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PROGRESS ON THE CHARACTERIZATION AND CONTROL OF CITRUS TRISTEZA VIRUS[1]

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Abstract. ***Toxoptera citricida*, the most efficient aphid vector of citrus tristeza virus (CTV), has moved rapidly northward from South America to Nicaragua in Central America and to Hispaniola, and now Cuba, in the Caribbean Basin. This poses a serious threat to citrus in this region, including the United States, especially if *T. citricida* is carrying the severe quick decline and/or stem pitting strains of CTV known to occur in South America. New techniques have enabled the cloning and sequencing of the CTV genome, development of CTV strain specific nucleic acid probes and production of polyclonal and monoclonal antibodies, now without the necessity of purifying the virus. Control methods being studied include mild strain cross protection, selection of CTV tolerant citrus genotypes and development of CTV resistant genotypes by conventional plant breeding, and genetic engineering through the introduction of CTV genes into citrus to produce transgenic plants. This increased knowledge and capability will facilitate a more rapid and accurate detection and characterization of CTV strains and possibly some amelioration of the impact on the Florida citrus industry caused by the probable arrival of *T. citricida* and new strains of CTV.**

Tristeza, caused by citrus tristeza virus (CTV), is the most economically important virus disease of citrus in the world (Bar-Joseph et al., 1989; Lee and Niblett, 1992). It is predicted to soon reach a new level of importance and destructiveness and threaten the 300 million citrus trees in the Caribbean Basin countries due to the influx from South America of high populations of *Toxoptera citricida* Kirkaldy, the most efficient aphid vector of CTV, possibly carrying new severe strains of CTV (Lee et al., 1992, 1993; Roistacher et al., 1991; Yokomi et al., 1993). The most damaging strains of CTV cause quick decline (QD) of scions on sour orange rootstock or stem pitting (SP) of grapefruit, limes and orange scions on all rootstocks (Garnsey et al., 1987). The QD strains are already endemic in Florida (Brlansky et al., 1986) and are being transmitted by *Aphis gossypii*, a common, but less efficient vector of CTV (Lee et al., 1992; Yokomi et al., 1993). The SP strains have not yet been reported affecting commercial citrus in Florida, but they may already exist as "sleeping strains" in citrus germplasm and old lines such as Meyer lemon introduced into Florida prior to initiation of the Florida Citrus Budwood Registration Program in 1952 (Lee et al., 1992). The more efficient transmission of existing severe strains of CTV and the introduction of additional severe strains by *T. citricida* pose significant threats to the Florida citrus industry. We report here some of the progress being made to further characterize and control CTV. For additional information on CTV and *T. citricida* , see Gottwald et al., Lee et al., and Yokomi in this volume.

CTV is the largest plant virus known. Its genome is comprised of a single-stranded, positive sense RNA of about 20,000 nucleotides (Bar-Joseph et al., 1989). This is three times larger than the average plant virus and may in part explain the existence of such great diversity in strains of CTV. The RNA genome of the virus was cloned as complementary DNA and the nucleotide sequence of portions of the genome was determined. Sequencing of the approximately 7,000 nucleotides of the 3′ end of the genome revealed the presence of eight open reading frames potentially encoding, in the 5′ to 3′ direction, proteins of 65, 61, 27, 25, 18, 13, 20 and 23 kilodaltons (kDa) (Pappu et al., 1994). The 25 kDa protein was identified as the coat protein (CP) (Sekiya et al., 1991). Comparisons of the coat protein genes (CPG) of geographically and biologically diverse strains of CTV showed about 90% homology and indicated that the coat protein (CP) sequences of the mild CTV strains (T4, T26, T30, and T55) are more closely

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related to one another than to those of the QD strains (T3, T36, T66, and B53) or the SP strains (B128, B185) (Figure 1; Pappu et al., 1993a). Thus from its CPG sequence we can reasonably predict whether a new strain of CTV might be a mild, QD or SP strain.

