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Differential gene expression for bacterial wilt incidence in tomato (*Solanum lycopersicum* L.) revealed by cDNA-AFLP analysis

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ABSTRACT

Bacterial wilt (BW) incited by *Ralstonia solanacearum* (Smith) – a soil-borne bacterium is a devastating disease of tomato cultivation in the tropical and subtropical humid regions of the world. The disease is widespread in India and is the main limiting factor for tomato cultivation in Kerala. Experiments were designed to trigger the pathogen-challenged disease responses in both susceptible (H24) and resistant (Anagha) tomato genotypes and monitor the expression of stress induced genes or gene fragments at the transcript level. cDNA-AFLP (Amplified Fragment Length Polymorphism) analysis was performed with the cDNA synthesized from resistant and susceptible genotypes at second and fifth days after inoculation. A total of 763 transcript-derived fragments (TDFs) were analyzed from 21 primer sets and 58 TDFs were detected to be differentially expressed during pathogen challenge. Upon cloning and sequence analysis of these differentially expressed TDFs, two of them showed homology to plant retrotransposons-putative gag-pol polyproteins and three-showed homology to aspartate kinase/homoserine dehydrogenase. Two TDFs have sequence identity to genes known to have function in plant defense. Three-showed homology to Secretin proteins. The results indicated the involvement of several such factors in plant–pathogen interactions. The expression of transcripts was further validated through quantitative real-time PCR. The present study would be helpful in elucidating the molecular basis of the infection process and identifying the defense genes that can be targeted for incorporating bacterial wilt resistance.

1. Introduction

Plant disease resistance and susceptibility are governed by host–pathogen interaction that results in a complex exchange of signals and responses under a given environmental condition [1]. The key difference between resistant and susceptible genotype is the timely recognition of the invading pathogen and the rapid and effective activation of host defense mechanisms. The activation of defense responses in plants is initiated by host recognition of pathogen-encoded molecules called elicitors [2]. During the initial steps of association, when a plant recognizes a potentially infectious pathogen, local defense responses aid to sequester the pathogen away from non-infected plant tissue. Events of recognition of defense by a host plant to its bacterial pathogen and ability of the pathogen to overcome the plant's defenses imply a complex, dynamic and interactive molecular network. Induction of these molecular responses necessitates up- and down-regulation of specific genes [3]. Differential gene expression analysis in plant–pathogen interactions has resulted in identification of several defense-related transcripts [4,5].

Tomato (*Solanum lycopersicum* L.) is considered as one of the most important and widely consumed vegetable crop in the world, second to potato. India is the sixth largest producer of tomato in the world with an area of 0.50 million hectares under cultivation. One of the major constraints of tomato production in many tropical and subtropical countries is the incidence of bacterial wilt caused by *Ralstonia solanacearum* (Smith) [6,7] – a soil-borne β-proteobacterium. *Ralstonia* has an extremely wide host range and affects more than 200 plant species [8]. In the natural process of infection, the bacterium can survive over long periods in humid soil, water and among the roots of non-susceptible plant hosts. During bacterial wilt pathogenesis, the bacterium enter the roots at sites of secondary root emergence [9,10] or at root tips [11] and then rapidly and effectively invades the xylem vessels of roots and disseminates into the stem where it multiplies causing wilt symptoms [12]. The disease is widespread in most of the tomato

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growing states of India causing yield losses to the extent of 90%. Kerala being characterized by warm, humid tropical climate and acidic soil is a hot spot for bacterial wilt disease. Chemical control for soil-borne disease is usually unsuccessful and furthermore no commercial pesticides are available for controlling *R. solanacearum* except the application of chemical fumigants. Thus, host plant resistance has been a major strategy for managing bacterial wilt. Use of resistant cultivars is one of the most practical and cost-efficient strategies for managing bacterial wilt. However, the efficiency of resistant tomato cultivars in wilt management is limited by various factors including, (i) pathogenic variability in the natural populations [13] and location-specific occurrence of races [14] which causes resistant cultivar to lose resistance over a period of time, (ii) susceptibility which limits exploitation of useful characters in certain varieties (iii) existence of specific races, which slows down progress in breeding program [15].

Unraveling the mechanism of wilt tolerance would be of great relevance in tomato, since resistance is available in wild relatives and few non-popular varieties. The identification of host genes, involved in defense responses, is important to understand plant resistance mechanisms against phytopathogens [16,17]. The cDNA-AFLP technique was successfully applied for identifying defense genes in crops such as Barley for powdery mildew resistance [18]. The differential gene expression analysis using cDNA AFLP is a highly reproducible technique and can be used to screen systematically a large number of differentially expressed cDNAs [19–22]. The sensitivity and specificity of the method allows detecting even the poorly expressed genes and distinguishes between homologous sequences [23]. The technique greatly reduces the number of false positives by ligating adapter molecules to the digested double-stranded cDNA. Furthermore, cDNA AFLP is also a flexile tool that can be used even when genomic sequence information available is not complete [24]. In contrast to other fingerprinting techniques the cDNA-AFLP analysis possesses several advantages: It can detect active regions of the genome and give information about DNA regions expressed after pathogen attack making it possible to identify genes related to pathogen defense or resistance. It is also possible to survey transcriptional changes with no prior assumptions about which genes are induced or repressed. Hence, cDNA AFLP has become one of the techniques for gene expression analysis in plants [25].

