MULTIPLE SPECIES OF DEFECTIVE RNAS IN PLANTS INFECTED WITH FLORIDA STRAINS OF CITRUS TRISTEZA VIRUS

M. Mawassi1, E. Mietkiewska2, M. E. Hilf3, L. Ashulin2, R. Gafny2, A. V. Karasev1, S. M. Garnsey3, W. O. Dawson1, M. Bar-Joseph2, and R. F. Lee1*

1Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850.
2The S. Tolkowski Laboratory, Department of Virology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel
3USDA-ARS, Horticultural Research Laboratory, 2120 Camden Road, Orlando, FL 33803

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Abstract. Multiple species of defective RNAs (D-RNAs) were found in citrus plants infected with several Israeli and Florida isolates of the citrus tristeza virus (CTV). These D-RNAs consist of 5' and 3' terminal fragments of the CTV RNA and lack most of the genetic information contained in the CTV genome. The overall organization of the CTV-specific D-RNAs is similar to the structure of defective-interfering RNAs (DIs) found in other plant viruses. These DIs are known to modulate symptom expression in infected plants mitigating or delaying development of the virus infection. Investigations aimed at finding possible association of the CTV-specific D-RNAs with particular symptoms in different citrus hosts are under way.

Literature Cited

Flexible filamentous particles of CTV contain a single positive-stranded genomic RNA (gRNA) which has recently been sequenced for the Florida T36 isolate and found to contain 19,296 nts (Fig. 1) (Karaes et al., 1995b; Pappu et al., 1994). The CTV genome encodes 12 ORFs potentially coding for at least 17 protein products which include replication-associated proteins, a homolog of the HSP70 proteins, the coat protein (CP), a duplicate of the CP, and several other protein products with unknown functions (Karaes et al., 1995; Pappu et al., 1994). The 3’-proximal ORF 11 was suggested to encode a 23.5-kDa protein with RNA-binding properties (Dolja et al., 1994). Studies of dsRNAs isolated from citrus plants infected with different CTV isolates (Dodds and Bar-Joseph, 1983; Dodds et al., 1987; Lee, 1984; Moreno et al., 1990) demonstrated the presence of numerous RNA fragments which could not be assigned to any of the CTV sgRNAs (Hilf et al., 1995).

Recently, we have reported that RNA preparations from CTV particles and those of ss- and dsRNAs from CTV-infected plants, contain an abundant 2.4 kb RNA species with features suggesting its designation as a defective RNA (D-RNA) molecule (Mawassi et al., 1995a). Further analyses of citrus infected with CTV revealed the presence of additional D-RNA molecules of 2.7 and 4.5 kb present in citrus infected with the VT strain in Israel (Mawassi et al., 1995b). Additionally, a short non-encapsidated ss-positive-sense RNA was also found in the infected plants. This ssRNA, which co-purified with dsRNAs was shown by hybridization to encompass the 5’ terminal part of the CTV genome and may have extensive secondary structure.

In this paper we demonstrate the presence of multiple D-RNA species in a number of plants infected with Florida strains of CTV. These D-RNAs differ in size and relative abundance within and between different isolates as well as the presence of an unusual single-stranded positive-sense 0.8 kb RNA corresponding to the 5’-terminal part of the CTV genome. Portions of this research have been previously reported (Mawassi et al., 1995b; Mawassi et al., 1995c).

Material and Methods

Florida strains of CTV T36, T64-1, and T66-1 and an isolate originating from Costa Rica, B181, were maintained in Madam Vinous sweet orange (Citrus sinensis (L.) Osb.) seedlings in a greenhouse in Beltsville, MD. The isolates all induce decline in trees on sour orange rootstock (Rosner et al., 1986; Garnsey et al., 1991). The dsRNAs were isolated from bark tissue of infected citrus by two cycles of CF-11 column chromatography (Dodds and Bar-Joseph, 1983) followed by an additional separation step using CC41 columns (Dulieu and Bar-Joseph, 1989). The dsRNA preparations were denatured by treatment with methylmercury hydroxide (Ashulin et al., 1992) and separated by electrophoresis in formamide/formaldehyde denaturing agarose gels prepared in MOPS buffer followed by treatment with 50 mM NaOH to enable the efficient transfer of gRNA. The denatured RNAs were transferred to nylon membrane (Hybond N-Amersham) and hybridized to cDNA probes corresponding to the 5’ and 3’ termini of CTV-VT according to Maniatis et al. (1982).

