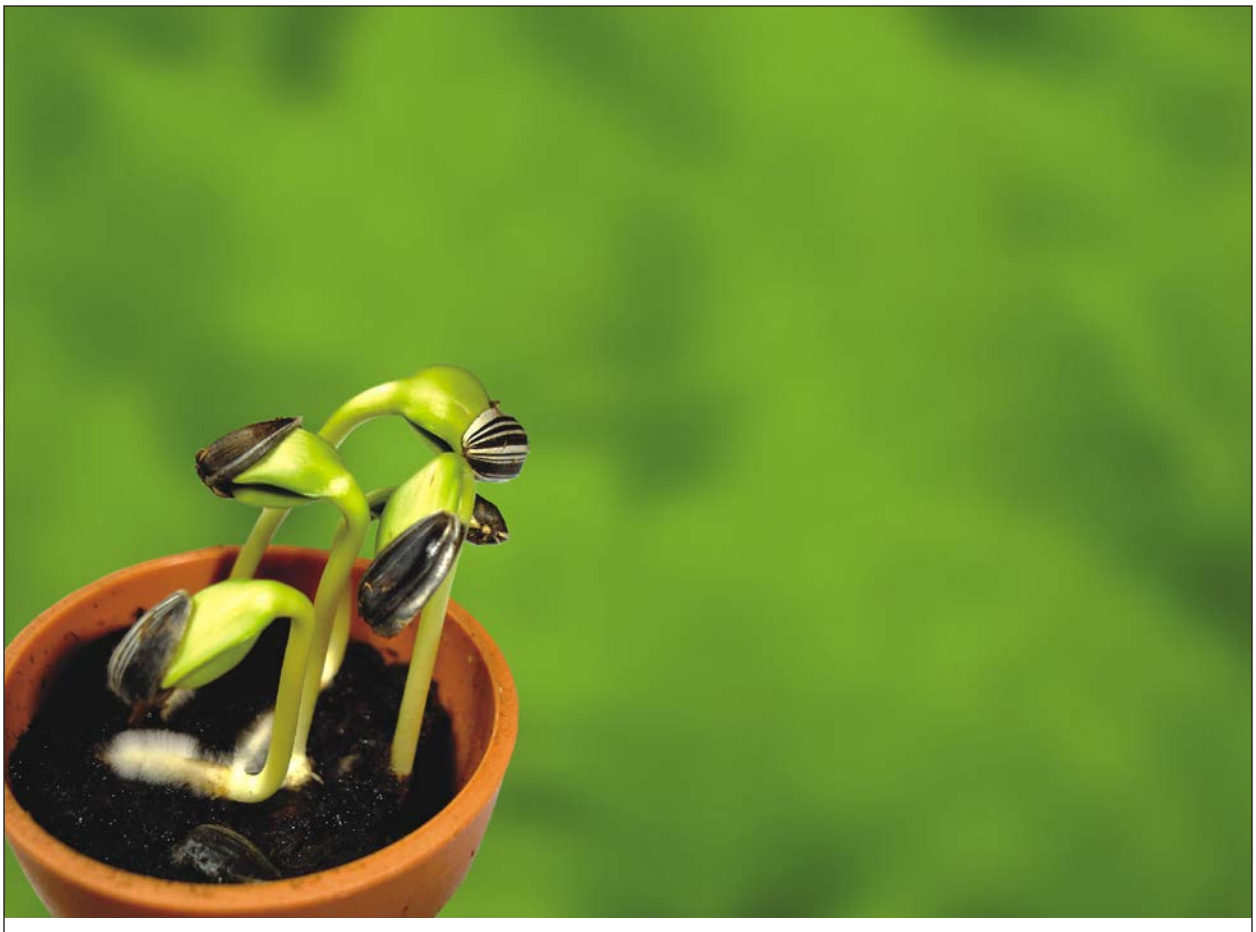

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Expression of *Sl-WRKY1* Transcription Factor During *B. cinerea* tomato Interaction in Resistant and Susceptible Cultivars

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Abstract: In this study, during the *B. cinerea* tomato interaction the regulation of *Sl-WRKY1* which encoding a putative member of the *WRKY* DNA-binding protein family and act as transcriptional activators co-regulated with PR-proteins expression was investigated in resistance and susceptible tomato cultivars. The studies were performed at the structural level (DNA level) and functional level (RNA level). Results showed that, in spite of the tested tomato cultivars varied in their resistance to grey mould disease, the gene encoding *Sl-WRKY1* was existed at DNA level. At RNA level, an induction of *Sl-WRKY1* expression was detected in resistant and susceptible cultivars after inoculation with *B. cinerea*, as compared to the healthy controls. These results indicated that, *Sl-WRKY1* gene was related to the defense mechanisms against grey mould disease in tomato. The expression of *Sl-WRKY1* gene in the resistant cultivar occurred earlier and stronger while being induced later in the susceptible cultivar. The phylogenatic analysis of 30 *WRKY* amino acid sequences in different 11 plant species, was constructed. Results showed that, some *WRKY* genes of *S. lycopersicum* are phytogenaticaly closed to *WRKY* genes of *A. thaliana*, *O. sativa*, *N. tabacum* and *S. tuberosum.*

