

Differential Expression of Potato *WRKY1* Gene Upon *Erwinia carotovora* Infection and Bioinformatics Analysis for a Novel Tomato WRKY-like Gene

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Abstract: Pathogen attack represents a major problem for potato and for agriculture in general. It is essential to control disease by having a thorough knowledge of resistance mechanisms. The present work focused on the regulatory genes potentially involved in the control of the plant defence response, the *WRKY* genes. *WRKY* genes encode transcription factors that are involved in the regulation of various biological processes. These zinc-finger proteins, especially those members mediating stress responses, are uniquely expanded in plants. Expression profile of *StWRKY1* gene was investigated, which regulate the defense-related PR-3 gene, during the infection by *Erwinia carotovora* bacteria. The *StWRKY1* gene was expressed in both Nicola resistant and Diamant susceptible potato cultivars. However, it exhibited variation in expression level. A detectable expression of *StWRKY1* transcription level was observed at 3 and 5 hpi in Nicola, and the expression was increased at 7 hpi then at 9 hpi till it reach the the maximum at 12 hpi. While the expression was observed in Diamant only at 7, 9 and 12 hpi. These results are consistent with a possible role for *StWRKY1* gene in potato defence against bacterial pathogens. The pattern of the phylogenetic tree revealed a close relationship between *StWRKY* and *AtWRKY* genes. We performed an exhaustive search in GenBank for *WRKY* genes and predicted a WRKY-like gene in tomato using bioinformatics tools.

Keywords: Potato *WRKY1* gene, *Erwinia* infection, bioinformatics analysis and tomato WRKY-like gene.

INTRODUCTION

Plants are exposed to various pathogens in nature. To face microbial attack, they have evolved multiple and complex defence strategies. Most of these microbes can be dealt with by the plant basal defence system that limits the growth of non-host pathogens. Plant disease resistance can be induced via host recognition of pathogen elicitors. Elicitor- or pathogen-activated transcription factors play an important role in controlling defence gene expression and plant resistance responses. Five major families of plant transcription factors (bZIP, WRKY, MYB, EREBF, and homeodomain proteins) have been shown to participate in the regulation of plant defence responses. In plants, many WRKY proteins are involved in the defence against attack by phytopathogens such as bacteria (Marchive *et al.*, 2007). WRKY proteins are zinc-finger proteins that bind to W boxes (TTGAC) of several genes encoding pathogenesis-related proteins, activating their transcription. W boxes have been identified in the promoters of many defense-related genes, including the well studied PR genes (Kalde *et al.*, 2003). The inducible defense of plants against pathogen attack requires expression of a large number of defense genes that encode diverse proteins, including cell wall proteins, hydrolytic enzyme (chitinases and β 1, 3-glucanases) and other pathogenesis-related (PR) proteins. Therefore, it is very important to study the elements involved in elicitor-responsive activation of transcription of genes and thereby elucidate defense mechanisms against pathogen invasion. Those elements are closely associated with the establishment of inducible defense response (Yamamoto *et al.*, 2004), such as a potato gene encoding a WRKY transcription factor which is induced in interactions with *Erwinia carotovora* (Dellagi *et al.*, 2000). It has been shown that a potato WRKY-like gene is induced by the treatment of potato leaves with *Erwinia carotovora* culture filtrate and pectate lyases (Dellagi *et al.*, 2000). *WRKY* genes encode transcription regulators with diverse functions that have been important for plant development and defense responses (Ross *et al.*, 2007).

This investigation presents the expression pattern of *WRKY1* gene in potato cultivars using the RT-PCR strategy, for the resistance against *Erwinia carotovora* infection which causes soft rot, along with the bioinformatics analysis data such as phylogenetic relationships and the annotation of *WRKY* genes. Our studies revealed aWRKY-like gene in tomato.

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MATERIALS AND METHODS

Plant and Bacterial Materials:

Nicola and Diamant potato cultivars were obtained from the International Potato Center (CIP) Kafr El-Zayat, Egypt. The *Erwinia carotovora* strain used in this study was obtained from the Department of Plant Pathology, Alexandria University.

