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Molecular cloning and expression analysis of jasmonic acid dependent but salicylic acid independent *LeWRKY1*

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ABSTRACT. Various plant genes can be activated or inhibited by phytohormones under conditions of biotic and abiotic stress, especially in response to jasmonic acid (JA) and salicylic acid (SA). Interactions between JA and SA may be synergistic or antagonistic, depending on the stress condition. In this study, we cloned a full-length cDNA (*LeWRKY1*, GenBank accession No. FJ654265) from *Lycopersicon esculentum* by rapid amplification of cDNA ends. Sequence analysis showed that this gene is a group II WRKY transcription factor. Analysis of *LeWRKY1* mRNA expression in various tissues by qRT-PCR showed that the highest and lowest expression occurred in the leaves and stems, respectively. In addition, *LeWRKY1* expression was induced by JA and *Botrytis cinerea* Pers., but not by SA.

Key words: LeWRKY1; Jasmonic acid; Salicylic acid; *Botrytis cinerea*

INTRODUCTION

Plants are typically sessile organisms, and therefore, cannot avoid the stressor when under biotic or abiotic stress. They have thus evolved the ability to respond to stress stimuli in a timely and specific manner. When exposed to stress, a set of genes can be activated or inhibited. These changes in gene expression are responsible for some of the specific responses observed under conditions of biotic and abiotic stress. The products of these genes are generally regulatory proteins, which may subsequently regulate cell responses and activate the expression of quick response defense genes. A transcription factor, WRKY, is strongly and rapidly upregulated in response to wounding, pathogen infection, and abiotic stresses in numerous plant species (Eulgem et al., 2000; Ulker and Somssich, 2004).

WRKY proteins comprise a large family of zinc finger type transcription factors (Park et al., 2005). The WRKY transcription factor superfamily consists of 74 and 109 members in *Arabidopsis* and rice, respectively (Eulgem and Somssich, 2007; Ross et al., 2007; Pandey and Somssich, 2009). WRKY proteins contain one or two WRKY domains, which are 60 amino acid regions containing WRKYGQK at the N-terminus, and a C-C-H-C/H zinc finger-like motif at the C terminus (Eulgem et al., 2000; Park et al., 2005). WRKY proteins bind specifically to W-box elements [TTGAC(C/T)] both *in vitro* and *in vivo* (Eulgem et al., 2000; Park et al., 2005). All known WRKY proteins contain either one or two WRKY domains. Based on the number of WRKY domains and the zinc finger motif sequence, WRKY proteins can be classified into three distinct groups (I, II, and III). Group I proteins contain two WRKY domains, whereas each of group II and III proteins possess one WRKY domain. Groups II and III differ in the structure of the zinc finger motif. In general, the WRKY domains of group I and II proteins contain the C_2-H_2 (C-X_{4-5}-C-X_{22-23}-H-X_1-H) pattern finger motif, whereas those of group III contain a C_2-HC motif (C-X_7-C-X_{23}-H-X_1-C) (Eulgem et al., 2000).

In this study, a WRKY transcription factor was cloned from tomato using reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). Furthermore, we characterized its expression patterns under various environmental stresses, such as low temperature (4°C), salt stress, jasmonic acid, salicylic acid, and *Botrytis cinerea* Pers. infection. The results showed that *LeWRKY1* expression is induced by jasmonic acid (JA), but not by salicylic acid (SA). The majority of the WRKY genes analyzed respond to pathogen attack, and to the endogenous signaling molecule SA (Eulgem and Somssich, 2007; Pandey et al., 2009). *LeWRKY1* also responded to pathogen attack (*B. cinerea*), but not to SA.

MATERIAL AND METHODS

Plant materials and treatment

Lycopersicum esculentum Miller seeds were obtained from a local garden and used for experiments shortly after harvest. *L. esculentum* plants were germinated and grown on MS medium. Seedling treatments were performed as described by Yamaguchi-Shinozaki and Shinozaki (1994) with slight modifications. The seedlings were grown for 3 weeks under photoperiod cycles of 16-h light (26°C) and 8-h dark (22°C). Three-week-old seedlings were treated with JA or SA, or allowed to be infected with *B. cinerea.* Next, they were grown hydroponically in MS solution containing 100 µM JA or 100 mM SA, or allowed to be infected with *B. cinerea* based on the method described by Ferrari et al. (2007). The seedlings were then harvested for RNA preparation.

Total RNA extraction

Total RNA was extracted using the UNIQ-10 column Trizol total RNA extraction Kit (SK1321), according to the manufacturer protocol.

