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Molecular cloning and expression analysis of *SpWRKY6* gene from *Solanum pimpinellifolium*

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Abstract

Ttranscription factors WRKY play vital roles in response to biotic and abiotic stresses, and previous studies have predominantly focused on model plants and fairly limited research has been performed with tomato. In the present study, a novel pathogen-induced *WRKY* gene named *SpWRKY6* was isolated from the late blight resistant tomato (*Solanum pimpinellifolium*) cultivar L3708 using *in silico* cloning and reverse transcription polymerase chain reaction (RT-PCR) methods. Multiple sequence alignment with other plant *WRKYs* indicates that *SpWRKY6* contains two WRKY domains and belongs to group I WRKY transcription factors. Furthermore, some *cis*-acting elements associated with responses to environmental stresses were observed in the promoter region of this gene. Gene expression patterns were determined by analyzing microarray data of *SpWRKY6* in tomato and of an orthologous gene from *Arabidopsis thaliana* using the *Genevestigator* tool. The results reveal a very strong biotic and abiotic stress responsive behaviour of this gene. Moreover, bioinformatics results were confirmed by real time quantitative polymerase chain reaction and show that *SpWRKY6* expression was rapidly induced after infection with *Phytophthora infestans* and *Botrytis cinerea*, respectively. Expression of *SpWRKY6* was up-regulated by application of various phytohormones including salicylic acid, methyl jasmonate, and abscisic acid. Likewise, the *SpWRKY6* expression was induced by NaCl, drought, heat, cold, and $HgCl_2$ treatments.

Additional key words: abscisic acid, *Botrytis cinerea,* cold, drought, heat, methyl jasmonate, *Phytophthora infestans*, tomato, salicylic acid, salinity, WRKY transcription factors.

Introduction

Plants are exposed to various biotic and abiotic stresses (Fujita *et al*. 2006). Late blight caused by the oomycete pathogen *Phytophthora infestans* causes a major threat to tomato production in cool and wet environments (Chen *et al*. 2014). The predominant method to control for late blight is through frequent use of fungicides (Evers *et al*. 2006), which is costly and has a negative impact on human health and environment. Therefore, many efforts have been focused on biological control.

Plants have developed intricate mechanisms to perceive and respond to several external signals *via* different signaling pathways (Vlot *et al*. 2008). This regulation is predominantly achieved by various genes, including *WRKY* genes, which encode a large family of regulatory proteins (Eulgem and Somssich 2007). The first WRKY transcription factor (TF) was identified in sweet potato and since then, WRKY TFs have been identified in a large number of higher plants (Rushton *et al*. 2010). They are defined mainly by highly conserved 60 amino acids comprising a highly conserved WRKY motif at the N-terminus and a zinc-finger motif at the C-terminus. According to the differences in the conservative amino acid sequences and the compositions of the zinc finger motifs, the WRKY TFs family can be divided into three groups. Group I WRKY TFs contains two typical WRKY domains: WRKYGQK and the type C_2H_2

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Abbreviations: ABA - abscisic acid; ABRE - ABA responsive element; EIRE - elicitor-responsive element; ETH - ethephon; hpi - hours post-inoculation; hpt - hours post-treatment; MeJA - methyl jasmonate; MRE - MYB binding site; NLS - nuclear localization signal; ORF - open reading frame; PDA - potato dextrose agar; PEG - polyethyleneglycol; qPCR - quantitative polymerase chain reaction; RT-PCR - reverse transcriptase polymerase chain reaction; SA - salicylic acid; TF - transcription factor; WT - wild-type.

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of zinc-finger motif C-X_{4-5}-C-X_{22-23}-H-X-H. Group II WRKY TFs possesses one typical WRKY domain and the same zinc-finger motif as group I. This type can be further divided into subgroups a - e based on the primary amino acid sequence. Group III WRKY TFs harbors a single WRKY domain and an altered C_2HC zinc finger motif C-X_7-C-X_{23}-H-X-C. The WRKY domain WRKYGQK can bind to the W-box motif containing a consensus sequence TTGAC (T/C) that presents in the promoter region of many defense-related genes and WRKY TFs themselves (Ciolkowski *et al*. 2008).