Treatment of Potato Leaves with *Erwinia carotovora*:

Bacteria were grown in Luria- Bertani medium (LB medium) (Miller, 1972) supplemented with ampicillin (100 µg/ml⁻¹). Bacterial culture filtrate (CF) was obtained by filter sterilization of the supernatant from *E. carotovora* subsp. *atroseptica* cultures. Plants grown in the glasshouse of each cultivar were sprayed with a 10⁸ CFU/ml of *Erwinia carotovora* in 10 mM MgSO₄ as described by Dellagi *et al.* (2000) and Norman *et al.*, (1999). Control treatments were sprayed with 10 mM MgSO₄ only. Leaves from each treatment were sampled at 1, 3, 5, 7, 9 and 12 (hpi) hour post- inoculation (Diatchenko *et al.*, 1996).

Reverse Transcription PCR (RT-PCR):

Isolation of Total RNA from CF-treated Potato Leaves:

Total RNA was isolated from CF-treated leaves using Biozol reagent (BioFlux). One hundred mg of the leaves were ground to a powder in a small mortar with liquid nitrogen and homogenized in 1 ml of Biozol solution. The lysate was transferred to a 1.5 ml tube and let stand for 5 min. at room temperature. Two hundred µl of chloroform was added and the mixture was centrifuged for 15 min. at 12,000 g. The upper aqueous phase was transferred to a new tube, and the volume was measured. While doing so, an equal volume of isopropanol was added, mixed, and left for 10 min. at room temperature. The precipitated RNA was collected by centrifugation for 10 min. at 12,000 g. The pellet was air dried for 10-15 min. and dissolved in sterilized distilled water.

Removal of DNA:

DNA contamination was removed from RNA preparation using DNase I. The RNA, 10 to 50 µg, was transferred to a 1.5 ml tube. DNase I reaction buffer, 5.7 µl, was added followed by the addition of 1 µl of DNase I (10U). The solution was mixed well and incubated for 30 min. at 37°C. Forty µl phenol: chloroform: isoamyl alcohol (25:24:1) was added, vortexed for 30 seconds, and the mixture was left for 10 min. on ice. The mixture was centrifuged for 5 min. at 12,000 g. The upper aqueous phase was transferred to a new tube and 5 µl of 3 M sodium acetate was added to the aqueous phase followed by 200 µl of ethanol. Samples were incubated for 1hr at -20°C and centrifuged for 10 min. The supernatant was removed and the pellet was washed with 0.5 ml of 70% ethanol. The final pellet was dissolved in distilled sterilized water. RNA was electrophoresed to check the quality of the RNA according to Sambrook *et al.* (1989).

Reverse Transcription Polymerase Chain Reaction (RT- PCR) Analysis:

Reverse transcription was done in 20µl total volume in RT buffer, 1mM dNTPs, 0.5 µg Poly-T Primer, and 0.2 µg of total RNA. The following PCR program was used to synthesize the first strand cDNA: 37°C for 5 min., 42°C for 60 min., and 70°C for 10 min. After the 5 min. at 37°C, the program was paused, 1 µl (200U) of M-MuLV reverse transcriptase (Fermentase) was added to each tube, and the incubation was continued. At the end of the reverse transcription, tubes were set on ice or stored at -20°C for later use. RNA synthesis of cDNA and PCR amplification were performed as described by Frohman *et al.* (1989). PCR was used to amplify specific regions of the *WRKYI* gene using specific primers.

The primers' sequence of *StWRKY* (Dellagi *et al.*, 2000) is as follows:

- Forward primer: 5' ACGTTTAAACCATTCTCAGAAATAGC 3'
- Reverse primer: 5' ACCTCGAGATACATGCCTTACTAGGC 3'
- PCR products were electrophoresed on 1.5 % agarose gel at 80 V for 2hr.

Phylogenetic Analysis:

The nucleotide sequences for *WRKY* genes of different plant species (Table 1) were obtained from the GenBank database. Phylogenetic analysis was carried out by neighbor-joining method using the MEGA package (Kumar *et al.*, 2004). In MEGA the gap extension and gap opening penalties were set at 6.66 and 15, respectively.

Table 1: Abbreviations for plant species referred in phylogenetic analysis.