RT-PCR and RACE-PCR

Degenerate forward (5'-TGGMGIAARTAYGGNCARA-3') and reverse primers (5'-TGRBYRTGYTTICCYTCRTAIGTDGT-3') were designed based on the conserved sequence of WRKY transcription factors. Using RT-PCR, a WRKY fragment from *L. esculentum* (*LeWRKY1* fragment) was obtained. The full-length sequence for *LeWRKY1* was obtained through RACE-PCR using the 3'-Full Race Cord Set and the 5'-Full Race Cord Set (Takara, Japan). The primers used for 3'-RACE were the 3'RACE adaptor primer and the gene-specific primer 5'-TGGAGGAAGTATGGGCAGAA-3'. The primers used for 5'-RACE were the 5' RACE adaptor primer, 5' RACE outer primer, 5' RACE inner primer (provided by 5' RACE Full kits), a gene-specific outer primer (5'-ATCGGCTGGCTGTGGAAG-3'), and a gene-specific inner primer (5'-GGTTCGTGGATGGTTATG-3'). PCR products were fractionated by electrophoresis on 1.2% agarose gels, and collected using a UNIQ-10 column DNA collection Kit (Shanghai Sangon Biological Engineering Technology & Services, Co., Ltd., China), after which the fragment was cloned into the pMD19-T vector (Takara, Japan) and sequenced (Shanghai Sangon Biological Engineering Technology & Services, Co., Ltd., China).

Sequence analysis

Sequence analysis was performed as previously described (Yang et al., 2003). Sequences were assembled using Seq Man II from DNASTAR, Inc. (Madison, WI, USA). Genes were identified using a combination of several methods. The genes in this region were predicted using GenScan (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin, 1997). The *Arabidopsis* settings were chosen for all programs. To identify *LeWRKY* genes, ClustalX was used to align *LeWRKY* with identified WRKY genes (Thompson et al., 1997). *LeWRKY1* was used for BLASTn and BLASTx homology searches against the GenBank database, which performed as described by Altschul et al. (1997).

Quantitative PCR

Based on the methods described by Yang et al. (2003), quantitative PCR experiments were performed using an Applied Biosystems ABI 7300 system and SYBR Green I was used as a fluorescent dye to quantify levels of DNA. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate CT values relative to endogenous controls. First-strand cDNA was prepared using the Prime Script™ RT Reagent Kit (Takara, Japan). To amplify gene-specific products, the following primers were used: *LeWRKY1* forward primer: 5'-CAA ATGGCTACTTCCTTGACC-3', *LeWRKY1* reverse primer: 5'-TGGACTTTTGTTTACCATCTCC-3', actin primers were also used, as previously described by Coker et al. (2005). To validate the quantitative PCR results, each experiment was repeated three times. Results are reported as means of biological replicates with the corresponding standard deviations. Significant differences among different treatments were evaluated using one-way ANOVA ($P \leq 0.05$).

RESULTS

Cloning of *LeWRKY1* cDNA using RT-PCR

We designed degenerate primers against the conserved sequence of WRKY. Using these primers, we cloned a cDNA fragment. The cDNA fragment was subjected to sequencing and nucleotide BLAST searches, and the results showed that the polypeptide encoded by the cDNA fragment contained a WRKY domain; this was named *LeWRKY1*.

The full-length cDNA of *LeWRKY1* was obtained using RACE-PCR method (accession No. FJ654265). A cDNA clone of approximately 1.7 kb was sequenced and consisted of an open reading frame beginning with an ATG start codon at position 226 and ending with a TAA stop codon at position 1308. It encoded a 360-amino acid protein with a predicted molecular mass of 39.77 kDa (Figures 1 and 2). This cDNA clone was designated *LeWRKY1*. Furthermore, it contained 5′ (225 bp) and 3' (312 bp) untranslated sequences. Sequence comparison showed that *LeWRKY1* contained one WRKY domain, and it had a C_2H_2 type zinc finger domain downstream of the WRKY domain (Figure 2). BLASTp analysis showed that the protein encoded by *LeWRKY1* is ~85 and 79% identical to CaWRKY (accession No. AAX20040.1) and WIZZ proteins (accession No. BAA87058.1), respectively.