The 5’ cDNA probe was labeled with [32P]dCTP by 30 cycles of PCR using the oligonucleotide primers #27196 (5’-CAAATTCAACCCTACCTCGGAAATC-3’) and #18243 (5’-AGCGAAGGATATCATCCA-3’) corresponding to nucleotides 1 to 27 and 686 to 703, respectively of the previously described 2.4 kb D-RNA molecule (Mawassi et al., 1995c). The 3’ cDNA probe was prepared using the primers #18168 (5’-TGCCCGCATATGTTAATGC-3’) and #26225 (5’-ATGGACCTATGTTGGCCCCCCCATAG-3’) representing the nucleotides 1811 to 1828 and 2400 to 2424, respectively of the previously reported 2.4 kb D-RNA molecule (Mawassi, 1995b).

Results

Figure 2 shows the hybridization pattern of the 3’- (Lanes 1-4) and the 5’- (Lanes 5-8) terminal probes with dsRNA preparations from CTV isolates from Florida. Extensive variations were observed both in the sizes and the relative abundance of the D-dsRNA molecules from the tested CTV strains. The results indicated that the D-RNAs are present in the T64 and T66 isolates included in this study as well as in the isolate originating from Costa Rica.

Using the 5’-terminal region of the CTV genome as a probe for Northern blots reveals the presence of a distinct RNA band with a size of ca. 0.8 kb associated with all the isolates studied (Fig. 2). This RNA molecule was detectable in dsRNA extracts prepared from CTV infected plants, but not in RNA extracted from purified CTV particles (not shown). This is similar to the low molecular weight tristeza 5’ RNA (LMT 5’-RNA) reported to be associated with the VT strain of CTV from Israel (Mawassi et al., 1995b). The use of the 3’ and 5’ probes on the Florida stains of CTV revealed extensive variations both in the sizes and the relative abundance of the D-dsRNAs.

Discussion

The first report of defective RNA from CTV was a 2.4 kb D-dsRNA from the VT strain of CTV from Israel (Mawassi et al., 1995b). This D-dsRNA contained a 14 nt segment, possi-
with DMSO and glyoxal, separated on a 1% agarose gel, blotted to nylon membrane and hybridized with \(^{32}P\)-labeled cDNA probes. The hybridizations were carried out with \(^{32}P\)-labeled 3'-cDNA probe (between primers \#18168 and \#26225) and lanes 5-8 with the \(^{32}P\)-labeled 5'-cDNA probe (between primers \#27196 and \#18245) as indicated. TMV RNAs (lanes labeled T) were used as size markers. The arrows on the right indicate the location and sizes (kb) of D-RNAs as indicated with hybridization with the 5'-cDNA probe.

The dsRNAs were denatured with DMSO and glyoxal, separated on a 1% agarose gel, blotted to nylon membrane and hybridized with \(^{32}P\)-labeled cDNA probes. The hybridizations were carried out with \(^{32}P\)-labeled 3'-cDNA probe (between primers \#18168 and \#26225) and lanes 5-8 with the \(^{32}P\)-labeled 5'-cDNA probe (between primers \#27196 and \#18245) as indicated. TMV RNAs (lanes labeled T) were used as size markers. The arrows on the right indicate the location and sizes (kb) of D-RNAs as indicated with hybridization with the 5'-cDNA probe.

The LMT 5'-RNA which was detected using the 5' probe appears to be present in all the CTV strains examined in this study. The LMT 5'-RNA is a positive-sense ssRNA molecule of about 0.8 kb representing the 5' terminus of the CTV VT strain genome (Mawassi et al., 1995c).

