CITRUS TRISTEZA VIRUS: CHARACTERIZATION OF TEXAS ISOLATES, STUDIES ON APHID TRANSMISSION AND PATHOGEN-DERIVED CONTROL STRATEGIES

A Dissertation

by

CAROLINE MARY HERRON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Plant Pathology

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Approved as to style and content by:

John V. da Graça (Co-Chair of Committee)	Herman B. Scholthof (Co-Chair of Committee)
T. Erik Mirkov (Member)	Karen-Beth G. Scholthof (Member)
Richard F. Lee (Member)	Dennis C. Gross (Head of Department)

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ABSTRACT

Citrus tristeza virus: Characterization of Texas Isolates, Studies on Aphid Transmission and Pathogen-Derived Control Strategies. (August 2003)

Caroline Mary Herron, B.Sc. (Hons.), University of Nottingham, UK;

M.Sc., University of Bristol, UK

Co-Chairs of Advisory Committee: Dr. John V. da Graça
Dr. Herman B. Scholthof

Citrus tristeza virus (CTV), an economically important graft-transmissible pathogen of citrus, causes major global declines in citrus production. In the commercial citrus region of the Lower Rio Grande Valley of Texas (LRGV), where red grapefruit on tristeza-decline sensitive sour orange rootstocks predominates, incidence of CTV is low. The efficient CTV vector, the brown citrus aphid (BrCA, *Toxoptera citricida* Kirkaldy) is now established in Mexico and Florida, thus information is needed on the severity of CTV, CTV aphid transmission and the performance of transformed citrus towards CTV before *T. citricida* arrives in Texas so that appropriate management strategies can be selected.

Biological indexing and molecular typing were performed on fifteen Texas CTV isolates. The majority of the CTV isolates tested contained the most severe CTV types known. In Florida, *T. citricida* were fed on crude CTV preparations *in vitro* and could transmit CTV to virus-free receptor plants with two CTV isolates, whereas a more

highly purified CTV preparation from one CTV isolate was not transmitted by *T. citricida*. There were no differences in the majority of treatments in infectivity neutralizations using three CTV-derived antibodies (p25, p27 and p20). CTV p20 antibodies significantly enhanced the occurrence of CTV transmission in one test. The CTV genome of isolate H33 was sequenced using 'shot gun' methods. The H33 major component and H33 minor components were phylogenetically compared to six other full-length CTV sequences. An untranslatable CTV coat protein gene was genetically transformed into the genome of the Texas commercial Rio Red grapefruit variety, and fifty-two independent transgenic lines were produced. CTV challenge responses by the transgenic lines were variable. Individual plants could be identified which had low virus titers by ELISA detection, a temporal decrease in virus titer, or a delay in virus titer accumulation. Comparing all wild types to all transgenic lines over every assessment revealed significant decreases in virus titer in the transgenic lines compared to that of the wild type. An RNA entity with similarities to marafiviruses was identified in a CTV infected plant. The entity appears non-graft transmissible to citrus, and non-mechanically transmissible to a range of herbaceous species.

This work is dedicated to my parents

John James Herron and Kathleen Nuvart Herron

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 SUMMARY

Tristeza is globally devastating disease of citrus caused by an infectious filamentous closterovirus, *Citrus tristeza virus* (CTV), which is phloem limited, aphid vectored and can be harbored asymptomatically in many citrus species. The virus has a very large single-stranded RNA genome, is not mechanically transmitted, and has been inadvertently spread to most citrus growing areas through the historical movement of citrus propagative material.

There are two major economically devastating symptoms caused by CTV. The first is a rapid decline and death of trees on sour orange (*Citrus aurantium* L.) rootstock. The second is stem pitting of scions regardless of rootstock causing reduced fruit production. Many species of aphid transmit CTV in a semipersistent manner, and this is important in the temporal and spatial spread of the virus within regions. *Toxoptera citricida* Kirkaldy, the Brown citrus aphid (BrCA), feeds and breeds mainly on citrus, is considered the most efficient vector of CTV, and has recently been introduced into North America.

This dissertation follows the style and format of *Virology*.

Management strategies for CTV usually involve shoot-tip grafting and thermotherapy, certification and eradication programs, deployment of tolerant cultivars, or mild strain cross-protection. Incorporation of CTV resistant genes into current commercial citrus cultivars is difficult and very lengthy by traditional crossing methods. Molecular pathogen-mediated strategies have been used to produce citrus plants. Such a strategy protects against infections by the virus from which the resistance gene is derived and closely related viruses (homology-dependent resistance).

1.2 INTRODUCTION

The center of origin for citrus is believed to be Southeast Asia and the Malaysian archipelago. Citrus has been cultivated since ancient times, in every civilization apart from those in North and South America. The citron, *Citrus medica* L., was recorded in pictures and models in the Karnak temple (15th century B.C. in northern Nigeria) and was recorded as early as 300 B.C. in Europe (Webber, 1948). Sweet orange, *C. sinensis* (L.) Osbeck, sour orange, *C. aurantium* L., and lemon, *C. limon* Burn. f. were cultivated in China long before their appearance in Europe. The times of the sea explorations in the 15th century proved to be a great time for the movement of propagative material of many crop species, particularly citrus to and from Europe. Columbus took citrus seeds to the New World (Haiti) on his second voyage in 1493. Grapefruit, *C. paradisi* Macf., has an unknown origin but most likely was first cultivated from a seedling of the Shaddock or Pummelo, *C. grandis* (L.) Osbeck in the Caribbean (Webber, 1948). In the 18th century, further dissemination of citrus from Europe to various places took place.

1.2.1 Origins of the tristeza disease and its viral nature

Citrus collections were established around the world for breeding and cultivation purposes and exotic citrus were introduced together with citrus viruses, *Citrus tristeza virus* (CTV) included (for example Meyer, 1911). Introduction of citrus viruses was unavoidable due to the lack of knowledge of such conditions at the time. As most citrus viruses are asymptomatic or inconspicuous in young trees, their distribution and propagation was guaranteed.

Citrus seeds and plants were brought to Australia with Cook's first voyage from Europe (1767-1771). Ports of call for subsequent voyages often included exchange of propagative material, for instance a second voyage of Cook (1772-1775) from England called at Rio de Janeiro, Brazil, and Capetown, South Africa (Bowman, 1955). The first indication of a grafting incompatibility or decline type problem associated with sour orange rootstocks comes from New South Wales (NSW), Australia, in 1890 at a Fruit growers' Conference in Sydney, where Mr. Thomas Pye stated "never use Seville (sour) orange stocks, as they have proved complete failure" (quoted by Bowman, 1955). Shaddock, Lisbon lemon and Seville orange were recorded as being very successful rootstocks in 1851 (Shepard, 1851 quoted by Bowman, 1955), therefore Fraser and Broadbent (1979) concluded the introduction into Australia of tristeza or its vector would be placed later than this. Fraser (Fraser and Broadbent, 1979) suggests that tristeza was probably present symptomlessly in the NSW coastal areas well before 1890 and possibly before 1874. In the mid-1860s *Phytophthora* root rot was causing damage to the citrus industry around Ryde, NSW. A committee of enquiry recommended that

sour orange rootstock should be tried, but it is not reported if growers changed husbandry practices *en masse* at that time (Report of the select Committee, 1866, Mackay, 1874, quoted by Bowman, 1955).

In the 19th century collar or foot rot caused by *Phytophthora* spp. destroyed seedlings of sweet oranges, tangerines and trees propagated from layering in South Africa, so much so that in 1896 the Cape Agricultural Department issued a circular instructing the grafting of scions to sour orange as a rootstock, justifying this change in husbandry practice by stating this was already a common practice in European countries (reported by Webber, 1943). After this change in practice in South Africa and elsewhere there were massive deaths of trees grafted to sour orange, and this was often termed a 'varietal incompatibility' in South Africa, Australia and Java. From extrapolation and retrospective reports, tristeza decline epidemics took place in Australia (pre-1890), South Africa (1910), Java (1928), Argentina (*ca.* 1938) and Brazil (1937).

Davis (1924) reported sweet oranges and mandarins died on sour orange rootstock whereas lemons did not, and related some of the observed trees had been grafted *ca.*1899 in Cape Colony in South Africa. Webber (1943) reporting on his comments from South Africa in 1924-5, had difficulty finding any trees on sour orange rootstocks under 20 years old in various locations. Those he could find were extremely stunted and worthless. Scions grafted to rough lemon or sweet orange rootstocks or lemons grafted to sour orange rootstocks, however were not affected by the problem.

Toxopeus, in Java, described disease symptoms on sour orange rootstocks similar to those in South Africa and ruled out environmental factors as the cause of the problem.

In elegant grafting and inter-stocking experiments Toxopeus (1937) concluded that sour orange did not injure sweet orange, but that the sweet orange scion produced some toxic substance which injured the sour orange. Bitancourt (1940a; 1940b; 1941) described a "Podridão das radicelas" or a rotting of citrus feeder roots and tested numerous scion-rootstock combinations in Brazil and suggested a viral origin for the syndrome. He suggested a latent virus in sour orange could be transmitted via the bud union to sweet orange, and that the virus was virulent in sweet orange. Webber (1943) was in a good position to evaluate the citrus disease symptoms in several countries. Webber had worked in Florida and published one of the first comprehensive publications on citrus diseases which is notable in the absence of any mention of tristeza-like symptoms (Swingle and Webber, 1896). Webber then studied citrus in 1924-5 in South Africa where he was exposed to the full impact of the symptoms from this 'new' problem. He then worked on a very similar problem causing death of citrus on sour rootstocks in California. Moreira (1942) used the term 'tristeza', Portuguese for sadness or melancholy, to describe the symptoms of canopy decline of scions grafted to sour orange in Brazil, and Webber (1943) suggested this term be used to describe the similar disease association in all the different countries. In his summary of all the scientific evidence for a cause to the tristeza problem, he concluded that only Toxopeus' hypothesis could not be disproved, since Bitancourt's hypothesis could not explain two other characteristic phenomena known to be associated to the disease. Important observations discussed by Webber (1943) were that sour orange scions grew without affect on sweet orange rootstocks and sour orange rootstocks failed whilst their respective sweet orange

scions recovered if inarched to rough lemon seedlings. Webber did have knowledge of the insect transmission of viruses from asymptomless carriers to susceptible plants but did not think this was important. Meneghini (1946) was yet to confirm the aphid transmission of the tristeza agent, and Fawcett and Wallace (1946), were yet to prove the viral nature of tristeza. The additional observation that sour orange rootstocks on declining sweet orange scions could often produce sprouts which did not become diseased lead to some confusion. Webber (1943) concluded that only a virus hypothesis could explain all instances of the disease. He also suggested that the foliage of sour orange and lemon produced a substance which inhibited the action of a virus in the sweet orange, and he notes there could also be different tissue specificities by the virus in various citrus species.

Tristeza was first confirmed in the United States in 1939 (Fawcett and Wallace, 1946; Wallace, 1956), where there was a 'quick decline' epidemic in California. This problem spurred research programs into the nature and control of the tristeza disease.

1.2.2 History of the grapefruit stem pitting and Mexican lime symptoms being associated with the tristeza disease

Only through the communications between scientists in different citrus growing countries did the various other symptoms come to be classified as being associated with the tristeza disease. At this time the aphid vector and transmissibility through propagative material also became apparent.

A disease of lime, (*C. aurantifolia* [Christm.] Swingle), in the West Indies with 'vein-clearing' leaf symptoms was reported as being caused by a pathogenic root fungus long before Ashby (1929) suggested another primary pathogen was the causal agent. This view was supported by Baker (1936) and Fennah (1942) studying similar lime problems in Montserrat, the Leeward and Windward Islands, respectively.

Lime production in the Gold Coast (now Ghana) had also been reported as being devastated by a disease in the Ghana since 1938. By 1947 limes could not be grown from seed in the open there and Hughes and Lister (1949) found symptoms included twig die-back, vein flecking of the young leaves and severe stem pitting of the trunks and branches. Costa and co-workers (1950) thought there was a possible link between the tristeza disease in Brazil and a 'stem pitting' disease of grapefruit in South Africa (Oberholzer *et al.*, 1949). Costa and co-workers (1950) used lime indicator plants in their experiments and noticed that after transmission of tristeza leaf vein clearing symptoms occurred, similar to those described in the lime disease from Ghana. A stem pitting disease of grapefruit was reported from plantings in Kenya and Ghana (Marloth, undated; Lister, pers. comm. quoted by Oberholzer *et al*., 1949). McClean (1950) used lime as an indicator plant for the stem pitting disease of grapefruit in South Africa after Lister visited him from Ghana. Hughes and Lister (1953) demonstrated that a virus caused the lime 'dieback' in the lime disease from Ghana. The virus was graft transmissible between citrus species, caused gummy hypertrophy in the xylem, impeded starch translocation from the leaves, and caused a growth reduction and chlorosis of leaves in sour orange seedlings ('seedling yellows' symptoms first formally attributed to

tristeza by Fraser, 1952) and, similar to tristeza, could be transmitted by the aphid *T. citricida*. Hughes and Lister (1953) also recognized different forms of the CTV caused different symptoms when graft inoculated to different citrus species.

1.2.3 CTV and human intervention

Graft transmission into new citrus growing regions is the primary route of introduction for CTV as the virus is not seed-borne (Bar-Joseph and Lee, 1989). Aphid transmission also can be important within citrus growing areas. The reaction of different citrus species, hybrids and citrus relatives varies to CTV infection considerably. Many citrus species are CTV-tolerant on their own or when grafted onto tolerant rootstocks; in both instances CTV particles are present in the phloem of such plants, but there are no symptoms caused by the virus in the plant. The virus causes damaging symptoms in some cultivars, however. Tolerance and susceptibility are complicated by the different behavior of various CTV isolates on citrus. The virus is recalcitrant to mechanical transmission, although this has been demonstrated experimentally (Garnsey *et al.*, 1977) after approximately forty knife slashes into the bark of single citrus seedlings.

1.2.4 Host-virus relationships

Histology

CTV particles occur in large numbers in phloem cells of *Citrus* and other members of the *Rutaceae*, *Passiflora* species being the only non-rutaceous hosts. CTV is generally reported as limited to the phloem cytoplasm and also to the young cortex

shoot tissue of plants. When tissue preparations are stained with Azure A, aggregates of virus particles or inclusion bodies are often found as purple stained, cross-banded 'chromatic cells' (Schneider, 1973) in the parenchyma-like cells adjacent to the sieve tubes in pollen. Such inclusions have also been associated with the cambium of newly developing stem cells in the ground meristem. Where this association is known to occur, xylem and phloem mother cells are not initiated - these are the regions of the 'pits' which later develop in the stems of infected trees. Necrosis at the bud union is a result of destruction of the phloem tissues in scions grafted onto sour orange rootstock which are undergoing 'quick decline'. CTV-associated inclusions can be observed by light microscopy (Brlansky, 1987), and are quantitatively related to the severity of CTV isolate involved (Brlansky and Lee, 1990; Broadbent *et al.,* 1996).

Leaf sap generally contains few virus particles, as has been reported for other systemic viral infections in woody plants (Schneider, 1973). No local lesion plant species are known for CTV. Yields of 0.1-2.5 mg virus per 100 g tissue can be obtained, depending upon the CTV isolate, tissue source and citrus species. Mexican lime and Etrog citron yield above average titers of the virus (Bar-Joseph and Lee, 1989). Such factors have implications for serological testing; young flush leaf midribs or young fruit peduncles are targeted for testing, as this rich in phloem tissues, and material is kept at 4˚C and processed for testing as soon as possible after collection (Lee, 1991).

Symptoms

Mixtures of CTV isolates in one plant have been hypothesized to occur (Grant and Higgins, 1957). Experimental work characterizing CTV is carried out in controlled conditions and/or on *in planta* viral cultures which usually have been passaged many times through experimentally reared aphids or graft transmission. CTV *in planta* cultures provide the basis for the many CTV isolates described (Garnsey *et al.*, 1987b; Bar-Joseph *et al.,* 1981; Roistacher and Moreno, 1992). The CTV isolates vary in their ability to be damaging to citrus plants after transmission through the various citrus aphids. CTV isolates vary in the symptoms induced in a standard set of citrus cultivar combinations under glasshouse conditions (see section biological characterization in section 1.3.2). Field symptoms of CTV are often more variable than those observed under controlled conditions.

Although a complex range of symptoms are produced under field conditions, as described in Table 1.1, there are three economically devastating field symptoms caused by CTV. The first is a decline of trees on sour orange rootstock. The second is 'stem pitting' of scions regardless of rootstock. Trees affected with CTV stem pitting strains decline, do not senesce, but have reduced fruit production and quality (Garnsey and Lee, 1988). A third type of symptom can cause losses in tree nurseries and is referred to as 'seedling yellows' (Fraser, 1952). Symptoms of seedling yellows are leaf chlorosis and stunting of sour orange, grapefruit or lemon seedlings.

Breeding for resistance to CTV

Genetic crossing methods to incorporate CTV resistance genes into citrus cultivars whilst retaining desirable characteristics of yield and quality have proved to be very lengthy and difficult in citrus. Genetic resistance to CTV has been found in citrus relatives *Severinia buxifolia* Poir. (Chinese box-orange), *Swinglea glutinosa* (Blanco) Merr. (the tabog or swinglea) and *Poncirus trifoliata* L. (Raf.) (the trifoliate orange) (Garnsey *et al.*, 1987a; Bar-Joseph *et al.*, 1989). The trifoliate orange is the only one of these species to be sexually compatible with *Citrus*. Nine genera within the *Aurantioideae* (orange sub-family) contain species where CTV introduction by inoculation through aphids and grafting techniques has been unsuccessful, leading to the conclusion these are resistant to CTV infection. Most of these genera are remote from citrus; however two genera, *Poncirus* and *Swinglea* can be crossed with *Citrus* (Williams, 1992).

1.2.5 Virus and vector interactions

Members of the *Aphididae*, *T. citricida* Kirkaldy (the brown citrus aphid or BrCA), *Aphis gossypii* Glover, and A. *spiraecola* Patch, are the principle vectors of CTV to citrus (Roistacher and Bar-Joseph, 1987a). Other vectors of minor importance have been recorded; *A. craccivora* Kock, *T. aurantii* Boyer de Fonsclombe, *Myzus persicae*, and *Dactynotus jaceae* L. (Bar-Joseph *et al.*, 1983). CTV is reported as being transmitted in a semi-persistent manner, with no latent period; aquisition and inoculation periods being at least 30 minutes in some cases (Bar-Joseph *et al.*, 1989). The rates of

TABLE 1.1

Field symptoms of *Citrus tristeza virus*

CTV DECLINE SYMPTOMS
1. Decline on sour orange rootstock
Within months the canopy of a mature tree suddenly wilts and dies
Scion/rootstock interface if bark is peeled back, may have 'pinholes' in the stem with corresponding 'pins' in the bark over the sour orange rootstock, called honeycombing, These symptoms are typical of decline on sour orange rootstock
2. Slow decline
3. Stunting
Mature trees do not grow
4. Bulge above the bud union
Gradually the scion has a greater diameter just above the bud union
5. No symptoms
CTV may be detected by serology but there are no detectible symptoms
CTV STEM PITTING SYMPTOMS
1. Stem pitting on main trunk, small branches and twigs
2. Small fruit size and stunting
3. Stunting
Mature tree does not grow
SEEDLING YELLOWS SYMPTOMS
Chlorosis and stunting in seedlings of lemon, grapefruit and lime May be associated with either stem-pitting or decline symptoms if mature infected trees are top-worked with susceptible citrus

Adapted from Garnsey and Lee (1988); Rocha-Peña *et al*. (1995).

aquisition have been found to be positively correlated with the length of acquisition period of up to 24 hours, at length of inoculation period 4-6 hours (Bar-Joseph *et al.*, 1989). Thus, time for the aphid to tap into the phloem is essential for viral transmission. Aphids can remain viruliferous for at least 24 hours. Infectivity is usually lost within 48 hours of acquisition.

1.2.6 Virus, vector, and plant interactions

Efficiency of CTV transmissibility is affected by the species of aphid, by the source plant at acquisition feeding and the CTV isolate. Several workers have reported that cultivars of sweet orange are more suitable for acquisition and more sensitive to infection than grapefruit or lemon seedlings (for instance, Bar-Joseph *et al.*, 1989). Grapefruit groves do not tend to undergo decline on sour orange rootstock where there is widespread decline of sweet orange on sour orange rootstock (Roistacher, 1983). Citrus aphids have a preference only for young lemon seedlings above sweet orange or young grapefruit seedlings (Bar-Joseph *et al.*, 1989). There is also a recognized decrease in transmission from plants kept at higher temperatures compared with a marked decrease in virus concentration in controlled experiments (Bar-Joseph and Lee, 1989). Red grapefruit varieties present problems with cross protection due in part to the slow distribution of protecting CTV isolates throughout the plant (Lee *et al*., 1987; Broadbent *et al.,* 1995). Pigmented grapefruits are more sensitive to stem pitting symptoms than

non-pigmented grapefruit (Marais and Breytenbach, 1996), and grapefruit also have been shown to influence the strain composition of CTV isolates (van Vuuren and van der Vyver, 2000).

1.2.7 Temperature and virus interactions

Ambient temperatures above 30˚C are known to surpress the field symptoms and detection of CTV through serology (Roistacher *et al.*, 1974; Mathews *et al.*, 1997). In hot desert areas, natural thermotherapy may well be important in field epidemiology and restrict disease spread. This may have a particularly pronounced effect on the detection and spread of CTV in tropical areas which have citrus regions in lowland and also areas in highland. Additionally, if a serological test is positive, then the tree is confirmed infected, even though at a later date another test from the same tree might be negative for CTV.

1.2.8 *Citrus tristeza virus* epidemiology

Temporal spread

Movement of virus in the early stages of the epidemic to trees in the late stages of the epidemic is reviewed by Thresh (1974). Measurement can take place on various scales of decreasing size; continent, country, area or individual citrus grove. Basically temporal spread considers how infected trees act as inoculum sources for the later viral infections. Variable data have been collected in the past on the rates of disease spread of CTV in North America, South America, and the Mediterranean area (Bar-Joseph *et al.*,

1983) based upon relative infection rate calculated using Vanderplank's compound interest equation (Vanderplank, 1960; Vanderplank, 1963). This is where inoculum is proportional to the initial inoculum plus the amounts subsequently produced during a season (polycyclic disease). Such data are confounded by the biology of CTV. For instance, not all trees infected with CTV decline or show any symptoms. Differences in the relative rate of disease spread are also explained by the variations of reaction by different CTV isolates present, citrus cultivars and fluctuations in the aphid field populations. Immigration of virus from neighboring groves, areas or regions also has to be accounted for, as well as natural thermotherapy, as CTV often spreads very slowly in the field.

With the advent of rapid and robust virus detection techniques, such as ELISA (Clarke and Bar-Joseph, 1984; Garnsey and Cambra, 1991), together with standardization of sampling techniques, useful data on the epidemiology of CTV can be collected. Many workers have used derivations of the compound interest equation to predict the efficiency of CTV eradication programs on a national scale (for instance, Bar-Joseph *et al.*, 1989; Fishman *et al.*, 1983, in Israel). Models applied to such data have provided very useful for analyses of future CTV epidemics. For instance, Allen (1983) developed a mathematical model for *Banana bunchy top virus* temporal spread and this model was used to investigate the suppression program of CTV in Israel in 1970-74. Fishman and co-workers' (1983) developed a mathematical model for CTV

which gave a prediction for the lag time from CTV infection to detection of CTV by ELISA (90-180 days) in field citrus trees. The proportion of trees found to be infected by ELISA and eradicated after discovery was used to indicate how good the control measure was.

Gottwald (Gottwald, 1992; Gottwald *et al*., 1996) has reported on epidemiological field trials in Hawaii, Costa Rica, the Dominican Republic, Spain and Taiwan using CTV assessments by ELISA. The Gompertz equation (Medawar, 1940) best describes the data, and is based upon the logistical model in that it incorporates early exponential-type growth, as well as incorporating the influence of healthy tissue. First there is a very low initial CTV incidence, and then this is followed by a sudden logistical increase in CTV infections, which in turn is followed by a period of stable, relatively high CTV incidence.

Spatial spread

CTV has been found to spread along rows in citrus groves rather than between rows (between row tree distance is usually greater in plantings) when *A. gossypii* is the vector. *T. citricida* tends to spread CTV 8-13 trees away from a source tree, whereas *A. gossypii* spreads CTV 2-3 trees away from a focus (Gottwald, 1992). Aphid patterns of CTV spread is indicated by a clustering nature of CTV infected trees in groves, and has been taken into account when planning large CTV detection surveys (Bar-Joseph *et al.*, 1989). CTV has been monitored for up to 14 years in experimental plots in Spain by ELISA (Gottwald *et al.*, 1996). CTV spreads a relatively long distance from the initial

viral source (Gottwald *et al*., 1996). There is also a suggestion that CTV spreads in a non-random fashion, either beyond the complexity of the analyses, or the plot size was not large enough to detect the spatial structure. These conclusions might also relate to aphid behavior and the nature of the interaction of CTV within the aphids. Aphid take-off, flight, landing and infectivity are difficult to study with respect to virus gradients and aphid distribution (Thresh, 1976). Statistical models of any benefit for prediction and eradication efficiency must take into account such factors. Sampling strategies for CTV detection depend upon systematic CTV sampling methods to take this problem into account (Lastra *et al*., 1991).

Spread of CTV into new areas

CTV spreads into new areas primarily via man transporting infected plant material (Thresh, 1980; Thresh, 1991). The extent to which long distance dispersal by aphids contributes to this is virtually unknown (Garrett and McLean, 1983). Aphids can survive flights by attaching to the clothing of humans. Citrus aphid vectors are thought to mainly contribute to secondary spread of CTV within a region. An exemption to this might be the introduction of a non-indigenous aphid pest as a virus vector into a new continent, however, as with the *T. citricida* into the Americas.