The function of several WRKY TFs has been gradually clarified using genetic and molecular biology methods. An increasing number of evidence have showed that they participate in plant stress responses (Li and Luan 2014). For example, *GhWRKY39* and *GhWRKY39-1* expressions are induced by salt treatment as well as overexpression of these genes in tobacco improves tolerance to salt stress (Shi *et al*. 2014a,b). Besides abiotic stress responses, WRKY TFs are also involved in plant defense responses. Pathogen infection and phytohormone treatments can rapidly induce their expression in various plant species. In *Capsicum annuum*, *CaWRKY27* and *CaWRKY40* expressions are induced after infection with *Ralstonia solanacearum* as well as by salicylic acid (SA), methyl jasmonate (MeJA), and ethephon (ETH) treatments. Moreover, overexpression of these genes in tobacco increases resistance to *R. solanacearum* compared with wild-type (WT) plants (Dang *et al*. 2013, 2014). Likewise in *Gossypium hirsutum*, *GhWRKY40*, *GhWRKY39*, *GhWRKY39-1*, *GhWRKY15*, and *GhWRKY3* expressions are induced after treatment with SA and jasmonic acid (JA), which are signaling molecules involved in plant defense response signaling pathways (Guo *et al*. 2011, Yu *et al*. 2012, Shi *et al*. 2014a,b, Wang *et al*. 2014). In *Arabidopsis thaliana*, *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* expressions are induced after infection with *Pseudomonas syringae* and *Botrytis cinerea* as well as by SA treatment (Dong *et al*. 2003, Xu *et al*. 2006). Likewise, *AtWRKY33* is also involved in SA signaling pathways and ethylen-JA mediated cross-communication (Birkenbihl *et al*. 2012). Huang *et al*. (2012) identified 81 SlWRKY TFs, but only a few WRKY TFs from tomato have been isolated, and their functions have been studied. It was reported that *SlWRKY70* and *SlWRKY72* functions in defense against aphid and nematodes, and *SlWRKY72* against *P. syringae* (Bhattarai *et al*. 2010, Atamian *et al*. 2012). Overexpression of *SlWRKY* in tobacco exhibits improved tolerance to abiotic stresses and increased expression of defense-related genes (Li *et al*. 2012). Recently, overexpression of *SpWRKY1* in tobacco enhances resistance to *Phytophthora nicotianae* as well as tolerance to salt and drought stresses (Li *et al*. 2015a). Moreover, overexpression of *SpWRKY1* in tomato also enhances resistance to *P. infestans* as well as tolerance to salt and drought stresses (Li *et al*. 2015b). Silencing a *B. cinerea*-responsive *WRKY* gene named *SlDRW1* results in an increased severity of disease and a decreased tolerance to oxidative stress (Liu *et al*. 2014). Likewise, overexpression of *SlWRKY39* in tomato shows an enhanced resistance to *P. syringae* as well as tolerance to salt and drought stresses (Sun *et al*. 2015). However, the biological function of the majority of tomato WRKY TFs remains unknown.

Bioinformatics tools have been successfully used for research of WRKY TFs (Eulgem *et al*. 2000). Recently, *Genevestigator* offered an online platform to access large and well-annotated datasets of curated microarray samples testing thousands of genes (Zimmermann *et al*. 2004, Hruz *et al*. 2008). For instance, the expression patterns of *TaWRKY68* were determined by analyzing microarray data in wheat and of orthologous genes from maize, rice, and barley using the *Genevestigator* tool (Ding *et al*. 2014).

Solanum pimpinellifolium (L.) Mill. cv. L3708 has been identified as special source of late blight resistance in tomato (Kim and Mutschler 2005). In the present study, we isolated a *WRKY* gene named *SpWRKY6* during the incompatible interaction between *S. pimpinellifolium* and *P. infestans*. The expression profiles of *SpWRKY6* and its homolog were analyzed under a broad range of treatments using the *Genevestigator* tool. Real time quantitative polymerase chain reaction (qPCR) was then carried out to validate the results. This study will provide a key clue in understanding the role of *SpWRKY6* in tomato.

Materials and methods

Cloning *SpWRKY6* sequence: The cloning method was performed as described previously (Li and Luan 2014). To verify the assembled sequence, specific primers (forward primer: 5′-ATGGGTGGATTTGATGAT CATG-3′ and reverse primer: 5′-TTACATCTGAGGTC CAAGCGA-3′) were used to amplify the open reading frame (ORF) region. The template was a mixture of the first strand cDNA reverse transcribed from the total RNA in the incompatible interaction. The PCR product was purified, cloned into a pMD18-T vector (*TaKaRa*, Dalian, China) and sequenced (*Sangon*, Shanghai, China). We obtained cDNA which was initially termed *SpWRKY6* and actually corresponds to *SlWRKY2* (Solyc07g 066220.2.1) of Huang *et al*. (2012) classification.