Key words: *Botrytis cinerea*, tomato resistance, *Sl-WRKY1* transcription factor, phylogenatic analysis

INTRODUCTION

Plants have the ability to defend themselves against pathogens by activating a series of defense responses to constrain pathogen invasion (Feys and Parker, 2000; McDowell and Dangl, 2000; Thomma *et al*., 2001). Transcriptional regulation of plant host genes plays a central role in the activation of plant inducible defense responses. Therefore, an important step towered understanding the regulation of plant defense mechanisms is to identify regulatory components and to establish transcriptional regulatory pathways of plant defense system. So far, several families of transcription factors, which are likely involved in the transcriptional regulation of plant defense genes, have been identified, such as ethylene-responsive element binding proteins, myb-like proteins, bZIP proteins, and *WRKY* proteins (Rushton and Somssich, 1998).

The *WRKY* proteins have been identified in many plants and appear to be encoded by a large gene family, with more than 74 members in *Arabidopsis thaliana* (Ulker and Somssich, 2004). This family is defined by the presence of one or two *WRKY* conserved domains of 60 amino acids characterized by tZhe sequence WRKYGQK, followed by a Cys2His2 or Cys2HisCys zinc-binding motif (Eulgem *et al*., 2000). This domain binds specifically to a

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DNA sequence called Wbox, which contains the core sequence TGAC. This DNA motif is frequently present in the promoter sequence of numerous plant defense genes, including those encoding PR proteins (Maleck *et al*., 2000; Rushton *et al*., 1996).

The *WRKY* proteins have been implicated in physiological responses to biotic and abiotic stress as well as in trichome and seed development, senescence and the biosynthesis of secondary metabolites (Devaiah *et al*., 2007; Johnson *et al*., 2002; Kato *et al*., 2007; Ulker and Somssich, 2004; Xie *et al*., 2006; Xu *et al*., 2004; Zou *et al*., 2004). In the past few years, much progress has been made on the characterization of *WRKY* proteins involved in regulating plant defense responses (Eulgem, 2006; Journot Catalino *et al*., 2006; Knoth *et al*., 2007; Ulker *et al*., 2007; Xu *et al*., 2006; Zheng *et al*., 2007). In this study, we investigated the role of *Sl-WRKY1* transcription factor, in relation to resistance to gray mold disease during *B. cinerea* tomato interaction in resistant and susceptible cultivars.

MATERIALS AND METHODS

The present study was carried out in 2008-2009 at the Plant Protection Department, Faculty of Food and Agriculture Sciences, King Saud University, Kingdom of Saudi Arabia.

Plant Material and Growing Conditions

Six tomato cultivars (*Solanum lycopersicum* L.) Almpra, Antra, Farha, Gala Neotol and Red-gold were used in this study. Seeds of each tomato cultivar were planted in 25 cm diameter pots containing 4 kg steam-sterilized sandy-loam soil and kept in an environmentally controlled greenhouse at 24±2°C with 16 h of light and 8 h of dark till desired stage. The plants were kept in a growth chamber at 23°C for 16 h photoperiod for 6 weeks prior to inoculation.

Fungal Isolate

One aggressive *Botrytis cinerea* isolate (isolated from samples of tomato plants showing symptoms of grey mould) was used in the present study.

Interaction Between Tomato Cultivars and *B. cinerea*

Fully expanded leaves of 6 week-old tomato plants were inoculated with *B. cinerea* by the detached leaf method, as described by Audenaert *et al*. (2002). Leaves of each tomato cultivar (placed in Petri-dish on moist tissue paper) with one centric droplet of 20 μL (1×10^6 conidia mL^{-1}) or 20 μL drops of water. Plates of the inoculated leaves were incubated at 23°C. After 24 h of inculcation, droplets were blotted with tissue paper to discourage bacterial growth on the leaf surface. Disease development was estimated as diameter of lesion extension within 7 days after inoculation.