1.2.9 Importance of the movement of the brown citrus aphid through the Americas

With the movement of citrus plants and propagative material in sea voyages in the eighteenth century (Bowman, 1955), there is no doubt that both CTV and *T. citricida* were introduced into the New World, most likely on several occasions. CTV damage most probably did not occur until *T. citricida* displaced the indigenous citrus aphids (Lee *et al*., 1994) causing the severe epidemics of CTV which began in Brazil and Argentina in the 1940s (Müller and Costa, 1992). *T. citricida* was first identified in southern Venezuela in 1976, but was widespread by 1979 (Mendt, 1992). The first CTV decline in Venezuela appeared in 1980. Venezuela had an estimated 6.5 million productive trees, the majority on sour orange rootstock. By 1987, 6 million trees were estimated to have died due to CTV decline. CTV had been present many years before 1987 (Roistacher *et al*., 1991). However, with the introduction of *T. citricida*, CTV decline-inducing strains were rapidly disseminated, and long term problems ensued (reviewed in Rocha-Peña *et al.*, 1995). CTV-stem pitting on sweet orange and grapefruit are now damaging after growers changed to CTV-decline tolerant rootstocks. Stem pitting also occurs on Cleopatra mandarin rootstock and on rough lemon and Volkamericana rootstocks.

The majority of citrus grown in the Caribbean Basin and North America is cultivated on sour orange rootstock (180 million trees estimated in the Caribbean Basin). Surveys for the CTV and *T. citricida* have taken place in Central America, the Caribbean and North America over the last decade in order to geographically map the

TABLE 1.2

Incidence and severity of *Citrus tristeza virus* in a 1991 survey and first reports of the Brown citrus aphid for various Central American and Caribbean countries

Country	Samples tested in 1991 ELISA survey	CTV+	MCA-13+	BrCA first identified
Panama	207	16	14	ND
Costa Rica	433	25	4	1989
Nicaragua	307	6	4	1991
El Salvador	231	2	0	ND
Honduras	473	4	>1	ND
Guatemala	170	2	0	ND
Belize	2,725	13	>1	1995
Mexico	2,900	>1	0	2000
Trinidad	18	11	11	1985
St. Lucia	0	-	-	1992
Martinique	0	-	-	1992
Guadeloupe	0	-	-	1992
Jamaica	113	2	2	1993
Puerto Rico	218	6	2	1992
Dominican Rep.	200	15	9	1992
Haiti	0	-	-	-
Bahamas	14	-	-	-
Cuba	30	0	0	1993
Bermuda	770	31	11	ND

Compiled from Lastra *et al*. (1991); Lastra *et al*. (1992); Rocha-Peña *et al*. (1991); Michaud and Alvarez (2000). CTV+, positive in ELISA tests using polyclonal CTV antibodies; MCA-13, positive in ELISA tests using monoclonal antibodies; BrCA, brown citrus aphid; ND, not detected; -, not determined.

spread of *T. citricida* and incidence of CTV as summarized in Table 1.2. The first identification of the BrCA in the continental USA was in Florida (Hardy, 1995). The northernmost limit from Central America is currently southern Mexico (Michaud and Alvarez, 2000).

1.2.10 Brief history of *Citrus tristeza virus* with respect to Texas

Rootstock diseases were reported as causing citrus losses in Texas (Olson, 1952). Olson and Sleeth (1954) first identified CTV in Texas by observing leaf flecking in Mexican lime seedlings graft inoculated with Meyer lemon buds (*C. meyeri* Tanaka, the Beijing or Hsien Yuang lemon). Meyer lemons were introduced to the Rio Grande Valley before 1923 and grew more vigorously on their own roots than when grafted to sour orange rootstock. Rickett's Meyer lemon (Friend, 1954) was found to be CTV-free at this time (Olson and Sleeth, 1954). Olson (1955) conducted a CTV survey using field symptoms and indexing to Mexican lime. Many Meyer lemons and satsumas (*C. unshiu* Marc.) were found to harbor CTV. Eight varieties (14 trees) in a citrus collection with tissues from New Zealand (originally from Japan), Australia and India caused leaf vein flecking in the Mexican lime indicators. In commercial citrus trees only two Valencia sweet orange scions out of 250 grapefruit and Valencia sweet orange trees tested were CTV infected. One infected lime tree was also found, and this was situated adjacent to a CTV-infected Meyer lemon (Olson, 1955). Olson (1956) found damaging CTV in a Sueoka satsuma tree which originated from Japan, and determined that Meyer lemon CTV could cross-protect against the damaging CTV symptoms in certain cultivars

(Olson 1956; Olson, 1958). CTV was found in the upper Gulf coast area of East Texas in Meyer lemon, satsuma and grapefruit (Malouf, 1959). Dean and Olson (1956) tested *A. spiraecola* and *A. gossypii* but could not transmit CTV. Smith and Farrald (1988) confirmed *A. gossypii* could not transmit Texas CTV, whilst *A. spiraecola* could transmit CTV from infected Mexican lime.

A CTV survey by Davis *et al*., (1984) did not detect CTV in commercial citrus using ELISA. Dooryard Meyer lemon, satsuma and three other varieties from a citrus collection were confirmed to have CTV. More extensive CTV surveys in Texas over the last decade using ELISA (Solís-Gracia *et al*., 2001) have concluded that the commercial Lower Rio Grande Valley citrus plantings had very low (*ca.* 1%) incidence of CTV, whereas the dooryard plantings in East Texas had relatively high incidence (approximately 18%) of CTV.

In conclusion, CTV has been present in Texas for at least 80 years. The presumption is the relatively inefficient CTV vectoring aphid species present in Texas have not widely distributed the virus. Additionally, periodic freezes to commercial crops and subsequent re-planting of newly propagated trees has likely purged CTV from commercial citrus. Re-growth of CTV infected, freeze damaged dooryard citrus rootstocks provides the majority of CTV inocula.

1.3 *CITRUS TRISTEZA VIRUS* CHARACTERIZATION

Many CTV isolates have been described (Garnsey *et al.*, 1987a; Garnsey *et al*., 1987b; Garnsey *et al*., 1991; Bar-Joseph *et al.,* 1981; Roistacher and Moreno, 1992). The isolates vary in their ability to be damaging to citrus plants usually after passage through the various citrus aphids and indicator plants (Roistacher and Bar-Joseph, 1987b).

1.3.1 Closterovirus taxonomy

Closteroviruses (Family: *Closteroviridae)* have very large single-stranded, positive-sense RNA (ssRNA) genomes and many members are recalcitrant to mechanical transmission. Three viral genera have been described based the RNA genome and the type of insect involved in viral transmission. The genus *Closterovirus,* type species *Beet yellows virus*, BYV, have aphid vectors (Homoptera: Aphididae), and an ssRNA genome of up to *ca.* 20 Kb. The genus *Crinivirus,* type species *Lettuce infectious yellows virus,* LIYV, is transmitted by whiteflies (Homoptera: Alyrediidiae), and the genome is composed of two ssRNAs (Klaassen *et al.*, 1994; Klaassen *et al*., 1995). The genus *Ampelovirus* (Mayo, 2002) type species, *Grapevine leafroll-associated virus 3*, GLRaV-3, are vectored by mealy bugs (Homoptera: Pseudococcae). CTV belongs to the genus *Closterovirus* having long flexuous virion filaments of ca. 2000 x 11 nm, a monopartite genome, and is transmitted by aphids (Martelli *et al*., 2000).

1.3.2 Assessment of the biological activity of *Citrus tristeza virus*

For a comprehensive review of the conditions, husbandry, tools, seeds, grafting techniques, assessment of symptoms and indexing methods used for CTV consult Roistacher (1991). Briefly, lateral buds or "blind buds" lacking meristematic tissue from the donor plant is graft-inoculated onto the stem of a receptor or indicator plant, and usually this is repeated in quadruplet. Uninoculated receptors are kept under the same conditions as control plants. Well characterized CTV isolates can be used also as controls in tests, and these can be obtained from the world CTV collection housed at USDA-ARS in Beltsville, MD. A panel of five citrus cultivar or species combinations (see Table 1.3) is generally used for the strain characterization (Garnsey *et al*., 1987b). Visual assessments of the subsequent growth from the receptor plants are noted at approximate times after inoculation as given in Table 1.3 for each of the five citrus cultivar or citrus species combinations. One method applies an economic weighting to each citrus cultivar or species and combines this with the severity score to obtain a number or cumulative index for each CTV isolate (Garnsey *et al*., 1987b), as described for a hypothetical CTV isolate in Table 1.4. The second method places the CTV reaction in one of eleven reaction type or biotype groups which is based upon the reactions of isolates within the world CTV collection (Lee *et al*., 1994; Garnsey *et al.,* 1995; Rocha-Peña *et al*., 1995) as in Table 1.5. Generally, the higher the cumulative index score or the biotype rating, the more severe the CTV isolate.

TABLE 1.3

Citrus indexing of *Citrus tristeza virus*

Citrus receptor	Symptoms	Evaluation period*			
		Months post-inoculation			
		2	4	6	12
Mexican lime	VC, LC	X	X	X	
Mexican lime	SP			X	X
Sweet/Sour	CH, FL, ST		X	X	X
Sour orange seedlings	CH, ST	X	X	X	
Duncan grapefruit	CH, ST	X	X	X	
Duncan grapefruit	SP				X
Madam Vinous	SP, ST				X

Mexican lime (*C. aurantifolia)*; a clonal propagation on alemow (*C. macrophylla*), Sweet/sour; Hamlin or Valencia sweet orange (*C. sinensis*) grafted to sour orange (*C. aurantium*) seedlings, Duncan grapefruit; seedlings of Duncan grapefruit, Madam Vinous; seedlings of sweet orange Madam Vinous. All plants have a stem diameter of 5-7 mm at grafting. VC; leaf vein clearing, LC; leaf cupping, CH; leaf chlorosis, FL; flowering, ST; stunting, SP; stem pitting. *Approximate assessment times. Plants are trimmed after inoculation and foliar symptoms are assessed during successive flushes. Stem pitting symptoms are made by peeling the bark from the main stem and branches. After Garnsey *et al.* (1991).

TABLE 1.4

Severity rating of a hypothetical *Citrus tristeza virus* isolate

	ML 1	SW/SO 2	SO 3	DG 4	MV 5	Cumulative Index
CTV isolate A **Mean raw assessments**	1	2	0	0	3	
CTV isolate A **Relative indices**	1	4	0	0	15	20

ML; Mexican lime (*C. aurantifolia)* clonally propagated on alemow (*C. macrophylla)*, SW/SO; sweet orange (*C. sinensis*) grafted to sour orange (*C. aurantium*) seedlings, DG; Duncan grapefruit seedlings, MV; seedlings of sweet orange Madam Vinous. Economic weighting factors are the numbers under the receptor designations. Mean raw assessments are scoring all symptoms per receptor on a 0-3 scale with 0 as no symptoms, 3 as the severest symptoms. Relative indices are multiplying the mean raw assessment per receptor by the economic weightings. The cumulative index for each CTV isolate is the sum of all the relative indices. After Garnsey *et al*. (1987b).

TABLE 1.5

Categories of *Citrus tristeza virus* isolates recognized by indexing

CTV Biotype	ML	SW/SO	SY	GFSP	SWSP
0	-	-	-	-	-
I	+	-	-	-	-
II	+	+	-	-	-
III	+	+	+	-	-
IV	+	+	+	+	-
V	+	+	+	-	+
VI	+	-	+	+	-
VII	+	-	-	+	+
VIII	+	-	-	+	-
IX	+	-	-	-	+
X	+	+	+	+	+

ML; Mexican lime leaf vein clearing, stunting and stem pitting, SW/SO; decline symptoms on sweet orange grafted to sour orange rootstock, SY; seedling yellows symptoms, GFSP; stem pitting symptoms on grapefruit, SWSP; stem pitting symptoms on sweet orange. After Lee *et al*. (1994).

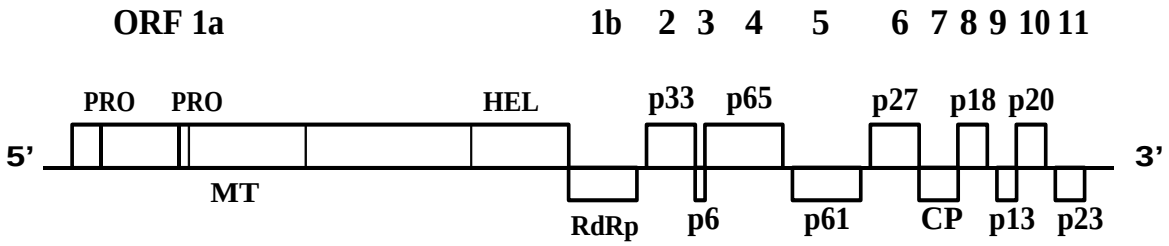

FIG. 1.1. The *Citrus tristeza virus* genome. Open reading frame (ORF) numbers are at the top and represented by rectangles. ORF 1a has identified domains of two leader proteases (PRO), a methyltransferase (MT), and helicase (HEL). The putative PRO cleavage sequences are denoted by thick vertical lines; the MT and HEL domains are delimited by the narrow vertical lines. Putative protein products are described in the text and are above the appropriate ORFs in the diagram. Total length represented is approximately 20 kb. Diagram re-drawn from Mawassi *et al.* (2000), not to any scale.

1.3.3 The *Citrus tristeza virus* genome

The capped *ca.* 20 kb RNA of CTV contains 12 open reading frames (ORFs), as described in Fig. 1.1., and these potentially encode at least 19 protein end products (Pappu *et al.*, 1994). The CTV genome has a 'Sindbis-type' replication block at the 5'-end, whereas there is a 'closterovirus hallmark' block at the 3'-end (Karasev *et al.*, 1997). In the core replication block, ORF 1a contains two tandem domains encoding related variants of the papain-like thiol proteases (PRO). The ORF 1a product is expressed as a polyprotein from which the two PROs can be released autocatalytically. Within ORF 1a there is also encoded a putative methyltransferase (MT) and a RNA helicase (HEL) domain. The polymerase (RdRp) is thought to be expressed by a +1 frameshift resulting in an ORF 1a-1b fusion protein (Karasev *et al.*, 1995). BYV has a similar organization apart from possessing only one papain-like thiol protease. Thegenome size, organization and expression resembles that of *Coronaviruses* (Family: *Coronaviridae,* Order: *Nidovirales*), however the mechanism of subgenomic expression may differ. CTV has no complementarity between the 5'-UTR and any of the subgenomic promoters; therefore the expression is thought to be similar to alpha viruses.

The CTV RNA world

The 3' 10 ORFs, including the 'closterovirus hallmark' block of ORFs 2-7 (Karasev *et al.*, 1997), are expressed by a nested set of 3' coterminal messenger RNAs (mRNAs: Hilf *et al.*, 1995; Navas-Castillo *et al.*, 1997), with corresponding negative-sense RNA versions of these sub genomic RNAs (sgRNAs). Double-stranded (ds)

replicative form (RF) RNA can be detected in CTV infected plants, this has been identified as ds versions of the gRNA and 3'-coterminal sgRNAs (Hilf *et al*., 1995). The 3' sgRNA promoters are dissimilar to the 5' genomic UTR and proximal region, as with the alphavirus supergroup-type viruses. The highly expressed 3' genes have distinct non-coding regions 5' to the respective ORFs (Gowda *et al*., 2001). The whole 'closterovirus hallmark block' sequence can be deleted without affecting replication of the virus in protoplasts (Satyanarayana *et al.,* 1999). A nested set of 10 positive-sense 5' coterminal sgRNA are also produced, with each RNA terminating upstream of the sequence for the respective corresponding 3' sgRNA, leading to the suggestion that the 3'-sgRNA promoter sequences are control elements. The 5'-coterminal subgenomic RNAs are suspected as being replication termination products, but as yet have unknown function (Gowda *et al*., 2001).

Three other classes of 5'-coterminal sgRNA have been characterized: Low-molecular-weight-tristeza-RNA-1 and -2 (LMT1, LMT2), and large-molecular-weight-tristeza-RNA-2 (LaMT) (Che *et al*., 2001). LMT1 and LMT2 are equivalent to approximately 0.7 kb of the CTV gRNA, with LMT2 being approximately 100 bp larger than LMT1. LMT1 and LMT2 are each more abundant than the gRNA in infected cells. LaMT is equivalent to the CTV ORF1a and 1b, is approximately 11 kb in size and is found in lower proportion than the gRNA in infected protoplasts.

CTV defective RNA (D-RNA) is composed mainly of sequences from the 5' proximal region fused to the 3' distal region of the gRNA, and is usually 2.0 to 5.0 kb size range (Mawassi *et al*., 1995, Karasev *et al*., 1997, Yang *et al*., 1997). Large D-

RNA species (~12 kb) constructed with intact ORF1a and 1b genes fused to variable length 3'distal region portions, are slash-transmitted to citrus plants, and also readily infect *Nicotiana tabacum* protoplasts (Che *et al*., 2002). D-RNA corresponding to ORF 1a and 1b or ORFs 2-11 inclusive have also been described (Che *et al*., 2003). No interspecific RNA viral associations have yet been documented with CTV although it is common to find CTV in combination with other graft transmissible RNA infective agents (Roistacher, 1991).

Putative 3' CTV proteins

ORF 3 encodes for p6, a putative 6-kDa hydrophobic protein, and is postulated as being membrane associated (Karasev *et al.*, 1995). A heat-shock protein 70 homologue (HSP70h) of *ca*. 65-kDa (p65) encoded by ORF 4 has been speculated to mediate CTV cell-to-cell movement via interaction with the cytoskeleton (Pappu *et al.*, 1995), and might stabilize or guide virions when moving through the plasmadesmata (Medina *et al.*, 1999). The HSP70h (p64) from BYV has been found to have an estimated 10 HSP70h molecules tightly attached to each virion (Napuli *et al*., 2000). The function of p61 (ORF 5; 61-kDa) may involve the assembly of multisubunit complexes or virions or in disarming the host defense response (Dolja *et al.*, 1994). The HSP70h and the p61 have been proposed to be involved in virion assembly and are also required for the minor capsid (CPm) tail assembly within the BYV virion (Alzhanova *et al*., 2001). In BYV the HSP70h has been postulated as providing a motor for the movement or directional entity, the CPm, whilst attached to the rest of the virion (Bukau

and Horwich, 1998). The BYV CP, CPm, 6-kDa protein (p6), HSP70h, 64-kDa protein (p64), which all have 3' gene analogues in CTV, are required for cell-to-cell movement (Alzhanova *et al*., 2002).

The CTV ORF 7 specifies the major CTV capsid protein (CP or p25; 25-kDa). ORF 6 encodes the related minor or duplicate or minor CP (CPm or p27; 27-kDa) which forms the unique closterovirus 'rattlesnake' structure on the 5'-end of the virion. This unusual virion structure was first discovered in BYV (Agranovsky *et al.*, 1995; Febres *et al.*, 1996). CPm accumulates in the host cell wall fraction (Febres *et al*., 1994). BYV p20 (ORF 7 product in BYV; 20-kDa) has been established as required for transport through the phloem but is dispensable for virion assembly and cell-to-cell movement (long distance movement factor). The BYV p20 interacts or 'docks' to the HSP70h molecules already attached to the virion thus providing a long distance transport factor to the complex to allow BYV trafficking through the phloem (Prokhnevsky *et al*., 2002). BYV p20 shows very little similarity to other ca. 20 –kDa proteins encoded by related closteroviruses. Deletion of the CTV p65, p61, p25 and p27 genes in a protoplast system does not prevent viral replication but prevents virion formation, suggesting the respective proteins are all needed for virion integrity (Satyanarayana *et al.*, 2000).

The ORF 10 product, p20, has been found to be the major cytoplasmic cell inclusion protein in a protoplast system (Gowda *et al.*, 1997; Gowda *et al.*, 2000). Recently p20 has been identified as a suppressor of post-transcriptional gene silencing in a transient assay system using *N. benthamiana* (Reed *et al*., 2003; Lu *et al*., 2003).

The putative ORF 11 product of 23-kDa, p23, is presumed to be involved in minus-strand synthesis and has predicted ribosome binding capacity (Dolja *et al.*, 1994). Accumulation of both positive and negative sense sgRNA and the respective products has been shown to be controlled by a 'master switch', the 3' terminal product (p23), and in particular delimited to a region including a RNA-binding and a zinc-finger domain (Satyanarayana *et al*., 2002a). The p23 is accumulated very early in cell infection and down-regulates negative-stranded RNA accumulation which indirectly increases expression of the 3' genes. Mexican lime plants transformed with the CTV p23 gene exhibited typical CTV symptoms of vein clearing in the leaves (Ghorbel *et al*., 2001). The p23 gene, when cloned into an *Agrobacterium tumefaciens* binary vector and used in *A. tumefaciens* co-infiltration assays into green fluorescence protein (GFP)-silenced *N. benthamiana* plants, was demonstrated as being the second CTV suppressor of post-transcriptional gene silencing (Lu *et al*., 2003).

Virus mutants from which the CTV p33, p18, and /or p13 genes are deleted infect and move in citrus plants (Dawson, 2000). None of these genes have an assigned function. This range of genetic diversity has been postulated as being evolved in CTV along with the expansion of genome size (Dolja *et al.*, 1994).

1.3.4 Laboratory methods used for the characterization of *Citrus tristeza virus* isolates

Cell inclusions

CTV induces amorphous or needle-shaped cell inclusions in phloem tissue of infected citrus plants (Christie and Edwardson, 1986). Thin sections of tissue may be stained with Azure-A to visualize the inclusions under light microscopy. Statistically the numbers of cell inclusions in replicate transverse sections from infected tissues has been found to correlate to CTV isolate severity (Brlansky, 1987; Brlansky and Lee, 1990; Broadbent *et al*., 1996).

Serological methods

Rapid tests for detecting CTV incidence and severity have been developed using serology, and have been tested during CTV regional, country and state surveys (Lastra *et al.,* 1991; Lin *et al.,* 2000). Many polyclonal antibodies raised against different isolates of the CTV CP can detect the virus (techniques reviewed by Rocha-Peña and Lee, 1991). Monoclonal antibodies (MAbs) have been found to be useful to determine between CTV isolates but only MCA-13 has been found to be associated to biological activity (Vela *et al*., 1986; Permar *et al.,* 1990; Shalitin *et al*., 1994; Nikolaeva *et al*., 1996; Lin *et al*., 2002a). Since only a single CP epitope is recognized by each MAb, usually tests of several antisera have to be used since the type of severity is difficult to determine (Cambra *et al.,* 2000). A rapid CTV CP purification method followed by peptide

mapping using six CTV specific antibodies allowed discrimination of four out of five CTV isolates which had similar biological activity, dsRNA profiles or reactivity with MAbs in an ELISA format (Albiach-Martí *et al*., 2000a).

The MAb MCA-13 has been widely utilized as this selectively reacts against a strain of CTV associated with severe sweet orange on sour orange decline in Florida (Permar *et al.,* 1990). Pappu *et al*. (1993) presented evidence that and introduced change from a tyrosine amino acid to a phenylalanine in the CP of a CTV isolate (T30, a biotype 1 CTV isolate from Florida) altered the reaction with MCA-13 from negative to positive. CTV stem pitting in sweet orange can also be detected now using a specific polyclonal antibody (OSP) against CTV CP expressed from *E. coli* (Nikolaeva *et al*., 1998).

Double-stranded RNA profiles

Plants not infected with RNA viruses or virus-like agents do not readily contain detectible amounts of high molecular weight (>0.1 x 10^6) double-stranded (ds) RNA (Morris and Dodds, 1979). Since most plant viruses have positive-sense, single-stranded RNA genomes, and corresponding viral replicative fraction dsRNA products accumulate in virus infected cells, dsRNA detection may be used as a non-specific virus detection technique. CTV dsRNA profiles have also be used to discriminate between certain damaging forms of CTV and non-invasive disease symptoms (Dodds and Bar-Joseph, 1983; Dodds *et al.,* 1984).

Genome-based methods

Full-length CTV cDNA sequences from the T36 isolate from Florida (Karasev *et al*., 1995), the VT isolate from Israel (Mawassi *et al.*, 1996), the SY 568 isolate from California (Yang *et al.*, 1999), the T385 isolate from Spain (Vives *et al*., 1999), the T30 isolate from Florida (Albiach-Martí *et al.*, 2000c) and the NUagA isolate from Japan (Gede *et al.*, 2001) are currently available in the Genbank for comparative CTV genomics. The techniques rely on the reverse-transcription polymerase chain reaction (RT-PCR) technique where the starting point is genomic RNA, cDNA copies are made and amplified for analyses.

The 5'-UTR and 5'-proximal coding region of the CTV genome have been found to be highly polymorphic when the full genomes of several CTV isolates were compared (for instance Yang *et al.*, 1999). However, two predicted 5'-UTR secondary stem loop structures are conserved between the different CTV isolates, suggesting this structure may be important for function. Based upon the 5'-UTR secondary structure of the gRNA from four CTV isolates, three genotypic groups (I, II and III) have been delimited (López *et al.*, 1998).

Expansion of this approach to analyze 58 5'-UTR clones from 15 CTV sources revealed all sequences could be placed into groups I, II or III (Ayllón *et al*., 2001). Most isolates were mixtures of sequences from different categories. CTV isolates containing solely type III sequences caused only mild to moderate symptoms in Mexican lime. CTV isolates causing stem pitting in sweet orange or grapefruit cultivars contained type II sequences. No isolate contained sequences of type I and type II alone.

Two CTV genotypic groups (VT and T36) have also been determined based upon hybridization with probes from 5' and 3' regions of three CTV Florida isolates (Hilf *et al.*, 1999), and these could be further subdivided into three genotype groups using three additional primer sets from the CTV 5'-proximal region. When the study was expanded to 13 CTV sources from different geographical regions, the same technique suggested one additional genotype could not be differentiated (Hilf and Garnsey, 2000).

Nine CTV strain discriminating CTV CP oligonucleotide probes have been developed based upon sequence analyses of the CTV CP gene from diverse biological and geographic backgrounds (Cevik, 1995; Nolasco *et al.,* 1999; Niblett *et al.,* 2000). CTV CP cDNA is immobilized on duplicated nylon membranes which are hybridized with each probe. The probes have been used to detect severe CTV and isolate mixtures of CTV from field samples in Portugal, Madeira and Florida (Nolasco *et al.*, 1997; Niblett *et al.,* 2000).