Bioinformatics analysis: Several bioinformatics analyses were performed as described previously (Li and Luan 2014). In brief, physico-chemical properties were

determined using the *ProtParam* tool (http://web.expasy.org/protparam/). Multiple sequence alignment and a phylogenetic tree were carried out by the *DNAMAN* and *Mega 4.0* software. Transmembrane domain and signal peptide predictions were analyzed using the *TMHMM* procedure (http://www.cbs.dtu. dk/services/TMHMM-2.0/) and the *SignalP* procedure (http://www.cbs.dtu.dk/services/SignalP/). Subcellular localization was predicted using the *Euk-mPLoc* program (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) and the *NLS mapper* program (http://nls-mapper.iab.keio.ac.jp). Putative *cis*-acting responsive elements present in the *SpWRKY6* promoter region were indentified with the *PlantCARE* software (bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Affymetrix probe identities (*IDs*) corresponding to the homologous gene from *Arabidopsis* were identified using the *PLEXdb Blast* tool. The expression profiles were analyzed by submitting the probe *IDs* to the corresponding organism databases in the *Genevestigator* online search engine. Gene function under different conditions can be showed through this online bioinformatics tools.

Experimental validation of bioinformatics analyses: *Solanum pimpinellifolium* (L.) Mill. cv. L3708 seeds were sown into plastic pots filled with soil and placed in a greenhouse under a temperature of 25 ± 3 °C, a 16-h photoperiod, an irradiance of 120 μmol m^{-2} s^{-1}, and a relative humidity of 50 %. Three-week-old seedlings were transferred into triangular flasks containing an aerated quarter-strength Hoagland nutrient solution and grown until they reached the five-leaf stage. For tissue-specific expression analysis, roots, stems, and leaves were collected separately.

Phytophthora infestans was grown on an oatmeal medium and kept in darkness at 20 °C. *Botrytis cinerea* was also incubated at 20 °C on a potato dextrose agar medium. Collection of sporangia and release of zoospores from *P. infestans* and *B. cinerea* were applied according to previous methods of Xiang and Judelson (2014) and Maruyama *et al*. (2013), respectively. Then, the seedlings of *S. pimpinellifolium* at the five-leaf stage were infected with *P. infestans* and *B. cinerea*, respectively. Briefly, 2-cm^3 aliquots of a pathogen suspension (1×10^6 conidia cm^{-3}) was applied to tomato leaves with a hand held sprayer until run off. The infected plants were maintained in the dark at a relative high humidity of about 85 % for 24 h and then moved to a growth room set at 23 °C. Leaf samples were harvested at 0, 3, 12, 24, 36, 48, 72, and 96 h post inoculation (hpi), immediately frozen in liquid nitrogen, and stored at -80 °C. Phytohormones were applied according to Yang *et al*. (2009) by spraying 2 mM SA, 100 μM MeJA, and 100 μM abscisic acid (ABA). Salt and drought treatments were applied according to Ma *et al*. (2015) with some modifications: the seedlings were transferred to the quarter-strength Hoagland nutrient solution containing 200 mM NaCl or 15 % (v/v) polyethylene glycol 6000. Heat and cold treatments were applied according to Dang *et al*. (2013) and Ma *et al*. (2015), respectively. The plants were exposed to 42 or 4 °C. Treatment with $HgCl_2$ was applied according to Yin *et al*. (2015) and the seedlings were transferred to the quarter-strength Hoagland nutrient solution containing 50 μM $HgCl_2$. Other conditions were as mentioned above. Leaves were harvested at 0, 2, 4, 8, 12, and 24 h post treatment (hpt), immediately frozen in liquid nitrogen, and stored at -80 °C. Experiments were repeated three times.

Tissue-specific expressions were determined using real time qPCR with the following primers; forward primer: 5′-ATATACAAGGGAGCCCACAATC-3′ and reverse primer: 5′-CTGACCGAGATCACCATTAACA-3′ with a *Rotor-Gene 3000* PCR instrument (*Corbett Research*, Australia) using a *SYBR Premix Ex Taq*™ kit (*TaKaRa*) under the following conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The tomato *actin* gene was amplified as internal control using the following primers; forward: 5′-ACCTTCAAC GTTCCAGCTATG-3′ and reverse: 5′-TCACCAGAG TCCAACACAATAC-3′. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Data acquisition and analysis were performed using the *Rotor-gene 6* software. The above primer sequences were obtained through the website http://www.idtdna.com/primerquest/Home/Index. Experiments were repeated three times.

