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Differentially expressed genes and temporal and spatial expression of genes during interactions between Mexican lime (*Citrus aurantifolia*) and a severe *Citrus tristeza virus* isolate

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ABSTRACT

Determining transcriptional responses of host plants to virus infection will help understand mechanisms involved in host–virus interactions. In this study, 430 genes differentially regulated by a severe CTV isolate in lime plants were identified by using SSH. The differential expression levels of 25 ESTs in CTV-N21 infected lime were validated by qRT-PCR. The largest set of those genes involved in primary metabolism (26.0%), and then in protein metabolism (15.1%). Our results revealed a large set of genes (51 ESTs) were commonly up-regulated by two severe strains CTV-N21 and CTV-T305 in lime. The quantitative analysis for temporal and spatial transcriptional levels of six genes in lime plants infected by CTV-N21 indicated that the transcriptional levels of those genes fluctuated during CTV infection and differed in different citrus leaves.

1. Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* in the family *Closteroviridae*, is a devastating virus in citrus industry. The virus is phloem-restricted and naturally transmitted by several species of aphids, and disseminated worldwide by infected propagating materials [1]. The genomic positive single stranded RNA (+ssRNA) of CTV is highly divergent [2], which contribute to the differentiations of its pathogenicity in citrus. Many CTV isolates, namely severe strains, are aggressive and can induce the decline and death of citrus trees propagated on sour orange (*Citrus aurantium* L.) rootstock or vein clearing and stem pitting (SP) on Mexican lime (*C. aurantifolia* [Christm.] Swing.). Only a few reported isolates, namely mild strains, induce slight leaf chlorosis or symptomless on Mexican lime [3]. Although the genome structure of CTV has been well characterized, its molecular determinants implicated in symptom development are rarely known. Previous studies suggested that the gene P23 was a potential pathogenicity factor [4,5]. Recently, the determining element of yellow seedling was located at the 3′-terminal region of CTV genomic RNA [6], and genes p13, p18 and p33 were considered to be involved in the determination of its host range and also in stem pitting symptom development [7,8].

The successful infection of a plant virus greatly depends on the complex molecular interactions between the virus and its host [9]. During the whole process of virus–plant interaction, the expressions of host genes are regulated by the virus infection, which then leads to the disease development [10,11]. The transcriptional response of plants to virus infection depends on host species, virus strains [12,13], and also plant tissues [14,15] and virus infection stages [16,17]. The systematic comparison of plant transcriptomes regulated by different viruses or virus strains during a time course [18,19], or in plants showing different resistances [20,21] is expected to underline gene functions involved in responses of host plants to virus infection. Studies of compatible interactions in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* [22] [23], and some economically important plants, including potato [24] and soybean [16], showed that different viruses trigged a wide range of plant gene expression changes [19,25].

Abbreviations: ESTs, expression sequence tags; qRT-PCR, quantitative reverse transcription PCR; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSH, suppression subtractive hybridization.

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The infection of viruses in perennial fruit trees usually causes persistent effect on host plants. The study of compatible interaction between a virus and grapevine revealed that numerous genes involved in a wide spectrum of biological functions, including translation (ribosomal protein etc.), transcription (auxin-responsive factor etc.), transport (ferredoxin-related protein etc.) and metabolism (callose synthase 1 etc.) were induced or repressed in viral infected grapevines [26]. To date, the most citrus cultivars are susceptible to the infection of severe CTV strains. Although previous studies showed that infections of both mild and severe CTV strains caused transcriptional changes in lime plants [27,28], the spatial and temporal dynamics of gene expression in citrus is rarely known. In this study, the transcriptional changes in Mexican lime plants caused by a severe CTV isolate were evaluated by using SSH and compared with results obtained from different interaction combinations between CTV strain and its host. We analyzed the expression profiling of six candidate genes involved in energy metabolism and defense reactions during a time course post CTV inoculation and in leaves at different growth stages. Results presented here would contribute to understand the physiological changes and molecular basis of citrus response to viral infection.

2. Materials and methods

2.1. Virus isolates and host plants

The CTV severe isolate CTV-N21, which induces stem pitting in Mexican lime and sweet orange, is a collection from south China [29] and preserved in Mexican lime seedlings. For SSH library construction, one-year old seedlings of Mexican lime were used for hosts of CTV. Each of three plants was graft-inoculated with two buds from N21-infected lime plants. Other three plants mock-inoculated with buds from healthy lime plants were used as control.

