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Mechanism of regulation of tomato *TRN1* gene expression in late infection with tomato leaf curl New Delhi virus (ToLCNDV)

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ABSTRACT

Tomato leaf curl disease caused by geminiviruses is manifested by curling and puckering of leaves and thickening of veins, resembling developmental defects. This is probably due to the long-term altered regulation of expression of development related gene(s). Our results show that in the infected leaves the transcript level of TORNADO1 (*SlTRN1*), a gene important for cell expansion and vein formation, increased significantly. *SlTRN1* is transcribed from two start sites. The preferential usage of one start site governs its expression in viral-stressed plants. To investigate the role of specific promoter elements in mediating differential expression of *SlTRN1*, we performed *SlTRN1* promoter analysis. The promoter-regulatory sequences harbor multiple W-boxes. The SlWRKY16 transcription factor actively interacts with one of the W-boxes. WRKY proteins are commonly induced by salicylic acid (SA), and consequently SA treatment increased transcript level of SlWRKY16 and SlTRN1. Further mutational analyses confirmed the role of W-boxes in mediating SlTRN1 induction during ToLCNDV infection or SA treatment. We postulate that the activation of SA pathway during stress-response in tomato induces WRKY16, which in turn modulates transcription of *SlTRN1* gene. This study unravels the mechanism of regulation of a developmental gene during stress-response, which may affect the severity of symptoms.

1. Introduction

Tomato leaf curl virus disease (TLCVD) is one of the major hurdle for tomato cultivation in tropical and subtropical regions of the world. This disease affects plants at any growth stage and almost irrespective of climatic conditions. In severe cases up to 100% yield loss have been reported from India [1] and other parts of the world [2]. In tomato, disease symptoms include upward or inward rolling of margins, interveinal yellowing, vein clearing, enations, crinkling and puckering of the leaves. Infected leaves usually develop thickening of veins and shortening of interveinal distance. Plants become stunted and bushy at late stage of infection and bear few or no fruit.

One of the causal organisms for this disease is the Tomato leaf curl New Delhi virus (ToLCNDV), a member of Geminivirus group. This group of viruses may have monopartite (DNA A) or bipartite (DNA A and DNA B) circular ssDNA genomes. The DNA A component encodes six Open Reading Frames (ORFs) namely, AC1, AC2, AC3, AC4, AV1, and AV2. BC1 and BV1 proteins are expressed from DNA B. Most of the monopartite geminiviruses need a β-satellite for their infectivity. Satellite DNAs are not commonly associated with bipartite geminiviruses [3].

Symptom development by a Geminivirus depends on the plant type infected. For example, ToLCV (Tomato leaf curl virus) can infect both tomato and tobacco; however, in tobacco it produces milder symptoms [4]. Thus, host factors are important determinants of the degree of symptom severity. Several host factors have been implicated as the interacting partner for virus proteins and having role in symptom severity. AL2 protein, a silencing suppressor, from Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV) interacts with TIFY4B, a cell cycle regulator of different dicotyledonus plants. AtTIFY4B overexpression delays symptom development probably due to the inhibition of cell cycle, which also affect viral replication. The virus may counteract this host defense response via interaction with and AL2-mediated capturing of TIFY4B protein [5]. Another host factor, SlSnRK1 (*Solanum lycopersicum* Sucrose-Nonfermenting 1-related kinase) could inhibit β-satellite mediated symptom manifestation and virus replication by specific phosphorylation of the βC1 protein [6]. The nuclear receptor karyopherin α1 mediates viral CP nuclear localization and prevention of its interaction with CP results in symptom-less mutant of TYLCV (tomato yellow leaf curl virus) [7]. Interaction of C4 gene of ToLCV-Australia virus and

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V2 protein of TYLCV (Tomato yellow leaf curl virus) with tomato shaggy-like Kinase (SlSK) and SlSGS3 respectively, was required for suppressing the virus silencing activity of the host and symptom appearance [8,9]. A detailed analysis using Arabidopsis plant depicted that the βC1 protein could interact with AS1 (Asymmetric Leaf 1) to alter leaf development [10]. In addition, host transmembrane transporter (Permease I-like protein), ubiquitin-conjugating enzyme (SlUBC3), acetyltransferase (AtNSI) etc., are also detected as interacting partner of viral proteins and manipulation of expression of these proteins affected symptom development [11–13]. From these results, it is evident that, Geminiviruses have adopted multiple modes of host gene manipulation [14] for its benefit and disease development.

Recent studies also indicate that deregulation of host development related miRNAs during Geminivirus infection might contribute to the developmental defect-like symptoms. V2 gene of monopartite, βC1 of β-satellite and multiple genes of bipartite viruses cause differential regulation of a set of miRNAs [15–17]. Notably, miR159/319 and miR172 level increased with progression of the disease in the tomato cultivar Pusa Ruby [18]. However, explicit link between a particular miRNA, virus and host gene in symptom development is yet to be established.