Statistical analysis: Values in figures are means of experiments conducted in triplicate and standard deviations (SDs) of means. Analysis of variance (*one-way ANOVA*) and multiple comparisons of differences were performed using Duncan's multiple range test ($\alpha = 0.05$; *SPSS v. 17.0* software).

Results

By using the *in silico* cloning method, we successfully harvested a *WRKY* gene named *SpWRKY6*; it had an ORF of 2 220 bp and encoded 739 amino acids. The estimated molecular mass was 182.98 kDa and the isoelectric point was 4.90. The predicted instability index was 42.51 and thus it was an unstable protein. The protein average hydropathicity was 0.811 suggesting that SpWRKY6 was a hydrophobic protein. The trans-membrane prediction with the *TMHMM* shows that the inferred amino acid sequence ofSpWRKY6 did not harbor any trans-membrane region. The *SignaIP* analysis shows that the protein did not possess any signal peptide. The

subcellular localization analysis predicts that SpWRKY6 might exist in the nucleus and its nuclear targeting was due to the presence of a nuclear localization signal at amino acids 495 - 507.

The multiple sequence alignment of SpWRKY6 with various plants was performed using the *DNAMAN* software. As shown in Fig. 1, consistent with other plant WRKYs, the SpWRKY6 protein contained two WRKY domains and C_2H_2-type zinc finger motifs. Therefore, it was classified as group I WRKY TFs. Nine available group I WRKY protein sequences from various species were used to establish a neighbor-joining phylogenetic tree. Previous studies showed that *NtWRKY4* plays a role in disease resistance (Ren *et al*. 2010). SpWRKY6 and NtWRKY4, which showed a very high similarity, were assembled into the same cluster (Fig. 2). Thus, we speculate that *SpWRKY6* might play an important role in response to pathogen infection.