Single-strand conformational polymorphism (SSCP) is a technique which can be used to detect polymorphisms or mutations in DNA between different individuals in a population. With ssRNA viruses RT-PCR is used for a specific region of the genome then the resultant cDNA is denatured and separated by non-denaturing polyacrylamide gel electrophoresis (PAGE). Separation of the cDNA depends upon the conformation taken up by the cDNA which in turn is dependent upon intra-molecular hydrogen bonding between the bases (rather than annealing to their complementary strands), thus different conformations are formed based upon the composition of the nucleotides in the

strands which migrate at different distances from the origin. Additionally, for separation of a greater number of individuals, the RT-PCR products can also be digested with restriction enzymes before denaturing and PAGE treatment (restriction fragment-length polymorphism or RFLP). Electrophoresis patterns of cDNA derived from various regions of the CTV genome has been found to be useful to quickly type CTV from field sources without the laborious sequencing of many samples of the CTV population from each tree or without hybridization procedures. Generally, the more numerous the bands obtained by SSCP (Rubio *et al.*, 1996; van Vuuren and van der Vyver, 2000; Sambade *et al.*, 2002) or RFLP analyses of the CP CTV gene (Valle *et al.,* 2000), the more severe the CTV in citrus plants. Banding patterns are related to mixed CTV isolate populations within single trees, and have also been used to study changes with CTV populations over time (Sambade *et al*., 2002).

1.4 APHID TRANSMISSION OF PLANT VIRUSES

Aphids are estimated to transmit over 200 plant viruses and are the most important vectors amongst insects for transmitting plant viruses (Harris, 1977b). The nature of the relationships between aphid, plant and virus involve specific interactions. The abundance, worldwide distribution and feeding behavior of aphids make them ideal vectors for dissemination of plant viruses. Most aphid-transmitted viruses do not reproduce themselves in the vector organism (non-propagative). In the persistent or circulative mode of transmission, the virus is translocated from the insect's alimentary canal to the accessory salivary gland and is injected with saliva into the recipient plant.

In noncirculative transmission the virus is retained in the foregut, and introduced into a new plant by an ejection-ingestion mechanism (Pirone, 1991). Foregut-borne, nonpersistent transmission involves brief feeding on the epidermal layers of plants as the virus remains in the aphid for a few minutes. Semipersistent transmission is thought to involve the continuous feeding by the aphid upon phloem tissue, thus the virus remains in the aphid for up to a few days. Semipersistent transmission and persistent transmission both involve continuous feeding of the aphid on the phloem to acquire the virus, and viruses which are transmitted in this manner usually exhibit strong phloem tropism. The differences and the similarities between the three categorized transmission types of nonpropagative aphid transmission are summarized in Fig. 1.2. Representatives of circulative, nonpersistent and semipersistent transmission are provided by the luteoviruses, potyviruses and closteroviruses, respectively.

1.4.1 Nonpersistent aphid transmission

Nonpersistent transmission is associated with the aphid's food canal within the maxillary stylets and involves the basal part of the feeding apparatus only (Harris *et al*., 1995). In the icosahedral *Cucumber mosaic virus* (CMV), transmission depends largely on the CP, suggesting direct attachment of virus particles to the insect foregut (Perry *et al.*, 1994). A single nucleotide change in the CP sequence can alter the physical properties of the CP and the aphid transmissibility of the virus is obliterated. A negatively-charged loop structure in CMV capsids which is conserved amongst

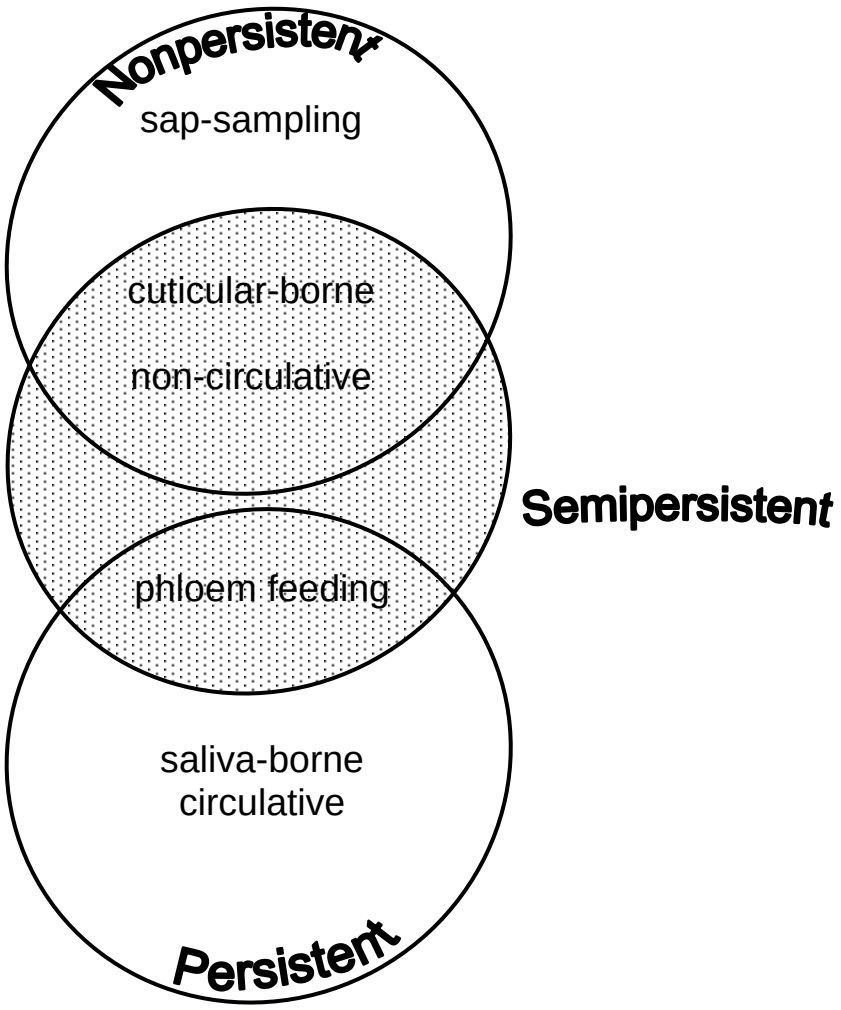

FIG. 1. 2. The differences and similarities between the types of nonpropagative aphid transmission of viruses. Nonpersistent aphid virus transmission is characterized by aphid plant sap-sampling or probing behavior with viruses being restricted to a cuticle lining interaction since they do not enter the hemolymph. Persistent aphid transmission of viruses involves a phloem feeding aphid behavior, with viruses passing into the digestive tract and circulating in the insect via the salivary glands. Semipersistent transmission of viruses involves aphid cutica interactions with phloem feeding aphid behavior.

cucurmoviruses, the βH-βI loop, has been shown to be essential for aphid transmission (Liu *et al*., 2002). The nonpersistent aphid transmission of filamentous potyviruses is mediated by the CP, and a helper component-proteinase (HC-Pro) that is found in virus-infected cells but not in virions (Pirone and Blanc, 1996; Thornbury *et al.*, 1985). The CP has a dual role; formation of the infective virion particles and aphid transmission. A short N-terminal domain of the CP, non-essential for particle assembly has been found to be indispensable for aphid transmissibility (Atreya *et al.*, 1991), and this has also been found to be required for long-distance movement of the virus within plants (Dolja *et al.*, 1994). HC-Pro has also been found to be multifunctional. The central domain is required for long-distance transport and virus replication , whilst the C-terminal domain represents a papain-like proteinase (Carrington *et al.*, 1989). A portion, P1/HC-Pro, acts as a viral suppressor of post-transcriptional gene silencing in plants (Anandalakshmi *et al.*, 1998). The direct interaction between the CP and HC-Pro cause a reversible binding of virions to the aphid foregut (Pirone and Blanc, 1996).

1.4.2 Persistent aphid transmission

The persistently transmitted luteoviruses are acquired by aphids from phloem sieve elements and are able to traverse the hindgut epithelium, diffuse through the hemocoel, and translocate into the salivary gland receptor-mediated transport, and then are secreted with the aphid's saliva when the aphid feeds on a plant (Harris *et al*., 1995). Most if not all aphids are thought to utilize bacterial symbionts to supplement their poor-quality diets (Dixon, 1998; Fukatsu, 1994)). The primary symbiont of most aphids

is *Buchnera aphidicola* (Subdivision: Proteobacteria) and the symbionts are contained within specialized cells in the aphid termed bacteriocytes (Dixon, 1998). Aphids have been found to perform better as far as feeding and growth on virus infected plants compared to non-virus containing plants (Dixon, 1998). Therefore the relationship between the aphid, its symbionts and virus infected plants might not be random. Aphids might derive a better diet if feeding from virus-infected plants, particularly if the virus produces vast amounts of proteins in the tissues upon which aphids feed.

Beet western yellows virus (BWYV, Family: *Luteoviridae*) minor CP has found to be a determinant of aphid transmission. The minor CP is produced by a read-through translation of the ORF for the major CP. Symbionin (Chaparonin 60), a protein secreted into the hemocoel by *B. aphidicola* is indispensable for luteovirus transmission (van den Heuvel *et al.*, 1997). The sequence of symbionin is similar to GroEL, a heat-shock like protein with chaperone function. The bacterial protein forms a complex with the minor CP of the virus within the aphid which is necessary for virus transmission. The symbionin production by the bacterium is controlled by the aphid, and other, secondary symbionins are produced in most aphids by secondary endosymbionts (Dixon, 1988).

The specific viral protein-aphid receptor molecule reactions which determine *Barley yellow dwarf virus* (BYDV; Family *Luteoviridae*) transmission are present in the aphid salivary gland (Gildow and Gray, 1993). Two proteins (SaM35 and SaM50) have been isolated from head tissues of the aphid vector, *Stobion avenae*, but not present in a

non-virus vectoring aphid species (Li *et al*., 2001). An anti-idiotypic antibody which mimics an epitope on the BYDV virions also bound to SaM35, SaM50 and six other aphid proteins, including a GroEL homologue.

1.4.3 Semipersistent and closterovirus aphid transmission

The semipersistent mode of CTV transmission by aphids is different from the nonpersistent transmission characteristic of cucurmoviruses and potyviruses. In semipersistent transmission the virus comes into contact with a far larger surface area of the aphid's cuticular lining, that is, the cibarial valve and pump. These structures are thought to be involved in tasting the quality of the feed, which is the 'functional mouth' (Harris *et al*., 1995). The exact mechanics of the semipersistent aphid transmission of the closteroviruses are unknown. A helper-component-like protein (s) may be required for aphid transmission, as shown for nonpersistent potyvirus transmission (Pirone and Blanc, 1996) and semipersistent caulimovirus aphid transmission.

For the icosahedral *Cauliflower mosaic virus* (CaMV), helper proteins P2 and P3 are needed for the reaction between the virions and aphid cuticle (Woolston *et al*., 1987; Leh *et al*., 2001), and the virus has been reported as being preferentially acquired from the phloem by the aphid vectors (Palacios *et al*., 2002). CaMV is documented as having a bimodal type of aphid transmission. In bimodal transmission, aphid virus acquisition can cluster around two periods, a short time period and a relatively long time period, and there is generally no change if aphids are pre-aquisitionally fasted or not (Lim and Hagedorn, 1977).

For CTV the leader protease (L1, the 5' Pro in Fig. 1.2.) shares homology with the potyvirus HC-Pro, whilst the CTV L2 leader protease (the 3' Pro in Fig. 1.2.) does not seem to function in the same manner (Peng *et al*., 2001). In the family *Closteroviridae*, the type of insect transmission to some extent is reflected by the number and type of leader protease sequences present. Therefore the CTV proteases may be considered strong candidates for aphid transmission CTV-helper proteins. The CTV HSP70h, as with the function of cellular heat-shock proteins, may be needed to halt the cell's defenses against stylet penetration. CTV CP, CPm, HSP70h, p61, p20 are also likely candidates to be involved in aphid transmission, since they are present on the outer surface of CTV virions. Since there are many species of aphids which can transmit CTV and at least 10 structural proteins encoded by the virus, different assortments of viral proteins may be involved with transmission, depending on the aphid species involved.

Crinivirus and closterovirus CPs and CPms are associated with virus transmission by insects. The CPm of *Lettuce infectious yellows virus* (LIYV) is a determinant of whitefly transmission when partially purified virus preparations are used for immunoneutralizations (Rochow and Muller, 1975) with antibodies generated to all the virus proteins (Tian *et al.*, 1999). The CP and CPm of BYV, in similar immunoneutralization tests were found to be essential for aphid transmission (He *et al.*, 1998).

1.4.4 *Toxoptera citricida* – the brown citrus aphid

'Citri-cida' in Latin means 'citrus killer,' and the aphid is a serious pest of citrus as well as the most efficient vector of CTV. Experimental transmission tests have been performed using four well characterized isolates of CTV from the CTV World Collection (Garnsey *et al.*, 1991), with *T. citricida* and *A. gossypii* (Yokomi *et al.*, 1994). These tests reveal that *T. citricida* is approximately 6-25 times more efficient at transmitting three CTV isolates than *A. gossypii*. Transmission of one CTV isolate is low (11%) by *T. citricida* and not transmitted by *A. gossypii* (0%). BrCA transmission of CTV has also been recorded as consistently very low or variable enough to produce inconclusive tests (Stubbs, 1964).

The BrCA probably originated in Asia, possibly China, within the same region as the center of origin for citrus. *T. citricida* has been reported on other plant species, *Rhododendron sp*., *Acerola sp*., *Malpighia punccifolia*, and *Eugenia uniflora* in Puerto Rico (Yokomi, 1992; Yokomi *et al.*, 1994), and on *Passiflora*, *Calodendron*, *Mangifera* and *Anacardium* genera elsewhere (Roistacher *et al.*, 1991). However, it is thought that feeding and breeding normally take place on *Citrus* (Yokomi *et al.*, 1994). The BrCA can survive a wide variety of climates in the tropics, from Sub-Saharan Africa to areas in Australia, India, Sri Lanka and Asia. Currently the BrCA is not present in Mediterranean areas where citrus is grown. CTV BrCA transmission has been

additionally classified as bimodal (Chalfant and Chapman, 1962), and this has not been reported for any other CTV-transmitting aphid species. Aphids using bimodal transmission of plant viruses are thought to be more important as virus vectors due to the variation in virus acquisition time needed (Lim and Hagedorn, 1977).

1.4.5 *Aphis gossypii* – the melon and cotton aphid

This aphid is the most efficient vector of CTV in the absence of *T. citricida*, but is far more polyphytophagous (Bar-Joseph *et al.*, 1983; Yokomi, 1992). *A. gossypii* has the potential to efficiently transmit some strains of CTV (Bar-Joseph *et al.*, 1983; Yokomi *et al.*, 1989; Rocha-Peña *et al.*, 1995), as noted with the stem pitting strain of CTV in California in the 1970s (Roistacher and Moreno, 1992). Where CTV and *A. gossypii* occur together in a citrus growing region, there seems to be a lag of approximately 30-50 years from the first citrus introduction into a new area and CTV damage due to *A. gossypii* movement (Rocha-Peña *et al.*, 1995).

1.4.6 *Aphis spiraecola* – the spirea or green citrus aphid

The spirea aphid is a far more serious pest of citrus than *A. gossypii*, but far less efficient at vectoring CTV. Yokomi and Garnsey (1987), observed a 29% CTV transmission rate with *A. spiraecola* compared to 76% with *A. gossypii* using different Florida CTV isolates. The overall transmission rate of CTV by *A. spiraecola* was 6.3% compared to 17.9% for *A. gossypii*. Dean and Olson (1956) conducted extensive transmission tests with *A. spiraecola* and *A. gossypii* in Texas, but could not demonstrate

any CTV transmission. Smith and Farrald (1988) reported a high transmission rate of CTV by *A. spiraecola* from Mexican lime yet *A. gossypii* did not transmit any of the Texas CTV isolates used. More recent transmission tests in Texas have suggested that *A. spiraecola* is the major CTV vector, with an overall transmission rate of 8% from known CTV sources (Cutrer, 1998).

1.5 PATHOGEN-DERIVED RESISTANCE IN PLANTS

Included within this subject is the classical cross-protection strategy which has been used to sustain citrus growing in areas where CTV would otherwise prevent economic citrus survival. Engineered pathogen-derived resistance involves processes which can be additionally used in the future to provide more durable resistance to CTV (Sanford and Johnston, 1985).

1.5.1 Cross protection

Cross protection is the use of a mild CTV strain to protect against economic damage by severe CTV strains (Gonsalves and Garnsey, 1989). Classical cross protection strategies have been used with success against stem pitting CTV strains on grapefruit in Brazil (Costa and Müller, 1980), Australia (Broadbent *et al.*, 1991), South Africa (van Vuuren *et al.*, 1991), and with decline inducing CTV in Florida and Venezuela (Lee and Rocha-Peña, 1992; Ochoa *et al.*, 1993). In the South African citrus clean-stock program, virus-free citrus propagative material is re-infected with a ‘mild’ CTV isolate (pre-immunized) before release to growers. CTV cross protection can delay

the onset of tristeza disease. Without CTV cross-protection grapefruit production would be uneconomic in South Africa (von Broembsen and Lee, 1988; van Vuuren *et al.*, 1993; van Vuuren and da Graça, 2000).

CTV isolates used for cross protection have usually been collected within the country of dissemination, and indexed in many different local commercial citrus cultivars. Experimental trials are usually conducted under different temperature regimes and field site conditions before such CTV isolates are evaluated for possible deployment as a management strategy (Powell *et al*., 1992). Only those CTV isolates which consistently give non-damaging CTV symptoms, for instance, CTV biotype I reactions (Mexican lime CTV symptoms) would be considered for cross-protection in certain circumstances, for instance in Florida.

The mechanism of the cross protection within the plant cell is unknown, even though virologists have known about the phenomenon since the 1920s (McKinney, 1929). One hypothesis is that there are a finite number of sites within a citrus plant available for CTV particles to uncoat or replicate. If 'mild' CTV isolates are artificially introduced, then attachment sites might be unavailable for the severe CTV virions to attach and uncoat at a later date. Thus the 'mild' CTV population is the dominant within the citrus plant, and protects the plant from the severe form of the virus (Matthews, 1991). Mechanisms of cross-protection have been elucidated by the study of plants transformed with viral genes.

1.5.2 Engineered pathogen-mediated resistance

Engineered pathogen-mediated resistance (Grumet *et al.*, 1987) can confer resistance in plants to pathogenic viruses without losing quality and yield aspects. For reviews on this subject see Wilson *et al*., (1993), Scholthof *et al.,* (1993), and Lomonossoff, (1995).

Protection is conferred by viral nucleic acid sequences (mainly defective or antisense) which are introduced into the plant genome by genetic engineering and this was first demonstrated using *Tobacco mosaic virus* (Powell-Abel *et al*., 1986; Beachy, 1990; Lomonossoff, 1995). This approach has been found to be applicable to a range of more than 20 virus species in different transgenic plant systems (Beachy, 1997), and has been applied to commercial crop species production. Protein-mediated resistance is the expression of a functional or dysfunctional viral gene product which does not support the viral disassembly, transcription, translation, replication or spread of the virus. The viral coat protein has been a target for this strategy (coat protein-mediated resistance or CP-MR) during experimentation, but viral movement and replicase proteins have also been used in this strategy.

Dysfunctional viral proteins interfere with the viral infection cycle in a dominant-negative manner as has been demonstrated with *Tobacco mosaic virus* (TMV*)* (Malyshenco *et al.*, 1993). This is similar to RNA-mediated resistance where untranslatable RNA sequences of the target viral gene have been found to confer resistant phenotypes with *Tomato spotted wilt virus* or *Tobacco etch virus* (Lindbo and Doughtery, 1992), and may now be considered all part of the same phenomenon as

discussed in section 1.5.3. below. That is, in the transgene, part of the replicase is expressed and acts in a dominant-negative manner. Transgenic plants developed by this approach are likely to be protected against infections by the virus from which the resistance gene is derived, and possibly closely related strains or viruses (homology-dependent resistance).

1.5.2 RNA-mediated virus resistance

Sense-RNA or homology-dependent virus resistance is distinct from CP-MR in that the resistance is conferred by a transgene which encode for untranslatable RNAs which are homologous to the corresponding viral RNA sequences (Baulcombe, 1996; Baulcombe, 1999; Prins *et al*., 1996). This strategy has lead to complete resistance by plants to certain viruses (Lindbo and Dougherty, 1992; Smith *et al*., 1994). Other RNA-mediated strategies have been categorized, for instance, antisense RNA, defective interfering (DI) RNA and satellite RNA (Harrison *et al*., 1987) but may not all be mutually exclusive in the mechanism or plant cell pathways utilized to obtain resistance.

Sense RNA leads to post-transcriptional gene silencing (PTGS) characterized by sense or co-suppression of virus replication (Baulcombe, 1996). The untranslatable RNA is generated by changing the initiation codon into a sense codon, deletion of the AUG initiation codon or by introduction of termination codons downstream of the initiation codon (Lindbo and Dougherty, 1992). The RNA sense strategy triggers the cell's machinery to specifically degrade target RNA sequences (Smith *et al*., 1994; Lindbo *et al*., 1993).

PTGS type resistance has been documented as plant immunity, since it is active in leaves of a plant known to be previously virus challenged (Dougherty and Parks, 1995). PTGS with plant viruses has been associated with virus-infected non-transgenic plants in 'recovery' phenotypes, is developmentally regulated, and can be induced from silenced rootstock to non-silenced scion or can systemic spread (Tanzer *et al*., 1997; Ratcliff *et al*., 1997; Voinnet and Baulcombe, 1997; Palauqui *et al*., 1997). Goregaoker *et al.* (2000) found that fragments of the RNA polymerase of TMV confer resistance in *N. benthamiana* by both PTGS and a protein-derived mechanism; therefore, several types of resistance mechanism in different plant-virus systems may act in concert or simultaneously.

PTGS has now been found to be a natural defense system against nucleic acid invasion with parallels in most living organisms. Scientists in different disciplines were describing phenomena, which were thought to be unrelated, thus the process has been referred to as RNA interference (*Drosophila melanogaster*, *Caenorhabditis elegans, Homo sapiens, Chlamydomonas* sp.), quelling (*Neurospora crassa* and other fungi) and PTGS or co-suppression in plants. In most organisms thus far described, the mechanics of the reaction have been partially unraveled, and analogous enzymes or nucleic acids have been found. Recent reviews of the PTGS mechanism include Voinnet (2001), Hamilton *et al*. (2002), and Cerutti (2003).

In brief, a specific RNA transcribed from a gene is targeted for degradation by the cell machinery by using RNA-dependent RNA polymerases to form dsRNA. The dsRNA is split into small fragments of 21-23 nt called small interfering RNAs (siRNAs)

by the action of a RNAse III-like enzyme, called Dicer in *Drosophila* (Zamore *et al*., 2000). The siRNAs guide a multi-component ribonuclease, the RNA-induced silencing complex or RISC (Hammond *et al*., 2000) which unwinds the siRNA (Nykänen *et al*., 2001) and uses the single-stranded siRNAs as a guide to recognize complementary RNAs (Nykänen *et al*., 2001; Martinez *et al*., 2002) whilst a putative endoribonuclease specifically degrades RNA from the center of the siRNA (Hammond *et al*., 2000; Elbashir *et al*., 2001; Martinez *et al*., 2002). There are clearly many so far undescribed cellular biochemical pathways acting upon this system.

The vast majority of plant viruses, including CTV, have positive-sense ssRNA genomes and it is likely that these viruses produce replicative form (RF) RNA (dsRNA) during replication within the plant cell, thus they can be the trigger to PTGS in plants. Another consequence is that plant viruses have evolved genes to suppress this plant defense system. The potyvirus helper-component protease (HC-Pro) interferes with silencing at the point of siRNA production, or upstream of this point (Anandalakshmi *et al.,* 1998; Kasschau and Carrington, 1998). The *Cucumber mosaic virus* (CMV) 2b protein also halts PTGS initiation in young flush by preventing PTGS long distance signaling (Guo and Ding, 2002). *The Potato virus X* (PVX) p25 suppresses PTGS at the mobile signal level (Voinnet *et al*., 2000), and the P0 of *Beet western yellows virus* has been demonstrated to have PTGS-suppressor activity (Pfeffer *et al.*, 2002).

1.5.3 Closterovirus pathogen-derived resistance

Several citrus species have been transformed with a either a functional or untranslatable CTV CP gene of CTV but presently there is little published information on the resistance of such plants to CTV infection (Gutiérrez *et al.*, 1992, Gutiérrez 1997; Moore *et al.*, 1992; Domínguez *et al.*, 2000; Ghorbel *et al.,* 2000; Yang *et al.*, 2000; Ghorbel *et al*., 2001; Febres *et al.,* 2003). Two groups have evaluated transgenic citrus plants for resistance to CTV. Domínguez *et al.,* (2000), used a translatable CTV CP transgene in Mexican lime and found approximately one third of transformants did not develop CTV symptoms when graft or aphid inoculated with CTV. The majority of transformed plants showed a significant delay in virus accumulation and CTV symptom onset. Febres *et al*., (2003), challenge tested Duncan grapefruit transformed with translatable CTV CP genes and an RdRp gene. All plants were susceptible to the CTV by ELISA, with a number of individual having lower titers of CTV compared to non-transformed controls.

PTGS-suppressor activity has been postulated for several closterovirus genes. BYV p21 and two analogous proteins from the closteroviruses, the p22 from *Beet yellow stunt virus* (BYSV; genus *Closterovirus*, Karasev *et al*., 1996) and CTV p20 (ORF 10 product) have recently been experimentally demonstrated to have PTGS-suppressor function in a transient *N. benthamiana* assays (Reed *et al*., 2003). Activity of these proteins is thought take place after the Dicer-mediated dsRNA cleavage step in the PTGS pathway (Reed *et al*., 2003). CTV also encodes two putative leader papain-like proteases, L1 and L2 (Karasev, 2000). CTV L1 shares sequence and possibly functional

similarity to BYV leader protease L-Pro, which in turn shares homology to the potyvirus HC-Pro (Peng *et al*., 2001). The CTV ORF 11 product, p23, been shown to possess an RNA-binding and a zinc finger domain (Satyanarayana *et al*., 2002a), and RNA-binding capacity seems to be general predictor of potential PTGS-suppressor activity (Lichner *et al*., 2003). The CTV p23 protein additionally is accumulated very early in cell infection and therefore is a strong candidate to have PTGS-suppressor activity, and this has been demonstrated recently in a *N. benthamiana* experimental system (Lu *et al*., 2003).

The *Sweet potato feathery mottle virus* (SPCSV: genus, *Crinivirus*) genome encodes a gene which putatively encodes an RNase III-family protein, most similar to *Arabidopsis thaliana* L. BAB02825 (46% similarity), potentially the first plant virus Dicer-like protein identified (Kreuze *et al*., 2002). Therefore closteroviruses might suppress plant antiviral machinery at several stages in the PTGS pathway, and might also have the capacity to silence other cellular nucleic acid 'invaders'.