Species	Abbreviation	Species	Abbreviation
<i>Arabidopsis thaliana</i>	At	<i>Nicotiana tabacum</i>	Nt
<i>Capsicum annuum</i>	Ca	<i>Oryza sativa</i>	Os
<i>Gossypium arboreum</i>	Ga	<i>Petroselinum crispum</i>	Pc
<i>Gossypium hirsutum</i>	Gh	<i>Solanum tuberosum</i>	St
<i>Hordeum vulgare</i>	Hv	<i>Triticum aestivum</i>	Ta
<i>Lolium perenne</i>	Lp	<i>Zea mays</i>	Zm

Database Search and Gene Annotation:

Tomato WRKY-like gene was obtained by multiple BLAST searches of GenBank (<http://www.ncbi.nlm.nih.gov>) database using the *WRKY* potato sequences. Nonredundant (nr) database was searched using blastn algorithm (Altschul *et al.*, 1990). The significantly aligned sequences ($E < 10^{-4}$) were then subject to further analysis. Splice sites were predicted by neural network algorithms using NetPlantGene Server (<http://www.cbs.dtu.dk/services/NetPGene>); (Hebsgaard *et al.*, 1996). Translation initiation sites were predicted by artificial neural network using NetStart server (<http://www.cbs.dtu.dk/services/NetStart>); (Pedersen and Nielsen, 1997). The SIWRKY-like gene was predicted with the stand-alone version of GENSCAN program (<http://genes.mit.edu/GENSCAN.html>); (Burge and Karlin, 1997). In this study the Arabidopsis option with default parameters was used. As GENSCAN results are based on gene prediction algorithms only, additional sequence annotations were carried out to identify sequence similarities with those in the public domain. The predicted genes were compared with their ESTs (Expressed Sequence Tags) in GenBank. An EST-hit was accepted if the identity of the alignment was = 100%. The alignment with $E > 0.0$ was discarded.

RESULT AND DISCUSSIONS

In the present study, we described the differential expression of a potato gene encoding a putative member of the WRKY DNA-binding protein family, *StWRKY1*, which is induced during the compatible interaction with the soft rot bacterial pathogen *Erwinia carotovora* subsp. *Atroseptica*, by using RT-PCR. In addition, we performed bioinformatics analysis on *WRKY* genes.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis:

Leaves of Nicola and Diamant potato cultivars were collected for RNA isolation at the time points indicated, before and after the inoculation (post-inoculated) with CF *E. carotovora* subsp. *atroseptica*. *StWRKY1* expression was analysed by RT-PCR using specific primers, whose design is based on the sequence encoding *StWRKY1*. This was performed in order to study earlier time points in the potato-*E. carotovora* interaction and to detect if the *StWRKY1* expression is detectable before 12 hour of post-inoculation (hpi). RNA was extracted before inoculation and considered as control and the extracts were performed after inoculation at 1, 3, 5, 7, 9 and 12 hpi. Results show that the level of *StWRKY1* transcripts increased progressively from 3 hpi and reached a maximum at 12 hpi in Nicola. While transcript accumulation commenced at 7 hpi and reached a maximum at 12 hpi in Diamant (Fig. 1).

Phylogenetic Relationship of WRKY Genes:

The phylogenetic tree of 54 *WRKY* genes was formed according to the neighborjoining method, with the sequences of *WRKY69* gene of *Arabidopsis thaliana* as the outgroup (Fig. 2). Nucleotide sequences of *WRKY* genes for diverse plant species were obtained from the GenBank. The lineages were grouped into several clusters. The phylogenetic analysis showed that *WRKY* genes of *Solanum tuberosum* are grouped among three different clusters. *StWRKY4* and *StWRKY1* are grouped in one cluster, while *StWRKY6*, *StWRKY2* and *StWRKY3* are grouped in another cluster. *StWRKY5* gene is included in a different cluster. *WRKY4* and *WRKY6* genes of *Solanum tuberosum* are claded with *WRKY57* and *WRKY38* genes of *Arabidopsis thaliana*, respectively. In addition, *StWRKY3* gene is phylogenetically close to *AtWRKY11*, *AtWRKY17* and *NtWRKY3*. Furthermore, *WRKY2* and *WRKY5* genes of *Solanum tuberosum* are phylogenetically close to *WRKY3* and *WRKY5* genes of *Petroselinum crispum*, respectively. *WRKY* genes of *Arabidopsis thaliana* and *Petroselinum crispum* are phylogenetically close to each other as it is revealed by *AtWRKY60* and *PcWRKY4* genes. *WRKY1* gene of *Solanum tuberosum* is phylogenetically close to *WRKY-b* gene of *Capsicum annuum*. *CaWRKY-a* is phylogenetically close to *NtWRKY1* and *NtWRKY6*, while *CaWRKY-c* is phylogenetically close to *NtWRKY4* and *NtWRKY9*. The phylogenetic tree placed *WRKY* sequences of *Solanum tuberosum* and *Triticum aestivum* close to each other. The constructed tree, grouped *StWRKY1* with *TaWRKY2-a* and *TaWRKY2-b* in the same cluster.