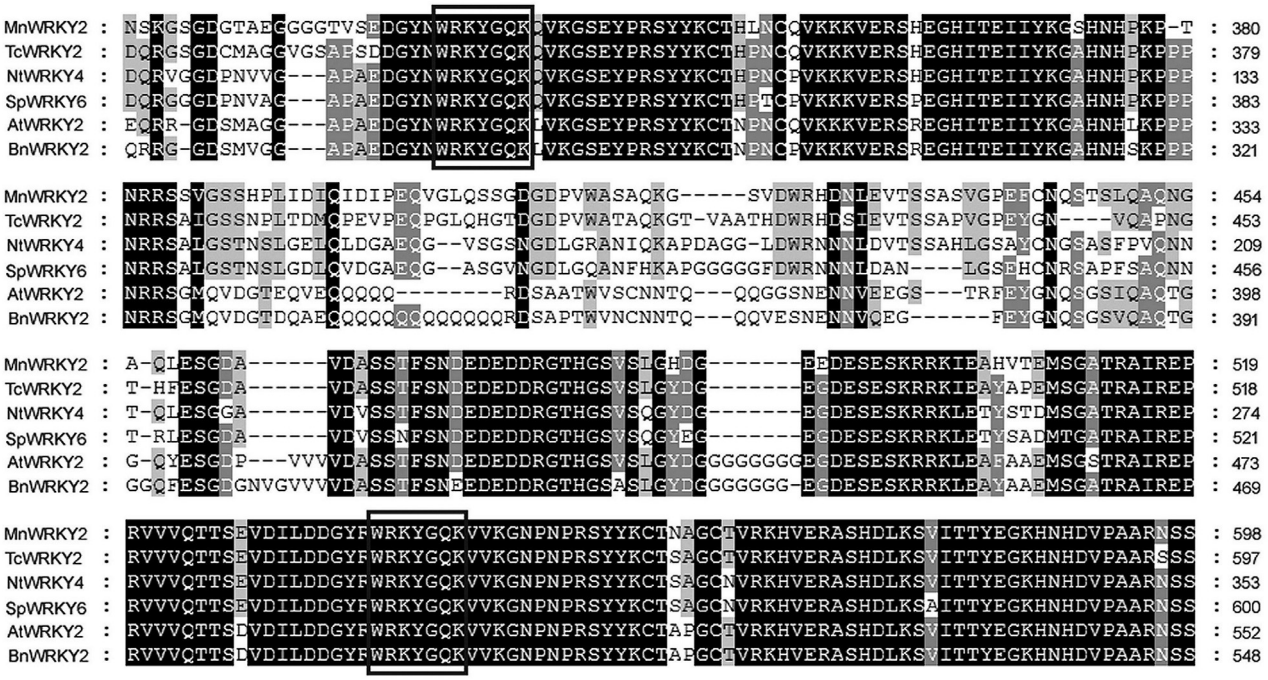

Fig. 1. The sequence analysis of *SpWRKY6*. Amino acid sequence alignment between SpWRKY6 and WRKYs from *Nicotiana tabacum* (acc. No. BAA86031.1), *Morus notabilis* (acc. No. XP_010092241.1), *Theobroma cacao* (acc. No. XP_007020303.1), *Arabidopsis thaliana* (acc. No. NP_200438.1), and *Brassica napus* (acc. No. ACQ76801.1). The alignment was performed using the *DNAMAN* software. Identical amino acids are *shaded in black* and a highly conserved amino acid sequence WRKYGQK is *boxed*.

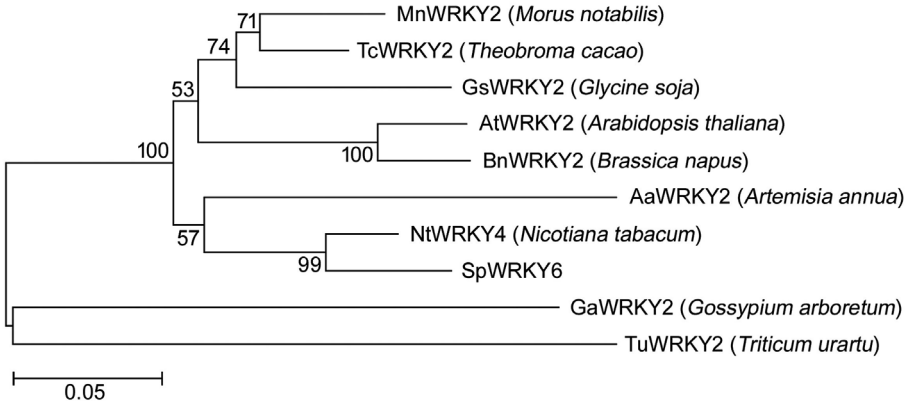

Fig. 2. The phylogenetic analysis of deduced amino acid sequences of SpWRKY6 and other WRKYs from various plants. The phylogenetic tree was generated by the neighbor-joining method using the *MEGA 4.0* software. The amino acid sequences used to build up the phylogenic tree were as follow: *Morus notabilis* (acc. No. XP_010092241.1), *Theobroma cacao* (XP_007020303.1), *Brassica napus* (ACQ76801.1), *Arabidopsis thaliana* (NP_200438.1), *Nicotiana tabacum* (BAA86031.1), *Glycine soja* (KHN45670.1), *Gossypium arboretum* (KHG07831.1), *Triticum urartu* (EMS67202.1), and *Artemisia annua* (AGR40498.1).

Table 1. Putative *cis*-acting regulatory elements identified in *SpWRKY6*. ABRE - abscisic acid responsive element; EIRE - elicitor responsive element; Skn - endosperm development responsive element; TC-rich repeats - defense and stress responsive elements; TGA - auxin responsive element.

Cis-elements		Position	Sequence (5′- 3′)
Elicitor-related elements	EIRE	-71 (+), -199 (+)	TTCGACC
	TC-rich repeats	-727 (-)	ATTTTCTTCA
	TGA	-32 (+), -1450 (+), -1278 (-)	AACGAC
	ABRE	-354 (+), -1187 (+), -1024 (+)	CACGTG/ACGTGGC
	CGTCA-motif	-189 (+), -898 (+), -839 (-)	CGTCA
	TGACG-motif	-189 (-), -898 (-), -839 (+)	TGACG
Development-related elements	CCGTCC-box	-371 (-)	CCGTCC
	Skn-1-motif	-190 (+), -838 (-), -238 (-), -1128 (+)	GTCAT

Table 2. The signal strength analysis of *SpWRKY6/AtWRKY2* arrays in the tomato/*Arabidopsis thaliana* genome under different conditions.

Infection or treatment	Tissue	Log(2)-ratio	Fold-change	*P*-value
P. infestans	leaf/leaf	-0.06/0.21	-1.04/1.16	0.570/0.030
B. cinerea	fruit/leaf	0.10/0.32	1.07/1.28	0.280/0.070
Salt	leaf/leaf	0.18/0.27	1.14/1.21	0.243/0.100
Drought	leaf/leaf	0.92/1.26	1.89/2.38	0.001/0.001
Heat	leaf/leaf	-0.21/0.14	-1.16/1.10	0.005/0.546

Several responsive elements were found in the promoter region such as elicitor responsive element, defense and stress responsive element, TC-rich repeats, and MYB binding site involved in light responsive element. Furthermore, some other regulatory elements associated with hormone responsiveness were also detected such as auxin responsive element, ABA responsive element, and JA-responsive (TGACG and AACCTAA) motifs. In addition, *cis*-acting regulatory elements associated with development also existed in this region (Table 1). These data imply that *SpWRKY6* might play a role in response to several biotic and abiotic stresses as well as in plant development.