Expression of *SL-WRKY1* Gene

Molecular Analysis at the Structural Level (DNA Level)

Genomic DNA Extraction

Total genomic DNAs were prepared from the tested tomato cultivars. Tomato leaf tissue was processed by freezing with liquid nitrogen and was ground into a fine powder using a mortar and pestle. Approximately 100 mg of that powder was transferred to 1.5 mL micro-centrifuge tube and 600 μL of warm (65°C) modified CTAB extraction buffer (100 mM Tris-Hcl [pH 8.0], 1.4M NaCl, 2% CTAB [hexadecyltrimethylammonium bromide], 20 mM EDTA [sodium salt, pH 8.0]). Tubes were vortexes for 1-3 sec. and incubated for 60-90 min, in water bath at 65°C. After that, the sample was allowed to cool to room temperature for 5 min. A volume of 700 μL chloroform/octanol (24:1) was added, the solution was gently mixed for

5-10 min. The mixture was centrifuged for 10 min at 8000 x g. Six hundred microliters of upper, aqueous layer were transferred to clean 1.5 mL micro-centrifuge tube, and a volume of 600 μL of cooled isopropanol was added to precipitate the DNA. The mixture was centrifuged at 5000 x g for 2 min at room temperature. The supernatant was decanted and 600 μL of 70% ethanol was added at room temperature and gently inverted the tube several times to wash the DNA. The mixture was centrifuged at 3000 x g for 2 min at room temperature. Carefully the ethanol was aspirated using a pipette. The tube was inverted onto clean absorbent paper and air dried the pellet for 15 min. DNA pellet was re-suspended in 100 μL TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA (pH 8.0) and stored at 20°C. DNA concentration was determined using spectrophotometer (Beckman DU-65) and was adjusted to 50 ng μL^{-1}.

PCR Amplification

PCR amplification was carried out in 25 μL volumes containing: 1 μL (50 pmol) of each primer, 0.3 μL Taq DNA polymerase (5 U μL^{-1}), 2.5 μL PCR buffer, 1 μL 10mM Mg Cl_2, 1 μL 2.0 mM dNTPs (for each), 1 μL of template DNA (approximately 40 ng) and 17.2 μL sterilized distilled water. The PCR amplification conditions were initial denaturation at 94°C for 5min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec annealing of primer for 40 sec at 55°C; extension for 1.5 min at 72°C and finally addition of 3 terminal A for 10 min at 72°C. The specific primer for *Sl-WRKY1* gene was designed based (GenBank accession No. FJ654265), the primers sequence: Forward primer: 5'-TCA ATT CCT CAG GAC CAA CC-'3'. Reverse primer: 5'-GAC CCA GTA TGG CTG CTG TT-3'. Amplified products were electrophoresed on 1.2% agrose gel containing 0.5 μL ethidium bromide with 1X TBE running buffer. 1 kb DNA Ladder (New England Biolabs) with size marker, ranged from 50 to 1000 bp was used as a molecular size standard.

Molecular Analysis at the Functional Level (RNA Level)

Expression of *Sl-WRKY1* gene was tested on the RNA extracted from leaves of two tomato cultivars differ in their levels of susceptibility to *B. cinerea*, Neotol (resistant cv.) and Red-gold (susceptible cv.), before and after the inoculation with *B. cinerea*, across a different time course (at 3, 6, 9, 12, 18 and 24).

Plant Inoculation

Tomato plants (45 days old) were artificially infected with *B. cinerea* conidial suspension (1×10^6 conidia mL^{-1}) using a fine mist hand sprayer. Inoculated plants were placed in moist chamber in a greenhouse at 23°C. Un-inoculated tomato leaves (treated with sterilized water and measured immediately) were served as control.

Reverse Transcription PCR (RT-PCR)

Isolation of Total RNA from Inoculated Potato Leaves

Total RNA was extracted from tomato leaves using RNA isolation system (GE Health care, illustra RNAspin Mini RNA isolation kit, Cat. No. 25-0500-71). The UV absorbance at 260 nm and the A260/A230 ratio were used to determine the concentration and purity of the tested RNA samples. The integrity of total RNA was further confirmed by formaldehyde-agarose electrophoresis. Leaf samples collected from the two separate sets of inoculations were used separately for RNA extractions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR was performed using One step RT-PCR Kit (QIAGEN Cat. NO. 210212) to detect the induction for the messages of *Sl-WRKY1* gene. The RT-PCR profile was as follows, 1 cycle of 50°C for 30 min, (for first strand syntheses), then initial denaturation step at 95°C

Table 1: Abbreviations and GenBank accession numbers of *WRKY* genes for plant species referred in phylogenetic analysis