Amino acid differences in the CP may generate a different epitope (antibody binding site) and elicit the production of a strain or group specific monoclonal antibody (MCA) such as MCA13, which reacts preferentially with severe strains of CTV (Permar et al., 1990) and be useful in the diagnosis of CTV infection. Comparisons of the CP amino acid sequences of several mild and severe strains of CTV revealed a consistent difference at amino acid position 124; in the mild strains the amino acid was always tyrosine (Y), whereas in severe strains it was replaced by phenylalanine (F) (Figure 2). By site-directed oligonucleotide mutagenesis, we changed the Y to F and conferred MCA13 reactivity on the expressed mutant CP of a mild strain, whereas an F to Y mutation abolished MCA13 reactivity of the expressed mutant CP of a severe strain (Pappu et al., 1993b). This required a single nucleotide change since the codons for Y and F are TAT and TTT, respectively. To confirm that the desired mutations had been introduced, it was necessary to distinguish between bacterial colonies carrying the wild type and the mutant CPG. This was accomplished by hybridizing a radiolabeled oligonucleotide probe of 19 bases containing the single T/A mismatch. This single nucleotide difference between the strains was reliably detected by nucleic acid hybridization. This observation provided an opportunity to develop CTV strain specific probes (Figure 3) which may be used for the detection of individual strains of CTV present in extracts of CTV-infected plants (naturally occurring or cross protected), or polymerase chain reaction-amplification products from the extracts of infected plants or *T. citricida* (Niblett et al., 1993) by nucleic acid hybridization. These probes will be useful in monitoring and quantitating virus replication in cross protected plants and the evaluation and selection of mild strains of CTV for cross protection.

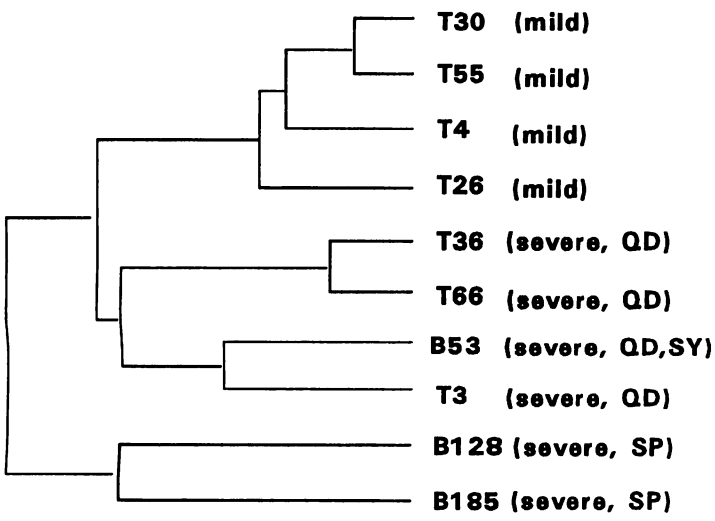

Figure 1. Cluster dendrogram generated from the multiple alignment of the amino acid sequences of the coat proteins of biologically different CTV strains. Strains with letter T are native to Florida while the exotic strains are denoted by the the letter B.

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                    103                                   145
          T3        ITYTREGVEVELSDKLWTDVVFNSKGIGNRTNALRVWGRTNDA
          T36       ITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDA
Severe    B53       ITYTREGVEVDLPDKLWTDVVFNSKGIGNRTNALRVWGRTNDA
strains   B128      ITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRSNDA
          B185      ITYTREGVEVDLSYKLWTDVVFNSKGIGNRTNALRVWGRSNDA
          ------------------------------------------------------
          T4        ITYTREGVEVDLSDKLWTDIVYNSKGIGNRTNVLRVWGRTNDA
          T26       ITYTREGVELDLSDKLWTDIVYNSKGIGNRTNALRVWGRTNDA
Mild      T30       ITYTREGVEVDLSDKLWTDIVYNSKGIGNRTNALRVWGRTNDA
Strains   T55       ITYTREGVEVDLSDKLWTDIVYNSKGIGNRTNALRVWGRTNDA
          B67       ITYTREGVEVDLSDKLWTDVVYNSKGIGNRTNALRVWGRTNDA
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Figure 2. Multiple alignment of a portion of the amino acid sequences deduced from the coat protein genes of several biologically distinct strains of CTV. Differences in the amino acid sequence are shown in large letters.