Different host may react differently to virus proteins. Expression of ToLCNDV nuclear shuttle protein (NSP) in *Nicotiana benthamiana* using a PVX vector resulted in leaf curl symptom; however, expression in tomato resulted in hypersensitive reaction. These findings demonstrate that NSP is a pathogenicity determinant and also a target of host defense mechanism [19]. To understand the host response towards individual viral proteins, six open reading frames of Tomato leaf curl virus (TLCV) was transiently expressed in *Nicotiana* plants [20]. The C4 gene, a cell cycle regulator, produced symptoms resembling leaf curl virus infection in all plants tested and V1 and C3 gene products caused severe stunting only of *N. benthamiana*, indicating that a complex interaction between viral genes and host factors occur during infection [20]. Ectopic expression of BCTV C4 gene in *Arabidopsis* induces procambial cell multiplication followed by vein thickening and severe branching [21].

All these studies suggest that symptom development depended on the modulation of functionality of several host factors [22] and interaction of viral proteins with particular development-related genes in host determined symptom severity. However, a comprehensive analysis of the significance and mechanism of deregulation of genes related to the different aspect of leaf development in symptom appearance is lacking. In addition, not much information is available regarding the cause of abnormal venation development during infection. In this study, we have analyzed the mechanism of regulation of expression of TRN1 gene coding for TORNADO1 protein during infection of tomato. Our data suggest that host cell hormonal response during infection is the probable cause of altered regulation of SlTRN1 expression. Hence, here we have proposed a new mechanism of leaf developmental gene regulation during viral infection that could have severe consequence in host cell physiology and might influence the severity of symptom manifestation.

2. Materials and methods

2.1. Plant material

Tomato (*S. lycopersicum* L.) cultivar Pusa Ruby used in this study. Plants were grown in glass houses (25 °C and natural light), in pots containing Soilrite (Keltech, India) with occasional addition of Suphala fertilizer (N, P and K each at 15% by weight). Similar growth environment also maintained for Tobacco (*Nicotiana tabacum* var. SR1) plants.

2.2. Agroinoculation and agroinfiltration of plant leaves

Agroinoculation technique was used to infect tomato plants with ToLCNDV. *Agrobacterium tumefaciens* LBA4404 strain harbouring clones of ToLCNDV-A (GenBank: DQ629101.1) and ToLCNDV-B (GenBank: DQ169057) genome (LBA4404:ToLCNDV) grown in LB media supplemented with Kanamycin (50 mg/l) and Rifampicin (25 mg/l) at 28 °C by continuous shaking at 200 rpm for 48 h. Cells were harvested by centrifugation. The pellet was resuspended in MES buffer (10 mM MES and 10 mM $MgCl_2$, pH 5.5) supplemented with 100 μM acetosyringone and adjusted to a final OD_{600} of 0.8. Ventral surfaces of top three fully expanded leaves of one-month-old plants were infiltrated at multiple spots, with approximately 100 μl of bacterial suspension in each spot, using 1 ml plastic syringe. Inoculated plants were maintained in glass house until symptoms appeared (~30 days) or as mentioned in individual experiments. Using similar protocol LBA4404 strains harbouring different constructs cloned in binary vectors pCAMBIA1301 or pPZPY112 were agroinfiltrated in tobacco leaves (~45 days old plants, 3rd -5th leaf from apex) for promoter activity assays.

2.3. Leaf imaging

Tomato leaves from both control and infected plants treated with 20% Acetone in Ethanol for 30 min. to remove chlorophyll. Subsequent treatments were with 5% NaOH solution, followed by three washes in water, and 10% Acetic Acid for another 1 h. Finally, the cleared leaves were stored in 100% Ethanol in an airtight container. Leaf images were taken using a molecular imager VersaDoc MP imaging system (Bio-Rad).

2.4. Total DNA isolation and Rolling circle amplification (RCA)

Total DNA was isolated from the leaves of both control and infected plants by Urea-lysis method. In brief, leaf tissues were ground under liquid nitrogen, and the powdered tissues suspended in urea-lysis buffer (0.2 M Tris–HCl pH 8.0, 0.5 M NaCl, 2% SDS, 50 mM EDTA and 7 M Urea; 1 μl/mg tissues) was extracted with an equal volume of Phenol:Chloroform:Iso-amyl alcohol mix (25:24:1). DNA was precipitated with 2.5 volume of cold ethanol from the aqueous phase. Isolated DNA was subjected to RNAse treatment to remove the contaminating RNA and further purification by phenol–chloroform extraction followed by ethanol precipitation. Viral coat protein specific PCR was done (CP-F and CP-R primers, Supplementary Table 1) using these DNA samples to screen infected plants. For further confirmation of virus replication, RCA reaction was carried out with RNA free high quality total DNA. The reaction mix contained 100 ng DNA, 1 mM dNTPs, 1.2 μM Random Hexamer primer, 1 X reaction buffer and 10 U Φ29 DNA polymerase (Thermo Scientific) in 10 μl volume and it was incubated at 30 °C over night. The amplified products were digested with *Eco*RI enzyme (New England Biolabs) to release the monomers from the concatamers of 2.7 kb. The digested products were resolved in 1% agarose gel electrophoresis.

2.5. RNA isolation and cDNA preparation

Total RNA was prepared from 300 mg tissue of systemic infected leaves of ToLCNDV- and mock-infected plants by TRIzol reagent (Invitrogen) following manufacturer's protocol. DNA contamination was removed from the RNA samples using RNase-free DNaseI (20 U/μg of DNA, Fermentas, USA) and RNA was further purified by phenol–chloroform extraction followed by ethanol precipitation using standard protocol. RNA integrity was checked by resolving the isolated RNA in denaturing Formaldehyde-MOPS agarose gel (1.5% agarose, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM