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Identification of transcription factors in tomato, potentially related to early blight resistance at invasion in host tissue, using microarray expression profiling

Priti Upadhyay [a,b,c,*], Showkat Hussain Ganie [a], Ashutosh Rai [b], Major Singh [b], Brajesh Sinha [c]

[a] University of Delhi, North Campus, Delhi, India
[b] Indian Institute of Vegetable Research Varanasi, Uttar Pradesh, India
[c] Banaras Hindu University Varanasi, Uttar Pradesh, India

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ABSTRACT

Tomato early blight is an important threat due to its capacity to reduce the production in all major tomato producing areas. Molecular mechanisms underlying resistance to the causal organism are not well known. Therefore, we aimed to study tomato – *Alternaria solani* system to search the transcription factors and pathways which, are responsible for resistance to this fungi using, affymetrix gene chip for tomato. three hundred ninety five transcription factors were found to be differentially expressed at 24 h after inoculation with *A. solani* in the resistant genotype, EC-520,061, of tomato. Also, Zinc Finger Proteins, Ribosomal binding unit S4 and Auxin responsive transcription factors were found to play significant role in resistance. Their expression has enhanced the pathogenesis related proteins and also other proteins as well, which, have direct role in stopping the penetration of mycelia in host plant.

1. Introduction

Early blight (EB) of tomato caused by *Alternaria solani*, a fungi, is economically the most important disease of tomatoes in USA, Australia, Israel, UK and India where, significant reductions in yield (35 to 78%) have been observed (Datar and Mayee, 1981; Jones et al., 1993). Whereas survey reports confirm that no resistant genotype for EB has been found yet, in different screening experiments few genotypes belonging to *S. hybrochaites* and *S. arcanum* have shown moderate to high resistance; however (Chaerani et al., 2007; Upadhyay et al., 2009). Tomato accessions possessing resistance to this microbe mostly belong to wild types for example, *S. arcanum, S. peruvianum*, *S. neorickii* and *S. chilense*. Accessions of *S. habrochaites* were found to possess both, susceptibility and resistance to EB. Lines resulting from crosses of tomato with these wild species do not have satisfying crop qualities.

EB disease control is therefore, mainly attempted with chemical protective agents. However, such agents do not always prevent the infestation of fruits thus, severe losses can still occur. Moreover, long-term effects of these chemicals e.g. fungicides on humans are still unknown though, they may contribute toward medicament resistance; with life-threatening consequences. Further, these fungicides may cause mutations by reprogramming normal genes or permanently silencing them, the effects may therefore, last for several generations (Weinhold, 2006).

EB resistance is a quantitatively governed genetic trait (Foolad et al., 2002), thereby making selection more difficult; compared to qualitative traits (Moody et al., 2003; Brown and Caligari, 2011). In order to understand genetic control of EB resistance and to facilitate its introgression in tomato, molecular markers and QTL analysis have been carried out. With discoveries of new technologies, researches in this field have progressed with the use of functional genomics tools to ascertain the mechanism of resistance to EB. Searching and determining the function of a set of resistance genes, helps us understand the pathways leading to the resistant reaction in a host plant.

Transcription factor (TF) is a molecule that controls the activity of a gene by determining whether the gene is up or down regulated. TFs control when, where, and how efficiently, RNA polymerases function and the proteins get translated from these transcribed RNAs. There are reports of genome-wide identification and phylogenetic analyses of the AP2/ERF TFs in plant genomes including *Arabidopsis*, rice, soybean, grapevine and tomato (Nakano et al., 2006; Zhang et al., 2008; Licausi et al., 2010; Sharma et al., 2010). Plants try to defend themselves against a wide array of microbes, this interaction may, or may not be

* Corresponding author at: University of Delhi, North Campus, Delhi, India.
E-mail address: priti.iasbhu@gmail.com (P. Upadhyay).

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pathogenic. The activation of defense responses in plants is initiated by, host recognition of primary pathogen-derived elicitors (Yang et al., 1997; Kim et al., 2002). Recognition of pathogen elicitors by plant cell receptors, activates signal transduction pathways which may involve: protein phosphorylation, ion fluxes, reactive oxygen species (ROS), secondary endogenous signals and the expression of TFs, and defense genes (Yang et al., 1997; Park et al., 2005). Responses of plants against microbes are regulated by multiple signaling pathways with, a significant overlap between gene expressions. The present study was aimed to see the transcription factors differentially expressed in resistant tomato accession at early stage of *A. solani* infection and observe the role of various TFs, in susceptible and resistant accessions.

2. Materials and Methods

2.1. Plant material

Plant materials utilized for this study were variety CO-3 and the accession EC-520,061, as susceptible and resistant respectively. The basis of this categorisation was the performance of the above mentioned genotypes against *A. solani*, during a screening experiment conducted earlier (Upadhyay et al., 2009). Seeds of the variety and the accession were provided by Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh, India. The plants were grown, in pots, in a growth chamber under temperature–controlled condition (25 °C).

2.1.1. Inoculum

Inoculum utilized for implanting the host (tomato) plants consisted of the Varanasi isolate of *A. solani* that, was isolated from tomato leaves showing EB symptoms. The culture was propagated on Potato Dextrose Agar (PDA) in 90-mm petri dishes. These dishes were incubated at 25 °C under a cool-white fluorescent diurnal light with a 12 h photoperiod for 15 d.