Species	Abbreviation	GenBank accession No.
Arabidopsis thaliana	*At-WRKY17*	AK227604
	At-WRKY38	AY568652
Capsicum annuum	*Ca-WRKY-c*	AY740531
Gossypium arboreum	*Ga-WRKY1*	AY507929
Lolium perenne	*Lp-WRKY1*	DQ145929
Nicotiana tabacum	*Nt-WRKY2*	AB020590
	Nt-WRKY6	AB063573
	Nt-WRKY12	DQ460475
Oryza sativa	*Os-WRKY62*	DQ298182
	Os-WRKY70	DQ298184
	Os-WRKY71	AB190817
Petroselinum crispum	*Pc-WRKY1*	AF121353
	Pc-WRKY3	U56834
	Pc-WRKY4	AF204925
	Pc-WRKY5	AF204926
Solanum lycopersicum	*Sl-WRKY1*	FJ654265
	Sl-WRKY2	FJ654264
	Sl-WRKY1000.3	EU755370
	Sl-WRKYIId-1	AY157058
	Sl-WRKYIId-5	AY157064
	Sl-WRKYIId-6	AY157065
Solanum tuberosum	*St-WRKY2*	EU056914
	St-WRKY3	EU056915
	St-WRKY5	EU056917
Triticum æstivum	*Ta-WRKY10*	EF368361
	Ta-WRKY19-a	EF368362
	Ta-WRKY68-a	EF368360
	Ta-WRKY71	EF368356
	Ta-WRKY74	EF368359
Vitis vinifera	*Vv-WRKY2*	AY596466

for 15 min (for activation of hotstart Taq polymerase) followed by 40 cycles consisting of denaturation at 94°C for 40 sec; annealing of primer for 50 sec at 50°C; extension for 1.5 min at 72°C and finally addition of 3' terminal A for 10 min at 72°C. PCR products were analyzed in 1.5% agarose gel.

Phylogenetic Analysis

The predicted amino acid sequences of 30 *WRKY* genes for 11 plant species (Table 1) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). Related sequences were aligned by using the program DNAMAN 6.0.40 (http://www.lynnon.com). A phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the DNAMAN program (Thompson *et al*., 1994).

Statistical Analysis

The data obtained were statistically analyzed using a personal computer according to the SAS (1988).

RESULTS

Interaction Between Tomato Cultivars and *B. cinerea*

To characterize the interaction between the tested tomato cultivars and *B. cinerea*, detached tomato leaves inoculated *in vitro* with one centric droplet of 20 µL of fungal conidial suspension. Results showed that, the first symptoms of infection-necrotic lesions were noticed 2 days post-inoculation and were increased in size within 7 days post-inoculation (Fig. 1a, b).

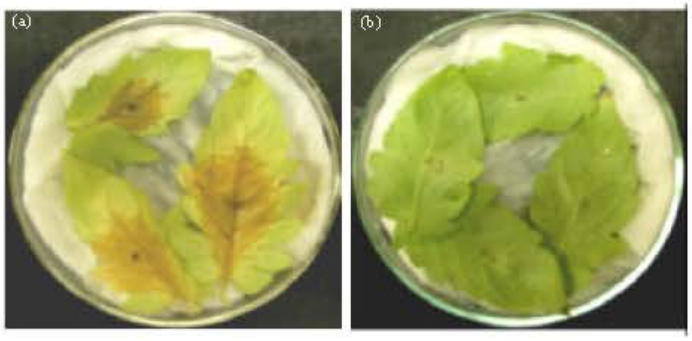

Fig. 1: Response of the susceptible (a) Red-gold cv. and resistant (b) Neotol cv. tomato leaves to inoculation with *B. cinerea* 7 days post-inoculation

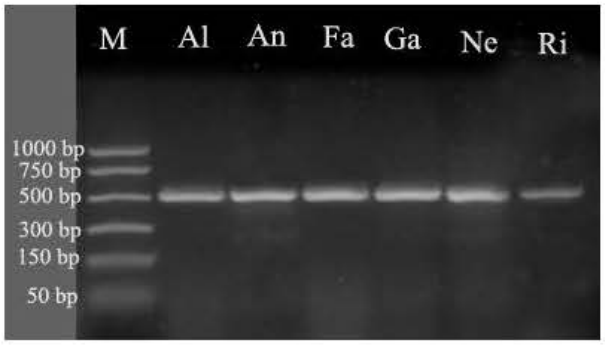

Fig. 2: Polymerase chain reaction product for *Sl-WRKY1* gene (525 bp) from different tomato cultivars (Al: Almpra cv., An: Antra cv., Fa: Farha cv., Ga: Gala cv., Ne: Neotol cv., Ri: Red-gold cv.) varied in their resistance to infection with *B. cinerea*

Table 2: Diameter of lesion extension (cm) related to leaves inoculated with *B. cinerea* on different tomato cultivars 7 days post-inoculation

Tomato cultivars	Diameter of lesion extension (cm)
Almpra	0.92c
Antra	1.10b
Farha	1.82a
Gala	0.85c
Neotol	0.35d
Red-gold	1.82a

*Mean of 10 replicates. Values followed by the same letter(s) are not significantly different at p = 0.05

Data in Table 2 reveal that diameter of lesion extension related to leaves infected with *B. cinerea* showed that, the tomato cultivar which had the highest significant resistance to the infection was Neotol (0.35). Tomato cultivars that showed moderate resistance were Gala (0.85), Almpra (0.92) and Antra (1.10). The lowest resistance was obtained in Farha and Red-gold (1.82).