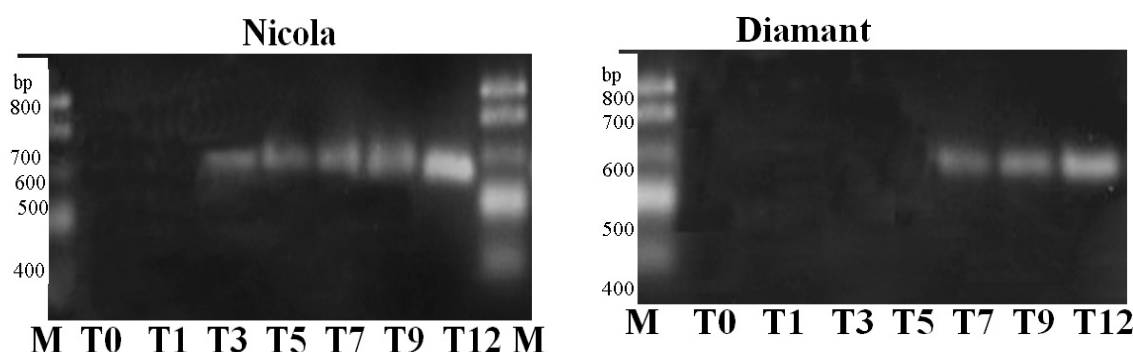


Fig. 1: RT-PCR involving *StWRKY1* gene (0.6 kb) for potato cultivars.

- * T0 = Control (without infection).
- * T1 = One hour post-inoculation.
- * T3 = Three hours post-inoculation.
- * T5 = Five hours post-inoculation.
- * T7 = Seven hours post-inoculation.
- * T9 = Nine hours post-inoculation.
- * T12 = Twelve hours post-inoculation.

Annotation and Prediction of a Novel SIWRKY-like Gene:

A bioinformatic analysis was carried out to identify *WRKY* genes in plants using the publicly available genomic sequences that were obtained from the GenBank. To identify *WRKY* genes from plants, we BLASTed potato *WRKY* genes against 'nr' and dbEST datasets. Alignment searches retrieved similarities of *StWRKY3* gene to genomic DNA sequences of *Solanum lycopersicum* (clone: C08HBa0216M19; acc. no. AP009283) as shown in Table (2). Some sequences were excluded from the analysis due to their low similarities with the *StWRKY* genes used. Similarity was determined using the BLAST program.

NetPlantGene predictions for the positions of splice sites in SIWRKY-like gene are given in Table (3) as first and last nucleotide in the intron.

NetStart predicted the translation start site)ATG(of SIWRKY-like gene at the position 54132 with score 0.795. Gene prediction was carried out by the gene-finding program GENSCAN. The predicted structure of the coding sequences has three exons (Table 4). The promoter includes TATA box / initiation site. The poly-A signal includes the consensus: AATAAA.

Table 2: Alignments of *StWRKY3* sequence with the sequence of tomato clone: C08HBa0216M19. Alignment was produced by the BLAST program.

Alignments	Potato	Tomato	Identities
Alignment 1	1-649	54125-54761	90%
Alignment 2	643-775	54843-54975	98%
Alignment 3	773-1132	55063-55419	89%

Table 3: Position of the splice site using NetPlantGene.

Position	Confidence
54761	0.93
54848	0.88
54975	1.00
55064	0.94

Table 4: The predicted locations, exon-intron structures and probability scores of SIWRKY-like gene using GENSCAN.

