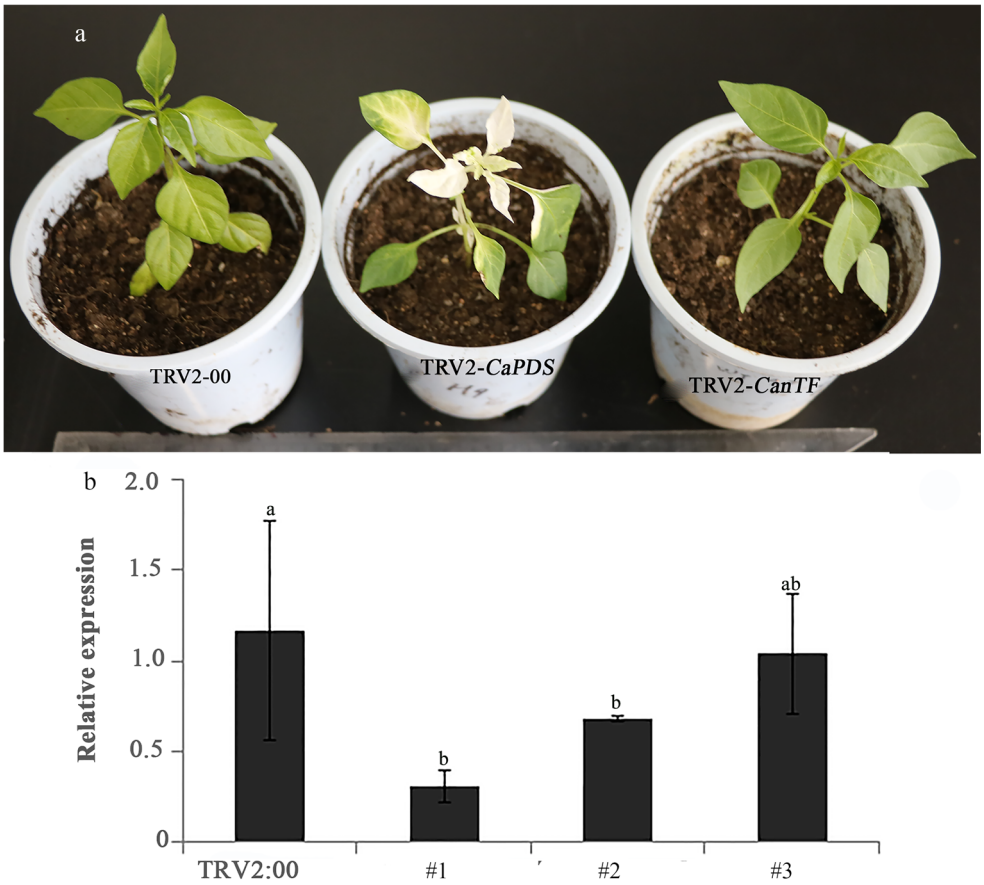

Fig. 6 Effect of *CanTF* gene silencing on pepper plants. Images were obtained 35 days after inoculation. **a** Phenotypes of gene-silenced pepper plants. Left: control plant (TRV2:00); middle: CaPDS-silenced plant (TRV2:*CaPDS*); right: *CanTF*-silenced plant (TRV2:*CanTF*). **b** Real-time RT-PCR analysis of *CanTF* expression levels in TRV2:00 line and TRV2:*CanTF* lines (#1, #2, and #3 indicate three biological levels of efficiency of *CanTF* gene silencing) 35 days after inoculation

significantly higher in non-silenced plants than in silenced plants. Furthermore, after *P. capsici* inoculation, we measured root activity in the silenced and control plants by the TTC method. Root activity in the silenced plants was lower than that in the control, and in the case of the HX-9 strain, a significant difference was observed at 3 and 7 days post-inoculation between silenced and control plants (Fig. 7d, e).