1.6 THE AIMS OF THIS STUDY

CTV can cause extreme economic losses due to death of trees or reduction in fruit size. At the moment in the citrus commercial area of the Lower Rio Grande Valley of Texas, the incidence of CTV is low with spread only possible via inefficient aphid vector species. Very soon the efficient CTV vector, the brown citrus aphid, *T. citricida* Kirk. will become established in Texas, and with this event, the risk of CTV economic losses becomes greater. Thus it is important to gather information concerning the severity type and location of the CTV in Texas before *T. citricida* arrives as this

determines the type of disease management strategy to be deployed and forms a base-line so that changes in CTV symptomology may be documented in future. Transgenic citrus plants containing genes to counteract virus proliferation must also be evaluated so that useful CTV resistance is demonstrated. In parallel with this more basic research on various molecular aspects of the virus, for instance the viral proteins needed for successful aphid transmission are essential in order to propose specific and adequate pathogen-mediated resistance strategies for further long-term control strategies.

With these thoughts in mind, the objectives of this work are to determine the severity of CTV in isolates collected from the different growing areas of Texas by typing the CTV sources on the standard host range of five citrus indicator species (Garnsey *et al.*, 1987b) in a controlled environment. The second aim is to type the CTV sources using some currently available molecular techniques and evaluate the tests for correlation with biological activity. The third aim is to gather more information on the role of the CTV CPm; for instance, is this protein crucial for the BrCA transmission of CTV? The fourth aim is to evaluate CTV untranslatable CP transgenic Rio Red grapefruit by challenges with CTV in a controlled environment.

CHAPTER II

SEVERITY OF *CITRUS TRISTEZA VIRUS* FROM TEXAS USING CITRUS INDICATORS, SEROLOGY AND MOLECULAR TECHNIQUES

2.1 SUMMARY

Citrus tristeza virus (CTV), an economically important viral pathogen of citrus reduces citrus production globally. For severity typing of CTV isolates, biological characterization is time consuming and costly. Therefore the intent of this work is to determine if molecular methods could reliably predict CTV severity were compared with biological indexing leading to faster, better and cheaper assays. The data were compared to characterize CTV isolates collected from the Lower Rio Grande Valley and East Texas. Fifteen CTV sources were indexed on Mexican lime, sour orange, sweet orange on sour orange, Duncan grapefruit and Madam Vinous sweet orange indicator plants. Additionally, some CTV sources were indexed on the Texas commercial cultivars Rio Red and Star Ruby grapefruit, and Marrs and N-33 sweet orange. Severity ratings were rated into biotype groups (0-X) or cumulative mean relative indices. Molecular characterization was carried out using poly- and monoclonal (MCA-13) antibodies, seven strain group specific probes and single-stranded conformational polymorphism (SSCP) all derived from the CTV major coat protein (CP) or gene. All

CTV isolates produced vein clearing symptoms on inoculated Mexican lime plants. Over half of the CTV isolates tested were placed biotype groups IX and X (causing decline of sweet orange on sour orange, seedling yellows on sour orange and grapefruit seedlings, and stem pitting of grapefruit and/or sweet orange), and one isolate was in biotype I (mild). Statistical analyses of the biological and molecular data were performed to determine any correlation of laboratory methods with the biological indexing.

2.2 INTRODUCTION

Citrus tristeza virus (CTV) induces a plethora of disease symptoms in citrus species. One major damaging CTV symptom is the decline of trees on sour orange rootstock, leading to tree death. The second major damaging symptom is stem pitting of citrus scions regardless of the rootstock, leading to reduced fruit size, quality, and production (Garnsey and Lee, 1988; Bar-Joseph *et al.*, 1989). Seedling yellows symptoms show dwarfing and chlorosis in sour orange, grapefruit and lemon seedlings, and can cause drastic tree losses in citrus nurseries (Fraser, 1952). CTV management practices depend upon the type or group of damaging symptoms present in any particular region.

CTV is an aphid-transmitted closterovirus with flexuous filamentous particles having a very large (~20 Kb) single-stranded, positive-sense RNA (ssRNA) genome. Observations and molecular studies indicate that the CTV occurring in one tree is usually a mixture of genotypes which may be more diverse than the quasispecies

concept implies. Full-length CTV cDNA sequences from the T36 and T30 isolates from Florida (Karasev *et al*., 1995; Albiach-Martí *et al.,* 2000c), the VT isolate from Israel (Mawassi *et al.*, 1996), the SY 568 isolate from California (Yang *et al.*, 1999), the T385 isolate from Spain (Vives *et al*., 1999), and the NUagA isolate from Japan (Gede *et al.*, 2001) are available in Genbank for comparative CTV genomics.

CTV has two putative capsid proteins, putatively; these are the major 25 k-Da (CP) protein and minor 27-kDa protein (CPm), which encapsidate approximately 95% and 5% of the virion length, respectively (Karasev *et al.*, 1995; Febres *et al.*, 1996). The CTV genomic RNA (gRNA) contains 12 distinct open reading frames or ORFs (Pappu *et al.*, 1994; Karasev *et al.*, 1995), potentially encoding at least 19 protein products and having two untranslated regions (UTR) at the 5'- and 3'-termini. The CTV gRNA has a 'Sindbis-type' replication block at the 5' proximal end, with proteins translated directly from the gRNA and other smaller proteins putatively produced from these by polyprotein processing. The RNA-dependent RNA polymerase (RdRp) is thought to be expressed by a +1 frameshift resulting in an ORF 1a-1b fusion protein (Karasev *et al.*, 1995).

The 3'-UTR from different CTV gRNA sequences are highly conserved in primary sequence, whereas the 5'-UTR and 5'-proximal region are highly polymorphic. Two predicted secondary stem loop structures for 5'-UTR sequence are conserved between the different CTV isolates, suggesting this structure may be important for function. Based upon the 5'-UTR secondary structure of the gRNA from four CTV isolates, three genotypic groups (I, II and III) have been delimited (López *et al.*, 1998).

Expansion of this approach to analyze 58 5'-UTR clones from 15 CTV sources revealed all sequences could be placed into 5'-genotype groups I, II or III (Ayllón *et al*., 2001). Most isolates were mixtures of sequences from different groups. CTV isolates containing solely genotype III sequences caused only mild to moderate symptoms in Mexican lime. CTV isolates causing stem pitting in sweet orange or grapefruit cultivars contained genotype II sequences. No isolate contained sequences of type I and type II alone.

Hilf *et al.* (1999) chose various regions across the CTV genome and compared these regions by RT-PCR from different CTV isolates. Two CTV genotypic groups (VT and T36) were determined based upon hybridization with probes from 5' and 3' regions of three CTV Florida isolates, and these were further subdivided into three genotype groups (VT, T36 and T30) using three additional primer sets from the CTV 5'-proximal region. When the study was expanded to 13 CTV sources from different geographical regions, the same technique suggested one additional genotype could not be detected (Hilf and Garnsey, 2000).

The 3'-UTR region does not contain a recognizable poly $(A)^+$ tract or t-RNA-like structure. Exchanging the 3' UTR between the different CTV isolates causes drastic changes in replication in protoplasts (Satyanarayana *et al*., 1999), and such a quantitative measure might relate to phenotype. The secondary structure for the 3'-UTR has been predicted as a complex of 10 thermodynamically stable stem loops of which three (SL4, SL8 and SL6) have been found to be necessary in primary structure for viral replication by mutational analysis of the genome in *vivo* (Satyanarayana *et al*., 2002b).

CTV infected tissue generally contains several variable gRNA species variable in sequence, at least 30 to 33 positive-sense subgenomic RNA (sgRNA) species (Gowda *et al*., 2001), and many types of defective RNA (D-RNA) species (Mawassi *et al*., 1995a; Mawassi *et al*., 1995b; Karasev *et al*., 1997; Yang *et al*., 1997; Che *et al*., 2001; Che *et al*., 2002). The 3' 10 ORFs, including the 'closterovirus hallmark' block of ORFs 2-7 (Karasev *et al.*, 1997), are expressed by a nested set of 3'-coterminal sub genomic (sgRNAs; Hilf *et al.*, 1995; Navas-Castillo *et al.*, 1997), with corresponding subgenomic double-stranded forms (dsRNAs) also being present.

Accumulation of both positive and negative sense sgRNAs and their respective products has been shown to be controlled by a 'master switch'- the 3' terminal product (ORF 11; p23) , and in particular delimited to a region including a RNA-binding and a zinc-finger domain (Satyanarayana *et al*., 2002a). The p23 protein, which accumulates very early in cell infection, down-regulates negative-stranded RNA accumulation which indirectly increases expression of the 3' genes (Satyanarayana *et al.*, 2002a).

The heat-shock protein 70 homolog (HSP70h) is postulated as having a cell-to cell movement function. In CTV the HSP70h, p61, CP and CPm are also required for efficient virion assembly (Satyanarayana *et al*., 2000). The HSP70h from *Beet yellows virus* (BYV), type member of the *Closterovirus* genus (Family: *Closteroviridae*), has been found to have an estimated 10 HSP70h molecules tightly attached to each virion (Napuli *et al*., 2000). These molecules have been proposed to be involved in virion assembly and are also required for the minor capsid (CPm) tail formation of the virion in BYV (Alzhanova *et al*., 2001). The BYV HSP70h has been postulated as acting as a

motor for the movement or 'steering' apparatus, the CPm on the virion end (Bukau and Horwich, 1998). The BYV CP, CPm, 6-kDa protein (p6), HSP70h, 64-kDa protein (p64) BYV, which all have 3' gene analogues in CTV, are required for cell-to-cell movement (Alzhanova *et al*., 2000). BYV p20 has been established as being dispensable for virion assembly and cell-to-cell movement but is required for transport through the phloem. The BYV p20 interacts or 'docks' to the HSP70 molecules already attached to the virion thus providing a long distance transport factor to the complex to allow phloem traffic (Prokhnevsky *et al*., 2002). CTV p20 protein is putatively expressed from the same position in the genome as BYV p20 but bears little similarity to the BYV protein in primary sequence, although this does not preclude functional similarity.

CTV p20 (ORF 10; 20-kDa) is found in abundance in CTV infected inclusion bodies, and infected protoplasts (Gowda *et al*., 2000). Mexican lime plants transformed with the CTV p23 gene exhibit typical CTV symptoms of vein clearing in the leaves (Ghorbel *et al*., 2001), suggesting p23 is a symptom determinant. p20 and p23 have also been found to have post-transcriptional gene silencing (PTGS) suppressor activity in heterologous transient plant assays (Reed *et al*., 2003; Lu *et al*., 2003).

No interspecific RNA viral associations have yet been documented with CTV although it is common to find CTV in combination with other graft transmissible RNA infective agents (Roistacher, 1991). CTV is disseminated into new citrus growing areas mainly through movement of CTV-infected nursery material (Permar *et al.,* 1990). The phloem-limited virus is mechanically-transmitted with difficulty, and often replicates to

low levels in infected tissues. CTV has a host range limited to *Rutaceae* and *Passiflora* species, and is not seed transmitted (Bar-Joseph and Lee, 1989). However, aphid dissemination is an important route of transmission in citrus growing areas, with the most efficient CTV vector being *Toxoptera citricida* Kirkaldy, the brown citrus aphid (BrCA).

Many CTV isolates have been described and characterized (Garnsey *et al.*, 1987b; Bar-Joseph *et al.,* 1981; Roistacher and Moreno, 1992). The isolates vary in their ability to produce symptoms in citrus; and the symptoms produced vary after passage through various aphids and/or plants. Generally isolates are biologically characterized according to symptoms induced in a standard panel of citrus indicator plants under greenhouse conditions, and based upon symptoms expressed, can be assessed according to two complementary methods. The first applies an economic weighting to the severity score (Garnsey *et al*., 1987b), the second method places the CTV reaction in one of eleven reaction type groups (Lee *et al*., 1994, Rocha-Peña *et al*., 1995), the higher either score or biotype, the more severe the CTV isolate. Mild CTV isolates, those considered as only causing disease symptoms in Mexican lime (biotype I), cause very little or no damage to commercial citrus and may be beneficial as they protect against severe CTV damage by cross-protection.

Cross protection is the use of a mild CTV strain to protect against economic damage by severe CTV strains (Gonsalves and Garnsey, 1989). Classical cross protection strategies have been used with success against stem pitting CTV strains on grapefruit in Brazil (Costa and Müller, 1980), Australia (Broadbent *et al.*, 1991), South

Africa (van Vuuren *et al.*, 1991), and with decline-inducing CTV in Florida and Venezuela (Lee and Rocha Peña, 1992; Ochoa *et al.*, 1994). The only geographic area where severe CTV and its efficient aphid vector, *T. citricida* did not co-exist upon the deployment of cross-protection was in Florida. In the South African citrus clean-stock program all virus-free propagative material was re-infected with a 'mild' CTV isolate (pre-immunized) before release to growers. Without this cross-protection grapefruit production would be uneconomic there due to CTV (von Broembsen and Lee, 1988; van Vuuren *et al.*, 1993; van Vuuren and da Graça, 2000). For many aspects of CTV management, therefore there is a need to differentiate between severe and mild CTV isolates within a particular region. In Florida, for instance, if certain severe CTV isolates are detected using MCA-13 (Permar *et al.,* 1990) antibody screening, then budwood from the trees cannot be used for propagation. A state legislated eradication program of CTV-infected trees is in place in California.

The BrCA was introduced into South America early last century, and severe epidemics of CTV have occurred in the wake of BrCA movement into various citrus growing regions of Argentina, Brazil, Paraguay and Uruguay (Roistacher *et al.*, 1991). Current interest in the CTV status of Texas citrus has been heightened by the recent northern movement of the BrCA into Florida (Hardy, 1995) and southern Mexico (Michaud and Alvarez, 2000). Presently there are no losses due to CTV reported in the commercial area of citrus production in the Lower Rio Grande Valley (LRGV) of Texas. In CTV surveys conducted the CTV incidence was found to be very low (*ca.* 1%) in the LRGV but relatively high (approximately 18%) in East Texas (Solís-Gracia *et al.*, 2001).

In concert with the threat of the BrCA to citrus production, a legislated Texas citrus certification program has been established which is soon to become ratified by The Texas State Dept. of Agriculture (Skaria *et al*., 1996, Kahlke *et al*., 2000).

The majority of the commercially grown citrus in Texas is red grapefruit (*C. paradisi* Macf.) on sour orange rootstock. Red grapefruit has been reported to have a relative slow spacial distribution of non-damaging CTV (Broadbent *et al.*, 1995), and pigmented grapefruit is also reported to be more sensitive to CTV stem pitting symptoms (Marais and Breytenbach, 1996). Sour orange rootstock makes the plants additionally susceptible to the CTV-declines. Once the BrCA arrives in Texas, is anticipated that CTV already present in the citrus will be temporally and spatially distributed to the majority of trees.

The established method for CTV biological indexing for strain discrimination is by graft inoculation of test tissue onto replicates of a panel of at least five different citrus indicator plants under controlled conditions. This takes at least one year to determine under optimal greenhouse conditions (Roistacher, 1991; Garnsey *et al*., 1995). The process is laborious and expensive in terms of greenhouse space and personnel. Rapid tests for detecting CTV incidence and severity have been developed using serology, and have also been tested during regional, country and state surveys for CTV (Lastra *et al.,* 1991; Lin *et al.,* 2000; Lin *et al.*, 2002a). Monoclonal antibody (MAb) MCA-13 has been found to be useful to determine if severe CTV is present (Permar *et al.,* 1990), other MAbs have been used for severity detection but are usually combined with other tests or antibody tests to differentiate different strains at a locality (Cambra *et al.,* 2000;

Vela *et al*., 1986; Lin *et al*., 2002a). Techniques based upon CTV dsRNA profiles have also been evaluated to discriminate between certain damaging and non-invasive CTV symptoms (Dodds and Bar-Joseph, 1983; Dodds and Lee, 1992). Single-strand conformational polymorphism (SSCP) of cDNA derived from various regions of the CTV genome after RT-PCR has been found to be useful to quickly type CTV from field sources without the laborious sequencing of many samples of the CTV population from each tree. Generally, the more complex the banding pattern obtained by SSCP (Rubio *et al.*, 1996; van Vuuren and van der Vyver, 2002; Sambade *et al.*, 2002) or restriction fragment-length polymorphism (RFLP) analyses of the CP CTV gene (Valle *et al.,* 2000), the more severe the CTV in citrus plants. CTV isolate banding patterns obtained from these analyses have been found to change in different hosts or after aphid transmission (Ayllón *et al.*, 1998). Strain group specific probes have been developed in Florida based upon sequence differences in the CTV CP gene from specific CTV isolates (Cevik, 1995; Nolasco *et al.,* 1999; Niblett *et al.,* 2000).

The aims of this study were to biologically characterize the CTV isolates collected in the various regions of Texas before the BrCA arrives. Additionally, various available rapid methods were assessed to characterize the CTV isolates and the results were compared with the severity based upon biological indexing. Both of these aims are congruent with the aim to develop a sound CTV management strategy for the Texas certification program.

2.3 MATERIALS AND METHODS

2.3.1 Indexing on citrus

CTV isolates collected from field sources (Solís-Gracia *et al.,* 2001) were maintained in a greenhouse on various citrus hosts since 1984. Donor buds or stem bark pieces (2-4 per seedling) were graft inoculated to citrus indicators in June 1997 (Experiment 1). Five citrus indicator plants were used; Mexican lime [*C. aurantifolia* (Christm.) Swing.], Duncan grapefruit, Pineapple sweet orange [*C. sinensis* (L.) Osbeck] grafted to sour orange, sour orange seedlings, and Madam Vinous sweet orange seedlings. Four plants of each indicator were used for each isolate, plus two plants were left uninoculated for controls. Rio Red and Star Ruby grapefruit, Marrs and N-33 sweet orange navel varieties, all grafted on sour orange rootstock were used as additional indicators for some of the CTV isolates. Plants were kept in the cool (27-30˚C maxima/18-21˚C minima) indexing facility. Visual assessments of symptoms were made periodically over a two year period according to methods detailed (Garnsey *et al*., 1987b). A severity score (0-3) was given to each symptom in each plant. The mean severity score was calculated for each donor original isolate in each citrus indicator. The mean severity score was multiplied by a weighting factor for each citrus cultivar according to the relative economic impact of the CTV symptom (Garnsey *et al*., 1987b). Mexican lime had a weighting factor of 1; sweet on sour, 2; seedling yellows symptoms, 3; stem pitting with Duncan grapefruit, 4; and stem pitting on Madam Vinous, 5. A second biological indexing (Experiment 2) was conducted in the same facilities with plants inoculated in August 2001. Six reference CTV isolates (B2, B4,

B5, B6, B28, B384) from the CTV Exotic World Collection maintained in the quarantine facilities, USDA-ARS Beltsville Agricultural Research Center, MD (BARC), were obtained under permit No. USDA 46874. Buds were graft inoculated onto Pineapple sweet orange seedlings in the Texas A&M University-Kingsville Citrus Center's indexing facility. These six reference CTV isolates were then compared in the evaluation with selected Texas CTV isolates (H11, H29, H33, and H41). The symptoms were assessed for 12 months. For all plants ELISA tests were used also to detect the extent of CTV infection.

2.3.2 RNA-blot analyses

Nucleic acids were extracted from leaf tissue of each test using a modified double phenol/chloroform extraction method in extraction buffer (0.1M NaCl, 0.01 M Tris-Cl, pH 7.5, 1 mM EDTA and 1% SDS; Sambrook *et al.,* 1989), and precipitated from the aqueous layer (using 0.1 vol of 3 M sodium acetate, pH 5.3 and 2.5 vol of 95% ethanol). After centrifugation, pellets were re-suspended in sterile ultra-pure water. Total RNA was precipitated by adding 2 vol 7 M LiCl and incubated for 12 h at 4˚C, after which pellets were obtained by centrifugation, washed with 70% ethanol and resuspended in sterile ultra-pure water. Total RNA was quantified by UV spectophotometry. Denatured total RNA (3 μg) was checked for integrity by electrophoresis through 1% agarose with TBE buffer (89 mM Tris-borate, pH 8.0, 2 mM EDTA). For size analyses and hybridization, formamide and heat-treated denatured RNA (10 μg) was electrophoresed in formaldehyde-1.6% agarose (Sambrook *et al.,*

1989). Gels were incubated with 2X SSC (20X SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 30 min, then the nucleic acids were transferred by downward capillary action onto Hybond-N+ nylon membranes (Amersham, Bucks., UK) using 20X SSC. Hybridization was performed at 65˚C according to Church and Gilbert (1984) with a ^{32}P-labeled dCTP DNA probe derived from the 3'-end of the CTV SY568 genome, made using a random primers DNA labeling kit and the supplier's instructions (GibcoBRL Life Technologies, Galthersburg, MD).

2.3.3 ELISA

Double antibody sandwich-indirect (DAS-I) ELISA was performed using two CTV polyclonal antisera (Garnsey and Cambra, 1991; Rocha-Peña and Lee, 1991). Sterile polystyrene flat bottom 96-well microtiter plates (Immulon) were incubated with CTV IgG CREC 28 (1μg/ml) in carbonate coating buffer (0.05M sodium carbonate at pH 9.6) for 4 h at 37˚C or overnight at 4˚C. Between each incubation step, plates were washed three times with phosphate-buffered saline with Tween 20 (PBST; 0.02 M phosphate, 0.14 M sodium chloride at pH 4.4, 0.1 % [v/v] Tween 20). Four near mature leaf mid-veins (0.5 g) for each test sample were pulverized in 5 ml extraction buffer (PBST with 2% [w/v] polyvinylpyrrolidone-40) using a tissue homogenizer. The resultant sap for each sample was added to duplicate test wells on the antibody-coated microtiter plates and incubated at 4˚C overnight. The secondary goat IgG antibody, G604-10, in conjugate buffer (PBST plus 2% w/v polyvinylpyrrolidone-40, and 0.2% [w/v] ovalbumin) at a dilution of 1:30,000, was added and incubated at 37˚C for 4 h or

overnight at 4˚C. Antigoat antibody conjugate with alkaline phosphatase (Sigma A-4187) at 1:30,000 dilution in conjugate buffer was added and incubated under the same conditions. Substrate (1µg/ml; ρ-nitrophenyl phosphate in 10% [v/v] triethanolamine, pH 9.8) was added and the hydrolyzed enzyme substrate extinction values were collected at 405 mm during the reaction. The data represent three separate duplicated experiments with uninoculated, CTV infected citrus controls and extraction buffer controls in each test.

2.3.4 Tissue blot

Reaction of the CTV source plants to CTV MCA-13 antibodies were tested and assessed as a tissue immunoblot (Nokomis Corp., Altamonte Springs, FL). MCA-13 was raised against a decline inducing CTV isolate collected from a sweet orange on sour orange rootstock in Florida (Permar *et al.,* 1990). Four young stems were taken from each plant and the cut stem end of each was blotted onto nitrocellulose paper. The paper was air dried and sent to Nokomis Corp. for assessment. Uninoculated citrus plants were included as test samples.

2.3.5 cDNA production

CTV CP gene specific cDNA was produced using reverse transcription polymerase chain reaction (RT-PCR) by two methods. One step RT-PCR contained 15 µl of a dsRNA enriched total RNA sample from each Texas CTV citrus source. The RNA was heated at 70˚C for 5 min then placed on ice. The reaction mix was added up

to a final volume of 50 µl which contained 100 pmole each of CN405 (plus-sense primer; 5'-GCCTTAAGGGTCGTTAATTG-3') and CN408 (minus-sense primer; 5'-GATTATATCACCCACGTTCACG-3'), 10 mM Tris-Cl pH 8.3 at 25C, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 5 U RNasin inhibitor (Promega), 10 U *Avian myeloblastosis virus* reverse transcriptase (AMV RT; Promega M5101), and 2.5 U AmpliTaq (DNA polymerase, Perkin Elmer N801-0060). The thermal cycle conditions were 45˚C for 60 min, 92˚C for 2 min, 30 cycles of 92˚C for 30 sec, 50˚C for 45 sec, and 72˚C for 1 min, then extension for 10 min at 72˚C, hold at 4˚C.

Reverse transcription for the synthesis of first strand cDNA in the two step RT-PCR method was made using 3 µg total RNA or 15 µl of a dsRNA-enriched sample from each CTV isolate, and the RNA was heated for 15 min at 65˚C, 10 min at 55˚C, then 5 min at room temperature. The reaction mixture was added to a volume of 25 µl, and this contained 50 pmoles primer CN408, 50 mM Tris-Cl, pH 8.3 at 25˚C, 50 mM KCl, 10 mM $MgCl_2$, 0.5mM spermidine, 10 mM DTT, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 5 U RNasin, and 10 U AMV RT. The contents were gently mixed then incubated at 47˚C for 1 h, after which 12 µl of nuclease-free water was added and the cDNA stored at -20˚C. Each of the two step PCR reactions contained 5 µl of the cDNA reaction, 100 pmoles each primers CN405 and CN408, 10 mM Tris-Cl pH 8.3 at 25˚C, 50 mM KCl, 2.5 mM $MgCl_2$, and 2.5 U AmpliTaq. PCR conditions were similar to the description of the one-step RT-PCR but the first incubation cycle of 45˚C for 60 min was omitted.

RNA samples were extracted from virus-free citrus plants and citrus plants infected with Citrus tatterleaf virus (Genus: *Capillovirus*, species, *Apple stem grooving virus*), and these samples were included in all tests. Nuclease-free water was included instead of the RNA as an additional RT-PCR control. PCR products (5 µl) were analyzed by gel electrophoresis through 1.2% agarose (Gibco-BRL) in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA), stained with ethidium bromide (0.5 µg/ml), and photographed under UV light.