Selected CTV CPGs were cloned into bacterial expression vectors which facilitated the expression and purification of large amounts of the CP from the bacterial host. This protein was used to raise mono- and polyclonal antibodies (Manjunath et al., 1994). This approach of cloning the gene directly obviates the necessity of the laborious, inefficient and expensive purification of CTV and yields a homogeneous product devoid of contaminating plant antigens. The expressed protein also is useful as a known positive standard for serological reactions and eliminates the need to carry desiccated or lyophilized tissue extracts of CTV into politically sensitive locations.

Generally the most efficient control measure for a virus disease is the development of plants which are highly resistant or immune to that disease. Frequently, however, disease resistance genes are not known or cannot be readily transferred into desirable plant genotypes. This is the case for CTV and *Citrus*. All desirable scion cultivars are susceptible to CTV, and resistance genes identified in *Citrus* relatives such as *Poncirus* and *Swinglea* are difficult or impossible to transfer due to the undesirable nature of the resulting hybrids or the sexual cross-incompatibilities of the species (Williams, 1992). Somatic hybridization between incompatible *Citrus* relatives has been successful (Grosser and Gmitter, 1990) and is now being tested as a method to transfer CTV resistance genes (Grosser, 1992). We also have begun a project to genetically engineer *Citrus* for resistance to CTV by incorporating various CTV genes into the *Citrus* genome. This approach has been successful with several plant viruses (for reviews see Beachy et al., 1990; Wilson, 1993), and citrus has been successfully transformed with the CTV CPG (Gutierrez-E. et al., 1992). To date we have performed citrus transformation experiments with CPGs of mild and severe strains of CTV and the p27 gene whose function is not yet known, but which shows significant protein sequence similarity with the CPG. Using p27-specific antibodies, the gene product was found to be expressed in CTV-infected, but not in uninfected citrus. Its role in the

```
          5'                                                3'
T36       TTCATCATTACAAAGCGATGACGACGCCACGGGTATAACGTACACTCGGG
T30       TTCATCATTGCAAAGTGATGATGACACCACGGGCATAACGTACACTCGGG
B128      TTCATCATTACAAAGCGACGATGATACCACGGGTGTGACGTACACTCGGG
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Figure 3. Partial nucleotide sequence (nucleotides 273-322) of the coat protein genes of three biologically distinct strains of CTV. Differences in nucleotide sequences are shown in large letters. The strain specific probe for each strain is underlined.

infection process and its ability to confer resistance are being investigated (Febres et al., 1994). Four additional genes of CTV have been expressed in bacteria, and polyclonal antibodies prepared to their gene products (p65, p61, p18, and p20). These genes have also been subcloned into plant transformation vectors and will be used to transform *Citrus*. Regenerated transformed plants will be multiplied and tested for resistance to CTV. However, any resistant plants obtained by these methods must still be tested for horticultural performance and quality. This it will require approximately 10 years of research and testing.

However, *T. citricida* and CTV obviously do not observe fiscal years, budget cuts or geographical boundaries! Something must be done now to protect citrus production in Florida. The only proven method is cross protection, the purposeful inoculation of plants with a mild strain of CTV to prevent damage by the subsequent infection with severe strains. In Brazil severe QD and SP strains of CTV and *T. citricida* are endemic, and therefore rootstocks other than sour orange are used exclusively. But the effects of SPstrains of CTV are ameliorated in over 80 million "Pera" sweet orange plants by cross protection (Muller and Costa, 1992). In South Africa, grapefruit cannot be produced commercially unless it is mild strain cross protected. All certified plants, whether grapefruit or orange, are inoculated with a known mild strain of CTV prior to their release (A. Lee et al., 1992). Cross protection also significantly increases grapefruit yields in Australia (Barkley et al., 1990). Experiments in South Africa (Van Vuuren, 1992), Brazil (Lee, 1992) and Florida (Rocha-Pena et al., 1990; Yokomi et al., 1990) have shown that cross protection also permits the use of sour orange as a rootstock where QD strains are endemic. In additional experiments in Florida, the effectiveness of cross protection was tested against QD strains of CTV in mature citrus trees on sour orange rootstock (R. F. Lee, unpublished). Protected trees were found to be generally more vigorous, and a lower percentage experienced decline. Experiments in Brazil, South Africa and Venezuela comparing local mild strains of CTV with mild strains from Florida showed obvious protection from the severe indigenous strains of CTV by the Florida mild strains (Lee et al., unpublished). The protection by the local mild strains persisted, but the heavy barrage by high populations of *T. citricida* finally "broke" the protection by the Florida mild strains (Lee, 1992). However, the promising results and the steady advance of *T. citricida* toward Florida encouraged Lee and his collaborators to develop large scale field tests in Florida. More than 20,000 trees are being used to further assess the protective ability of the Florida mild strains against natural and purposeful infection by severe indigenous strains of CTV. Preliminary evaluations show promising results (Lee et al., this volume).