Silencing of *CanTF* Weakened Tolerance to Abiotic Stress

Silencing of *CanTF* resulted in more severely bleached leaf discs compared to those of empty vector-treated plants after exposure to salt stress for 4 days (Fig. 9a). The same results were obtained for POD activity (Fig. 9b). There was no significant difference in POD activity at 0 h between the TRV2:*CanTF* and TRV2:00 lines, but a significant difference was observed at 6 h, 24 h, and 48 h after salt stress treatment. Although POD activity in the leaves of *CanTF*-silenced plants was increased at 6 h, an obvious decrease was observed at 24 and 48 h. These results indicated that the silencing of *CanTF* results in poor plant defense under long-term salt stress.

Exposure of the TRV2:00 and TRV2:*CanTF* lines to cold stress resulted in increased POD activity at 24 h post-treatment compared with that at 0 h (Fig. 10a). However, the increases were less substantial for the TRV2:*CanTF* line than TRV2:00. The opposite trend was observed for electrolyte leakage. Leaf conductivity increased throughout the experimental period after cold stress treatment in the TRV2:*CanTF* line. Furthermore, cold stress increased electrolyte leakage more significantly in leaves of the TRV2:*CanTF* line than the TRV2:00 line (Fig. 10b).

Mannitol stress resulted in lower POD activity in *CanTF*-silenced plants at 24 and 48 h post-treatment than in TRV2:00 (Fig. 10c). Under drought stress, electrolyte leakage increased gradually and a significant difference was observed at 6 and 24 h post-treatment (Fig. 10d).

Discussion

In response to different biotic and abiotic stresses, plants have a finely regulated and intricate defense system (Choi and Hwang 2012). The roles of TFs and proteins in controlling different biological processes, e.g., responses to biotic and

