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

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Additional index words. Polymerase chain reaction, hybridization.

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.

Citrus is a major world food crop in terms of nutrition and the generation of income and foreign trade. Estimates for the previous five years indicate that the United States produces 18% of the world’s citrus; 70% of it is produced in Florida, and the annual value of the Florida citrus crop is over $1.2 billion (Anonymous, 1994; Freie and Young, 1992). Tristeza, caused by citrus tristeza virus (CTV) is the most economically important virus disease of citrus in the world (Bar-Joseph et al., 1989; Rocha-Pena et al., 1995b). It is predicted to soon reach a new level of importance due to the influx from South America of high populations of Toxoptera citricida Kirkaldy, the most efficient aphid vector of CTV, and subsequent spread of new severe strains of CTV (Rocha-Pena et al., 1995b; Lee et al., 1992).

Tristeza was first recognized as a decline of citrus scions propagated on sour orange rootstock in South Africa about 1910 and in Argentina in 1930. In Brazil it was first called Tristeza (“sadness”, in Portuguese). Quick decline, the rapid death of all genotypes and ages of citrus grafted on sour orange rootstock, was first noted in California in 1939, and later in New Zealand, Australia, West Africa, Ceylon, and Hawaii (Bar-Joseph et al., 1989). In 1946 the tristeza disease was found to be caused by a virus and to be vectored by aphids (Bar-Joseph et al., 1989). The virus also is transmitted by budding and grafting during citrus propagation, but it is not seed transmitted (Rocha-Pena et al., 1995a). It has been speculated that CTV originated in the Orient and was distributed worldwide through the movement of citrus budwood and plants, in man’s quest for new citrus varieties (Bar-Joseph et al., 1989).

Tristeza of citrus is actually several different types of diseases, depending on the biological properties (that is, symptom type induced) of the tristeza strain and the host genotype involved (Rocha-Pena et al., 1995a). The biological properties of CTV strains can be separated into five major categories: mild, seedling yellows, decline (D) on sour orange, stem pitting (SP) on grapefruit, or SP on sweet orange (Rocha-Pena et al., 1995a) or the disease can be any combination of these symptoms if the host is infected by a mixture of strains. Strains of CTV which cause SP on grapefruit and orange have not been reported from commercial citrus in Florida (Brown et al., 1995), but we now know that such strains are harbored in the state in Meyer lemon and possibly other
citrus germplasm importations (Minutes of CTV/BCA Taskforce, Sept. 1995).

Tristeza is caused by a phloem-associated, aphid-transmissible citrus tristeza Closterovirus (CTV) (Bar-Joseph et al., 1989; Rocha-Pena et al., 1995a). 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) (Karasev et al., 1995, 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 (Karasev et al., 1995; Pappu et al., 1994). The 3'-proximal ORF 11 was suggested to encode a 23.5-kDa RNA-binding property (Dolja et al., 1994). Plants infected with CTV contain a nested set of 3'-coterminal single-stranded (ss) and double-stranded (ds) RNAs: a large RNA molecule corresponding to the CTV replicative form (RF) and at least nine smaller subgenomic RNA (sgRNA) species corresponding to 3'-terminal ORFs 2 to 11 (Mawassi et al., 1995a; Hilf et al., 1995). Three of the sgRNAs of 3.2, 1.6 kb and 0.9 kb were identified by in vitro translation as templates encoding protein products of ORFs 7 (CP), 10, and 11 (Mawassi et al., 1995a; Dulieu and Bar-Joseph, 1990). Extensive 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).

**Figure 1. Schematic illustration of the genome of citrus tristeza virus (CTV). Boxes represent open reading frames (genes). PRO, papain-like protease; MT, methyltransferase; HEL, helicase; RdRp, polymerase; HSP70, homolog of HSP70 proteins; CP, coat protein; RBP, putative RNA-binding protein.**

**Figure 2.** 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, possibly non-viral, at the junction of 1151 nt from the 5' end and 1259 nt from the 3' end of the CTV gRNA. Additional study of the VT strain of CTV has revealed the presence of two additional D-RNA molecules of 4.5 and 2.7 kb (Mawassi et al., 1995c). The 4.5 kb D-RNA is composed of 4,036 nt corresponding to the 5' terminus of the

**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 Vinos 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 formaldehyde/denaturing agarose gels prepared in MOPS buffer followed by treatment with 50mM 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'-CAAT-TCACCCGTACCTCCGGAATC-3') and #18243 (5'-AGCGAAGGATATCATTCA-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'-TGGCGCATATGGTCATTAG-3') and #26225 (5'-ATGACCTATGTTGGCCCCCATAG-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 5'- (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, possibly non-viral, at the junction of 1151 nt from the 5' end and 1259 nt from the 3' end of the CTV gRNA. Additional study of the VT strain of CTV has revealed the presence of two additional D-RNA molecules of 4.5 and 2.7 kb (Mawassi et al., 1995c). The 4.5 kb D-RNA is composed of 4,036 nt corresponding to the 5' terminus of the
CTV-VT gRNA and the remainder represent 442 nt from the 3' end. The 2.7 kb D-RNA molecule of the CTV-VT strain is composed of 1,818 nt from the 5' portion and the remainder containing 938 nt from the 3' terminus. There was not a non-viral segment at the 3' end. The presence of D-RNAs in various CTV strains originating from Florida strains T36 (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 denaturated with DMSO and glyoxal, separated on a 1% agarose gel, blotted to nylon membrane and hybridized with 32P-labeled cDNA probes. The hybridizations in lanes 1-4 were carried out with 32P-labeled 3'-cDNA probe (between primers #18168 and #26225) and lanes 5-8 with the 32P-labeled 5'-cDNA probe (between primers #27196 and #18243) 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 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**


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 the experiment are listed in Table 1. A multiple split plot design with 4 replications was used. Scion variety was the main plot factor followed by successively