Type	Begin	End	Probability
Promoter	54066	54105	
Initial exon	54132	54760	0.994
Internal exon	54849	54974	0.996
Terminal exon	55065	55296	0.915
poly-A signal	56384	56389	

Additional information, such as homology to EST hits, was determined by sequence database searches. We searched the EST database with blastn using the sequences of clone: C08HBa0216M19 as the query. The annotation result was supported when ESTs (DB727022, DB687452, DB695230 and DB700341) covering

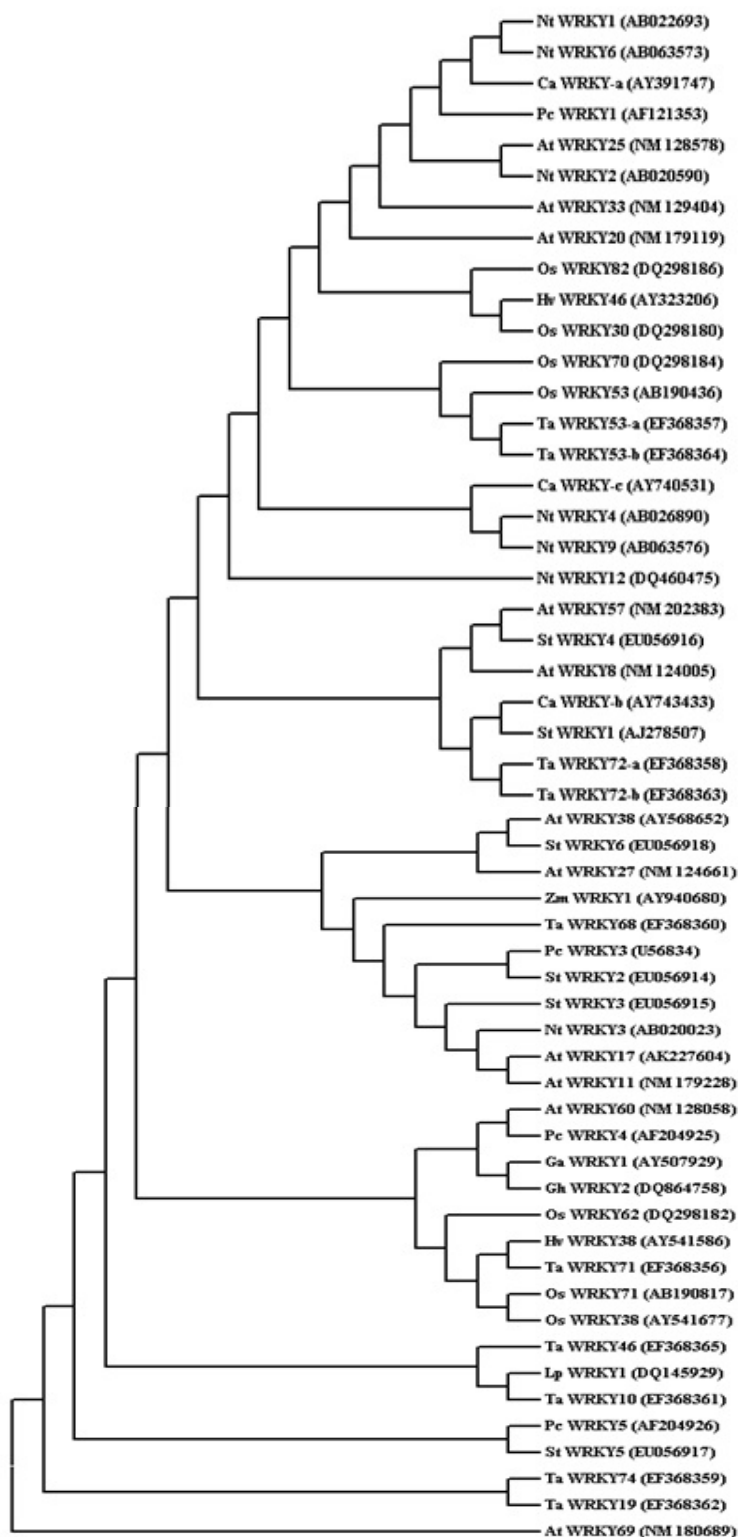


Fig. 2: Phylogenetic tree of *WRKY* sequences in 12 plant species. The phylogenetic tree was depicted by the *MEGA* program and constructed by the neighbor-joining method. The two letters at the beginning of *WRKY* gene names represents the initials of plant species. GenBank accession numbers (acc no) are given in brackets.