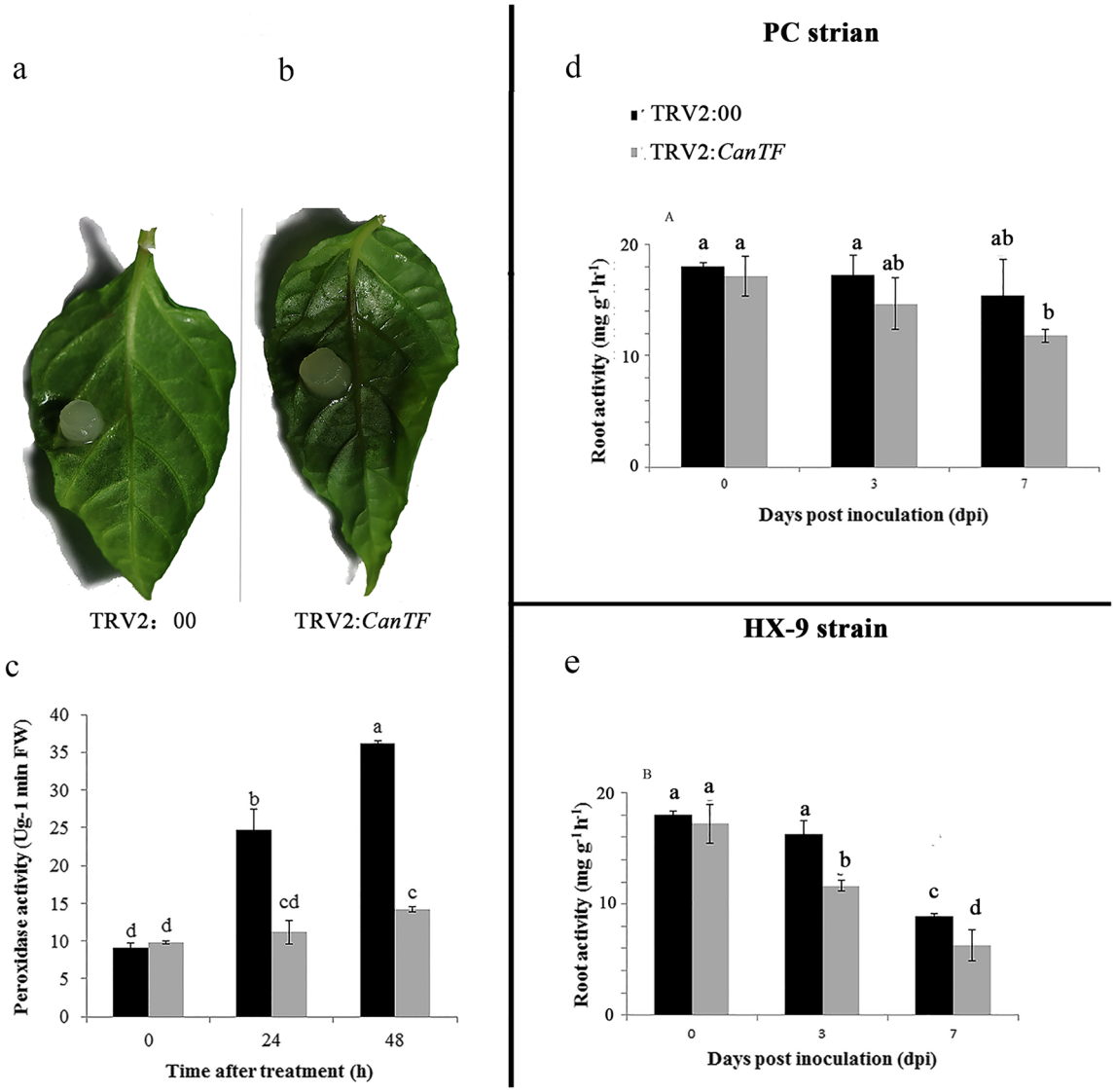

Fig. 7 Silencing of *CanTF* weakened the tolerance of pepper plants to *P. capsici* inoculation. **a**, **b** Disease symptoms in detached-leaves after inoculation with *P. capsici*. **a** Control plant (TRV2:00). **b** Silenced pepper plant (TRV2:*CanTF*). **c** Activity of POD after inoculation with *P. capsici*. **d** Root activity in *CanTF*-silenced and control plants after inoculation with the PC strain. **e** Root activity in *CanTF*-silenced and control plants after inoculation with the HX-9 strain

abiotic stresses, development, differentiation, metabolism, and defense, are clearly established (Ambawat et al. 2013). TFs can activate complex biological process (Fishburn et al. 2015) and can be affected by stress (Singh et al. 2002; Shoji and Hashimoto 2015). In higher plants, TF genes have been investigated in several species, including petunia, rice, cotton, maize, and *Arabidopsis* (Ambawat et al. 2013). In the current study, a new TF gene, *CanTF*, was isolated and identified from pepper. TFIIB-related genes are characterized by several intriguing features. The full sequence of *CanTF* in pepper cultivar A3 consisted of 1488 nucleotides. *CanTF* did not include a trans-membrane domain, demonstrating that the deduced protein does not contain a signal peptide. The putative amino acid sequence of CanTF showed high homology to other TFIIB proteins, such as those of *Solanum tuberosum* and *Nicotiana tomentosiformis*. A tissue-specific expression analysis showed that *CanTF* was expressed in all the tested tissues and was highly expressed in both the green and red fruits of pepper plants. In agreement with our results, previous studies have reported that AtTFB genes are widely expressed in different plant tissues, including vegetative nuclei, generative cells of pollen grains, pollen tubes, endosperm, and embryos. The results suggest that AtTFIIBs play important roles in the reproductive phase of a plant (Knutson 2013), and members of TFIIB exhibit sensitivity to oxidative stress (de Faria and Fernandes 2006).

CanTF showed early expression under cold, drought, and SA stresses as well as late expression under salt and H_2O_2.