Expression of *Sl-WRKY1* Gene During the *B. cinerea* Tomato Interaction

Molecular Analysis at the Structural Level (DNA Level)

PCR was conducted to confirm the presence of the *Sl-WRKY1* in the 6 tomato cultivars varied in their resistance to grey mould disease (Almpra, Antra, Farha, Gala, Neotol and Red-gold), using specific primer which is designed, based on the sequence encoding *Sl-WRKY1* (GenBank accession No. FJ654265).

As shown in Fig. 2 the *Sl-WRKY1* gene showed a band size of 525 bp which present in all resistant, moderate and susceptible cultivars such as Neotol (resistant cv.) and Red-gold (susceptible cv.). These results indicating that, in spite of the tested tomato cultivars varied in their resistance to grey mould disease, the gene encoding *Sl-WRKY1* was existed at DNA level.

Molecular Analysis at the Functional Level (RNA Level)

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis

Expression of *Sl-WRKY1* gene was tested on the RNA extracted from leaves of two tomato cultivars differ in their levels of susceptibility to *B. cinerea*, Neotol (resistant cv.) and Red-gold (susceptible cv.), before and after the inoculation with *B. cinerea*, across a different time course (at 3, 6, 9, 12, 18 and 24). As shown in Fig. 2 an induction of *Sl-WRKY1* expression was detected in both cultivars after inoculation with *B. cinerea*, as compared to the healthy controls.

When the resistant tomato leaves (Neotol cv.) inoculated with *B. cinerea* conidia, the *Sl-WRKY1* gene transcripts were detected at 3 hpi and increased gradually at 9, 12, 18 and 24. On the other hand, inoculated leaves of the susceptible tomato cultivar (Red-gold) with fungal conidia, showed a weak expression of *Sl-WRKY1* gene. The *Sl-WRKY1* gene was detected at 6 hpi and reached a maximum at 12 and 18 hpi (Fig. 3). These results indicated that, *Sl-WRKY1* gene was related to the defense mechanisms against grey mould disease in tomato. The expression of *Sl-WRKY1* gene in the resistant cultivar occurred earlier and stronger while being induced later in the susceptible cultivar (Fig. 3a, b).

Phylogenetic Analysis

Phylogenatic analysis of 30 *WRKY* amino acid sequences in different 11 plant species, was constructed from a matrix of pairwise genetic distances by the maximum-parsimony

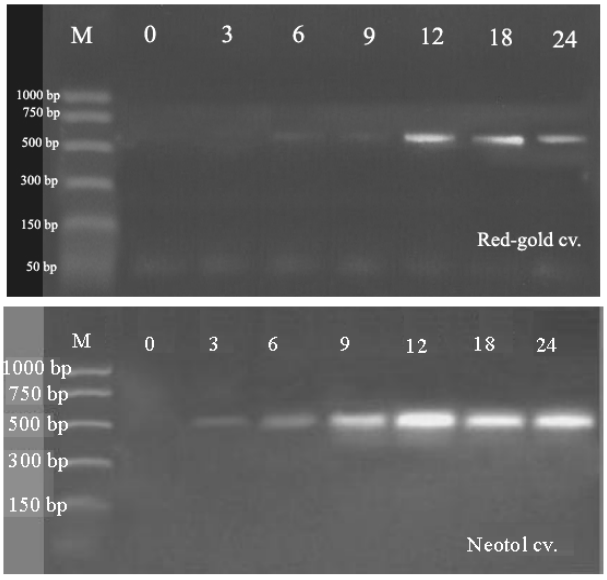

Fig. 3: Reverse-transcriptase polymerase chain reaction analysis showing expression of the *Sl-WRKY* gene (525 bp) in leaves of (a) susceptible (Red-gold cv.) and (b) resistant (Neotol cv.) tomato cultivars during 24 h post inoculation with *B. cinerea*. M: DNA marker. 0: Un-infected leaves (treated with sterilized water and measured immediately)

algorithm and the neighbor-joining method (Fig. 4). Homology matrix (Table 3) shows the homology percentage (H%) between the different *WRKY* genes.