The *Genevestigator* tool collects high quality gene expression data from tomato and *Arabidopsis*. The sequence obtained from the phylogenetic analysis was used to query the *Affymetrix* probe set database and the probes were identified as follows: Les.3512.1.S1_at (SpWRKY6) and 248008_at (AtWRKY2). To clarify the role of *SpWRKY6* in response to environmental stimuli, we analyzed gene expression profiles in *Arabidopsis* under different conditions using the *Genevestigator* tool. The results show that these genes might be responsive to biotic (*P. infestans* and *B. cinerea*) and abiotic (salt, drought, and heat) stresses (Table 2).

We then performed the real time qPCR assay to validate the results from the *Genevestigator* analysis. It was found that *SpWRKY6* was expressed in different tissues (Fig. 3). The highest expression appeared in tomato leaves and the lowest in stems. To further

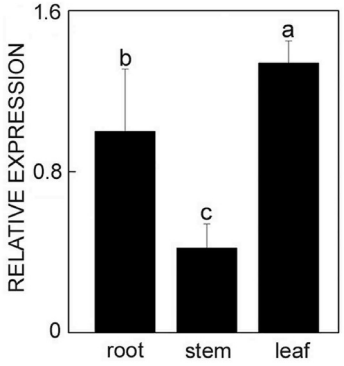

Fig. 3. The expression of *SpWRKY6* in tomato roots, stems, and leaves. The expression in roots was set as one. Means ± SDs of three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

understand whether the *SpWRKY6* was induced in the pathogen attack response, *P. infestans* and *B. cinerea* were inoculated to tomato seedlings. *SpWRKY6* exhibited similar expression patterns, reaching a peak at 24 h with 5.8-fold and 5.6-fold increases when infected with *P. infestans* and *B. cinerea*, respectively (Fig. 4). These data suggest that *SpWRKY6* might play a role in regulating plant pathogen disease responses.

To investigate the effects of phytohormones on *SpWRKY6* expression, three phytohormones, SA, ABA, and MeJA were used to treat the tomato leaves, and the expression patterns of *SpWRKY6* were examined. For SA

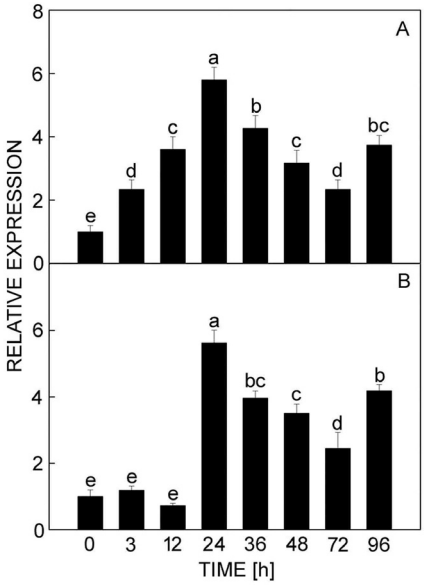

Fig. 4. Expression profiles of *SpWRKY6* in response to *P. infestans* (*A*) and *B. cinerea* (*B*) infection. The leaves were collected at 0, 3, 12, 24, 36, 48, 72, 96 hpi. The tomato *actin* gene was used as internal control. The expression of the control treatment (0 h) was set as one. Means ± SDs, $n = 3$. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Discussion

The WRKY TFs are one of the largest superfamily of regulatory proteins in plants. Recently, an increased attention has been focused on WRKY TFs as they are involved in the regulation of plant responses to various biotic and abiotic stresses (Rushton *et al*. 2010). However, functional analysis of WRKY TFs has been mostly focused on model plants, and little progress has been made toward understanding the function of WRKY TFs in tomato (Bhattarai *et al*. 2010, Atamian *et al*. 2012, Li *et al*. 2012, 2015a,b, Liu *et al*. 2014, Sun *et al*. 2015).