For the analysis of the temporal and spatial expression profile of selected genes in response to CTV-N21 infection, nine seedlings of two-year old lime were bud-inoculated with CTV-N21, and other nine plants were mock-inoculated. Apex leaf samples were collected from two branches at the upper and lower positions of tested plants. Sampling was performed from 17 to 177 days post inoculation (dpi) at an interval of 40 days. Each test consisted of three independent biological replicates, and each biological replicate consisted of three tested plants.

All the mock and CTV-N21 inoculated lime plants grown in pots were kept in an insect-proof greenhouse. CTV infection in inoculated plants was confirmed by RT-PCR before sample collection.

2.2. Total RNA and mRNA isolation for subtraction and gene expression analysis in a time course

For subtraction library construction, about 1 g of young shoots with 4–6 leaves from each of mock and CTV-N21 inoculated plants were collected at about one year after inoculation. Total RNA was extracted from each lime plant by using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Equal amount of total RNA from CTV-N21 infected and mock inoculated plants were pooled, respectively, and message RNA (mRNA) was isolated using PolyATtract® mRNA Isolation Systems (Promega, Madison, USA).

For gene expression analysis in a time course of CTV-N21 infection, leaves collected from branches at the same position (upper or lower position) of three individual plants were mixed and used as a biological replicate, respectively. The total RNA was extracted from leaf samples as described above, and digested with *DNase*I(TaKaRa, Dalian, China). About 2 μg total RNA was used in cDNA synthesis with six oligo nucleotide random primer (TaKaRa, Dalian, China).

2.3. PCR-select cDNA subtraction

Isolated mRNA was adjusted to a concentration of 1 μg/μl for PCR-Select cDNA subtraction. In forward subtraction, mRNA obtained from mock-inoculated plants and CTV-N21 inoculated plants were used as driver and tester, respectively. In reverse subtraction, mRNA obtained from mock-inoculated plants was used as a tester and mRNA isolated from CTV-N21 inoculated plants was used as the driver.

Two subtracted cDNA libraries were constructed by using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA). In brief, two μg mRNA from tester and driver samples was used for the first- and second-strand cDNA synthesis. The obtained cDNA was digested with *Rsa* I, and then ligated with adaptor 1 and adaptor 2R. After two rounds of subtractive hybridization and suppressive PCR, the amplified fragments were ligated to PMD18-T vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5α. Potential positive clones were selected and preserved in the medium containing 50% glycerol at −80 °C.

2.4. Screening of differentially expressed sequence tags (ESTs) by reverse-southern blot

The cDNA inserts in selected clones were amplified with the nested primer 1 and 2R provided by PCR-select cDNA subtraction kit (Clontech, USA). All amplified products were analyzed by agarose gel electrophoresis to check the presence and size of the inserts. Then, an aliquot of 0.2 μl of each product was spotted onto quadruplicated Hybond-N+ nylon membranes (Amersham, UK) by using Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). The membranes were air dried, and then the spotted PCR products were denatured in a solution containing 0.5 M NaOH and 1.5 M NaCl for 2 min, neutralized with a solution containing 1.5 M NaCl, 0.5 M Tris-Cl (pH8.0) for 5 min, and rinsed in 2 × SSC, 0.2 M Tris-Cl (pH8.0) for 1 min. The PCR products were cross-linked to membranes by UVC500 UV crosslinker (Hoefer, USA) in 70 mJ/cm^2 UV radiation intensity for 3.3 min. The cDNA without subtraction and subtracted cDNA resulted from mRNA of tester and driver samples were labeled with α-^{32}P-dCTP by using Random Primer DNA Labeling Kit (TaKaRa, Dalian, China), respectively. The spotted membranes were pre-hybridized in a solution containing 1 M Na_2HPO_4, 0.5 M EDTA (pH 8.0), 10% SDS, 1% BSA for 10 h at 65 °C, and then hybridized with four probes overnight at 65 °C, respectively. After two cycles of washing, hybridization signals were visualized on BAS-1800II (Fujifilm, Tokyo, Japan). The clones showing specific hybridization signals or three-fold greater difference of signal intensity between two hybridizations with probes from tester and driver as determined by densitometry were considered as positive and selected for sequencing.

2.5. Sequence analysis

Selected clones were sequenced at GenScript Corporation (Nanjin, China). Homology searches were performed by comparing all the obtained sequences to those in NCBI non-redundant (NR) protein database with the tool of BlastX (http://www.ncbi.nlm.nih.gov/BLAST). The sequences with BLAST scores <45 bits were designated as having no significant similarity and the ESTs hitting the gene with the same access number were considered as one gene. Gene ontology (GO) term annotation and function analysis were performed using the software Blast2GO (http://blast2go.org/start_blast2go).