The phylogenetic analysis showed that, *WRKY* genes were grouped into several clusters. *WRKY* genes of *S. lycopersicum* are grouped among three different clusters (1, 2 and 3). Cluster-1 included only *Sl-WRKYIId-1* gene, which is phylogenetically closed to *WRKY3* and *WRKY2* gene of *S. tuberosum* (H% = 97.6 and 65.6, respectively) and *WRKY17* gene of *A. thaliana* (H% = 62.1). while, Cluster-2 included *Sl-WRKYIId-5 and Sl-WRKYIId-6* genes are phylogenetically closed to *WRKY2* gene of *S. tuberosum* (H% = 72.2-72.7), *WRKY17* gene of *A. thaliana* (H% = 70.4-70.9), *WRKY68-a* of *T. aestivum* (H% = 64.8-65.5), *WRKY3* gene of *P. crispum* (H% = 62.7-72) and *WRKY* genes of *N. tabacum* (H% = 41.8-51.9). Furthermore, Cluster-3, included *Sl-WRKY1*, *Sl-WRKY2* and *Sl-WRKY1000.3* genes which are phylogenetically closed with *WRKY4* gene of *G. arboretum* (H = 56.4-57.5%) and *WRKY1* gene of *P. crispum* (H% = 45.5-47.1). The phylogenatic tree placed *WRKY* genes of *S. lycopersicum* in Cluster 1 and 2 closed to each other. Also, the phylogenatic tree predicted that, *WRKY* genes of *S. lycopersicum* closed to *WRKY* genes of *O. sativa* and *WRKY71* gene of *T. aestivum*. These results indicated that, some *WRKY* genes of *S. lycopersicum* are phytogenaticaly closed to *WRKY* genes of *A. thaliana*, *O. sativa*, *N. tabacum* and *S. tuberosum*.

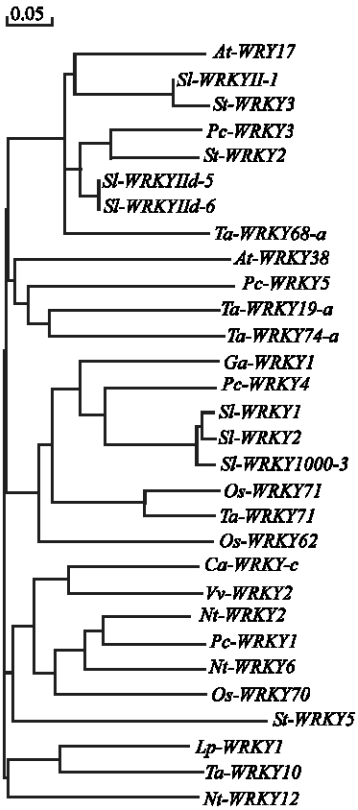

Fig. 4: Phylogenatic tree of 30 *WRKY* amino acid sequences in different 11 plant species, was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the DNAMAN program. The scale bar = 5% difference in amino acid sequences

Table 3: Homology percentage (H%) between the different *WRKY* genes

WRKY genes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
At-WRKY17	100																													
At-WRKY 38	20.3	100																												
Ca-WRKY -c	19.0	21.5	100																											
Ga-WRKY 1	21.6	14.3	19.1	100																										
Lp-WRKY 1	22.1	20.1	29.7	20.6	100																									
Nt-WRKY 2	21.1	15.7	43.5	16.8	27.6	100																								
Nt-WRKY 6	17.4	15.4	36.6	16.5	28.1	60.8	100																							
Nt-WRKY 12	24.6	17.4	22.9	22.1	28.4	23.3	24.7	100																						
Os-WRKY 62	20.0	14.0	21.0	31.8	20.2	18.6	17.4	23.0	100																					
Os-WRKY 70	18.8	15.0	32.9	16.8	24.9	53.6	38.7	22.7	19.5	100																				
Os-WRKY 71	19.5	14.6	18.5	36.5	22.3	17.0	14.9	20.9	30.5	17.2	100																			
Pc-WRKY 1	20.6	14.0	36.8	17.2	28.1	63.4	48.9	24.5	17.5	38.1	16.5	100																		
Pc-WRKY 3	43.7	21.2	18.2	22.5	21.1	20.8	17.8	21.1	21.2	17.8	17.6	19.2	100																	
Pc-WRKY 4	20.7	14.1	18.9	46.9	22.7	19.7	14.6	24.0	31.2	16.5	34.7	17.6	21.1	100																
Pc-WRKY 5	18.6	15.0	17.0	16.0	20.7	14.6	12.1	14.9	16.5	15.6	14.8	13.7	20.8	16.3	100															
Sl-WRKY IId-5	70.9	34.5	48.1	43.6	42.3	55.8	51.9	48.1	41.8	50.0	45.5	51.9	72.7	45.5	38.2	100														
Sl-WRKY 1	19.0	13.7	19.1	46.8	22.7	18.2	17.7	21.8	31.5	16.2	37.3	18.0	18.1	57.5	15.6	47.3	100													
Sl-WRKY 2	19.8	13.7	21.1	45.5	22.8	18.3	16.2	20.9	31.2	15.8	36.6	17.5	18.6	56.4	15.2	47.3	95.0	100												
Sl-WRKY1000.3	19.3	14.5	20.7	47.1	22.0	18.5	17.6	21.7	31.9	15.9	37.0	18.6	18.4	57.4	14.7	47.3	92.7	92.5	100											
Sl-WRKYIId-1	62.1	28.2	39.1	28.8	37.8	35.4	32.9	31.1	28.8	32.9	32.9	35.4	62.7	30.3	31.0	61.8	31.0	31.0	31.0	100										
Sl-WRKY IId-6	70.4	33.3	46.2	44.4	44.2	54.7	50.9	48.1	42.6	50.9	46.3	50.9	72.2	45.3	37.0	100	48.1	48.1	48.1	61.1	100									
St-WRKY 2	48.5	21.9	17.0	20.7	22.9	21.4	18.3	24.4	25.3	18.9	16.9	20.4	64.9	20.9	20.6	72.7	19.2	19.3	18.7	65,6	72.2	100								
St-WRKY 3	47.4	21.1	18.6	21.4	22.9	21.6	18.3	19.6	22.0	18.6	19.5	18.9	45.9	19.8	19.5	63.6	21.8	21.4	21.3	97.6	63.0	44.6	100							
St-WRKY 5	11.7	8.3	12.7	7.6	11.8	17.0	16.1	7.9	7.3	15.1	6.5	16.7	14.9	6.7	2.60	19.5	8.70	9.90	9.10	19.5	20.0	11.7	10.4	100						
Ta-WRKY 10	15.9	18.8	25.9	19.4	47.3	23.2	24.2	24.5	18.9	26.3	18.6	25.6	19.2	15.0	18.3	40.7	17.2	16.8	17.6	32.9	41.5	17.3	18.1	10.6	100					
Ta-WRKY 19-a	21.2	19.4	19.8	15.7	20.6	18.2	18.5	14.3	20.2	17.2	16.8	15.5	20.4	14.6	23.7	34.4	15.0	15.4	16.1	29.5	33.3	21.1	21.5	7.2	18.2	100				
Ta-WRKY 68-a	47.9	19.0	17.3	18.9	19.7	22.4	19.5	20.1	20.7	21.0	19.1	21.1	43.8	20.3	17.0	65.5	19.0	20.2	19.3	49.4	64.8	47.7	40.0	15.4	18.8	22.6	100			
Ta-WRKY 71	18.5	13.5	20.1	36.0	20.4	19.3	16.4	22.7	31.3	18.3	71.4	18.1	17.6	33.7	14.3	47.3	35.8	35.7	36.4	38.5	48.1	17.8	20.0	7.6	18.7	16.9	18.6	100		
Ta-WRKY 74-a	22.2	18.6	21.6	16.9	19.3	16.3	17.8	16.3	18.7	16.9	13.9	15.2	20.2	16.2	21.7	40.0	16.2	16.2	16.2	32.1	38.9	23.1	21.5	6.9	14.5	33.2	20.4	14.0	100	
Vv-WRKY2	17.9	18.0	48.9	18.1	27.6	48.8	36.2	22.7	19.0	30.1	16.8	35.1	17.9	16.7	14.3	49.1	17.2	17.0	18.4	35.9	50.0	17.7	17.1	12.8	23.7	18.3	18.6	19.2	17.5	100

1: *At-WRKY17*, 2: *At-WRKY 38*, 3: *Ca-WRKY -c*, 4: *Ga-WRKY 1*, 5: *Lp-WRKY 1*, 6: *Nt-WRKY 2*, 7:*N t-WRKY 6*, 8: *Nt-WRKY 12*, 9: *Os-WRKY 62*, 10: *Os-WRKY 70*, 11: *Os-WRKY 71*, 12: *Pc-WRKY 1*, 13: *Pc-WRKY 3*, 14: *Pc-WRKY 4*, 15: *Pc-WRKY 5* 16: *Sl-WRKY Iid-5*, 17: *Sl-WRKY 1*, 18: *Sl-WRKY 2*, 19: *Sl-WRKY1000.3*, 20: *Sl-WRKYIId-1*, 21: *Sl-WRKY Iid-6*, 22: *St-WRKY 2*, 23: *St-WRKY 3*, 24: *St-WRKY 5*, 25: *Ta-WRKY 10*, 26: *Ta-WRKY 19-a*, 27: *Ta-WRKY 68-a*, 28: *Ta-WRKY 71*, 29: *Ta-WRKY 74-a*, 30: *Vv-WRKY2*

DISCUSSION

During early stages of plant-pathogen interaction, the activation of defense responses may be elicited by signals derived either directly from the pathogen or generated by degradation of the plant cell wall (Davis *et al*., 1984; Palva *et al*., 1993; De Wit, 1995; Kalde *et al*., 2003). Czernic *et al*. (1999) supported a model in which such pathogen induced signals which transmitted through a membrane receptor and coupled with a kinase activity (receptor-like kinase; RLK) that then initiates a phosphorylation cascade leading to the induction or repression of target genes. Among the target genes, a variety of transcription factors that mediate the regulation of a variety of plant defense responses, including the up-regulation of *PR* genes, through recognition of specific DNA sequences in their promoter regions (Rushton and Somssich, 1998).

WRKYs are elicitor-induced proteins that bind to the sequence TGAC, or W box, in the promoters of PR genes (Rushton and Somssich, 1998; Du and Chen, 2000; Yu *et al*., 2001; Dong *et al*., 2003) and appear to be responsible for upregulation of these genes (Rushton *et al*., 1996). Yamamoto *et al*. (2004) reported that, WRKY proteins have regulatory functions in plant response according to pathogen infection. Such findings has been attributed to two reasons; first, several WRKY genes from a number of plants are rapidly induced by pathogens, elicitors, or treatment of Salicylic Acid (SA) (Eulgem *et al*., 1999; Dellagi *et al*., 2000; Chen and Chen, 2002) and second, a number of defense-related genes, including PR genes, contain W box elements in their promoter regions (Rushton *et al*., 1996; Yang *et al*., 1999).

During the *B. cinerea*-tomato interaction we investigated the regulation of *Sl-WRKY1* gene which encoding a putative member of the WRKY DNA-binding protein family and act as transcriptional activators co-regulated with PR-protein expression. Results showed that, at DNA level the *Sl-WRKY1* gene showed a band size of 525 bp which present in all resistant, moderate and susceptible tomato cultivars. These results are in agreement with Ghazy (2006), who found that, *Sl-WRKY1* gene present in all potato cultivars, resistant and susceptible to infection with *Erwinia carotovora* sub sp. *atroseptica*. At RNA level our results indicated that, in resistant tomato leaves inoculated with *B. cinerea* conidia in tissues under inoculation drops, the *WRKY1* gene transcripts were detected at 3 hpi and increased gradually at 9, 12, 18 and 24. On the other hand, inoculated leaves of the susceptible tomato cultivar (Red-gold) with fungal conidia, showed a weak expression of *Sl-WRKY1* gene. The *Sl-WRKY1* gene was detected at 6hpi and reached a maximum at 12 and 18 hpi. These results indicated that, *Sl-WRKY1* gene was related to the defense mechanisms against grey mould disease in tomato. The expression of *Sl-WRKY1* gene in the resistant cultivar occurred earlier and stronger while being induced later in the susceptible cultivar. Similar results were obtained in the studies reported by Eulgem *et al*. (1999), Dellagi *et al*. (2000), Chen and Chen (2002), Yu *et al*. (2001), Ghazy (2006) and El_Komy (2007), who reported that, *WRKY1* gene is related to the plant defense mechanism, in which *Sl-WRKY1* gene is rapidly and locally activated in infected plant tissues. Difference in the *Sl-WRKY1* gene expression due to resistant and susceptible tomato cultivars suggested that, the promoter elements that represent the target for *WRKY1* (W- box in PR gene promoter) may differ in location and/or number. Trognitz *et al*. (2002) found that, resistant potato cultivars had high copy numbers of WRKY genes compared with susceptible ones. Also, *WRKY1* promoter may have different combinations of cis/trans acting elements in both cultivars, which may cause different *WRKY1* expressions (Ghazy, 2006).

To better understand the functions of *WRKY* transcription factor of *S. lycopersicum*, a phylogenatic analysis of 30 *WRKY* amino acid sequences in different 11 plant species, was constructed. Results showed that, some *WRKY* genes of *S. lycopersicum* were phytogenaticaly closed to *WRKY* genes of *A. thaliana*, *O. sativa*, *N. tabacum* and *S. tuberosum*. Asai *et al*. (2002) reported that, two Arabidopsis *WRKY* transcription factors (*At-WRKY22* and *At-WRKY29*) have been identified as important downstream compounds of the MAPK pathway that confer resistance to both bacterial and fungal pathogens. Li *et al*. (2004, 2006) found that, Over-expression of *At-WRKY70* increased resistance to virulent pathogens in Arabidopsis. Also, a *WRKY* transcription factor (*Os-WRKY71*) was over-expressed in rice, and transgenic plants showed an enhanced resistance to virulent bacteria (Liu *et al*., 2007). In summary, it was confirmed that *Sl-WRKY1* transcription factor was related to the defense mechanisms against grey mould disease in tomato. Such information may lead to the development the tomato breeding programs against diseases.

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