D-RNAs are defined as nonautonomous, truncated versions of viruses whose replication by the viral replicase usually competes with the intact viral genome (Simon and Bujarski, 1994). They are probably the result of template switching of the replicase during RNA transcription (Holland, 1991). The D-RNAs are most likely the result of recombination that occurs either by the replicase enzyme detaching-reinitiating on different molecules or on the same molecule due to a looping back of the template RNA. D-RNAs usually compete with the helper virus with a resultant interference of replication of the helper virus, resulting in reduced virus concentration and an attenuation of virus symptoms. However, some D-RNAs have been found which intensify the viral symptoms (Cascone et al., 1993).

The use of probes specific for the 3' and 5' termini of the CTV gRNA indicates the presence of D-RNA molecules in Florida strains of CTV, also from the B181 strain of CTV which originated from Costa Rica. The use of the 5' probe also revealed the presence of a small dsRNA-like molecule similar to the LMT 5' RNA reported for the VT strain of CTV is present in all the CTV extractions.

The role of D-RNAs and the LMT 5' RNA in CTV is unclear. It is clear that the presence of D-RNAs in various CTV strains occurs with different CTV strains from different geographic areas. Research is underway to determine the biological significance of the phenomenon, and to determine if D-RNAs may be useful for cross protection purposes and/or determination of the function of specific CTV genes.

**Literature Cited**


**Figure 2. Northern blot analysis of dsRNAs from CTV strains originating from Florida strains T56 (lanes 1 & 5), T64-1 (lanes 2 & 6), and T66-1 (lanes 3 & 7) and Costa Rica strain B181 (lanes 4 & 8). The dsRNAs were denatured with DMSO and glyoxal, separated on a 1% agarose gel, blotted to nylon membrane and hybridized with \(^{32}P\)-labeled cDNA probes. The hybridizations were carried out with \(^{32}P\)-labeled 3'-cDNA probe (between primers \#18168 and \#26225) and lanes 5-8 with the \(^{32}P\)-labeled 5'-cDNA probe (between primers \#27196 and \#18245) as indicated. TMV RNAs (lanes labeled T) were used as size markers. The arrows on the right indicate the location and sizes (kb) of D-RNAs as indicated with hybridization with the 5'-cDNA probe.**

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Since 1991, there have been renewed concerns about harvesting for the same reasons stated above (Whitney, 1995). The average cost of harvesting (tree to processing plant or packinghouse) almost equals the cost of production and is expected to escalate to $2.33/box of oranges by the 2002-03 season (Polopolus et al., 1993). Total Florida citrus production is expected to increase 48% to a record 362 million boxes in the decade ahead, and 75% of this will be oranges. These increases in production will require 10,000 additional pickers, and with foreign competition, are expected to depress on-tree fruit prices by 33%.

Since the early 1960s, Florida citrus growers have continued to plant and interset orange trees at higher densities to achieve high yields early and throughout the life of the tree. In 1980, the Lake Alfred Citrus Research and Education Center (CREC) initiated a cooperative experiment with the Coca Cola Company to investigate the management of orange trees planted at densities ranging from 150 to 360 trees/acre. One research objective in this experiment was to study effects of high-density planting variables on harvesting systems for processed oranges. Whitney et al. (1994) has discussed how various characteristics of this high-density grove may affect harvesting by manual means, with picking aids, and by machine.

The objective of the research reported in this paper was to quantify the effects of scion variety, rootstock, tree height, tree spacing, and other pertinent variables in this planting on the manual (conventional) harvesting rate.

**Materials and Methods**

**Test site.** The experimental orange grove used for the harvest tests has been described by Wheaton et al. (1986), Whitney et al. (1994), Wheaton et al. (1995), and Whitney et al. (1995). The trees were planted in 1980 on a 25-acre site in Polk County between Frostproof and Babson Park. Factors in