Identification of differentially expressed genes in response to infection of a mild *Citrus tristeza virus* isolate in *Citrus aurantifolia* by suppression subtractive hybridization

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ABSTRACT

Citrus tristeza virus (CTV) is the most serious viral pathogen of citrus. Pathogenicity differentiation among CTV isolates has been found in some countries. In order to better understand the responses of Mexican lime (*Citrus aurantifolia*) to the infection of a mild CTV isolate (CTV-N4), we constructed two suppression subtractive hybridization (SSH) libraries. A total of 589 expressed sequence tags (ESTs) were screened from two SSH libraries by dot blot analysis. Sequencing results showed that these ESTs represented 202 genes, of which 111 and 91 genes were up-regulated and down-regulated, respectively. The differential expression of 10 out of 13 selected genes was confirmed by quantitative real-time RT-PCR (qRT-PCR). All differently expressed genes were functionally categorized into eight groups, of which the majority was associated with stress and disease defenses. The transcriptional levels of three genes in lime plants with the infection of a mild isolate CTV-N4 and a severe isolate CTV-N21 were comparatively analyzed by qRT-PCR over a 120 days time course. Results indicated that transcriptional changes of three studied genes were more greatly induced by CTV-N21 than those induced by CTV-N4. The obtained results provided novel information on genes potentially involved in citrus responses to CTV infection.

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1. Introduction

Plants respond to virus infection with a number of physiological alterations, which are accompanied by changes in plant gene expression. Responses of plants to virus infection have been studied on different plant-virus interactions (Love et al., 2005; Sajnani et al., 2007; Havelda et al., 2008; Alfenas-Zerbini et al., 2009). Previously, researchers have reported differential responses of host plant to related or unrelated virus isolates (Whitham et al., 2003; Dardick, 2007; Garcia-Marcos et al., 2009; Kogovšek et al., 2010). Responses of different host plants to the same virus isolate have also been compared (Shalitin et al., 2002; Love et al., 2005; Baebler et al., 2009). All these results indicate that the response of plants to virus infection depends on host species and virus strains, or even plant tissues of virus accumulation (Yang et al., 2007). However, data on the effects of viral infection on gene expression of host plants were mainly restricted to some viruses in model plants like *Arabidopsis thaliana* or *Nicotiana benthamiana* (Golem and Culver, 2003; Senthil et al., 2005; Yang et al., 2007) and in a few economically

important crops like soybean (Babu et al., 2008), potato (Baebler et al., 2009), grapevine (Espinoza et al., 2007) and citrus (Cristofani-Yaly et al., 2007; Gandía et al., 2007). Meanwhile, previous studies were mostly performed on the interactions between symptom-inducing virus strains and their susceptible hosts (Kogovšek et al., 2010; Satoh et al., 2011).

Citrus tristeza virus (CTV) is a member of the genus *Closterovirus* in the family *Closteroviridae*. It has a large single-stranded, positive-sense genomic RNA (gRNA) of ~20 kb, which consists of 12 open reading frames (ORFs) and untranslated regions at the 5′ and 3′ ends (Karasev et al., 1995). The virus is phloem-restricted and naturally transmitted by aphids in a hemi-persistent manner (Bar-Joseph et al., 1989). Most citrus species and varieties are susceptible to CTV infection. Symptoms induced by CTV depend on virus strains and citrus species or scion-rootstock combinations (Bar-Joseph et al., 1989). Many CTV isolates can induce the decline and death of citrus trees propagated on sour orange (*Citrus aurantium* L.) rootstock or vein clearing and stem pitting on Mexican lime (*C. aurantifolia* [Christm.] Swing.). Some mild strains induce very mild leaf chlorosis or symptomless on Mexican lime. The well characterized mild strains include T30 from Florida (Albiach-Martí et al., 2000) and T385 from Spain (Vives et al., 1999).

Citrus is a perennial plant, in which CTV can replicate and spread to whole plants, and then persistently affect the development of citrus plants during its whole life. Previous studies showed that

transcriptional changes were induced in susceptible lime plants (Gandía et al., 2007) and resistant *Poncirus trifoliata* (Cristofani-Yaly et al., 2007) in response to the infection of severe CTV strains. Our previous studies also revealed different physiological changes of citrus plants in response to the infection with a mild and a severe CTV isolate (Wang et al., 2009; Fan et al., 2010). In this work, we employed SSH to construct cDNA libraries for identifying differentially expressed genes in Mexican lime plants infected by a mild CTV isolate. The information provided from this work will enhance the understanding of the complex interaction between a mild virus and its host plant and the improvement of citrus resistance to the viral infection.

2. Materials and methods

2.1. Virus isolates and host

A CTV mild isolate (CTV-N4) and a severe isolate (CTV-N21) previously identified from southern China (Jiang et al., 2008) were maintained in Mexican lime plants and used for virus inoculation in this study. The isolate CTV-N4 did not induce visible symptoms on Mexican lime, sweet orange, and grapefruit seedlings. The severe isolate N21 induced stem pitting or vein clearing on those plants. Healthy Mexican lime plants on seedlings of sweet orange were used as host plants.