2.3.6 Hybridization to CTV ORF 7 specific oligonucleotides

A subset of seven of the Texas CTV isolates evaluated by the citrus host-range indexing were analyzed using a technique called strain group specific probes (SGSP) developed by Niblett and co-workers (Niblett *et al.,* 2000, personal communication and patent No. 6,140,046). For these analyses 20 µl of PCR product was mixed with 30 µl nuclease-free water, 50 µl 20X SSC and 2 µl bromophenyl blue then vacuum-dot blotted onto nylon membranes. Membranes were incubated in 4 M NaOH for 10 min at room temperature with gentle shaking, then neutralized twice by incubating the membrane in 0.2M Tris-Cl pH 8.0, 0.1%SDS and 1X SSC at room temperature for 10 min. Prehybridization was at 37˚C in a sealed bag for 1 h containing 7 ml of prehybridization solution (5X SSC, 5X Denhardt's solution, 1% SDS, 0.01 M phosphate buffer, pH 6.8, 1 mM ATP, 1m M EDTA, 1.4 mg of denatured salmon sperm DNA). After 1 h, 700 ng of a CTV strain specific probe was added, the bag resealed and further incubated for with gently agitation for 1 h. The membrane was cut out of the bag and rinsed in 6X SSC at

RT for 5 min. For probes 0, I, II, III, IV, VIII the membranes were then washed twice at 45˚C for 10 min in 4X SSC, 0.5% SDS. For probe V conditions with two changes of the same solution were 10 min at 55˚C and 10 min at 50˚C. Detection was by CDP Star streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim). Each sample was assessed visually on a 1, weak hybridization to 5, strong hybridization rating for each probe reaction, including trace. Probe and uninfected controls were included in each assessment.

2.3.7 SSCP

For these analyses, 1 µl of the amplified PCR product was mixed with 9 µl of denaturing solution (95% HPLC grade formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene-cyanol), heated for 10 min to 99˚C and immediately chilled on ice. The DNA strands were separated by electrophoresis in a non-denaturing polyacrylamide (8% acrylamide), using TBE (1X) as electrophoresis buffer, and a constant voltage of 200 V for 3 h at 4˚C (Rubio *et al.*, 1996; Sambade *et al.*, 2002). Gels were stained with 1:10,000 diluted SYBR Gold (Molecular Probes) for 10 min, destained for 10 min, and then photographed under UV light.

2.3.8 Statistical analyses

Correlation and multiple stepwise linear regression analyses were performed according to Zar (1999) and using the SAS statistical program, version 6.12 (SAS Institute Inc., Cary, NC). Included in this data were four additional Texas CTV isolates for which the biological indexing data and Florida SGSP data were collected in the same manner and time period.

2.4 RESULTS

2.4.1 Biological indexing

In the first biological indexing test all CTV isolates produced leaf vein flecking in Mexican lime three months after inoculation. With the most severe isolates there were many leaf flecks in every leaf, whilst with other CTV sources there were one or two flecks on one or two leaves compared to the uninoculated plants. Leaf cupping was noted from some CTV isolates, but this symptom was not rated since it is not diagnostic for CTV alone. Some CTV isolates produced numerous stem pits in Mexican lime whilst the milder CTV isolates had minor stem pitting. CTV isolates inoculated onto the sweet orange grafted to sour combination caused stunting of the scions relative to controls, and in the most severe cases a brown smear under the bark on the sour orange side of the bud union was present where the scions were undergoing senescing and typical CTV decline. Duncan grapefruit stunting symptoms ranged from severe to very mild compared to the uninoculated controls. Madam vinous sweet orange seedlings were stunted with or without stem pitting as compared to the uninoculated controls.

Distinct chlorosis usually associated with the seedling yellows symptoms (sour orange or grapefruit seedlings), was not observed. Table 2.1 summarizes the biological indexing data from indexing experiment 1 and the corresponding serological data. Only two CTV isolates (H10 and H11) of the fifteen were mild in symptoms, that is they produced symptoms in Mexican lime only. Six of the CTV isolates produced symptoms in every indicator host. The most severe CTV isolate originated from a Meyer lemon (*C. meyeri* Tan.). Considering the biotype rating method (Lee *et al.,* 1994), the majority of the CTV isolates were in biotype groups IX or X or contained either two or three of the severe groups of CTV symptoms. Six of the CTV isolates which were inoculated onto Texas commercial scion varieties produced symptoms only on sweet orange (two isolates), one on grapefruit scions only, and three isolates on both sweet orange and grapefruit scions (Table 2.2). The most severe CTV isolates on the Texas commercial varieties were from Meyer lemon plants. Generally the commercial varieties were more susceptible to CTV with greater stem pitting and reduction in height than the Pineapple sweet orange and Duncan grapefruit indicator plants.

Experiment 2 bioindex compared four Texas CTV isolates to six control BARC CTV isolates (Table 2.3), three of the Texas isolates produced disease symptoms in all indicator hosts whilst one Texas CTV isolate produced symptoms only in Mexican lime and sweet orange on sour orange. Ranked according to the cumulative total of all the mean relative disease assessments (in descending order of severity) for the BARC CTV isolates, there was B6, B28, B384, B4, B5 then B2. B6 also produced leaf corking on sweet orange and severe dieback of grapefruit seedlings. B2 produced the mildest CTV

TABLE 2.1

Summary of the citrus indexing and serology for Texas *Citrus tristeza virus* isolates in Experiment 1

		Citrus indexing						Serology	
CTV SOURCE	LOCATION/TISSUE	ML Rx1	SW/SO Rx2	SO Rx3	DGFT Rx4	MV Rx5	CUM	ELISA POLY	MCA-13
H6	EAST/Nippon orangequat	1	NT	0	0	1	2	+	+
H8	LRGV/grapefruit	3	0	0	0	6	9	+	-
H9	LRGV/Bell tangerine	2	2	1	1	2	8	+	-
H10	LRGV/Cara cara sweet orange	1	0	0	0	0	1	+	-
H11	LRGV/?	1	NT	0	0	0	1	+	-
H12	LRGV/variegated lemon	1	NT	0	0	2	3	+	-
H19	EAST/Armstrong early satsuma	2	NT	1	0	0	3	+	+
H29	LRGV/citrangeuma	1	NT	1	2	6	10	+	-
H31	LRGV/Thornton tangelo	1	NT	1	1	2	5	+	-
H41	LRGV/Meyer lemon	3	1	2	6	4	15	+	+
H42	EAST/satsuma	2	NT	2	2	0	6	+	NT
H45	EAST/Armstrong early satsuma	1	NT	1	2	1	5	+	-
H47	LRGV/Meyer lemon	1	1	1	4	1	7	+	+
H48	EAST/Hamlin sweet orange	1	NT	2	0	5	8	+	-
H49	LRGV/Meyer lemon	1	NT	2	3	0	6	+	-

LRGV; collected in the Lower Rio Grande Valley, EAST; collected in East Texas. Citrus indexing**:** Mean relative weighted indices in citrus cultivars, ML; Mexican lime, SW/SO; Pineapple sweet orange grafted to sour orange, DGFT; Duncan grapefruit, SO; sour orange, MV; Madam Vinous sweet orange, Cum; cumulative total of all mean relative weighted indices for the particular CTV source. Each number represents the relative mean assessment (R) of symptoms on a 0-3 scale (0-no symptoms to 3- severe symptoms), with extrapolations, multiplied by a weighting factor (Rx1 for ML, for example) for each particular citrus cultivar or species combination (Garnsey *et al*., 1987b). Serology**:** Poly; using polyclonal CP CTV antisera in a DAS ELISA format, MCA-13 (Permar *et al*., 1990); using CTV MCA-13 in an immunoblot format, +; reaction with antibodies, -; no reaction with antibodies, NT; not tested. All serology tests were replicated three times.

TABLE 2.2

Indexing of six Texas *Citrus tristeza virus* isolates in Experiment 1 with four Texas commercial cultivars on sour orange rootstocks

CTV SOURCE	RIO RED GFT Rx4	STAR RUBY GFT Rx4	MARRS SW Rx5	N-33 SW Rx5
H8	0	0	2	4
H9	3	2	1	1
H19	4	1	0	0
H41	2	2	3	6
H47	6	4	4	4
H48	0	0	3	2

Mean relative weighted indices in commercial citrus cultivars: GFT; grapefruit variety grafted to sour orange rootstock, SW; sweet orange variety grafted to sour orange rootstock. Each number represents the relative mean assessment (R) of symptoms on a 0-3 scale (0-no symptoms to 3- severe symptoms), with extrapolations, multiplied by an economic weighting factor (Rx4 for GFT, Rx5 for SW) for each particular citrus species combination (adapted after Garnsey *et al*., 1987b).

TABLE 2.3

Summary of the citrus indexing of four Texas *Citrus tristeza virus* isolates compared to six reference isolates from the World CTV Collection in Experiment 2

CTV SOURCE	ML Rx1	SW/SO Rx2	SO Rx3	DGFT Rx4	MV Rx5	CUM
H11	1	2?	0	0?	0	3?
H29	1	2	3	4	5	15
H33	3	4	6	8	5	26
H41	2	2	6	4	5	19
B2	1	0	0	0	0	1
B4	2	4	0	4	5	15
B5	1	2	0	0	0	3
B6	3	6	6	12	10	37
B28	2	4	9	12	5	32
B384	2	6	3	8	0	19

CTV isolates: H prefix refer to Texas CTV isolate, H33, this derives from the same original Meyer lemon tree as H41. B prefix refers to CTV isolates obtained from USDA, Beltsville, which are used in this experiment to compare the severity of CTV to these isolates under Texas conditions. Citrus indexing: Mean relative weighted indices in citrus cultivars: ML; Mexican lime, DGFT; Duncan grapefruit, SW/SO; Pineapple sweet orange grafted to sour orange rootstock, SO; sour orange, MV; Madam Vinous sweet orange, CUM; cumulative total of all mean relative weighted indices for the particular CTV source,?; reactions do not fit with any known isolate, symptoms very slow to develop and always in the lowest reaction group. Each number represents the relative mean assessment (R) of symptoms on a 0-3 scale (0-no symptoms to 3- severe symptoms), with extrapolations, multiplied by a weighting factor (Rx1 for ML, for example) for each particular citrus cultivar or species combination (Garnsey *et al*., 1987b).

symptoms observed with only one or two inoculated Mexican lime leaves showing occasional leaf vein flecking. H33 was used in Experiment 2 and ranked as the most severe Texas CTV isolate to date, with a cumulative index of 26. This isolate produced all of the severity components of stem pitting on sweet orange and grapefruit, sweet on sour decline and seedling yellows in sour orange and grapefruit seedlings. H33 was originally collected from a Meyer lemon tree.

Several CTV isolates (H33, H47, H41, H49) induced stem pitting on sour orange rootstock distinct from the classical decline symptom of honeycombing (Cohen and Knorr, 1954). The Capao Bonîto CTV isolate from Brazil causes stem pitting on sour orange, and another infectious disease of citrus, cristacortis can also cause this symptom (Roistacher, 1991).

2.4.2 Se**rological and RNA-blot analyses**

All CTV isolates consistently reacted with CTV CP polyclonal antibodies from their original source plant tissues over three assessment periods (Table 2.4, serology panel). In tissue blots using MAb MCA-13 four CTV isolates consistently reacted, suggesting H6, H19, H41 and H7 contain decline-inducing CTV strains. H6 CTV did not give a sweet orange on sour orange decline symptom in the biological indexing, additionally six other CTV isolates (Table 2.1) gave sweet on sour orange decline type symptoms but they did not react with MCA-13. Northern hybridizations using total RNA from each CTV source probed with the 3'-end of the SY568 CTV genome also confirmed that the same plants contained CTV (Fig. 2.1).

TABLE 2.4

Summary of the Texas *Citrus tristeza virus* isolate Florida group specific probe hybridizations and single-strand conformation analyses

		Florida SGSP							SSCP
CTV SOURCE	LOCATION/TISSUE	0	I	II	III	IV	V	VIII	SSCP bands
H6	EAST/Nippon orangequat	4	3	0	1	0	3	5	4
H8	LRGV/grapefruit	1	1	0	2	0	1	5	4
H9	LRGV/Bell tangerine	NT							4
H10	LRGV/Cara cara sweet orange	3	3	0	0	0	0	5	4
H11	LRGV/?	NT							4
H12	LRGV/variegated lemon	3	3	0	0	0	1	5	2
H19	EAST/Armstrong early satsuma	NT							4
H29	LRGV/citrangeuma	1	1	0	T	0	0	3	4
H31	LRGV/Thornton tangelo	T	T	0	0	0	0	2	4
H41	LRGV/Meyer lemon	1	1	0	1	0	4	4	4
H42	EAST/satsuma	NT							4
H45	EAST/Armstrong early satsuma	NT							4
H47	LRGV/Meyer lemon	1	T	0	T	0	5	4	4
H48	EAST/Hamlin sweet orange	NT							4
H49	LRGV/Meyer lemon	1	T	0	2	0	4	2	4

Florida strain group specific probe (SGSP) hybridizations: 0, I, II, III, IV, V and VIII are the different oligonucleotide probes generated to the CTV CP gene and hybridized to cDNA from each source, NT; not tested. Hybridization assessments were made on a visual 0-5 scale for the intensity of the different samples, 0-no hybridization to 5-strong hybridization, (Niblett *at al*, 2001). Single-strand conformational polymorphism (SSCP): number of bands obtained during SSCP analyses of the CTV CP gene, all SSCP tests were repeated three times.

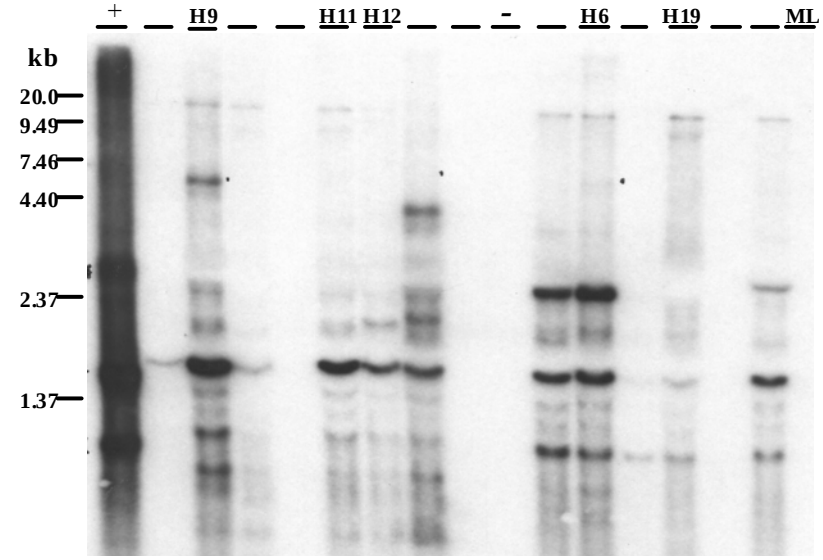

FIG. 2.1. RNA analyses of Texas *Citrus tristeza virus* isolates. An example of an autoradiograph of an RNA blot probed with a CTV SY568 3' cDNA. +; CTV isolate SY568 total RNA, ML; total RNA extracted from a virus-free Mexican lime, -; total RNA extracted from a Mexican lime plant harboring Citrus tatterleaf virus, H; prefix of CTV Texas isolate codes (see text).

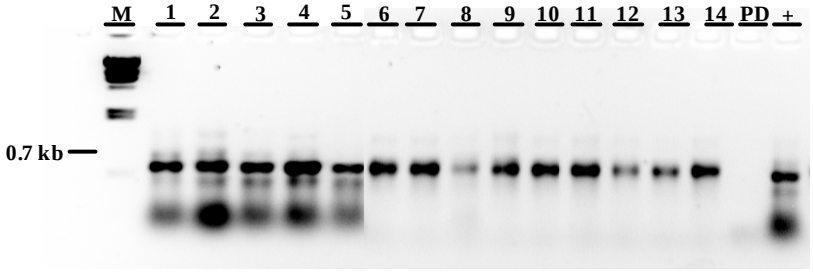

FIG. 2.2. Reverse-transcription polymerase chain reaction (RT-PCR) products from the different *Citrus tristeza virus* isolates. Examples of the RT-PCR products separated by electrophoresis, M; DNA marker, with the position of 0.7 kb, the approximate size of the CTV CP cDNA is 672 bp, 1-14; CTV Texas isolate samples, PD; nuclease-free water replacing sample in the RT-PCR reactions, +; plasmid containing CTV T36 CP used as a sample in the PCR reaction.

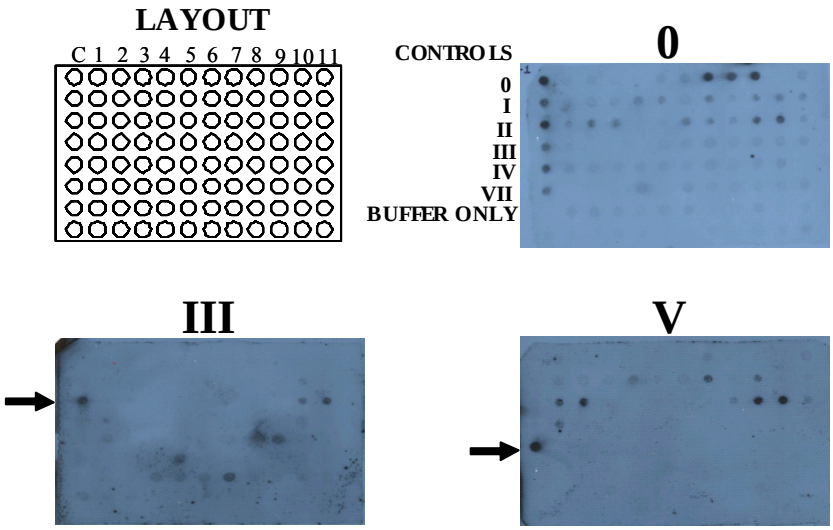

FIG. 2.3. Florida strain group specific probe (SGSP) tests. CTV isolate CP cDNAs were blotted onto replicated membranes using a 96-well slot blot apparatus, with controls always in the first column, marked C in LAYOUT, and test samples in columns 1-11 in LAYOUT, O; blot probed with SGSP O, III; blot probed with SGSP III, V; blot probed with SGSP V, CONTROLS; control samples loaded onto every blot (only shown for O blot) include all probes (0, I, II, IV, VII), BUFFER ONLY; sample of blotting ingredients without cDNA only. Arrows on blots III and V indicate control sample SGSP III and SGSP V, respectively.

2.4.3 cDNA production and CTV CP strain group probes

Using CTV CP specific primers an RT-PCR product of the approximate predicted size (672 bp) was amplified from all CTV isolates tested (Fig. 2.2.). Examples of the CTV CP SGSP hybridizations are shown in Fig 2.3., with the assessments in Table2.4 under the Florida probe hybridizations panel. Probes II and IV did not hybridize to any of the CTV isolates tested. Visual assessments appeared to be associated with certain probes and CTV isolate biological activity.

The Florida SGSP hybridization assessments were compared to the biological indices for each CTV isolate to find if there were any significant statistical associations between the phenotype and the probe scores. Since the dependent variables were not known, correlation analyses were done to measure the degree of association between any two variables in the whole data block. These analyses revealed that there were significant correlations between certain host indicators and SGSPs. Logarithmic (base 10) transformation of the indices strengthened some of these trends. The disease index for every citrus cultivar or species combination was significantly positively correlated to all other citrus cultivar or species combination indices (greater than $p \geq 0.050$, df=17). Probe 0 correlated to log Duncan grapefruit index ($p \geq 0.05$ df=11); probe I correlated to log sour orange index, log sweet on sour orange index, log Duncan grapefruit index ($p \geq 0.05$, df=11). Probe VIII correlated to log Duncan grapefruit index, where all significant correlations were negative. There were also significant associations between individual probe assessments. Probe 0 and Probe I were highly associated (p=0.001, df=11). Probe I and probe VIII (p=0.06, p=df=11) indicated strong positive correlation.

On the basis of these initial associations, a multiple stepwise linear regression was performed using the type of disease index (log transformation for each citrus cultivar or species combination) as dependent variables verses all probe scores (excluding probes II and IV). The summary statistics for the best regression models for the data obtained with each type of disease index is shown in Table 2.5. Note that the sweet orange on sour orange (SW/SO) data set was incomplete as far as the indexing was concerned since this indicator was not used for every CTV isolate in the biological indexing (Table 2.1). The SW/SO data set were removed before analyses, then reintroduced and re-analyzed. There were no significant differences to the overall summary for either analysis, therefore the SW/SO data were included in the final analyses. From the regression disease indices of sweet orange on sour orange and Duncan grapefruit most reliably describe the variation in the probe data (R^2 of 0.673 and 0.712, respectively). All other disease symptoms in the indexing cultivars or species cannot be reliably described by the probe data, even though the regressions are significant. Probe I has a significant interaction ($p=0.014$) with the disease indices from SW/SO and sour orange seedlings, respectively, and has a near significant contribution to the Duncan grapefruit disease indices model ($p=0.06$), and a minor role in the Madam Vinous sweet orange disease indices model ($p=0.114$). Collectively this means that one specific probe can describe some of the variation in particular indexing receptors with relative precision, however not all symptoms in all indicators could be adequately or reliably described by any one probe or group of probes.

TABLE 2.5

Summary statistics of the step-wise linear regression analyses for the mean disease assessments per citrus host, with the Florida strain group specific probe assessments for Texas *Citrus tristeza virus* isolates

Dependent variable	Independent variable	Intercept	SE	t	tprob
Mexican lime index	Intercept	0.795	0.106	7.513	<0.001**
$R^2 = 0.285$	FPIII	0.178	0.085	2.093	0.060
Sweet orange on sour orange index	Intercept	1.728	0.189	9.158	<0.001**
$R^2 = 0.673$	FPI	-0.52	0.148	3.516	0.013*
Sour orange index	Intercept	1.278	0.269	4.754	0.001**
$R^2 = 0.332$	FPI	-0.41	0.171	2.341	0.039*
Duncan grapefruit index	Intercept	2.302	0.379	6.069	0.001**
$R^2 = 0.712$	FPI	-0.378	0.177	2.135	0.059
	FPVIII	-0.216	0.135	1.597	0.142
Madam Vinous sweet orange index	Intercept	1.751	0.292	6.007	<0.001**
$R^2 = 0.211$	FPI	-0.318	0.186	1.715	0.114

R^2, coefficient of determination, SE; standard error, t; t statistic, tprob; probability in the t distribution, FPI, III, VIII; Florida strain group specific probes included in the models, **; significant at ≤ 0.001 probability, *; significant at ≤ 0.050 probability.

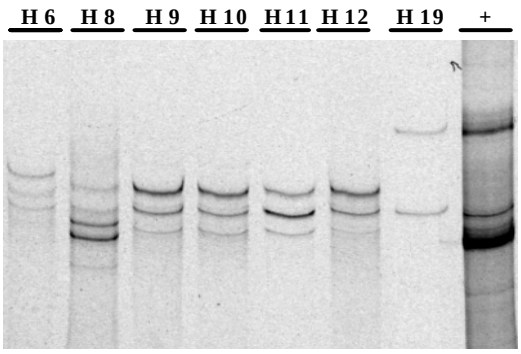

FIG. 2.4. Single-strand conformational polymorphism (SSCP) analyses. CTV isolate CP cDNAs were denatured and separated by PAGE, H; prefix for CTV Texas isolate code (see text), +; cDNA amplified from a plasmid containing T36 CTV CP gene.

2.4.4 SSCP analyses

The SSCP profiles for the PCR amplified CP gene product for each CTV isolate are shown in Fig 2.4. In some isolates, two or three DNA bands were delimited, but in the greater majority four or six bands were observed with similar staining intensity. Some of the banding patterns were indistinguishable. For instance, H9, H10, H11, and H12 all separated three bands at similar distances to each other although the citrus indexing data suggests different biology of the CTV source. There were no associations between geographical origin or cultivar type of the CTV sources and SSCP profile. Meyer lemon tissue samples generally had similar profiles, even though individual samples were collected in different geographic regions and at different times.

2.5 DISCUSSION

2.5.1 Texas CTV isolates and biological indexing

CTV isolates from Texas had cumulative scores ranging from 1 to 26. In comparison to the BARC CTV control isolates indexed under our conditions, the most severe Texas CTV isolate, H33, ranks less severe than B6 and B24 but more severe than B384, B4, B5 and B2. The BARC CTV control isolates have been widely used by other scientists and biologically tested extensively at BARC. At BARC, B6 has a cumulative index of 31 or 41 (two tests), and a score of 42 in California. B2 has a cumulative index of 1 in Florida and 0.5 or 1 at BARC (Garnsey *et al*., 1987b). Our tests with the BARC isolates showed profiles similar to those detailed at BARC; CTV isolate B6 had a cumulative index of 37 and B2 had a cumulative index of 1.

Five Texas CTV isolates contained the full complement of severe CTV components of SW/SO decline, seedling yellows and stem pitting in grapefruit, seedling yellows symptoms in sour orange seedlings, and stem pitting on sweet orange, placing these isolates in the most severe biotype group of X (Lee *et al*., 1994). The CTV isolates from Texas contained two or all of the groups of economically severe CTV symptoms (biotypes IX or X). When collecting CTV isolates for this biological testing, it was difficult to find isolates from the LRGV commercial CTV sources since the CTV incidence is extremely low (Solís-Gracia *et al.,* 2001). Three of the CTV isolates, H8, H10, and H11 originated from commercial sources in the LRGV. H10 and H11 produced the mildest CTV symptoms in the biological indexing. The majority of the CTV isolates were collected from non-commercial citrus, and these isolates harbored the most damaging CTV biotype X. The majority of the CTV isolates used in these tests were essentially field CTV isolates, with no sub-isolation or passaging *in planta*.

In the biological tests on Texas commercial scions and six Texas CTV isolates, CTV symptoms were more severe than symptoms produced in the respective standard index citrus species. Stem pitting symptoms were more severe in the red grapefruit scions (more pits per stem surface area), as reported elsewhere (Marais and Breytenbach, 1996), and the sweet on sour orange decline symptom was induced in a shorter time.

2.5.2 Implications to Texas citrus production

Reservoirs of damaging CTV isolates exist in Texas, mainly within dooryard citrus, and surveys indicate greater CTV incidence in East Texas (Solís-Gracia *et al*., 2001). The majority of commercial citrus in Texas is on CTV-sensitive sour orange rootstock; this citrus has low CTV incidence at the moment. The vector situation will likely change when the BrCA arrives. Currently BrCA is spreading westward from Florida along the Gulf of Mexico. Additionally, Texas is the 'front line' in the USA for arrival of the BrCA through Mexico. When the BrCA arrives, the damaging Texas CTV isolates will likely get distributed from dooryard plants to commercial citrus.