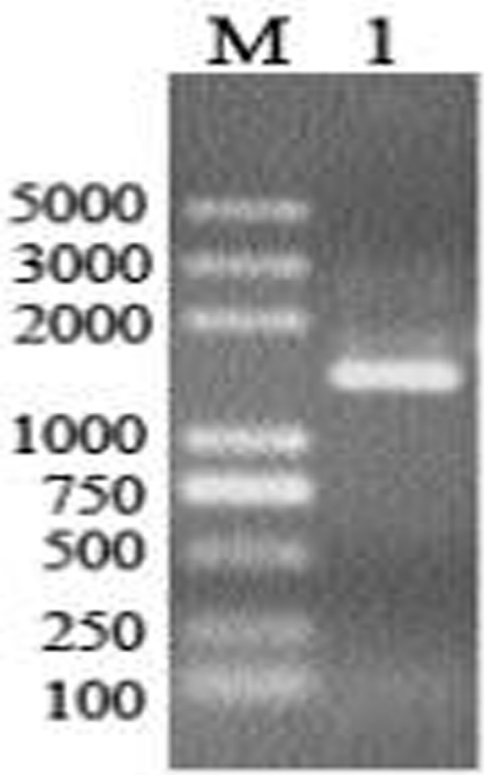

Figure 1. Agarose gel electrophoresis (1.2% gel) of the full-length cDNA of *LeWRKY1* from RT-PCR amplification. *Lane M* = Trans 2K plus DNA Marker; *lane 1* = *LeWRKY1* cDNA.