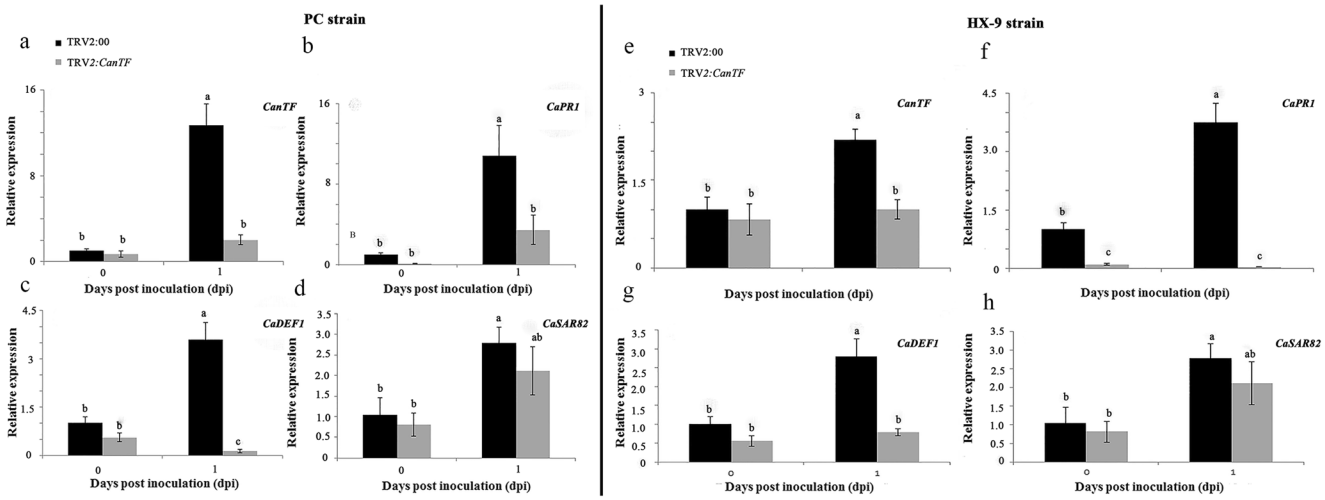

Fig. 8 Silencing of *CanTF* reduced the expression of defense-related genes (*CaPR1*, *CaDEF1*, and *CaSAR82*) in pepper leaves at 1 d post-inoculation with PC (**a**–**d**) and HX-9 (**e**–**h**) strains of *P. capsici*. Values are means ± SD from three independent experiments. Small letters represent significant differences ($p < 0.05$)

These results suggested that *CanTF* can be induced by abiotic stress and signaling molecules, and this may be related to the characteristics of the TFIIB family. Previous studies have reported that TFIIB genes have potential roles in controlling the cell cycle (Gibson et al. 1994), and the response of the cell cycle to stress is usually regulated in a time-dependent manner (Reichheld et al. 1999; Solé et al. 2015). Our results clearly indicate that *CanTF* expression increased in pepper plants exposed to abiotic stresses. Cold stress significantly increased *CanTF* gene expression at 4 h post-treatment, salt stress at 12 and 24 h, and drought stress at 2 h post-treatment. As a result of exposure to various abiotic stresses, the expression level of *CanTF* increased, suggesting that this gene is directly related to abiotic stress tolerance. Our results are in agreement with those of Denekamp and Smeekens (2003), who found that *AtMYB* in *Arabidopsis* is up-regulated by drought stress. Furthermore, *AtMYB2* was induced by drought and salt stresses, which indicates that *AtMYB2* is responsive to water deficiency at the transcriptional level (Abe et al. 2003). Yi et al. (2004) also revealed that the transcript level of *CaPF1* is stimulated by several treatments, including cold stress, ethephon, and methyl jasmonate.

Various plant hormones are important signaling molecules with a vital role in the resistance of plants to biotic and abiotic stresses (Choi and Hwang 2011). In the present study, SA and H_2O_2 signaling molecules were used to treat pepper plants, resulting in significantly increased *CanTF* expression. These results suggest that *CanTF* has a potential role in the hormonal response to biotic and abiotic stress. In *Arabidopsis*, various TFs are stimulated by salt and dehydration stress (Shinozaki et al. 1992). Moreover, Lim et al. (2015) have found that the *CabZIP2* gene is induced by defense-related hormones, such as SA and MeJA.

The results of the current study revealed that infecting pepper plants with two strains of *P. capsici* (HX-9 and PC) affected *CanTF* expression. These results suggested that *CanTF* is involved in pathogen defense. Our results are also in line with the findings of Yi et al. (2004). In *Arabidopsis*, MYB-TF genes play roles in the defense response against pathogen infection (Li et al. 2009). Furthermore, functional analyses of pathogen-induced TFs have revealed key roles in plant immunity (Reddy et al. 2011). In response to *X. campestris* pv. *vesicatoria* infection, *CabZIP2* transcripts accumulate more rapidly and strongly for the incompatible interaction compared with the compatible interaction (Lim et al. 2015). Thus, the difference in stress response by *CanTF*, a member of the TFIIB family, may be closely related to the cell cycle, which is affected by the TFIIB structure. Higher expression levels of *CanTF* were observed when pepper plants were exposed to *P. capsici*, especially the PC strain, suggesting that *CanTF* also enhances the pepper defense response against *P. capsici*. Generally, the PC strain post-inoculation exhibits higher expression levels of *CanTF* in roots than in leaves, while lower expression of *CanTF* was observed in both roots and leaves of the HX-9 strain post-inoculation.