2.5.3 Comparison of biological indexing to rapid methods

The serological methods used in these tests were effective at identifying CTV and provided the information in the shortest time for relatively little expense. MCA-13 gave an indication of the presence of CV isolates causing SW/SO decline, similar to other earlier reports (Permar *et al.*, 1991). MCA-13 also reacted with one Texas non-CTV decline isolate, and other reports suggest MCA-13 cannot detect all stem pitting CTV isolates. Sequencing data is still needed on these CTV isolates. If such non-decline activity is common, MCA-13 may not be useful to differentiate between destructive and non-destructive in sour orange CTV isolates in Texas. However, should there be an urgent need to identify severe CTV sources over the commercial citrus growing region, then this would be the fastest method of identification.

The Florida CTV SGSP gave good indications of certain severe symptoms, with sweet on sour orange declines and grapefruit stem pitting being the most reliably predicted. Probe I gave the best all round performance at prediction of all types of severe CTV symptoms. Probe I was developed using CTV isolates T36, T66, 202B-1, T10 and PB53DRF1. CTV T36 and T66 are Florida isolates, causing decline of scions on sour orange rootstock (Pappu *et al*., 1993). Florida isolate 202B-1 causes decline, seedling yellows and grapefruit stem pitting. Isolate PD3DRF1 is a grapefruit stem pitting isolate from Australia. From the statistical analyses in this study, the reliability of detecting stem pitting in sweet orange, seedling yellows of sour orange seedlings were poor. CTV symptoms in Mexican lime were also not significantly correlated to any of the probe assessments, but this would be expected as Mexican lime CTV symptoms indicate CTV presence and not severity (all the Texas CTV isolates in this study gave CTV symptoms in Mexican lime). An improvement to the detection could be quantitative measurements of the hybridizations rather than the visual assessments, followed by testing hundreds of CTV isolates with different but known biological activity through this process, and subjecting the resultant data to statistical treatment to determine out how reliable the test is at predicting particular disease combinations. A drawback to using a hybridization technique would be variability depending on the stringency of the hybridization conditions. Thus the test might be highly subjective if carried out by different laboratories.

SSCP profiles of the CTV CP gene were diverse and similar to that obtained by other workers (Rubio *et al*., 1996). Usually two to six bands are obtained when using

cDNA generated to the CTV CP gene. The procedures are relatively robust and easy to perform and banding patterns were reproducible. Staining gels with SYBR Gold substantially decreased the time taken for the tests and was more sensitive compared to silver staining (data not shown). The mildest CTV isolate H10 (biotype I) showed three bands and the severe isolates showed sometimes two or four or more bands (all biotype 10 isolates). The three band profile can be accounted for in the stable configuration of two forms for one of the DNA strands (Rubio *et al.,* 1996) or that two bands cannot be discerned (electrophoretic mobility is approximately equal) being visualized as one band. The intensity of the three bands in the H11 profile is different from the H10 profile, for instance. Enzymatic digestion of the RT-PCR products then electrophoretic separation could further delimit differences in the profiles between the CTV sources (Valle *et al.,* 2000). Multiple DNA bands in the SSCP profile reflect the sequence variants present in the RNA population down to sequence variants forming 10% of the RNA population (Rubio *et al*., 2000; Sambade *et al*., 2002). The technique is useful therefore to study the dominant population components, for instance in initial selection of cross-protection CTV isolates, monitoring the process of cross-protection (Sambade *et al*., 2002) or virus passaging experiments. The phenomenon of gRNA 'clusters' around a sequence variant is a well established aspect of positive-stranded RNA plant virus populations in nature (Roossinck, 1997; Holmes, 2003) and may account for the biological nature of certain CTV isolates. Similar observations have been made by other workers when analyzing the p18 gene or the 5'-UTR of various CTV isolates (Ayllón *et al*., 1998; Ayllón *et al.,* 2001). In such studies, it was found that mildly pathogenic CTV

isolates contained one sequence type whereas severely pathogenic isolates contained two or three sequence types. There was no clear-cut association between band number and biological activity of the Texas CTV isolates in this study. Meyer lemon CTV sources all had 4 bands, even though samples were collected at different times and geographic regions, and this might be more indicative of the clonal nature of Meyer lemon trees, since some propagations in the continental USA can be traced back to one introduction in 1905 (Meyer, 1911; McKee, 1926), but this also indicates that the CTV population in the tree is stable over time.

Six full length CTV sequences are now available in Genbank and each isolate represents different geographic areas of origin or different biological activities (Karasev *et al*., 1995; Albiach-Martí *et al.,* 2000c; Mawassi *et al.*, 1996; Yang *et al.*, 1999; Vives *et al*., 1999; Gede *et al.*, 2001). The search for genetic markers for the different symptoms can be done across the whole genome now using the full length sequence data and various algorithms at different settings to firstly search for regions of the genome with predicted low, medium and high nucleotide mutation rates (Moonan *et al.*, 2000; Moonan and Mirkov, 2002). Such regions can be sampled by RT-PCR, with polymorphisms detected, and a model(s) predicted. Many field CTV isolates could then be subjected to the tests and phenotyped so that the proposed model(s) may be verified or modified. The intricacies of the behavior between different RNA of the CTV population within one tree relates to symptom development and severity since making sub-isolates by grafting or aphid transmission separates some of the variety of CTV biology in citrus plants (Roistacher and Bar-Joseph, 1987b; Albiach-Martí *et al.,* 2000b).

Thus more CTV sequencing data from field isolates are likely needed in order to sample the biological activity of CTV in field trees.

CTV management strategies include eradication (Bar-Joseph *et al*., 1989), quarantine and certification programmes, use of resistant or tolerant rootstocks, or cross-protection with mild isolates (Rocha-Peña *et al*., 1995). Deployment of each strategy is highly dependent upon the incidence and severity of CTV present in a region; therefore practices often change over time. Severity typing using the standard set of citrus indicators and determining CTV biotype category or cumulative index can only be done on small groups of samples, is very subjective, lengthy, requires skill and knowledge of each citrus species or cultivar and the many symptoms which may be induced by CTV. A rapid reliable test to differentiate mild and severe CTV sources with great reliability still eludes the citrus indexing process.

CHAPTER III

EFFICIENCY OF ACQUISITION AND TRANSMISSION OF *CITRUS TRISTEZA VIRUS IN VITRO* USING *TOXOPTERA CITRICIDA* KIRKALDY, AND INFECTIVITY NEUTRALIZATION TESTS USING THREE VIRALLY-DERIVED ANTIBODIES

3.1 SUMMARY

Citrus tristeza virus (CTV) is transmitted by several aphid species with *Toxoptera citricida* Kirkaldy, the brown citrus aphid (BrCA) reported to be the most efficient. The transmission of CTV by the aphid vectors is semipersistent, no helper proteins from CTV have been identified yet. *T. citricida* were fed on crude tissue preparations of CTV across artificial membranes and were able to inefficiently transmit CTV to virus-free receptor plants. CTV p20, p27 and p25 proteins could be detected by immunoblot assay in all crude tissue preparations. A more highly purified CTV preparation was not transmitted by the *T. citricida* in this manner. Infectivity neutralization transmission experiments were performed against CTV proteins using three antibodies against p25, p27 and p20 CTV proteins. Aphids were fed on CTV-infected source plants, then transferred for *in vitro* feeding on the diluted antibody, then placed on virus-free receptor plants. There were no differences in the rates of transmission between the majority of treatments and the control samples. However, in

one transmission experiment the CTV p20 antibodies significantly enhanced the occurrence of CTV transmission compared to buffer only, pre-immune antiserum or no antibody treatments. This data suggests the CTV p20 antibody might neutralize a factor which results in the higher *T. citricida* rate of transmission of CTV.

3.2 INTRODUCTION

Closteroviruses (Family; *Closteroviridae)* have very large positive-sense, single –stranded RNA (ssRNA) genomes, and many members are recalcitrant to mechanical transmission. Three viral genera have been delimited based upon the type of insect involved in viral transmission and genome partite status (Martelli *et al*., 2000; Mayo, 2002). The genus *Closterovirus,* type species *Beet yellows virus* (BYV), has aphid vectors (Homoptera: Aphididae). The genus *Crinivirus,* type species *Lettuce infectious yellows virus* (LIYV), is transmitted by whiteflies (Homoptera: Alyrediidae). The genus *Ampelovirus,* type species, *Grapevine leafroll-associated virus 3* (GLRaV-3), is vectored by mealy bugs (Homoptera: Pseudococcae).

Citrus tristeza virus (CTV) is the most economically important viral pathogen of citrus, and has killed many trees and caused great losses to citrus production worldwide (Bar-Joseph *et al*., 1981; Bar-Joseph *et al.*, 1983; Bar-Joseph *et al.*, 1989). CTV belongs to the genus *Closterovirus* having long flexuous virions of ca. 2000 x 11 nm, a monopartite genome, and aphid transmission. The virus is phloem limited in citrus plants and is spread into new areas by movement of infected propagating material. Aphid dispersion of the virus is important within a citrus growing region. *Toxoptera*

citricida Kirkaldy, the brown citrus aphid (BrCA), *Aphis gossypii* Glover, the melon-cotton aphid, and *A. spiraecola* Patch, the spirea or green citrus aphid are the principle vectors of CTV on citrus (Roistacher and Bar-Joseph, 1987a; Stoetzel, 1994). There is also one report of a mealy bug (*Ferrisia virgata* Cockerall) transmitting CTV after 30 hours feeding on a CTV infected plant (Hughes and Lister, 1953).

Since the introduction of the BrCA into the New World, specifically Brazil and Argentina early in the 20th century, CTV has caused major citrus declines on sour orange rootstock in the wake of the northward movement of the BrCA (Roistacher *et al.,* 1991; Rocha-Peña *et al*., 1995). Within the last decade the BrCA has moved northward through Central America and the Caribbean Basin to first arrive in North America in Florida in 1995 (Lastra *et al.*, 1991; Lastra *et al.*, 1992; Lee *et al*., 1994; Hardy, 1995). The BrCA is now established in Mexico in the Yucatan peninsula (Michaud and Alvarez, 2000). Texas is threatened by BrCA spread across the Gulf of Mexico from Florida and by a continued northward movement in Mexico.

T. citricida is a serious pest of citrus in addition to being the most efficient vector of CTV. The aphids both feed and reproduce on citrus plants (Roistacher *et al.,* 1991). BrCA transmission of CTV is semipersistent with no latent period and with acquisition and inoculation periods being as short as 30 minutes (Costa and Grant, 1951; Roistacher and Bar-Joseph, 1987a; Bar-Joseph *et al.*, 1989). However, other reports indicate the acquisition and inoculation periods of CTV by the BrCA may take place in seconds (Retuerma and Price, 1972). CTV was first demonstrated as being aphid transmitted by Meneghini (1946) using hundreds of BrCA to transmit the tristeza disease in Brazil. The

semipersistent nature of CTV BrCA transmission was recognized, and additionally CTV transmission was classified as bimodal (Chalfant and Chapman, 1962). In bimodal transmission, aphid virus acquisition can cluster around two periods, a short time period and a relatively long time period, and there is generally no change if aphids are preaquisitionally fasted or not (Lim and Hagedorn, 1977). Variable single aphid transmission rates for specific CTV isolates by the BrCA of 0-55% have been recorded (Broadbent *et al*., 1996; Tsai *et al*., 2000). In parallel testing comparisons of transmission rates for BrCA and *A. gossypii* indicate the BrCA is about 25 times more efficient (Yokomi *et al*., 1994). CTV isolates can also vary considerably in their ability to be transmitted experimentally by the BrCA (Yokomi *et al.,* 1994). CTV transmission rates have also been noted to increase with increasing the numbers BrCA used (Costa and Grant, 1951), and have been recorded as consistently very low or variable enough to produce inconclusive tests (Stubbs, 1964). Factors such as tissue age, CTV isolate, day light time, aphid colony make it difficult to compare all literature sources as not all transmissions are performed the same way.

For such an economically important virus-aphid association, very little is known about the mechanics of the specific CTV-BrCA interaction. For many different taxa of plant viruses the viral coat protein (CP) has been found to facilitate aphid transmission (Perry *et al.*, 1994; Atreya *et al.*, 1991). Evidence that a virus-encoded helper-component type protein may be a requirement for aphid transmission in closteroviruses comes from classical *in vitro* aphid feeding experiments. Aphids fed with purified closteroviruses *in vitro* could not transmit these viruses to plants (Murant *et al*., 1988).

This is similar to the situation described for other aphid-transmitted, non-circulative viruses such as potyviruses (nonpersistent) and caulimoviruses (semipersistent or bimodal) where viral helper-factor components are needed for efficient aphid transmission. For potyviruses, the helper component-protease (HC-Pro) is needed for aphid transmission of the virus (Pirone, 1964; reviewed in Pirone and Blanc, 1996). For *Cauliflower mosaic virus* (CaMV), helper proteins P2 and P3 are needed for the reaction between the virions and the aphid cuticle (Woolston *et al.,* 1987, Leh *et al*., 2001), additionally CaMV has been reported as being preferentially acquired from the phloem by aphid vectors (Palacios *et al.,* 2002). Differences between the non-persistent and semi-persistent aphid-borne viruses are postulated to be related to the retention of the virus in the foregut of the aphid (Harris, 1977a; Harris, 1977b), which most likely relates to particular cuticular anatomical characteristics of the aphid combined with virion physical dimensions, as well as to specific virus-vector interactions, such as those between the proteins on the surface of the virion and aphid foregut proteins.

Candidates for CTV encoded aphid-transmission helper proteins are numerous since the genomic RNA of CTV potentially encodes at least 19 protein products. Two papain-like protease domains in the 5' end of CTV ORF 1a share some homology to the potyvirus HC-Pro which have been speculated to be involved in aphid transmission. Proteins which are known to be on the outer surface of the virions may also interact with aphid surfaces, thus aiding viral transmission. In closteroviruses, there may be up to 10 structural proteins which could play a role in aphid transmission.

A unique characteristic for the closteroviruses amongst filamentous RNA viruses is that the virions possess two coat proteins. CTV has the major capsid (CP), which has a predicted molecular mass 25-kDa (p25), and the minor capsid (CPm), with a predicted molecular mass of 27-kDa (p27), which encapsidate approximately 95% and 5%, respectively, of virions (Karasev *et al.*, 1995; Febres *et al.*, 1996). In BYV the CPm structure or virion tail needs another virally encoded protein, the heat shock protein-70 homologue (HSP70h or p65), in order to correctly assemble at one end of the virion; both the CPm and HSP70h have been implicated in an ATP-driven long distance movement apparatus (Alzhanova *et al*., 2001). The BYV HSP70h has also been delimited to plasmadesmata within infected cells (Napuli *et al*., 2000), suggesting an additional role in virion cell-to cell movement. The BYV CP, the CPm, the 6-kDa protein (p6), the HSP70h, and the 64-kDa protein (p64) , which all have 3' gene analogues in CTV, are required for cell-to-cell movement (Alzhanova *et al*., 2002).

BYV p20 has been established as being dispensable for virion assembly and cell-to-cell movement but is required for transport through the phloem. The BYV p20 is thought to interact with the virion attached HSP70h molecules thus providing a long distance transport factor to the complex to allow phloem trafficking (Prokhnevsky *et al*., 2002). CTV p20 protein is putatively expressed from the same position in the genome as BYV p20 but bears little similarity to the BYV protein in primary sequence, although this does not preclude functional similarity. CTV p20 is found in abundance in amorphous inclusion bodies formed in infected protoplasts (Gowda *et al.*, 1997; Gowda *et al.*, 2000; Napuli *et al.*, 2000).

There also is supporting evidence that closterovirus CP and CPm are involved in virus-vector interactions. The CPm of LIYV has been found to be a determinant of whitefly transmission when partially purified virus preparations were used in serological infectivity tests (Rochow and Muller, 1975) and with antibodies generated to all the virus proteins (Tian *et al.,* 1999). The CP and CPm of BYV, in similar immuno-neutralizations, were found to be essential for aphid transmission (He *et al.*, 1998). LIYV has also been reported as being transmitted *in vitro* using an artificial infected protoplast diet, in this case a viral defective RNA (D-RNA) has been implicated in efficient whitefly transmission (Ng *et al*., 2002). CTV D-RNAs are also known to be encapsidated (Mawassi *et al*., 1995a), and they might provide a specific avenue for more efficient aphid transmission of the helper virus alone or in specific combination with one or more CTV structural proteins.

Once the CTV determinants of aphid transmission are known, these may be targeted for development of pathogen-mediated control strategies to counteract spread of CTV by the BrCA. The aim of this work was to test purification methods which would enable CTV to be acquired by BrCA through an artificial membrane, and additionally, through the use of immuno-neutralization techniques, to determine if the CTV CP, CPm or p20 are possible CTV-BrCA specific interacters during virus transmission by the aphid.

3.3 MATERIALS AND METHODS

3.3.1 Virus isolates and receptor plants

All experiments were conducted in Florida where CTV and the BrCA co-exist. Six Florida isolates were used: T3, T36, T11a, T66a, T3800 and T66a subisolate "H". CTV isolates T3 (Grant and Higgins, 1957), T36 (Garnsey and Jackson, 1975), T11A and T66a were used for membrane transfer with crude viral preparations, and additionally T66a was used for preparation of a purified viral preparation. Single sweet orange plants harboring CTV T3800 and T66a were used in the infectivity neutralization transmission tests. CTV T3800 is a severe CTV isolate, known to have a very low BrCA single aphid transmission rate (F. Ochoa, pers. comm.). T66a is an *A. gossypii* sub-culture of field isolate T66 collected in Florida in 1985, and has a single aphid BrCA transmission rate of approximately 6% (Tsai *et al*., 2000). A single aphid transmission isolate from previous BrCA single aphid transmissions, T66a subisolate "H", has a single aphid transmission of estimated at 40% (R. Lee, personal communication), and causes stem pitting in sweet orange and grapefruit (Tsai *et al.,* 2000). The reported biological activity and BrCA transmissibility of the CTV isolates used in this study is summarized in Table 3.1. Aphids were maintained on virus-free Carrizo citrange (*Poncirus trifoliata* L [Raf.] x *Citrus sinensis* L.) or rough lemon (*C. jambhiri* Lush.) plants for at least 48 h before the start of each test. The aphid colony was from the Ft. Lauderdale collection by Tsai *et al.* (2000) in 1995. Receptor plants were virus-free 3-6 month-old Madam Vinous sweet orange (*C. sinensis* L.) seedlings.

TABLE 3.1

Biological classification and aphid transmissibility reported for the *Citrus tristeza virus* isolates used in the brown citrus aphid transmission experiments

CTV source	ML	SW/SO	SO	MV	DG	Biotype	SAT (%)
T66a	+	+	+	-	-	III	6[1]
T66a "H"	+	+	+	+	+	X	40[2]
T3800	+	+	+	-	+	IV	0[3]
T11a	+	-	-	-	-	I[4]	ND
T36	+	+	+	-	-	IV	1-2[5]
T3	+	+	+	-	-	VI[6]	ND

Reaction in citrus indicator is denoted by +, non-reaction by -. The five citrus indicator plants are ML; Mexican lime (*Citrus aurantifolia*) seedlings, SW/SO; sweet orange (*C. sinensis*) grafted to sour orange (*C. aurantium*) rootstock, SO; sour orange seedlings, MV; Madam Vinous sweet orange seedlings, DG; Duncan grapefruit (*C. paradisi*). SAT; single BrCA transmission rate. Biotype; CTV biotypes are those according to Lee *et al.,* (1994), ND; not reported. T 66a, T66a "H", T3800, T11a, T36, and T3 are different CTV isolates. Biotype and aphid transmission data compiled from [1]Tsai *et al.,* (2000); [2] R. Lee, unpublished; [3] Halbert , (pers. comm..); [4]Ochoa *et al.,* (2000); [5] *Aphis gossypii* transmission, Yokomi *et al.,* (1989); Garnsey and Jackson, (1975); [6] Grant and Higgins, (1957).

3.3.2 *In vitro* feeding procedure

Aphid feeding chambers were constructed using a Parafilm M (Menash, WI) membrane stretched to four times the original area placed on one end of a 1.5 cm diameter plastic tube, the opposite end of the tube was then covered with a snap plastic lid, essentially as described by Duffus (1989). All virus and comparable control preparations contained a final concentration of 20% (v/v) concentration sucrose in phosphate-buffered saline (PBS: 0.02 M phosphate, 0.14 M sodium chloride at pH=7.4). Antiserum preparations were diluted to 1:20 in 20% sucrose made up in PBS. One drop of yellow food coloring was added to each solution then 500 µl aliquots were immediately dispensed onto the feeding chamber membranes. A second piece of Parafilm M was then stretched over the liquid on each chamber, so that there was a layer of liquid between two Parafilm layers.

3.3.3 Immunoneutralization aphid transmissions

T. citricida were allowed to feed on CTV-infected plants (48 h) and were then introduced into chambers to feed on CTV p25, p27 or p20 antiserum preparations diluted to 1:20 and containing 20% sucrose in PBS (12-14 h). The aphids were then transferred to virus-free receptor plants for 48 h. Control treatments included transferring aphids direct from CTV donor to receptor plants. Five aphids per recipient plant were used except in T66a and T3800, where one aphid per recipient was used. BrCA alates and apterans were both used throughout as previous studies did not find a significant transmission difference in the ability to transmit CTV (Tsai *et al.*, 2000). After

completion of aphid transmission feeding, all aphids were exterminated using a proprietary insecticide. Receptor plants were moved to an insect-screened, air-cooled greenhouse and assessed using CTV CP polyclonal antibodies in an indirect ELISA format at least ten weeks after aphid transmissions, using methods and materials previously described (Garnsey and Moreno, 1991).

3.3.4 Viral purifications

Crude preparations were prepared using a modification of the method used by Garnsey *et al*., (1985). Stem bark (2.0 g) was pulverized in liquid nitrogen, then 10 ml buffer was added (0.05 M Tris, pH 8.0, 10% sucrose) and the mixture allowed to set for 10 min at room temperature. The mixture was filtered through four layers of sterile cheesecloth. The homogenate was centrifuged for 10 min at 8,000 rpm at 4˚C (Beckman model J2-21, JA-20 rotor). The supernatant was collected and used immediately to feed to virus-free aphids through a Parafilm M membrane as described in 3.2.2.

Purified preparations (T66a subisolate "H") were prepared using liquid nitrogen titurated stem bark tissue (5.0 g) mixed with 25 ml buffer (0.05M Tris, pH 8.0, 5% sucrose), and left to set for 15 min. The procedure was then followed as for the crude preparation. Supernatants were then layered in sterile centrifuge tubes containing a sucrose cushion consisting of 1 ml 60% sucrose and 1 ml 25% sucrose. Samples were centrifuged at 38,000 rpm for 1 h at 4˚C (Beckman L7-55, SW41Ti rotor). Two opalescent bands were visualized and drawn off with a wide bore syringe. The fractions

were dialyzed (tubing was Spectra/Por MWCO 6-8,000, Spectrum Medical Industries, CA) three times for 20 min at 4˚C against 0.05M Tris, pH 8 with 5% sucrose. The fractions were used immediately for *in vitro* aphid transmission experiments.

3.3.5 Serological neutralization of infectivity

Infectivity neutralization experiments used proteins from purified antisera raised against polyclonal CTV CP (CREC 28, 1µg/µl , R. Lee, unpublished), CPm (Febres *et al.,* 1994) and p20 (Gowda *et al.,* 2000), and were conducted using CTV T3800 and T66a as described (Hunt *et al.,* 1988; Rochow and Duffus, 1978). Virus-free aphids were allowed to feed on CTV source plants for 48 h before being transferred to antiserum-primed feeding chambers (as described in 3.3.2). After 12-14 h, the aphids were then transferred to ten virus-free citrus seedlings (receptors) per treatment. T66a tests were repeated on three periods, transfers taking place on June 20th, 2000 (T66a-1), June 22nd, 2001 (T66a-2) and June 25th, 2001 (T66a-3).

3.3.6 Immunoblots

Samples from the crude CTV preparations were mixed with equal volumes of 2X extraction buffer (Læmmli, 1970), boiled for 5 min, then the supernatants were estimated for total protein content using the Bradford assay (Bradford, 1976). Sample proteins were electrophoretically separated (20 µg total protein) on four gels using 16% SDS-PAGE with 4.5% stacking gels. One gel was stained with Coomassie brilliant blue R-250 (Fisher Scientific, BP 101-25), whilst proteins from the three remaining gels were

electrotransferred to nitrocellulose membranes (390 mA for 65 min). Each nitrocellulose membrane was blocked with 3% gelatin in Tris-buffered saline or TBS (0.1 M Tris-HCl, pH 7.5, 0.15 M sodium chloride). Each CTV antibody (anti-p25, p27 or p20) was then incubated with one blot in 1% gelatin in TBS. The same antibodies used in the aphid infectivity neutralization tests were used at these dilutions 1:2000 for p25 and p20 antibodies, and 1:1000 for p27 antibodies, each in 1% gelatin/TBS. Secondary antibody was goat anti-rabbit IgG-alkaline phosphatase (Sigma A4187) used at 1:2000 in 1% gelatin/TBS. Between each incubation step there were three washes with TBS with added 1% (v/v) Tween 20 . Specific proteins were visualized using bromochloroindolyl-nitro blue tetrazolium substrate in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM magnesium chloride). A broad range pre-stained standard protein marker (BioRad 161-0314), an extracted sample from virus-free citrus leaf tissue, and also a purified preparation of CTV (Texas CTV isolate H33) were included on each gel.

3.4 RESULTS

3.4.1 *In vitro* feeds of viral preparations

From the five CTV sources used in crude viral preparations there were two ELISA positive receptors; one plant for T11a and one plant for T66a. None of the T66a purified preparations yielded CTV infected receptor plants (Table 3.2). Extraction buffer treatments used as controls in both crude and purified preparations did not result in any infected receptor plants.