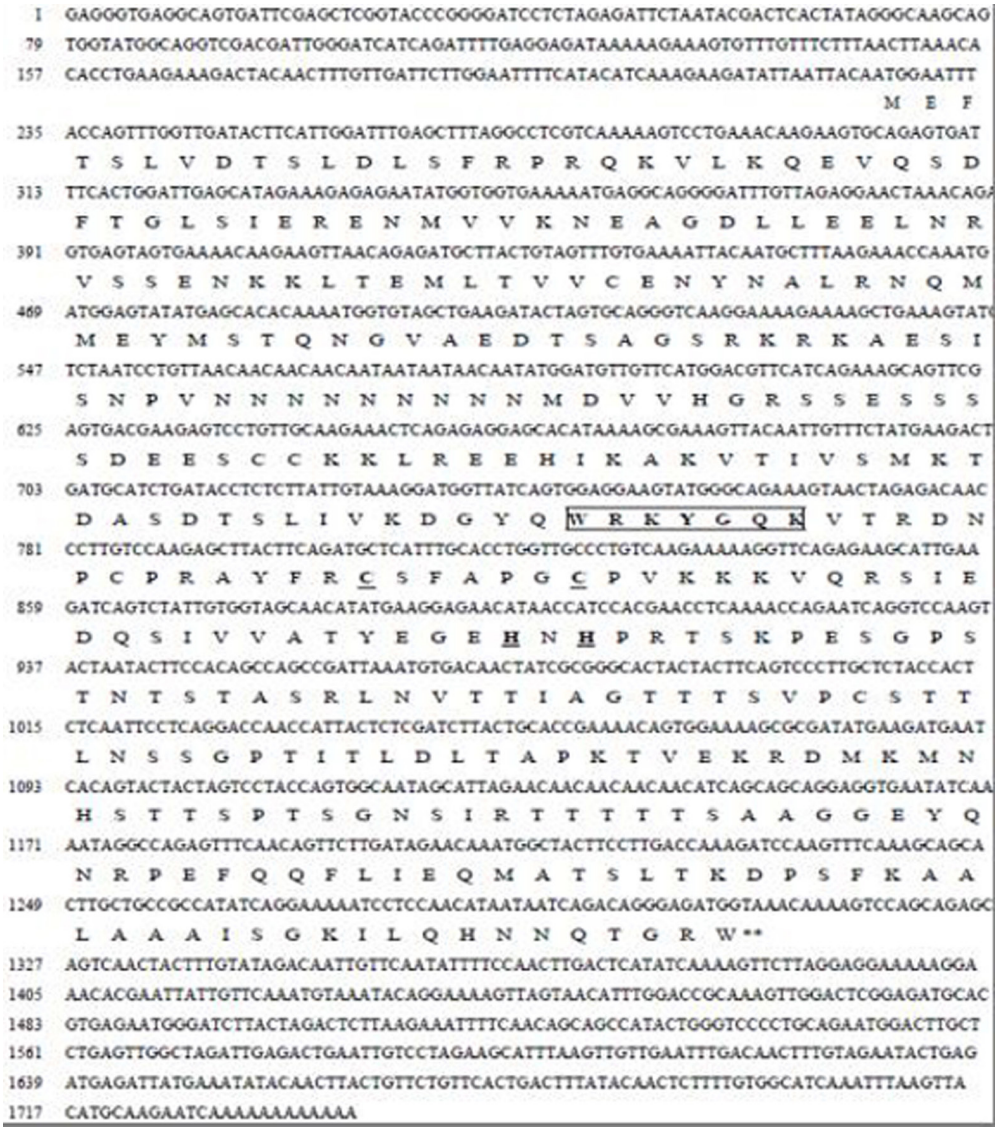

Figure 2. Nucleotide and predicted amino acid sequence of *LeWRKY1*. The rectangular box shows the conserved amino acid sequence WRKYGQK. The underlined sequence is the C_2H_2 zinc finger.

LeWRKY1 expression profiles in different tissues of tomato seedlings

Expression levels of *LeWRKY1* in three tomato seedling tissues (root, stem, and leaf) were measured by qRT-PCR, and data were normalized using *LeWRKY1* transcript levels in roots by using the $2^{-\Delta\Delta Ct}$ method. The relative expression of *LeWRKY1* in stems and leaves were 0.89 and 1.6 times that in roots (normalized as 1), respectively (Figure 3).

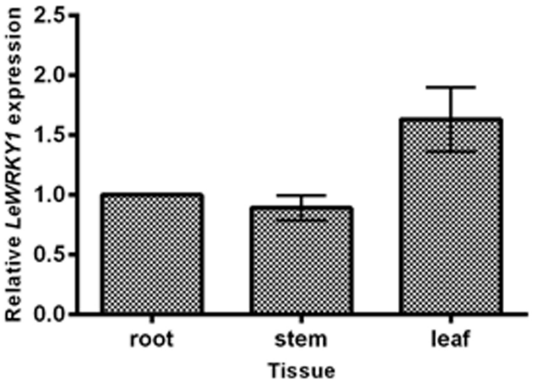

Figure 3. Relative expression of *LeWRKY1* in different tomato seedling tissues. Roots, stems, and leaves from three-week-old seedlings were collected separately, and total RNA was extracted from each tissue for qRT-PCR. Data were normalized to the *LeWRKY1* transcript.

Regulation of *LeWRKY1* expression by JA and SA in tomato seedlings

To assess the effects of JA and SA on *LeWRKY1* expression, tomato seedlings were treated with 100 μM JA or 100 μM SA, respectively, for different indicated times (Figure 4). A quantitative PCR experiment using actin as the endogenous control showed that JA could induce *LeWRKY1* expression. When treated with JA, statistically significant differences in *LeWRKY1* expression were observed. A 50% increase in *LeWRKY1* expression was induced 30 min after JA treatment, and the expression peaked at 12 h (4-fold increase) (Figure 4a). SA treatment had no significant effect on *LeWRKY1* expression (Figure 4b).

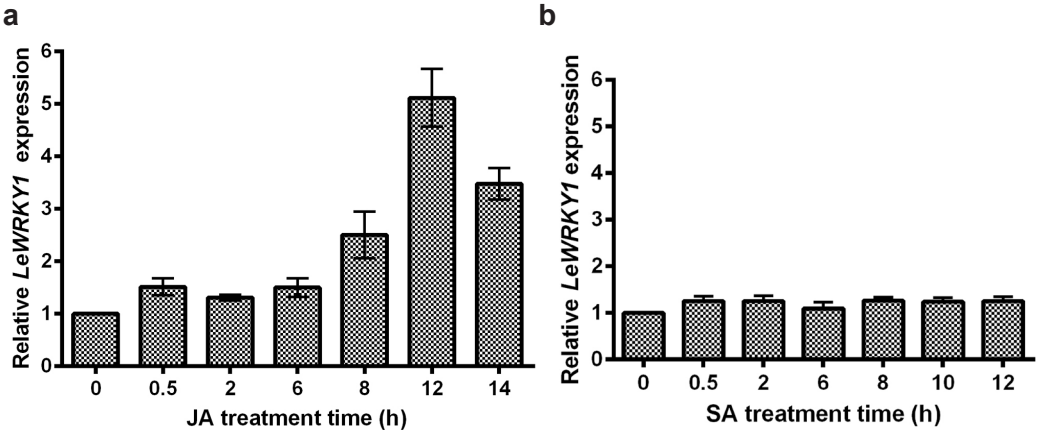

Figure 4. Effect of JA on levels of *LeWRKY1* transcription in *Lycopersicum esculentum* seedlings. **a.** Seedlings were treated with 100 μM JA for 0, 0.5, 1, 2, 6, 8, 12, or 14 h. **b.** The seedlings were treated with 100 μM SA for 0, 0.5, 2, 6, 8, 10, or 12 h. *Actin* (U60480.1) was used as the internal control. Data are reported as means ± SD (N = 3).