After fifteen days, culture was scraped and macerated together with sterile pestle and mortar. Although the culture was free from conidia, thickening of conidiogenous hyphae and chlamydospore like structures were observed. Before the formation of these structures, cultures did not possess their usual aggressiveness and the potential for implantation. One and half month old tomato plants were implanted by spraying a suspension solution, 157 cfuml^{-1} spore load, of *A. solani*; under control condition. The implanted plants, in pots, were kept at 28 °C and more than 95% humidity, to create proper epiphytotic conditions. Plants sprayed with sterile distilled water were treated as control.

After 24 hai of implantation of plants with *A. solani* when, leaves were not curled and the only symptom detected was - initial appearance of black spots, leaves were sampled for RNA isolation. Control samples were also collected for RNA isolation and immediately stored in liquid nitrogen at −80 °C. While germination of spores start and reaches its maximum level between 6 and 12 h of inoculation, interaction with plant cells begin at the stage when penetration starts. This stage is very important as it indicates the time when the spores of *A. solani* start penetrating the leaf tissue (Dita et al., 2007) besides, it is one of the earliest cellular stages where, changes in the host tissues start in response to the fungi.

2.1.2. Microarray Experiment

RNA isolation was done using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The RNA was quantified using Nanodrop spectrophotometer and its quality was checked on 1% agarose gel containing formaldehyde.

Double stranded cDNA synthesis, *in vitro* transcription to synthesize biotin labeled cRNA, purification and fragmentation of cRNA, and hybridization of arrays were performed according to Affymetrix technical manual. The Affymetrix GeneChip, Tomato Genome Array, contains 10,038 probe sets representing about 4600 unigenes. Hybridized chips were washed, stained and scanned using, GeneChip scanner to generate the CEL files.

These CEL files were imported into GeneSpring GX v12 (Agilent Technologies). Signal intensities were recorded for all probe sets. The data has been deposited at NCBI (http://www.ncbi.nlm.nih.gov), with, an accession number - GSE71428. Signal intensities were normalized using, Robust Multi-array Average (RMA) algorithm (Irizarry et al., 2003). The Principal Component Analysis (PCA), GeneSpring GX 10.1, established the close location of all three biological replicates. A high correlation coefficient was observed among the three replicated samples indicating, less genetic background noise. It shows that the molecular fluctuations inherent in samples that cause random switching of genes on and off are very less (Hana and Weissman, 2011). To correct the variability in the normalized expression values, probe sets with coefficient of variation <50% were retained while, rest were discarded.

2.1.3. Functional annotation of the differentially expressed probe sets

Tables of significant transcripts were generated at p values <0.05 and fold change (FC) values >2.0. For annotation of transcripts an annotated probe file was referred, that, was generated at Cornell University, USA: (ted.bti.cornell.edu/*TFGD/array/Affy_probe_annotation.xls)* and NCBI website. Among those significantly differentially expressed transcripts, we selected the transcripts, which functions as regulators of transcription.

2.2. Screening of TFs from microarray data

Tomato TFs analyzed in this experiment have been described in the TF database (http://planttfdb.cbi.pku.edu.cn/). According to the annotation of Affymetrix genome microarray, we screened for TF genes that were differentially induced or repressed after *A. solani* implantation in CO-3 and EC-520,061 with, a FC value >2.0 and a *P*-value <0.05(Suppl Table 1). The results have been shown as a (http://bioinformatics.psb.ugent.be/webtools/Venn/ *website*). Further probe filtering for TF genes that were significantly induced by *A. solani* or constitutively expressed in the resistant accession, RHT was performed with the FC tool in GeneSpring - GX 11.5.

2.2.1. Quantitative Real Time PCR validation

Total RNA was extracted from leaves of the two genotypes: CO-3 and EC-520,061 after 24 h of implantation, in three biological replications. First strand cDNA for each sample was synthesized using, SuperscriptTMIII first-strand synthesis system for RT- PCR (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. Primers for quantitative real-time RT-PCR were designed using, web based primer designing tool from IDT (http://eu.idtdna.com/Scitools/Applications/Primerquest/default.aspx). The sequences of all primers are enlisted in Table 1.

Quantitative real time PCR was performed in three biological replications using, SYBR Green (Qiagen, USA) fluorescence dye and, analyzed by iQ-SYBR Green Supermix (Bio-Rad, CA, USA) according to the manufacturer's protocols on iQ5 thermo cycler (Bio-Rad, CA, USA) with, iQ5Optical System Software version 2.0 (Bio-Rad, CA, USA). To normalize the target gene expression, differences between the C_T of the target gene and the C_T of Actin (constitutive control) for the respective template were calculated (ΔC_T value). To calculate FCs in gene expression, the ΔC_T value was calculated as follows: $\Delta C_T = C_T$ (target gene)–C_T (constitutive control gene). Relative transcript levels were calculated as: $1000 \times 2–\Delta C_T$.

3. Results

3.1. Potential regulator genes associate with EB resistance

For understanding the mechanism behind host responses to *A. solani*, in the resistant and susceptible genotypes EC-520,061 and