3.4.2. Infectivity neutralizations

In every pre-immune antiserum, buffer only and no membrane feeding treatments with T3800 or T66a CTV isolates, there was a consistency of between 20-30% rates of CTV transmissions (Table 3.3). The antiserum treatments were inconsistent over time with T66a. Generally there were relatively low CTV detection levels amongst all recipients in experiments -1 and -2 with p27, p25 and p20 antibodies, and a markedly different pattern with relatively high recipient CTV detection T66a-3, in particular using p20. In p20 treatments using T3800 and in experiments T66a-1 and T66a-2, transmission was 20%, 0%, and 20% respectively, then the rate was 80% in T66a-3. The T66-a p20 antiserum test was the only treatment which was significant compared to the control treatments using Chi-squared with correction for continuity tests (Zar, 1999), $\chi^2_{0.05,1} \geq 3.841$, thus the null hypothesis that the antiserum has no effect on CTV transmission rate compared to the controls must be rejected, and the alternative hypothesis that the p20 antiserum did have an effect on aphid transmission of the virus must be accepted. All other antiserum treatments were non-significant ($\chi^2_{0.05,1} \leq 3.841$), therefore the null hypothesis must be accepted; all p25, p27 treatments and T66a-1 and T66a-2 p20 antiserum treatments were indistinguishable from the controls. The CTV p27 derived antibodies in the tests with T3800 and T66a-1 resulted in no positive CTV recipient plants. In T66a-2 and T66a-3 there were 30% and 50% recipients ELISA positive, respectively. For the CP antibodies, there were 10% ELISA positive recipients for T3800, T66a-1 and T66a-2 tests then with T66a-3 this rose to 30%.

TABLE 3.2

***Citrus tristeza virus* ELISA positive recipient citrus plants with *in vitro Toxoptera citricida* feeds of different viral preparations**

Treatment	Crude CTV extracts	Purified CTV extracts
T3	0/6	NT
T36	0/7	NT
T11a	1/9	NT
T66a "H"	1/9	0/6
Extraction buffer	0/10	0/5

All scores are numbers of CTV infected recipient plants/numbers of recipient plants used in treatment detected by ELISA. T3, T36, T11a, T66a "H" are different CTV isolates. Extraction buffer; buffer used during virus extraction used as a treatment.

TABLE 3.3

***Citrus tristeza virus* ELISA positive recipient plants with *in vitro Toxoptera citricida* infectivity neutralization tests, using three CTV antibodies**

Antibody treatment	T3800	T66a-1	T66a-2	T66a-3
p27	0	0	3	5
p25	1	1	1	3
p20	2	0	2	8
Pre-immune	3	2	2	2
Buffer only	2	3	1*	3
No treatment	2	2	3	2

Each score represents the number of CTV infected recipient plants detected by ELISA out of 10 recipient plants tested, except * where nine plants were used. T3800 and T66a are different CTV isolates; -1, -2, -3, indicate three separate tests for T66a. Pre-immune; pre-immune antiserum from CTV p27 antibody development, Buffer only; buffer treatment only, No treatment; aphids transferred direct from the CTV source plants to recipient plants

3.4.3 Immunoblots

The Coomassie brilliant blue stained gel of the total proteins from the samples is shown in Fig. 3.1.A. For the p27 antibodies, specific bands at approximately 26 –kDa were visualized in T3, T36, and T66a (a doublet) and the H33 CTV viral preparation (Fig. 3.1.B.). A specific band of approximately 27-kDa was distinguished in sample T11a and no bands of this size were detected in the virus-free citrus tissue (lane C). For the p20 antibody blot (Fig. 3.1.C.), faint specific bands of approximately 20-kDa could be visualized after a long incubation period with T66a, T36, T3 and T11a, but not in the H33 CTV viral preparation nor in the virus-free citrus extracts (lane C). With p25 antibodies (Fig. 3.1.D.) vague bands of approximately 25-kDa were detected in all samples except the virus-free tissue (lane C). The p27 blot was re-probed with the p25 antibodies and the band size categories of the p27 and p25 specific bands were confirmed.

3.5 DISCUSSION

T. citricida transmitted CTV isolate T11a and T66a subisolate "H" from crudely prepared plant sap acquired through membranes. Further purification of the virus preparations, including high speed centrifugation, rendered the CTV non-transmissible. When the crude CTV-infected tissue preparations were examined by immunobloting, the CTV p20, p27 and p25 proteins were detected (Fig. 3.1.). The H33 CTV viral preparation was used in the immunoblots and had been subjected to further high speed centrifugations (not described in the methods) comparable to the T66a subisolate "H"

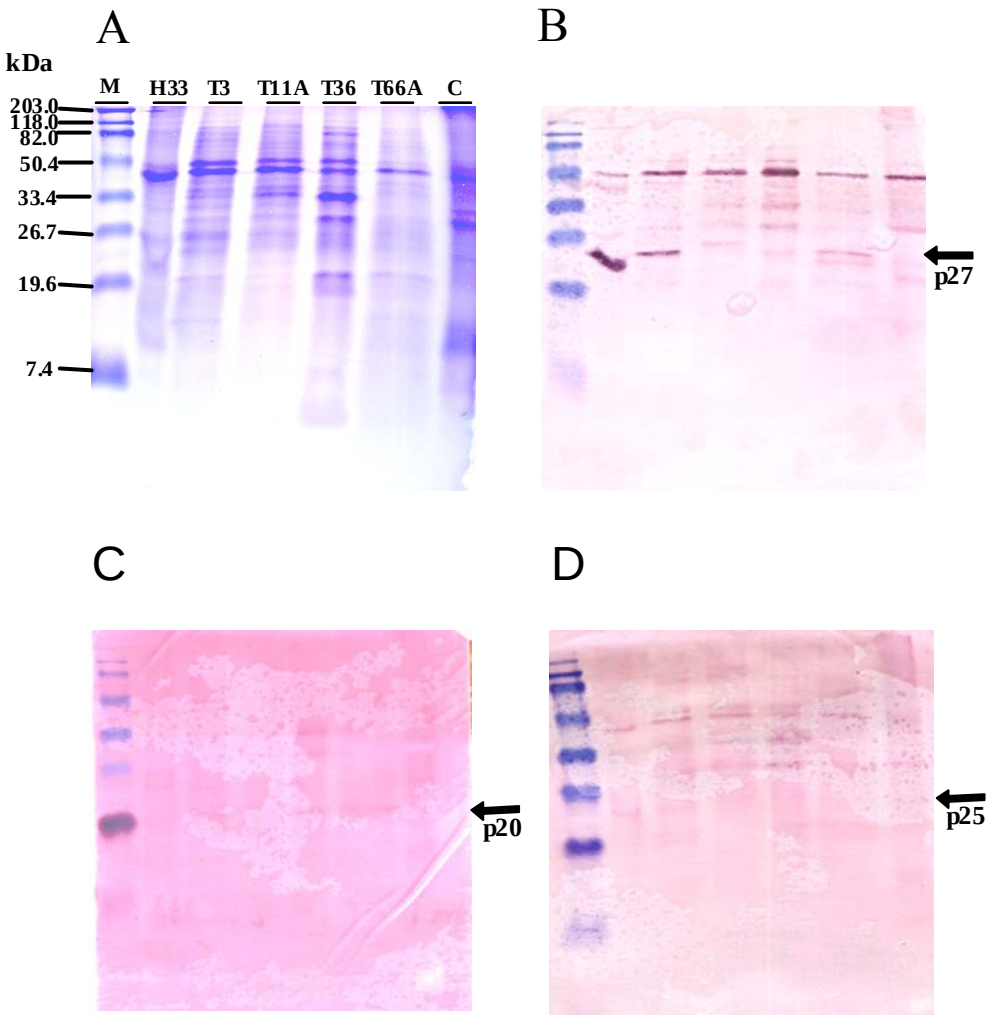

Fig. 3.1. Protein analysis of *Citrus tristeza virus* crude viral preparations. A. Coomassie stained gel showing protein marker and sample loading configuration for all blots, A-D, B; immunoblot using CTV p27 antibodies, C; immunoblot using CTV p25 antibodies, D; immunoblot using CTV p25 antibodies, arrows indicate the respective specific bands on the immunoblots.

purified viral preparation used in this study. The CTV specific p25 and p27 proteins, but not the p20 proteins, were detectible by immunoblot with the H33 CTV viral preparation. This leads to the suggestion that CTV p20 is more readily disassociated from CTV virions during further virus purification steps. Virion and protein integrity might be important for BrCA transmission. This has been alluded to by other workers. For instance for preparation of CTV tissues for transmission electron microscopy of whole particles, preparations of CTV infected crude plant sap yields abundant particles (Garnsey *et al*., 1977; Lee *et al.,* 1987), whereas further purification procedures are known to break the viral thread-like particles. The CTV T36 isolate is reported to have very low aphid transmission (Lin *et al.*, 2002b). Thus associated viral components or structural aggregations of viral protein complexes may be needed for CTV to be transmissible using *in vitro* BrCA acquisition feeding.

This is the first report of transmission of CTV by *in vitro* transmission by aphids. The experimental conditions described here provide the basis for further tests using different CTV isolates and changing various experimental conditions, such as pH of buffer and sucrose concentration of the aphid feeds. Aphid feeding is known to be affected by a vast range of environmental and behavioral factors (Harris, 1977b) all which can make *in vitro* experiments inconsistent. Thus many more tests may be needed in order to observe definite trends.

CTV p25, p20 and p27 antibodies in general could not be considered as having any effect upon BrCA CTV transmission by CTV T3800 or T66a, except for p20 antibodies in experiment T66a-3. Generally there was consistently low CTV rate of

transmission throughout the tests using both T3800 and T66a, even though T66a is known to be transmitted by BrCA at higher rates by other workers (Tsai *et al*., 2000). *In vitro* feeding with a p27 pre-immune antiserum or buffer alone was no different from transferring aphids direct from the CTV infected plants to receptor plants. Thus the membrane feeding, buffer or antiserum used could not account for this transmission rate. Using five aphids (T66a-2, T66a-3) per citrus receptor plant instead of single aphids (T3800, T66a-1) per receptor did not affect CTV transmission incidence in the controls. Blocking the p20 function by feeding the aphids with the p20 antibodies significantly increased CTV transmission by the BrCA *in vitro*. There was a similar trend with p27 antibodies in -3, but this was not significant. CTV p20 can be detected in CTV virion preparations (data from a Texas CTV isolate, H33, not shown), therefore p20 is presumed to form a structural component to the virus particle or to be bound to virions. The p20 protein is reported to be the major constituent of CTV amorphous inclusion bodies formed in infected protoplasts (Gowda *et al.*, 2000) and in BYV the corresponding protein has been proposed as having a long distance phloem movement role for the virus (Prokhnevsky *et al*., 2002).

CTV p25 and p27 proteins are virus capsid proteins, additionally CTV virions may have molecules of the CTV HSP70h and CTV p63 protein attached, and there may be additional CTV proteins thus far not detected on the surface of the virions. The HSP70h for instance encoded by CTV may also be necessary to block the plant cell defenses against stylet penetration in aphids, as cellular HSP70s are known to be produced as a protective cellular response to stress. Since the CTV HSP70h is thought

to be attached to the virions, there is the possibility for aphid interactions for this protein. Aphid heat-shock proteins or symbionins produced by bacterial endosymbionts are thought to be ubiquitous amongst aphid species (Dixon, 1998). Symbionin (Chaparonin 60) has been implicated in *Beet western yellows virus* (Family: *Luteoviridae*) aphid transmission (circulative, non-propagative) together with viral components (van den Heuvel *et al.*, 1997). The specific luteoviral protein determinants of aphid transmission in *Barley yellow dwarf virus* have now been indicated outside the hemocoel, in the accessory salivary gland (Li *et al*., 2001).

Semipersistent, aphid transmitted viruses such as the closteroviruses and caulimoviruses may have complex and very different specific reactions with their vectors. Transmission of CaMV by two aphids (*Brevycorine brassicae* L. and *Myzus persicae* Sulzer) has been found as semi-persistent, and even though virus could be acquired from non-phloem tissues, the probability of acquisition rose significantly when aphids reached committed ingestion from the phloem phase (Palacios *et al*., 2002). In their tests, Palacios and co-workers suggest epidermal and mesophyll probing first loaded aphids with the CaMV P2 transmission factor and then CaMV virions were acquired during phloem feeding (where only P3-virions could be detected by immuno-electron microscopy).

Many CTV multifunctional proteins aggregated in different conformations may be needed to provide virion integrity, and this may give a disadvantage or size exclusion limit to specific reactions occurring in the aphid's cuticular lining of the foregut including the cibarial valve and pump. Disassociation or disaggregation of some of the

CTV structures may be needed so that specific interactions can take place between the aphid cutica and virus components in order that virus transmission occurs. Alternatively, consecutive or sequentially formed viral-aphid associations based upon aphid behavior may be needed before aphid transmission of the virus occurs.

CHAPTER IV

CHARACTERIZATION OF A SEVERE *CITRUS TRISTEZA VIRUS* (CTV) ISOLATE FROM MEYER LEMON (*CITRUS MEYERI* TANAKA) IN TEXAS AND PHYLOGENIC COMPARISONS TO ALL OTHER COMPLETE CTV GENOMES

4.1 SUMMARY

A Texas *Citrus tristeza virus* (CTV) isolate from Meyer lemon (*Citrus meyeri* Tan.), H33, was characterized by indexing on citrus species, serology and sequencing. H33 contains all the severity components of CTV; sweet orange (*C. sinensis* L.) or grapefruit (*C. paradisi* Macf.) decline symptoms on sour orange (*C. aurantium* L.) rootstock, seedling yellows of sour orange and grapefruit seedlings, stem pitting of sweet orange and grapefruit scions and also an additional symptom of stem pitting of sour orange. Long flexuous filaments near to the full-length CTV size (2000 nm) were visualized by transmission electron microscopy (TEM) in preparations from the infected plant, and the preparations also reacted in immunoblots to CTV coat protein and CTV p20 antibodies. A cDNA library from infected plant dsRNA was generated in a 'shot-gun' manner. The majority of sequences (346) were aligned into a consensus CTV genome of 19,232 nt, the H33 major component. Six other sequences (H33 minor components) were analyzed and phylogenically compared to the H33 major component,

and T36, VT, T30, T385, NUagA, and SY568 CTV genomes at the 5'-UTR, RdRp, p6, p20 and 3'-UTR regions. This study is the first to give an insight into the population structure of a severe CTV isolate not biased by primer walking strategies. Since CTV-infected Meyer lemon propagative tissue has been grown in all the major citrus US growing states, the sequence diversity data is important when considering pathogen-mediated strategies.

4.2 INTRODUCTION

Citrus tristeza virus (CTV) is a major graft-transmissible virus of citrus (Bar-Joseph *et al.*, 1981) which has inflicted extreme economic losses to citrus crops world-wide (reviewed by Roistacher and Moreno, 1992). Three types of CTV symptoms can be damaging to citrus. Tristeza decline is tree canopy wilt and death of scions grafted to sour orange rootstock (*Citrus aurantium* L.). Small, poor quality fruit can result from CTV stem pitting tristeza symptoms on scions regardless of the rootstock. Additionally, juvenile seedlings of sour orange, grapefruit (*C. paradisi* Macf.), and lemon (*C. limon* L.) can be rendered worthless due to tristeza seedling yellows symptoms which delay growth. Other strains of CTV are symptomless in some citrus hosts and can pose threats to others. CTV can remain relatively dormant in an area for many years and then suddenly become damaging (Roistacher and Moreno, 1992; Rocha-Peña *et al*., 1995). Often this may be related to the introduction of a more efficient aphid vector (*Toxoptera citricida* Kirkaldy) or to different citrus varieties being introduced to a region, but little is known about the molecular basis for these changes in CTV severity.

CTV is an aphid-borne, monopartite, closterovirus,which is recalcitrant to mechanical transmission and is limited to the phloem in members of the *Rutaceae* and *Passiflora* species. Particles are long flexuous filaments of ca. 2000 x11 nm dimensions (Bar-Joseph and Lee, 1989). The viral genome is a single-stranded positive sense RNA which can vary from ca. 19-20 kb in size depending on the particular isolate. Unique to the filamentous plant viruses, the closterovirus viral coat protein consists of two subunits. In CTV this is a major unit of putatively 25-kDa (CP; p25) which covers the majority of the virion and a minor unit of predicted 27-kDa (CPm; p27) molecular weight, which covers one end of the virion, referred to as the rattlesnake structure (Agranovsky *et al*., 1995; Febres *et al*., 1996). The genome consists of 12 ORFs (Pappu *et al.*, 1994), which can potentially encode at least 19 protein products (Karasev, 2000).

Six CTV isolates have been fully sequenced which vary in the severity of symptoms caused in citrus or in their geographical collection. All have been sequenced using primer walking methods. The first full-length CTV genome to be sequenced was the T36 isolate from Florida (Karasev *et al.,* 1995), which causes a severe sweet orange [*C. sinensis* (L.) Osbeck] decline when grafted to sour orange rootstock. VT is a severe seedling yellows tristeza isolate from Israel (Mawassi *et al*., 1996). The T380 isolate from Spain (Vives *et al*., 1999) and T30 isolate from Florida (Albiach-Martí *et al*., 2000c) have been found to be nearly identical to each other in sequence across the genome; both are considered mild CTV isolates in that they cause symptoms only in

Mexican lime [*C. aurantifolia* (Christm.) Swing.]. SY568 is a severe seedling yellows isolate from California (Yang *et al.*, 1999), and the NUagA isolate is reported to be a severe seedling yellows CTV isolate from Japan (Gede *et al.*, 2001).

From the genomic analyses, the CTV isolates have been found to be highly similar in the 3'-UTR, but are highly polymorphic in the 5'-proximal region. Using this diversity in the different 5'-prominal regions, at least two genotype groups can be distinguished (López *et al*., 1998; Hilf *et al*., 1999; Hilf and Garnsey, 2000; Ayllón *et al*., 2001), and as a result CTV severity detection can be predicted by rapid laboratory tests.

Genomes of RNA viruses, CTV included, are expected to rapidly accumulate mutations due to the error-prone nature of RNA polymerases (Domingo and Holland, 1994). Such diversification may lead to loss of fitness due to the build up of deleterious mutations or to rapid and unpredictable fitness gains (Clarke *et al*., 1993; Roossinck, 1997). This genetic variation can be altered by CTV being tissue grafted to different citrus hosts or by different aphid species when feeding on CTV infected plants. From the earliest documented observations of the symptomology of CTV in citrus, it has been known that the CTV in one plant consists of a mixture of variants which can be separated by grafting to different citrus species. Such experiments have now been confirmed by insect transmission studies and analysis of the viral components in sub-cultures from one plant (for example, Fraser, 1952; Grant and Higgins, 1957; Jarupat and Dodds, 1991; Albiach-Martí, *et al*., 2000b). CTV is a known quasispecies in that those related, nonidentical genomes constitute a replicon population (Eigen *et al*., 1988).

CTV infected tissue at a minimum is known to contain different gRNA species variable in sequence with possible chimeras, at least 30 to 33 positive-sense subgenomic RNA (sgRNA) species (Gowda *et al*., 2001), and many types of defective RNA (D-RNA) species (Mawassi *et al*., 1995b; Yang *et al*., 1997). An improved understanding of the molecular nature or structure to CTV populations is needed in order to use pathogen-mediated strategies for CTV protection purposes, since these methods use virus sequence homology as their targets.

Meyer lemon (*C. meyeri* Tan.) was first introduced to the continental US with CTV from China in 1908 to the plant introduction station at Chico, California (Meyer, 1911; McKee, 1926). Buds were propagated from this source to all major citrus growing areas of the USA. We sought to characterize this CTV isolate biologically and to observe the CTV population in one tree in a random manner using a shot-gun cloning and sequencing approach from a plant infected with the virus for almost thirty years.

4.3 MATERIALS AND METHODS

4.3.1 Virus source, plant indexing and characterization

The original CTV-infected material was collected as budwood from a mature Meyer lemon tree in the Lower Rio Grande Valley of Texas in 1972 (L. W. Timmer, pers. comm.) CTV H33 represents a third passage of the original tissue onto a Mexican lime seedling inoculated in 1987. Since 1987 the tree has been retained in the Texas A&M University-Kingsville Citrus Center *in planta* virus collection. H33 leaf mid-ribs and young stem bark tissue samples for the CF11 extraction were taken on May 9, 2000.

H33 leaf tissue samples for total RNA extractions and northern analyses were taken in 1998, 2000 and, 2001. A virus-free Mexican lime seedling was obtained from the Texas citrus budwood certification program (Kahlke *et al*., 2000); samples were extracted as for H33 and were used as controls in the CF11 extraction procedure (Morris and Dodds, 1979; Dodds, 1993), and the northern analyses.

4.3.2 Citrus indexing

Donor buds or stem bark pieces (2-4 per seedling) were graft inoculated to citrus indicators in August 2001. Five citrus indicator plants were used; Mexican lime, Duncan grapefruit, Pineapple sweet orange grafted to sour orange, sour orange seedlings, and Madam Vinous sweet orange seedlings. Four plants of each indicator were used for each isolate, plus two plants were left uninoculated for controls. Plants were kept in an environmentally controlled (27-30˚C maxima/18-21˚C minima) indexing facility. Visual assessments of symptoms were made periodically over one year according to methods detailed by Garnsey *et al*., (1987b). A severity score (0-3) was given to each symptom in each plant. The mean severity score was calculated for each donor isolate in each citrus indicator. The mean severity score was multiplied by a weighting factor for each citrus cultivar according to the relative economic impact of the CTV symptom (Garnsey *et al*., 1987b). Mexican lime had a weighting factor of 1, sweet on sour 2, seedling yellows symptoms 3, stem pitting with Duncan grapefruit 4, and stem pitting on Madam Vinous, 5. Six reference CTV isolates (B2, B4, B5, B6, B28, B384) from the CTV Exotic world collection maintained in the quarantine facilities,

USDA-ARS Beltsville Agricultural Research Center (BARC), MD, were obtained under permit No. USDA 46874 and graft inoculated onto Pineapple sweet orange seedlings in the Texas A&M University-Kingsville Citrus Center's indexing facility, and used as controls. The symptoms were assessed for 12 months. For all plants, ELISA tests were used to detect CTV infection.

4.3.3 Virus purification

Liquid nitrogen-titurated leaf mid-ribs and stem bark (100 g) were stirred for 10 min with 500 ml extraction buffer (0.1 M Tris-Cl, pH 7.4, 0.5% [w/v] sodium sulfite and 0.5% [v/v] β-mercaptoethanol). The mixture was strained through four layers of cheesecloth, 2% Triton X 100 was added (v/v), and this was placed at 4˚C for 1 h with continuous agitation. Supernatants (after 5,000 *g,* 10 min, 4˚C) were layered onto 20% (w/v) sucrose in TE buffer (0.01 M Tris-Cl, pH 7.4, 1 mM ETDA), then centrifuged for 4 h at 93,000 *g*. Pellets were resuspended in 1 ml TE containing 2% ethylene glycol (v/v), and stored at -80˚C until used.

4.3.4 ELISA

Double antibody sandwich-indirect (DAS-I) ELISA was performed using the methods outlined previously (Garnsey and Cambra, 1991; Rocha-Peña and Lee, 1991), and replicated three times. Coating antibody was CTV polyclonal CREC IgG 28 (1μg/ml) in carbonate coating buffer (0.05 M sodium carbonate at pH 9.6) incubated for 4 h at 37˚C or overnight at 4˚C. Detecting antibody was G604-10 in conjugate buffer

(PBST plus 2% w/v polyvinylpyrrolidone-40, and 0.2% [w/v] ovalbumin) at a dilution of 1:30,000, incubated at 37˚C for 4 h or overnight at 4˚C. Antigoat antibody conjugate with alkaline phosphatase (Sigma A-4187) at 1:30,000 dilution in conjugate buffer was added and incubated under the same conditions. Substrate (1μg/ml; ρ-nitrophenyl phosphate in 10% [v/v] triethanolamine, pH 9.8) was added and the hydrolyzed enzyme substrate extinction values were collected at A_{405} during the reaction.

4.3.5 Tissue blots

The reaction of the CTV H33 to CTV MCA-13 monoclonal antibodies was tested and assessed as a tissue immunoblot (Nokomis Corp., Altamonte Springs, FL) on three occasions. MCA-13 was raised against a decline inducing CTV isolate collected from a sweet orange on sour orange rootstock in Florida (Permar *et al.,* 1990). Four young stems were taken from each plant and the cut stem end of each was blotted onto nitrocellulose paper. The paper was air dried and sent to Nokomis Corp. for assessment. Uninoculated citrus plants were included as test samples.

4.3.6 Immunoblots

CTV H33 virion preparations (10 μl) were combined with 10 μl 2X extraction buffer (Læmmli, 1970), then placed at 100˚C for 5 min, and the resultant supernatants (20 μl) were separated by 12% SDS-PAGE with 4.5% stacking gels. One gel was stained with Coomassie brilliant blue R-250 (Fisher Scientific, BP 101-25), and proteins from the two remaining gels were electrotransferred to nitrocellulose membranes. Each

nitrocellulose membrane was blocked with 3% gelatin in Tris-buffered saline or TBS (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl). CTV antibodies raised to the CP (p25) or p20 were used to probe the blots at 1:2000 dilution in 1% gelatin/TBS. Secondary antibody was Goat anti-rabbit IgG-alkaline phosphatase (Sigma A4187) used at 1:2000 in 1% gelatin/TBS. Specific proteins were visualized using bromochloroindolyl-nitro blue tetrazolium substrate in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM magnesium chloride). A broad range pre-stained standard protein marker (BioRad 161-0314), and similarly prepared samples from a virus-free Mexican lime tissues were used as controls.

4.3.7 Electron microscopy

Virion preparations (H33 and virus-free Mexican lime samples) were placed onto Formvar-coated copper electron microscope grids for 4 min, and then washed three times in sterile distilled water. The grids were then stained with uranyl acetate (1%) for 3 min, then excess stain was absorbed with sterile filter paper (Whatman, 3MM), and the grids were visualized immediately using a transmission electron microscope (Zeiss 10C).

4.3.8 dsRNA isolation

Enriched dsRNA fractions were obtained using methods previously described (Morris and Dodds, 1979; Moreno *et al*. 1993). Leaf mid ribs or stem bark (7 g) were removed from the plants and ground in liquid nitrogen to a fine powder, combined with

17 ml of extraction buffer (2X STE ,10% [w/v] SDS, 1% [w/v] PVP40, 0.02% [v/v] β-mercaptoethanol) , and vortexed. After one phenol:chloroform extraction at 4˚C, the aqueous phase was collected and adjusted to 16.5% ethanol at room temperature. The sample was passed over a CF11 cellulose (Whatman, Clifton NJ) column equilibrated with column buffer (16.5% ethanol in 1X STE) once, and the enriched dsRNA fraction was eluted using 1X STE. Nucleic acids were precipitated with 95%. After centrifugation at 3,000 *g* for 30 min, pellets were washed with 70% ethanol and resuspended in 200 µl sterile water and treated with RQ DNase (Promega Corp., Madison, WI) at 37˚C for 30 min using the manufacturer's protocol. Samples were electrophoretically separated on a 6% non-denaturing polyacrylamide gel, stained with ethidium bromide, and visualized under UV light.