In the present study, a novel pathogen-induced *WRKY* gene named *SpWRKY6* was isolated from tomato. The deduced SpWRKY6 protein contained two typical WRKY domains, and two zinc finger motifs show that it belongs to group I WRKY TFs. The two WRKY domains of group I members play distinct functions: the C-terminal WRKY domain is a predominant DNA-binding domain, whereas the N-terminal WRKY domain may take part in binding process and enhance the DNA binding to their target genes (Eulgem *et al*. 2000). The subcellular localization analysis predicts that *SpWRKY6* may exist in the nucleus.

The promoter and *Genevestigator* analysis show that *SpWRKY6* might be affected by several biotic stresses. So the expression patterns of *SpWRKY6* were examined treatment, the *SpWRKY6* expression was more rapidly induced at 8 h and reached a maximum at 24 h with a 4-fold increase (Fig. 5*A*). For MeJA treatment, the expression of *SpWRKY6* increased at 4 h and also reached a peak at 24 h with a 2.6-fold increase (Fig. 5*B*). For ABA treatment, the *SpWRKY6* expression was rapidly induced and already after 2 h it reached a maximum with a 4-fold increase (Fig. 6*A*). These results imply that *SpWRKY6* might be involved in complex signaling pathways and might play an important role in plant defense responses mediated by phytohormones.

To examine expression patterns of *SpWRKY6* under abiotic stresses, the tomato seedlings were exposed to salt, drought, heat, cold, and $HgCl_2$. For salt treatment, the *SpWRKY6* expression was rapidly induced at 2 h and reached a maximum at 12 h with a 3.52-fold increase (Fig. 6*B*). For drought treatment, the *SpWRKY6* expression was induced at 2 h, and then reached its highest level at 4 h with a 5.99-fold increase (Fig. 6*C*). For heat treatment, the *SpWRKY6* expression reached a maximum at 8 h with a 5.76-fold increase (Fig. 6*D*). For cold treatment, the *SpWRKY6* expression also reached a maximum at 8 h with a 4.76-fold increase (Fig. 6*E*). Moreover, the *SpWRKY6* expression was up-regulated all the time after the $HgCl_2$ treatment (Fig. 6*F*). Our studies suggest that *SpWRKY6* might be involved in various abiotic stress responses.

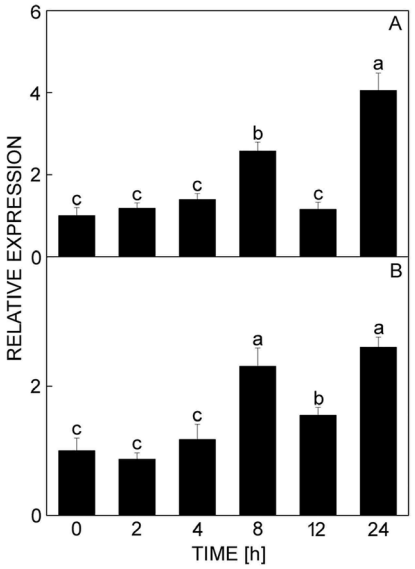

Fig. 5. Expression profiles of *SpWRKY6* in response to 2 mM SA (*A*) and 100 μM MeJA (*B*) treatments. The leaves were collected at 0, 2, 4, 8, 12, and 24 hpt. The tomato *actin* gene was used as internal control. The expression of the control treatment (0 h) was set as one. Means ± SDs, $n = 3$. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

in tomato after infection with a biotrophic pathogen *P. infestans* and a necrotrophic pathogen *B. cinerea*. As result, the expression of *SpWRKY6* was induced significantly. Previous studies on WRKY TFs have displayed their roles in both SA- and JA-mediated signaling pathways. Generally, SA-dependent defenses are often triggered by biotrophic pathogens, whereas JA-dependent plant defenses are generally activated by necrotrophic pathogens (Mur *et al*. 2006). For instance, *AtWRKY3* and *AtWRKY4* play a positive role in JA-mediated resistance to necrotrophic pathogens, but they play a negative role in SA-mediated resistance to biotrophic pathogens (Lai *et al*. 2008). In this study, the expression patterns of *SpWRKY6* were also examined after the treatment with SA and MeJA. The results show that the *SpWRKY6* expression was induced significantly. We speculate that *SpWRKY6* might be involved in SA and JA signaling pathways.