Some genes in pepper showed low expression under PC strain inoculation than HX-9 strain inoculation (Jin et al. 2016; Zhang et al. 2016). However, in our study, *CanTF* showed the opposite response to *P. capsici* strain inoculation, i.e., higher expression of *CanTF* was observed under PC strain inoculation as compared to HX-9 strain inoculation. However, the mechanisms underlying the interaction between *CanTF* and the pepper defense response remain elusive. Thus, we speculate that *CanTF* responses to both biotic and abiotic stress may regulate the plant defense response. Therefore, we further investigated the function of *CanTF* by VIGS.

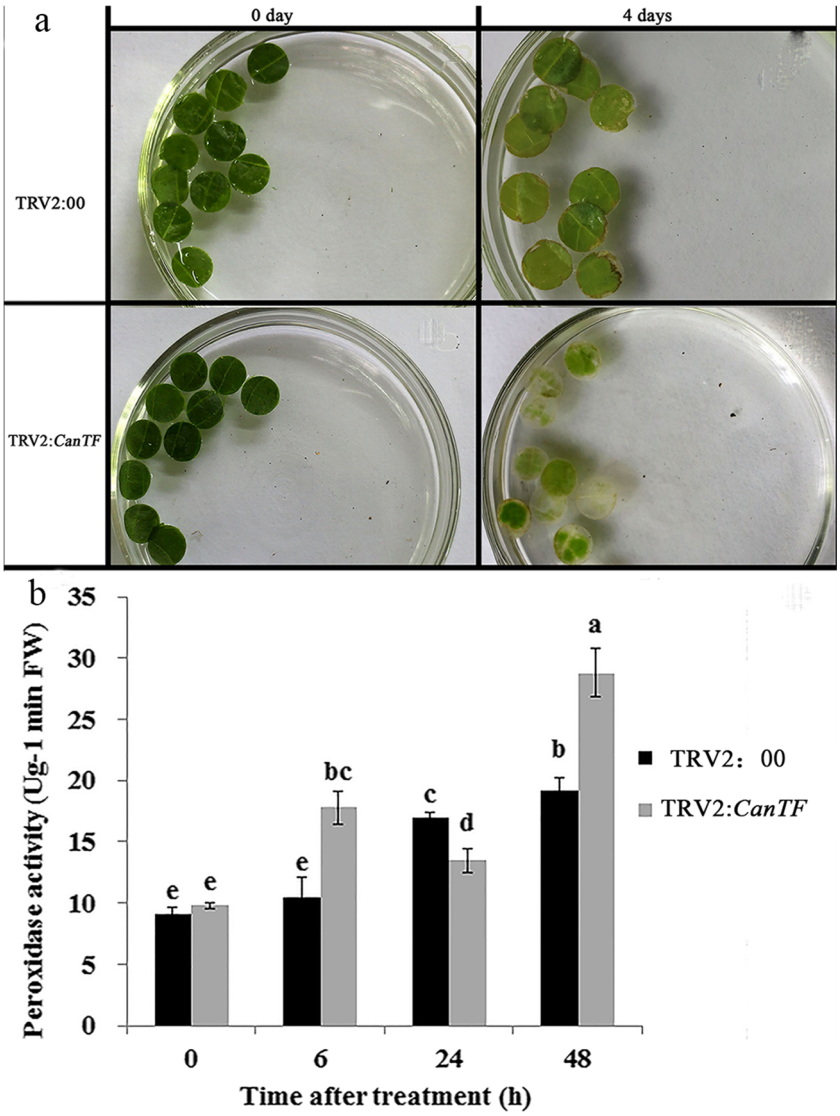

Fig. 9 Weakened tolerance of *CanTF*-silenced pepper plants to salt stress. **a** Phenotypes of the silenced and control plants after salt stress. Leaf discs from the gene-silenced pepper leaves and control plant leaves were floated in 400 mM NaCl solutions for 4 days at 26 °C with continuous fluorescent lighting. **b** Activity of POD after salt stress. Error bars represent SD for three biological replicates. Small letters represent significant differences ($p < 0.05$)