Cross protection is a complex biological phenomenon, and the process for selecting mild strains of CTV has been empirical and consumptive of time, labor and space in the greenhouse and field. For example, selecting the strains now used widely in Brazil and those showing promise in Florida required the testing of over 300 strains of CTV and 7-10 years. A more rapid and efficient method is necessary for the identification and selection of CTV mild strains useful for cross protection. Also, to effectively implement cross protection in Florida it is necessary to know the biological characteristics of the CTV strains being transmitted and advanced toward Florida by *T. citricida*. First, are they new severe strains to this region, and secondly, are there mild strains available to cross protect against those new strains which pose the greatest threat?

Using the sequence information now available for CTV, we are developing molecular methods to rapidly detect and identify new strains of CTV in the Caribbean Basin, to classify them as mild or severe, and to assess the protective ability of new mild strains. Toward that goal, we have succeeded in detecting and cloning the CPG of CTV from as few as three *T. citricida* (Niblett et al., 1993). We believe our increased knowledge and capabilities will enable a more rapid and accurate detection and characterization of CTV strains being vectored in the Caribbean Basin by *T. citricida*, and hopefully the protection of our citrus industry, which is vital to all Floridians.

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POSTBLOOM FRUIT DROP OF CITRUS—SYMPTOMS, DISEASE CYCLE AND CONTROL[1]

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Additional index words. Colletotrichum gloeosporioides.

Abstract. **Postbloom fruit drop (PFD) appeared in Florida in 1983 and has since become widespread in the state. The disease appears as necrotic spots on petals and produces fruit drop and the formation of persistent buttons. PFD is caused by the slow-growing orange (SGO) strain of *Colletotrichum gloeosporioides* which is readily distinguished pathologically, morphologically and physiologically from the common, saprophytic fast-growing gray strain. However, the SGO strain is nearly identical to the strain of *C. gloeosporioides* which causes Key lime anthracnose. All isolates from Key lime tested caused PFD. The fungus is spread primarily by rain splash of conidia produced on infected petals. It appears to overwinter as appressoria on the surface of leaves, twigs and buttons which germinate at flowering to produce new conidia. The only fungicide currently registered for control of PFD is benomyl. An equation has been developed based on the current number of affected flowers and rainfall for the past five days to predict disease incidence and assist in timing of fungicide applications.**

In 1983, a new disease—postbloom fruit drop (PFD) hit Florida citrus groves for the first time. Initially, it was confined to Tahiti lime plantings and a few sweet orange groves in the Immokalee area. Subsequently, outbreaks occurred in various areas with damage ranging from minor to locally severe. Rainfall was high during the bloom period in 1988 and damage was more widespread and more severe. The causal agent, a strain of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz., had become widely distributed and significant outbreaks occurred on the East Coast, South Ridge, and North Ridge production areas. Rainfall during the blossom period was minimal in many areas in subsequent years and outbreaks were localized. In 1993, the bloom period was extended and rainfall abundant throughout citrus production areas through March. Losses were severe particularly on Navel oranges and some Valencia plantings, but some damage occurred on most citrus types.

The disease was described first in Belize by Fagan (1979) and most of what was known about PFD was from research conducted there (Denham, 1979; Denham and Waller, 1981; Fagan, 1971, 1979, 1984a, 1984b). Following the 1988 outbreak, my laboratory initiated an extensive research program to determine the nature of the causal organism, the disease cycle, the effects of environmental factors on the disease and to develop effective control measures. Much of that research is summarized herein.

Symptoms

Symptoms of the disease first appear as peach to brown-colored necrotic spots on petals of flowers which

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