Effect of *B. cinerea* treatment on *LeWRKY1* expression

LeWRKY1 expression was increased in a time-dependent manner over 48 h in *B. cinerea*-

infected tomato seedlings. *LeWRKY1* expression increased to about 4.1-fold at 48 h after treatment (Figure 5). After 72 h, *LeWRKY1* expression began to decrease.

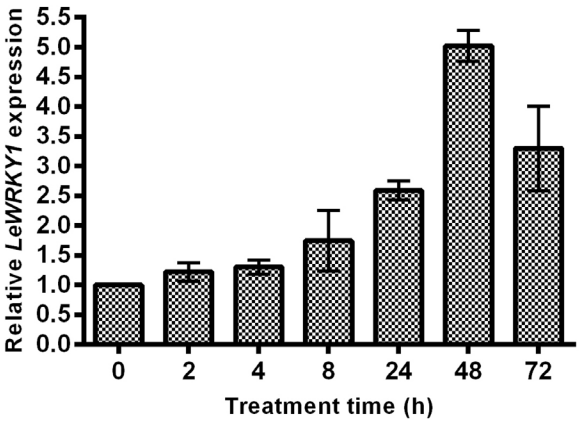

Figure 5. Induction of *LeWRKY1* after *Botrytis cinerea* infection. The seedlings were treated with *B. cinerea* for 0, 2, 4, 8, 24, or 72 h. *Actin* (U60480.1) was used as the internal control. Data are reported as means ± SD (N = 3).

DISCUSSION

We cloned *WRKY* cDNA from tomato, and determined that it encodes the complete amino acid sequence of this transcription factor. The number of WRKY domains and the type of zinc finger motif suggest that *LeWRKY1* belongs to group II WRKY transcription factors. *LeWRKY1* was detected in the root, stem, and leaf of tomato (Figure 3). *LeWRKY* is expressed throughout the intact plant, with the highest expression observed in the leaf (Figure 3).

Plants have two interconnected innate immunity pathways. The first involves pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), which is initiated by the recognition of molecular signatures of many pathogens, and often activates downstream mitogen-activated protein kinase cascades and defense genes. The second involves effector-triggered immunity (ETI), which is driven by plant-disease resistance proteins that directly or indirectly recognize specific pathogen-derived effectors (Chisholm et al., 2006; Pandey and Somssich, 2009). PTI and ETI activate local and systemic defense responses, which are modulated by phytohormones, especially JA and SA (Durrant and Dong, 2004; Bostock, 2005; Pandey and Somssich, 2009). These responses to pathogen attack require large-scale transcriptional reprogramming and include WRKY transcription factors (Eulgem, 2005; Ryu et al., 2006; Naoumkina et al., 2008; Pandey and Somssich, 2009). *LeWRKY1* was induced by JA (Figure 4a), but not by SA (Figure 4b). JA-dependent plant defenses are generally activated by necrotrophic pathogens and chewing insects, whereas SA-dependent defenses are often triggered by biotrophic pathogens (Pandey and Somssich, 2009). Synergistic and antagonistic interactions between JA and ethylene signaling have been reported and depend on the stress conditions under investigation (Kazan and Manners, 2008). JA and SA signaling usually act antagonistically, but synergism between these two phytohormones has also been observed (Mur et al., 2006). Our results show that *LeWRKY1* can be induced by JA, but not by SA (Figure 4).

Various plant defense responses against microbial pathogens are regulated by SA and JA pathways (Ferrari et al., 2007). These signaling pathways have been extensively studied, but how the SA, JA, and ET signaling pathways are related to those activated by OGs and other PAMPs remains unknown (Ferrari et al., 2007). *B*. *cinerea* infection increased *LeWRKY1* expression (Figure 5). Ferrari et al. (2007) indicated that OGs increase *Arabidopsis* resistance to *B*. *cinerea* through the activation of defense responses that are independent of SA, JA, and ET, but that SA, JA, and ET are also involved in defense pathways that confer resistance to *B*. *cinerea*. It is crucial to elucidate the cellular/nuclear components of *LeWRKY1* interaction when plants are infected with *B*. *cinerea*, and to determine how *LeWRKY1* is regulated.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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