In the present study, genes differentially expressed in interactions between tomato genotypes and *R. solanacearum* were identified using cDNA AFLP. Earlier attempts in analyzing the molecular profiles of different resistant and susceptible genotypes with different molecular marker techniques like RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats) and AFLP did not yield any clear demarcation for resistance/susceptibility [26] within the cultivated genotypes of tomato. This highlights the fact that the resistance/tolerance might be due to variations in gene expression rather than the difference at the sequence level. Here we report, comparison of transcript profiles generated from the tomato genotypes, viz., Anagha (resistant) and H24 (susceptible) at second and fifth day after inoculation by subjecting them to cDNA-AFLP analysis. The quantitative real-time PCR (RT-PCR) analysis was used to validate the expression pattern of some important up-regulated genes. The present study would be helpful in elucidating the molecular basis of defense mechanism in tomato with respect to bacterial wilt incidence.

2. Materials used

2.1. Tomato genotypes

Seedlings of the selected tomato genotypes were raised in sterile sand and one month old seedlings transplanted to earthen pots (12 inch dia) filled with sterile potting mixture. The planting medium was sterilized with 40% formaldehyde solution in order to avoid bacterial inoculum at the time of planting. Fresh bacterial ooze collected from wilted tomato plants in the open field was used as inoculum for infecting the experimental plants. The potted plants (30 for each variety) were maintained in open conditions and irrigated once in a day. Ten days after transplanting, the seedlings (15 plants in each genotype) were inoculated with the fresh bacterial ooze using soil drenching and wounding inoculation technique as suggested by Hussain and Kelman [27]. The leaf samples were collected from resistant and susceptible genotypes at 0, 2, and 5 days after inoculation, for cDNA-AFLP analysis.

2.2. RNA extraction and cDNA synthesis

Young, tender leaves were collected between 6 am and 8 am for RNA isolation. To eliminate all residual DNA contaminations, the leaf samples were washed with DEPC treated water prior to RNA isolation. Total RNA was isolated from about 100 mg leaves, following the procedure described by Chomczynski and Sacchi [28], using TRIzol (Invitrogen) reagent with some modifications. Quality and quantity was determined on 0.8% agarose gels in MOPS buffer and using a Nanodrop spectrophotometer. cDNA was synthesized using M-MuLV RT-PCR kit (Genei, Bangalore) following the manufactures instruction and the concentration checked in a Nanodrop spectrophotometer.

2.3. cDNA-AFLP analysis

The cDNA-AFLP assay was carried out using AFLP® Analysis System I and AFLP® Starter Primer Kit of Invitrogen, USA. cDNA (25 ng) was subjected to digestion for 2 h at 37 °C with EcoRI/MseI (1.25 U/μl) restriction enzymes and ligated to EcoRI and MseI Adapters. Ligated samples were diluted 10 fold and from this 2.5 μl of diluted cDNA samples were used for each pre-amplification reaction. Twenty cycles of pre-amplification with pre-amp primer mix (Invitrogen) were performed in Eppendorf master cycler with the PCR program – 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. The amplified products were then diluted to 50 fold with sterile water for selective amplification. A total of 21 EcoRI/MseI primer combinations were used for further analysis. The primers that were used for selective amplification were, MseI + CNN and EcoRI + NN, in which N correspond to A, G, C, T. Touch down PCR was carried out for selective amplification with different temperature profile (1st cycle – 30 s at 94 °C, 30 s at 65 °C, 60 s at 72 °C; next 13 cycles – the annealing temperature was reduced by 0.7 °C per cycle; next 23 cycles – 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C). Amplicons were separated on 4% denaturing polyacrylamide gel run at 1200 V and 100 W for 3 h and visualized after silver staining [29]. The transcripts developed on the gel were scored for their presence or absence in the healthy and susceptible genotypes at different stages of infection. The differentially expressed transcripts were detected for elution and further analysis.

2.4. Isolation and cloning of transcript-derived fragments

The polymorphic TDFs were excised separately from the gel and DNA eluted as per procedure suggested by Wu [30]. The recovered transcripts were reamplified using the same set of selective primers and PCR conditions. The PCR products were further analyzed on 0.8% agarose gel and purified using Axygen Gen elute DNA extraction kit (Axygen Biosciences). The purified TDFs were cloned in pGEMT-easy vector of size 3.0 kb (Promega, USA). Competent *Escherichia coli* (JM 109) cells were prepared using $CaCl_2$ method and transformation carried out with the recombinant pGEMT-easy