For the construction of SSH libraries, three healthy Mexican lime plants were graft-inoculated with two buds from a lime plant infected with CTV-N4 or from a healthy lime plant. For comparative analysis of transcriptional changes induced in Mexican lime plants infected by different CTV isolates, three Mexican lime plants were inoculated with CTV-N4 and other three plants were inoculated with CTV-N21. Three healthy lime plants which were mock-inoculated with healthy buds were used as control.

All plants were kept under an insect-proof greenhouse conditions. CTV infections were confirmed by RT-PCR before sampling.

2.2. Extraction of total RNA and purification of mRNA

Young shoots about 3–4 cm long collected from three Mexican lime plants inoculated with CTV-N4 and mock-inoculated with healthy buds were pooled, respectively. The total RNA was extracted from 5 g sample using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The mRNA was isolated from approximately 1 mg of total RNA by using the PolyA Ttract® mRNA Isolation Systems III kit (Promega), according to the manufacturer's instructions.

2.3. Construction of subtracted cDNA libraries

Two subtracted cDNA libraries were constructed using the PCR-Select cDNA Subtraction Kit (Clontech, USA) as described by the manufacturer. Briefly, 2 μg mRNA from both non-inoculated and CTV-N4 infected Mexican lime tissues was reversely transcribed into cDNA, respectively. Each cDNA preparation was divided into two portions, which were ligated with adaptors 1 and 2R, respectively. For forward subtraction, cDNA from the non-inoculated Mexican lime tissues was used as driver, and cDNA from the CTV-N4-infected Mexican lime tissues was used as tester. In contrast, for reverse subtraction, the cDNA from the non-inoculated Mexican lime tissues and CTV-N4-infected Mexican lime tissues was used as tester and driver, respectively. After two subtracted hybridizations, the PCR products obtained from the forward and reverse subtractions were separately cloned into the pMD18-T vector (TaKaRa, Dalian, China), and transformed into *Escherichia coli* DH5α for the construction of SSH libraries.

2.4. DNA dot blot analysis

For the preparation of α-^{32}P-labeled cDNA probes, the second subtracted PCR products as the same used for the construction of forward and reverse libraries were digested with restriction enzyme *Rsa* I, and purified using a Fragment Recovery Kit (TaKaRa, Dalian, China) to remove adapters. The purified products were labeled by using α-^{32}P and Random Primer DNA Labeling Kit (TaKaRa, Dalian, China) according to the protocol provided by the manufacture.

Recombinant clones were randomly picked up and individually inoculated into 1 ml of LB medium containing 100 μg/ml ampicillin. The specific insert in each recombinant was amplified by PCR using the nested primers Nested 1 and Nested 2R, followed by analysis on 1.2% agarose gel. Aliquot of 0.2 μl of each PCR product was arrayed onto Hybond™-N$^+$ nylon membranes (Amersham Biosciences, USA) using robotic printing (BioRobotics, UK) in duplicate and fixed using Mini UV crosslinker 230 V (Pharmacia, USA). The duplicate membranes were separately hybridized with two probes at 65 °C for 16 h. Hybridization signals were visualized on BAS-1800II (Fujifilm, Tokyo, Japan). The clones showing specific hybridization signals with the tester probe or 3-fold greater signal intensity over the control as determined by densitometry were considered as positive and selected for sequencing.

2.5. Sequence analysis

All the selected clones were sequenced at the GenScript Corporation (Nanjing, China). Sequence alignment and homology searches were completed by using TIGR (http://blast.jcvi.org/euk-blast/plantta_blast.cgi) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) BLAST search database. *E*-value scores below 10^{-5} were considered as significant and used to indicate homology between obtained sequences and database sequences. All differentially expressed genes were grouped into functional categories according to the classification method of Bevan et al. (1998).

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Expression levels of selected genes in CTV-infected and healthy plants were evaluated by qRT-PCR using the SYBR Green I (TaKaRa, Dalian, China) and the LightCycler System (CFX 96, Bio-rad). Primers for qRT-PCR were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) based on available sequences of selected ESTs. An actin gene (GenBank ID: CF837237) was used as an internal control. Total RNA was extracted individually from three lime plants by the method mentioned above and used as templates for cDNA preparation. qRT-PCR in a final volume of 20 μl was performed in technical triplicates. The thermal profile was: an initial 95 °C pre-denaturation step for 3 min followed by denaturation for 20 s at 95 °C, annealing for 15 s at 60 °C, and extension for 20 s at 72 °C for 40 cycles. Values for relative ESTs expression were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and were normalized to the transcriptional value of an actin gene.

3. Results

3.1. Screening of SSH libraries and functional categorization

A total of 589 clones, 349 clones from the forward library and 240 clones from the reverse library, were isolated and sequenced. All the single-read DNA sequences were processed by removing repeated sequences. The sizes of inserts ranged from 150 to 1000 bp. Some of the clones were redundant due to the use of *Rsa* I-restricted cDNA fragments in the SSH procedure, and in