In the current study, photobleaching symptoms were observed on *CaPDS*-silenced plants leaves, indicating that the VIGS assay was successful. Silencing of *CanTF* revealed that the transcript levels of *CanTF* in silenced plants (TRV2:*CanTF*) were drastically reduced compared to the levels in control plants (TRV:00), indicating that *CanTF* was effectively silenced in pepper plants.

Furthermore, after inoculation with compatible and incompatible strains of *P. capsici*, the expression levels of defense-related *CaDEF1* (JA-dependent), *CaPR1* (SA-dependent), and *CaSAR82* (systemic acquired resistance) genes in the leaves of *CanTF*-silenced plants were lower than those in control plants. Additionally, root activity in the silenced plants was reduced as compared with that of the control plants, indicating its role in *P. capsici* resistance. Similarly, the silencing of *CaPTI1* also compromised the expression levels of *CaPR1*, *CaDEF1*, and *CaSAR82* as well as the root activity of silenced plants as compared to those in control plants (Jin et al. 2016). The transcript levels of defense-related *CaPR1* and *CaDEF1* were significantly lower in leaves of *R. solanacearum*-inoculated *CabZIP63*-silenced than in the control pepper plants (Shen et al. 2016). Another study supporting our results showed that the silencing of *CaPAL1* suppresses the expression of *CaPR1* in silenced plants as compared to control plants during *Xcv* infection (Kim and Hwang 2014). The silencing of *CaMLO2* significantly suppresses the induction of *CaDEF1* transcripts in the leaves of *CaMLO2*-silenced plants as compared to the control plants after inoculation with virulent *Xcv* Ds1 (EV) or avirulent *Xcv* Ds1 (*avrBsT*) (Kim et al. 2014). For infection with Xcv (especially the virulent strain),

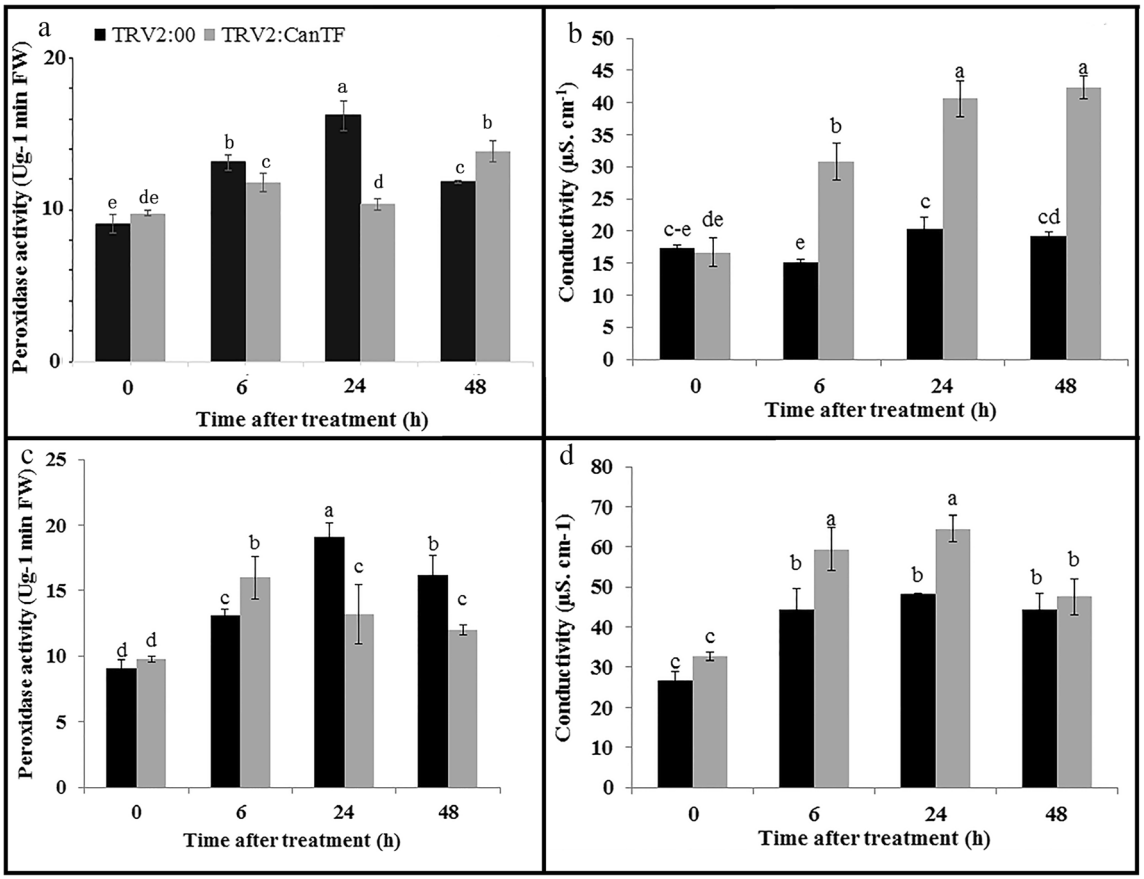

Fig. 10 Weakened tolerance of *CanTF*-silenced pepper plants to cold and drought stress. **a** Activity of POD after cold stress. **b** Conductivity of leaf discs after cold treatment. **c** Activity of POD after drought stress. **d** Conductivity of the leaf discs after drought stress. Error bars represent SD for three biological replicates. Small letters represent significant differences ($p < 0.05$)

CaPO$_2$-silenced plants exhibited significantly less induction of *CaDEF1* and *CaSAR82* (Choi et al. 2007). Yeom et al. (2011) used the cell vitality of the root as an indicator of the degree of injury in response to *P. capsici* infection and measured TTC reductase activity in the roots of pepper cultivars after *P. capsici* inoculation; they observed significant differences in root activity from 24 to 48 hpi, and concluded that the activity of CM334 is 2–3 times greater than that in Chilsungcho.

Disease symptoms were observed on leaves detached from the *CanTF*-silenced plants on the 4th day of *P. capsici* inoculation, while no disease symptoms were observed on the detached leaves of the control (TRV2:00) plants. This indicates that *CanTF* has an important role in the defense response to stress (Choi et al. 2007). In agreement with the present results, Wang et al. (2013b) also found that control plants were more resistant to *P. capsici* infection than TF-silenced plants. Our results are similar to those of Lim et al. (2015) who found that *CabZIP2*-silenced pepper plants are susceptible to infection by a virulent strain of pathogen. In addition, it was reported in the same study that TF genes are involve in the pepper plant defense response against different stresses. These results demonstrate that *CanTF* has an important role in the pepper defense response against different types of stresses.

Conclusion

A novel TF gene belonging to the TFIIB family, *CanTF*, was identified in pepper by a bio-informatics analysis, with a potential role in plant development. *CanTF* is induced by abiotic and biotic stresses and may be responsible for plant defense. The expression of *CanTF* in pepper organs, especially roots, exhibits high sensitivity to avirulent strains compared with virulent strains, while differences in responses to abiotic stresses may be explained by differences in the expression of *CanTF*. Early expression under cold, drought, and SA stresses and late expression under salt and H_2O_2 were observed in our study. VIGS technology showed that *CanTF* may positively regulate the responses to biotic and abiotic stresses, which indicates that *CanTF* plays a key role in plant defense. Our results provide important information regarding the regulatory

role of TFIIB genes for plant defense, and additional work should focus on the mechanisms underlying the interaction between the *CanTF* gene and environmental stresses.

Author Contributions YMH, DXL, and ZHG conceived the research. YMH, KKL, HXZ, AK, GXC, and XM performed the research. YMH, HXZ, and MHA performed statistical analyses. YMH and AK wrote the paper. YMH, MHA, AK, and ZHG revised the paper. DXL and ZHG provided the materials and resources for the research. YMH, KKL, and ZHG performed the integrity of the work. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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