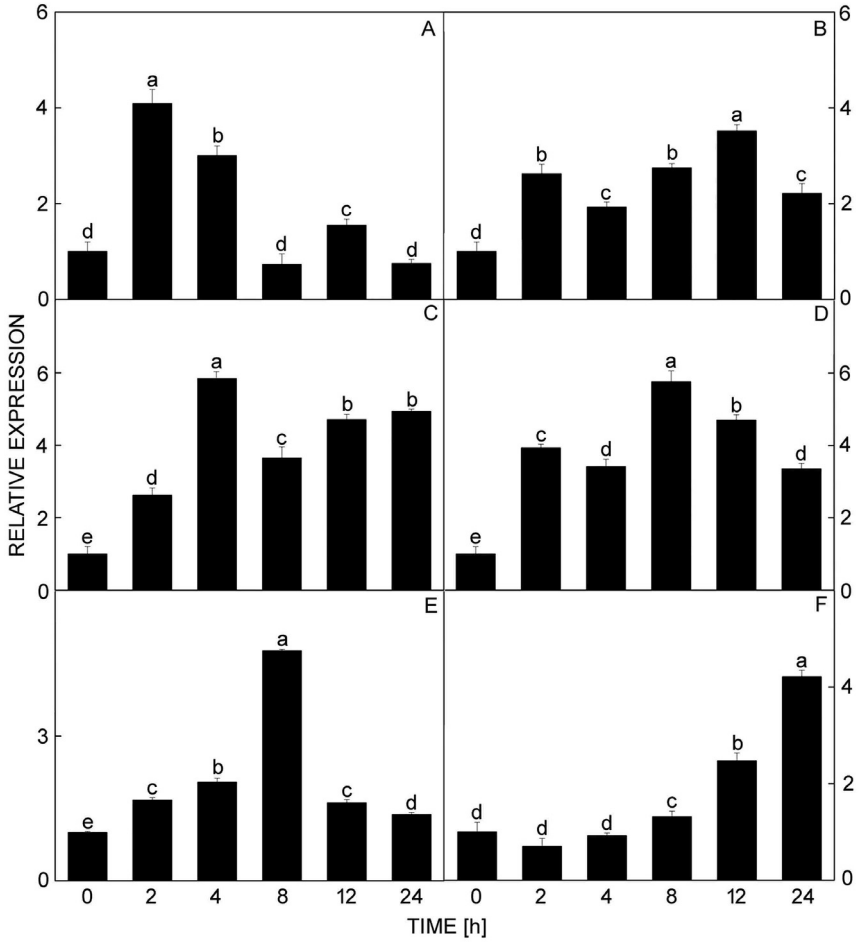

Fig. 6. Expression profiles of *SpWRKY6* in response to 100 μM abscisic acid (*A*), 200 mM NaCl (*B*), 15 % (v/v) polyethylene glycol (*C*), heat stress of 42 °C (*D*), cold stress of 4 °C (*E*), and 50 μM $HgCl_2$ (*F*) treatments. The leaves were collected at 0, 2, 4, 8, 12, and 24 hpt. The tomato *actin* gene was used as internal control. The expression of the control treatment (0 h) was set as one. Means ± SDs, $n = 3$. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

The expression of *SpWRKY6* was also induced by the ABA treatment. Moreover, the ABA responsive elements also existed in the promoter region. Generally, ABA is regarded to play an important role in regulating plant abiotic stress responses (Chinnusamy *et al*. 2004). There are many evidences indicating that ABA also plays vital roles in plant defense against pathogens (Chen *et al*. 2013, Sun *et al*. 2014). According to our results, we speculate that *SpWRKY6* might be involved in ABA-mediated signaling pathways that regulate plant defense against pathogens. So far, there is limited evidence about WRKY TFs involved in ABA-mediated defense responses (Chen *et al*. 2013). Therefore, *SpWRKY6* involved in defense against pathogens through ABA-mediated signaling pathways will be further examined. According to our results, the expression of *SpWRKY6* was induced by the SA, MeJA, and ABA treatments. We speculate that *SpWRKY6* might play an important role as integrator among SA-, JA-, and ABA-mediated signaling pathways.

An increasing body of evidence suggests that WRKY TFs participate in plant defense responses as well as in

plant development. For example, *GhWRKY15* is involved in disease resistance and plant development (Yu *et al*. 2012). Similarly, *AtWRKY70* affects defense signaling pathways and plant senescence (Ülker *et al*. 2007). In this study, *cis*-acting regulatory elements associated with development were found in the promoter region. We speculate that *SpWRKY6* might participate in plant defense responses as well as plant growth or development, and further experiments may verify this possibility.

In conclusion, *SpWRKY6* may be one of the key regulatory genes involved in tomato plant defense and stress responses. Thus, the biological function of *SpWRKY6* in the corresponding signaling pathways in tomato needs to be further investigated using over-expressing and silencing methods.

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