the SIWRKY-like gene, were found. The *SIWRKY*-like gene was located on chromosome 8 of tomato genome, as the source of the clone is chromosome 8. The predicted gene structure of SIWRKY-like gene is supported by the results of NetPlantGene and NetStart. As a consequence of this annotation analysis, we identified a WRKY-like gene in tomato.

Discussion:

The present study includes an attempt for characterization of a potato gene that is up regulated in leaves after inoculation with *E. carotovora* subsp. *atroseptica*. *StWRKY1* encodes a putative group 2 member of the WRKY DNA binding protein family (Rushton *et al.*, 1996). WRKYs are elicitor-induced proteins that bind to the W box in the promoters of PR genes (Rushton and Somssich 1998) and appear to be responsible for upregulation of these genes (Rushton *et al.*, 1996). The results indicated that Diamant responds to the inoculation later than Nicola, which is more resistant to the bacteria. This is indicated in the expression of *StWRKY1*. The two cultivars, the resistant and susceptible, have the WRKY gene. However, in the resistant cultivar, the gene is expressed in response to infection earlier than in the susceptible one. Such results may disclose different signal transduction pathway components in the two cultivars that control the expression of *StWRKY1*. In addition, the promoter elements that represent the target for *WRKY1* (W-box in PR gene promoter) may differ in location and/or number. This could lead to a delay or fast expression response for PR proteins. Similarly, *WRKY1* promoter may have different combinations of cis/trans acting elements in both cultivars, which may cause differential *WRKY1* expression.

Functional studies have only been performed in *A. thaliana* for a few WRKY transcription factors. Two Arabidopsis WRKY factors (AtWRKY22 and AtWRKY29) have been identified as important downstream compounds of the MAPK pathway that confer resistance to both bacterial and fungal pathogens (Asai *et al.*, 2002). Overexpression of *AtWRKY70* increased resistance to virulent pathogens in Arabidopsis (Li *et al.*, 2004 and 2006). A WRKY transcription factor (OsWRKY71) was overexpressed in rice, and transgenic plants showed an enhanced resistance to virulent bacteria (Liu *et al.*, 2007). Overexpression of *VvWRKY1* in tobacco, and transgenic plants exhibited reduced susceptibility to various fungi (Marchive *et al.*, 2007). Our results for the *WRKY* phylogenetics are in good agreement with what others have published earlier. The phylogenetic tree showed that some of potato *WRKY* genes are grouped with the *WRKY* genes of Arabidopsis. This is in agreement with the phylogenetic trees of Dellagi *et al.* (2000) and Lambais (2001). *StWRKY1* was grouped with *AtWRKY75*; similar results have been shown with a phylogenetic tree constructed by Marchive *et al.* (2007). *OsWRKY70* is phylogenetically close to *OsWRKY53*. These findings are in agreement with the phylogenetic tree reported by Ross *et al.* (2007). The constructed phylogenetic tree indicated that *NtWRKY3* was grouped with *AtWRKY11* and *AtWRKY17* genes in the same cluster. Park *et al.* (2005) found similar results.

In this study a WRKY-like gene of tomato was identified. We aligned the sequences in the dataset of GenBank with potato *WRKY* genes using BLAST program. GENSCAN was used to predict the structure of the SIWRKY-like gene. The dbEST dataset was also used to survey the expressed *WRKY* genes in plant species. Comparative genetic mapping experiments yielded evidence for the conservation of gene repertoire and colinear chromosome segments for related species. An extensive conservation of marker order was found for the tomato and potato chromosomes. Five chromosomal inversions could explain differences in marker organization (Tanksley *et al.* 1992). No doubt that those studies of WRKY transcription factor genes will not only further our understanding of the fundamental processes that are controlled by these genes which are important in pathogen resistance. Thus agricultural productivity will be enhanced.

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