4.3.9 Library construction and sequencing

The H33 dsRNA samples (2 µg) were denatured in 10 mM methyl mercury hydroxide (Alfa Aesar, Ward Hill, MA) for 10 min, then neutralized by the addition of β-mercaptoethanol to 120 mM, incubated for 5 min, then used directly for cDNA synthesis. All incubation steps were at room temperature. cDNA was synthesized from the denatured RNA sample using the Superscript Choice System for cDNA Synthesis kit (Invitrogen Life Technologies, Carlsbad, CA) using the manufacturer's protocols for random hexamer-primed first strand DNA synthesis. cDNA products were size fractioned, ligated into *Eco*RI digested and phosphatased pBluescript II SK (+), and electroporated into XL-1 Blue *E. coli* cells (Stratagene, La Jolla, CA). A small fraction

of the cells were plated in serial dilution to determine the best plating frequency, whilst the majority of the cells were combined with 30% glycerol and stored at 5˚C. After the appropriate plating density was determined to provide the growth of independent colonies on 150 x 15 mm Petri plates, colonies were spread on 20 Petri plates and allowed to grow overnight at 37˚C. Individual colonies were picked from the plates and used to start 2 ml overnight cultures for automated plasmid isolation.

4.3.10 Sequencing

Plasmids were prepared from overnight Luria broth cultures containing 100 μg/ml penicillin in 96-deepwell plates using the Qiagen 9600 liquid handling robot and the QIAprep 96 Turbo mini prep kit following the manufacturer's protocol. Sequencing reactions were performed using ABI PRISM BigDye Primer Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) at $1/16^{th}$ the standard total volume reaction. Reactions were prepared in 96-well format using the Biomek 2000 liquid handling robot (Beckman Coulter, Inc.). Sequencing reactions were ethanol-precipitated and resuspended in 15 μl sterile water, and then loaded onto a 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Base calling was performed by TraceTuner (Paracel, Pasadena, CA) and quality trimming, vector trimming and sequence fragment alignments were performed using Sequencher Software (Gene Codes, Ann Arbor, MI). Sequence identity was initially determined based on blast homology using the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nlm.nih.gov/) and comparison to the nucleic acid and protein databases

(Altschul *et al*., 1997). Overlapping sequence data from the CTV clones were aligned and edited using Sequencher version 4.1.4.

4.3.11 Comparative sequence analysis

Nucleotide multiple sequence alignments using the six full-length CTV genomes from the NCBI databank (Table 4.1), were analyzed with the neighbor-joining (NJ) method of ClustalX (Thompson *et al*., 1997) and the NJ and maximum-likelihood method of PAUP* 4.0 beta 10 (Swofford, 2000). Nucleotide and amino acid pairwise comparisons from the alignments were determined using GeneDoc version 2.6.02 (Nicolas and Nicolas, 1997), and additional sequence editing was done using BioEdit version 5.0.9 (Hall, 1999). Deduced amino acid sequences were analyzed with the NJ method of ClustalX and the quartet maximum-likelihood based method of Tree-puzzle (formerly Puzzle; Strimmer and von Haesler, 1996). Tree-puzzle analyses were done with Dayhoff and Jones, Taylor, and Thornton phylogenic models with 10,000 quartets. The sequence of a woody plant closterovirus, *Grapevine leafroll associated virus-2* (GLRaV-2, accession number AF039204, genus; C*losterovirus*), a 15,000 bp near full-length genome (Zhu *et al.,* 1998) was used as an outgroup in all the analyses.

4.4 RESULTS

4.4.1 Biological characterization, molecular characterization, electron microscopy and serology

CTV H33 was found to cause severe leaf vein clearing, stem pitting and stunting in Mexican lime, a severe decline on sweet orange or grapefruit grafted to sour orange, severe seedling yellows in sour orange and Duncan grapefruit seedlings, and moderate stem pitting of Duncan grapefruit, Madam Vinous. Additionally, sour orange rootstock had moderate stem pitting. The cumulative index was 26, which was lower than SY568 (B6) and far higher than T30 (B2) BARC obtained CTV isolates. Thus, H33 is in the highest rated biotype group as is SY568 (biotype X), which means all CTV economically damaging severity components are present. T30 is categorized as biotype I, causing symptoms only in Mexican lime seedlings (mild).

H33 purified virions were visualized by transmission electron microscopy as numerous near complete length particles of ca. 2000 nm, with no distinct 'rattlesnake' feature delimited (Fig. 4.1.). H33 viral preparations consistently reacted with both polyclonal and MCA-13 antibodies at different times of the year and with different development stage tissues being tested by ELISA. Purified virion preparations reacted in immunoblots with CTV CP and p20 antisera giving specific bands of ca. 25-kDa and 20-kDa, respectively (Fig. 4.2.).

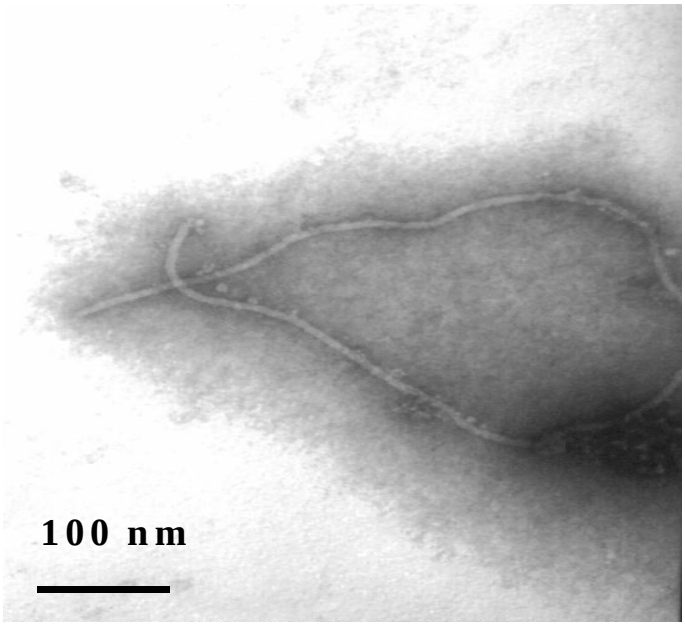

FIG. 4.1. A *Citrus tristeza virus* (CTV) particle from Texas CTV isolate H33. A near full-length virion, negatively stained with uranyl acetate, from a purified preparation of H33.

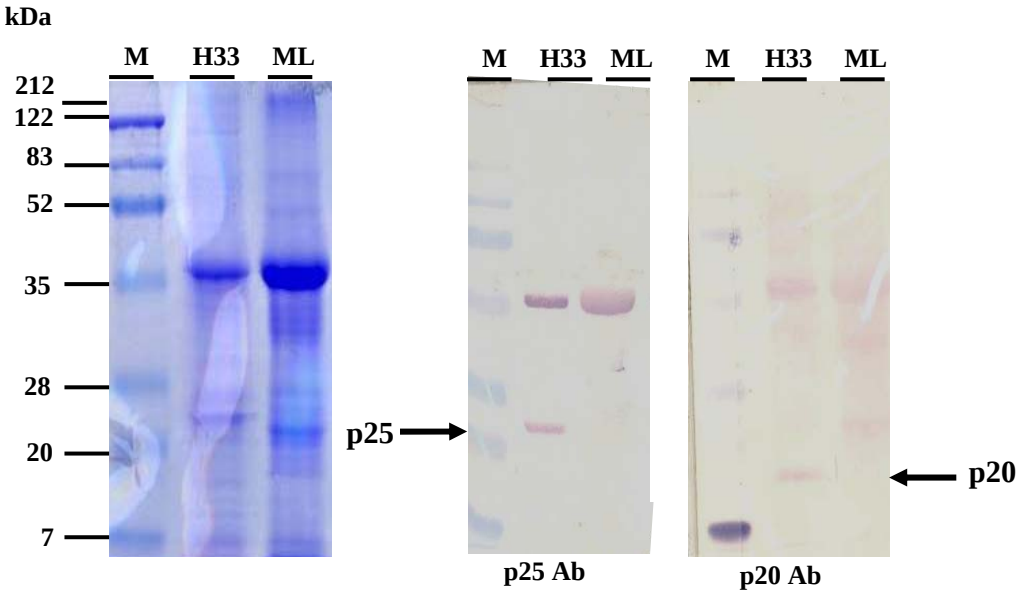

FIG. 4.2. Immunoblots of *Citrus tristeza virus* (CTV) isolate H33 virion preparations. Immunoblots (right) probed with CTV p25 or p20 antibodies (Ab), and a Coomassie blue-stained gel (left) of the same samples.

TABLE 4.1

Biological activity and accession numbers for the six *Citrus tristeza virus* (CTV) isolates for which the full-length genomes were used in sequence comparisons

CTV isolate	ML	SW/SO	SY	GSP	SSP	Biotype	NCBI Accession numbers
T30	+	-	-	-	-	I	AF260651
T385	+	-	-	-	-	I	Y18420
VT	+	+	+	?	?	III	U56902
NUagA	+	?	+	?	?	VI?	AB046391
T36	+	+	+	+	(+)	IV (X)	NC_001661
SY568	+	+	+	+	+	X	AF001623
H33	+	+	+	+	+	X	-

ML; Mexican lime vein clearing and stem pitting symptoms, SW/SO; sweet orange (*Citrus sinensis*) grafted to sour orange (*C. aurantium*) stock decline symptoms, SY; seedling yellows symptoms in sour orange, grapefruit or lemon seedlings, GSP; grapefruit (*C. paradisi*) stem pitting , SSP; sweet orange stem pitting, +; presence of symptoms, -; no symptoms, ?; not known. Parentheses indicate different reports of symptoms for the T36 isolate. Biotype designations are on a 0-X scale, as described by Lee *et al*., (1994).

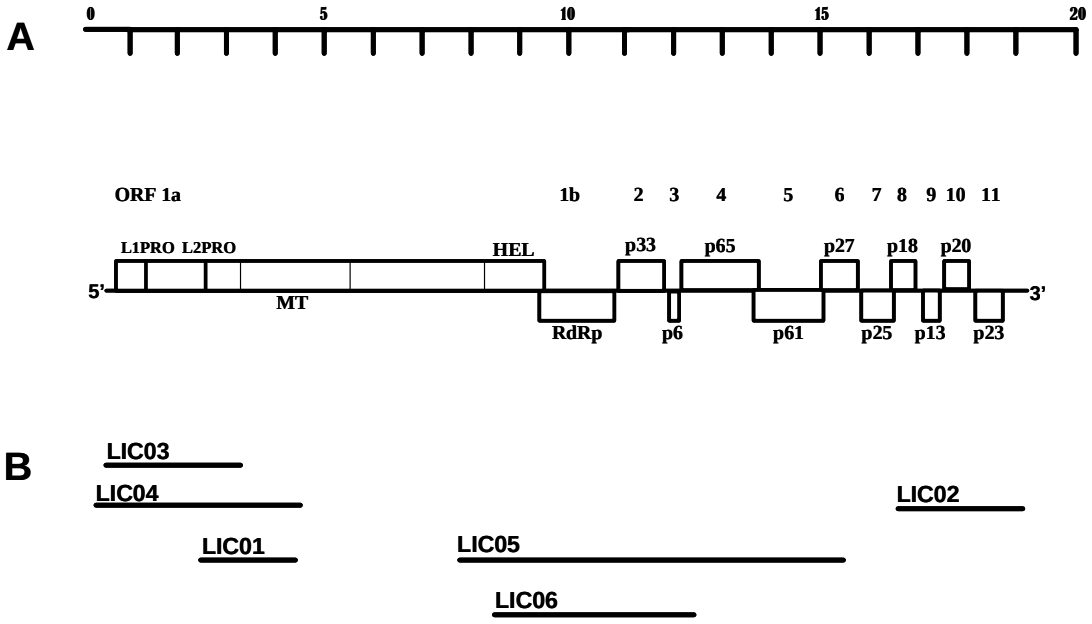

FIG. 4.3. The *Citrus tristeza virus* isolate H33 genome. A; H33 major component consensus to scale, scale bar (top), where divisions are 1000 bp, and a schematic diagram of the genome structure (below) including the 11 CTV ORFs with their appropriate predicted protein labels. B; H33 minor components, LIC01 to LIC06 inclusive, with approximate positions relative to the H33 major component.

TABLE 4.2

Biological characterization of *Citrus tristeza virus* (CTV) isolate H33 compared to six BARC CTV isolates under Texas conditions

CTV isolate	ML Rx1	SW/SO Rx2	SO Rx3	DGFT Rx4	MV Rx5	CUM	Biotype
H33	3	4	6	8	5	26	X
B2 (T30)	1	0	0	0	0	1	I
B4	2	4	0	4	5	15	NC
B5	1	2	0	0	0	3	II
B6 (SY568)	3	6	6	12	10	37	X
B28	2	4	9	12	5	32	X
B384	2	6	3	8	0	19	IV

ML; Mexican lime seedlings, SW/SO; sweet orange (*Citrus sinensis*) grafted to sour orange (*C. aurantium*) seedlings, DG; Duncan grapefruit (*C. paradisi*) seedlings, MV; seedlings of sweet orange Madam Vinous. Mean raw assessments are scoring all symptoms per receptor on a 0-3 scale with 0 as no symptoms, 3 as the severest symptoms. Relative indices are obtained by multiplying the mean raw assessment per receptor by the economic weightings (RxN). The cumulative index for each CTV isolate is the sum of all the relative indices. After Garnsey *et al*., (1987b). Biotype designations are on a 0-X scale, as described by Lee *et al*., (1994). NC; this reaction type has not been classified in the Biotype system.

4.4.2 H33 genome major component

Over 350 sequences of the sequenced cDNAs were found to be from CTV. The majority of these sequences (346) could be aligned into an overlapping, non-gapped consensus sequence corresponding to a CTV genome of 19,232 nt, henceforth called the H33 major component. Coverage of the genome was estimated to be approximately 8.9X. Three small regions were present where both DNA strands were not sequenced at least once, in these areas there were multiple sequences in one direction only. The H33 major component consensus was aligned with the other full-length CTV sequences in the NCBI databank and the CTV genome structure was found to be identical to the organization of the six other full-length CTV genomes (Table 4.1.). The genome has 12 putative ORFs, with 5' and 3'untranslated regions (UTRs), as illustrated in Fig. 4.3. The translated products of each ORF were similar in size to the corresponding ORF productsof the other CTV isolates. From the alignments of H33 major component genome to the other full-length genomes, it is estimated that between 20-24 nt may be missing from the 5'- UTR and 3'-UTR, putting the estimated size of the H33 major component genome to 19,252-19,256 nt.

From the alignment data comparing H33 sequences to the six other CTV genomes (Tables 4.3. and 4.4.), the isolates had a high polymorphism in the 5'-UTR, ORF 1a and ORF1b (RdRp), compared to the 3'-UTR, ORF 8 and ORF 9. Amino acid identity and similarity to the six isolates was most variable in the RdRp, ORF1a and

TABLE 4.3

***Citrus tristeza virus* (CTV) isolate H33 major component details; nucleotide differences between the CTV genomic sequences from H33 and CTV isolates T30, T385, SY568, NUagA, VT and T36**

H33 genome				Nucleotide comparisons (%)					
ORF	Size (nt)	Start	Stop	T30	T385	SY568	NUagA	VT	T36
5'-UTR[1]	89	1	89	82	82	89	88	90	71
1a	9350	90	9440	86	86	90	90	89	73
1b	1434	9364	10798	89	89	88	94	95	78
2	912	10847	11758	85	85	85	92	94	84
3	156	11828	11983	92	92	92	94	98	88
4	1785	11989	13773	89	88	89	96	97	88
5	1608	13697	15304	88	88	88	93	92	88
6	15279	15279	16001	91	91	87	87	88	91
7	672	16093	16764	91	92	95	96	95	92
8	504	16730	17233	88	89	90	91	91	90
9	360	17267	17626	90	90	88	91	89	90
10	549	17702	18250	89	89	94	94	94	89
11	630	18333	18962	91	92	92	93	92	91
3'-UTR[2]	270	18963	19232	98	98	98	98	97	97

[1] Comparisons to 1-89 nt in H33 only all other isolates; [2]Comparisons to the 270 nt only in the 3'UTR of H33 all other isolates.

TABLE 4.4

***Citrus tristeza virus* (CTV) isolate H33 major component deduced amino acid sequence comparisons to CTV isolates T30, T385, SY568, NUagA, VT and T36**

H33 genome	Amino acid identity (%)						Amino acid similarity (%)					
ORF	T30	T385	SY568	NUagA	VT	T36	T30	T385	SY568	NUagA	VT	T36
1a	74	74	80	78	81	54	77	77	81	81	82	62
1b	72	73	69	89	89	52	81	81	70	92	92	66
2	87	86	87	93	88	82	91	90	91	95	92	89
3	96	96	96	100	92	92	96	96	96	100	94	94
4	93	93	93	96	94	93	96	96	96	97	95	95
5	90	90	90	91	91	89	94	93	93	93	93	92
6	91	91	76	92	89	92	92	92	76	93	91	93
7	93	92	94	93	92	92	94	94	95	95	94	93
8	85	86	86	88	86	87	87	88	86	88	88	88
9	91	90	86	89	91	89	96	95	91	94	95	94
10	86	86	91	91	90	89	90	90	91	91	90	90
11	89	90	92	93	92	89	96	97	97	97	98	96

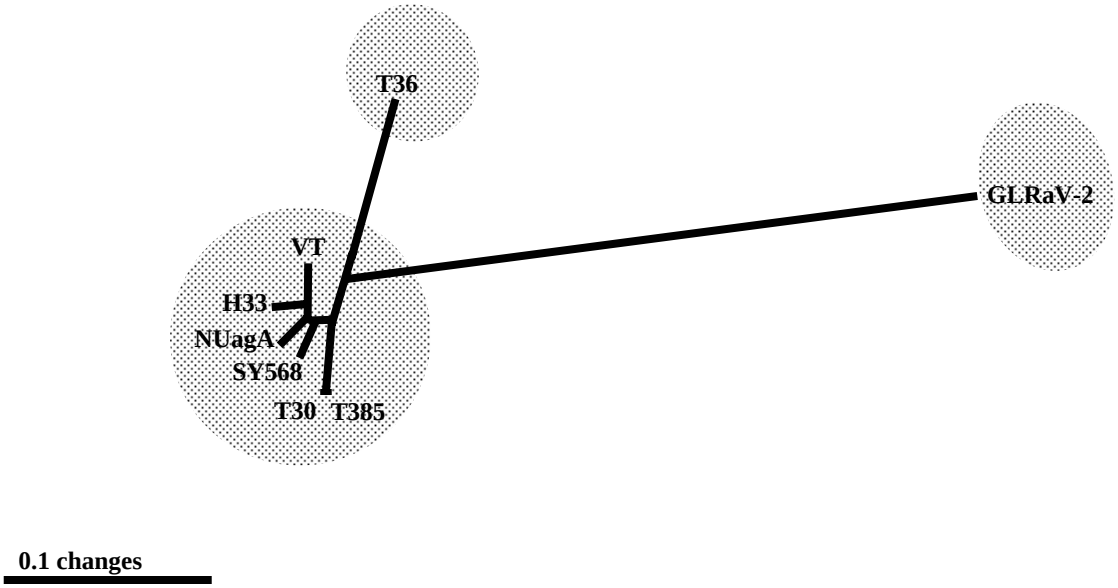

FIG. 4.4. Unrooted phylogram of the nucleotides from seven *Citrus tristeza virus* genomes. H33; H33 major component, T36, VT, NUagA, SY568, T30, T385 and GLRaV-2 genome using neighbor joining methods in ClustalX, and maximum likelihood methods in PAUP*.

ORF 6 compared to relatively low diversity between other 3' ORFs. Other workers have noted this trend of variability amongst the CTV isolates between the 5' regions compared to the 3' regions (Karasev, 2000; Yang *et al.,* 1999; Albiach-Martí *et al*., 2000c).

Phylogenetic trees constructed from the 5'-UTR and ORF 1a nucleotides, ORF1a amino acids, were all near identical to that for the whole genome . All phylogenic trees generated to the full-length genome were consistent, and a maximum-likelihood phylogram is illustrated in Fig. 4.4. The CTV genome sequences are in a tight clade with relatively few nucleotide changes between each CTV isolate, except for CTV T36 which has relatively more differences to any of the other CTV isolates. GLRaV-2 nucleotide differences are far greater than those between any CTV genomes, as predicted.

4.4.3 H33 minor components

Five CTV clones were found (LIC01, LIC02, LIC03, LIC04, LIC06) which had relatively long CTV sequence inserts, and three sequences formed a contig (LIC05). All were polymorphic to the H33 major component sequences. A diagram of where these sequences are in relation to the H33 major component consensus is detailed in Fig 4.3.B. These sequences henceforth are termed the H33 minor components. BLASTN searches indicated that there was diversity in these sequences to H33 (Table 4.5).

Phylogenetic analyses of 5 regions, the 5'-UTR, RdRp, ORF 3 (p6), HSP70h, ORF10 (p20) and the 3'-UTR were undertaken in order to delimit the diversity between

TABLE 4.5

***Citrus tristeza virus* (CTV) isolate H33 minor components; details of the clones and the highest nucleotide identities amongst existing CTV isolates by BLASTN**

CTV code	Type	Size (bp)	Position in genome (isolate)	CTV ORFs represented	BLASTN searches	
					CTV isolate	identity (%)
LIC01	long insert	1782	2582-4361 (VT)	ORF 1a incomplete	VT	97
LIC02	long insert	2444	16832-19273 (T36)	ORF 7 partial, ORF 8, 9,10,11 and part 3'-UTR	T36	91
LIC03	long insert	2754	456-3192 (VT)	ORF 1a incomplete	SY568	96
LIC04	long insert	4359	34-4365 (VT)	ORF1a incomplete	T36	93
LIC05	contig	7958	7097-15034 (VT)	ORF 1a incomplete, ORF 1b, 2,3, 4 and partial ORF 5	VT/NUagA	96
LIC06	long insert	4010	8477-12486 (VT)	ORF 1a incomplete	NUagA	97

CTV isolate; the isolate which gives the highest nucleotide identity to each clone, identity (%); percentage nucleotide identity of each sequence to the respective CTV isolate, reported by BLASTN.

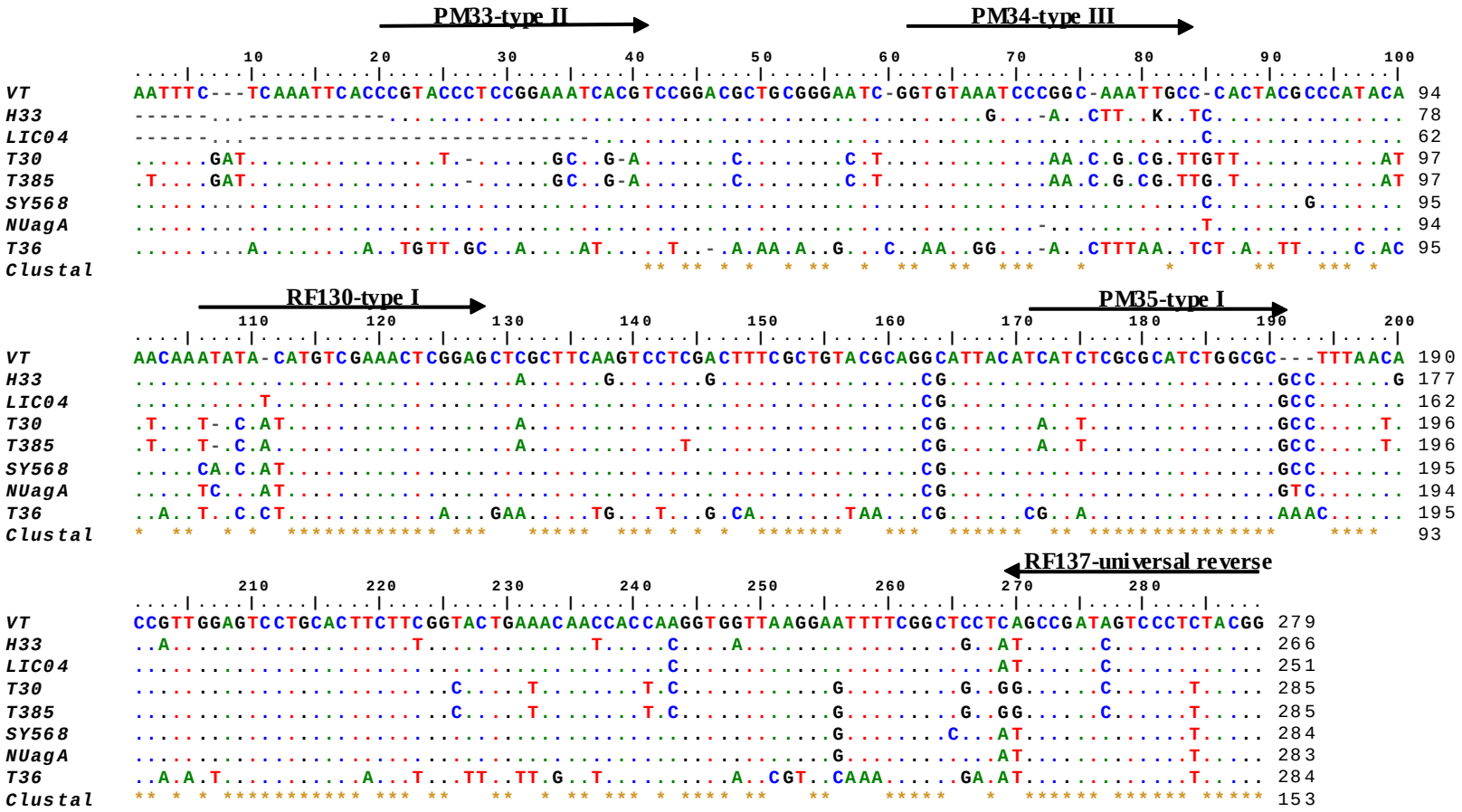

FIG. 4.5. Alignment of the 5'-UTR of *Citrus tristeza virus* (CTV) to show the position of five primers designed to discriminate between different phenotypes. Primer designations and phenotypes are marked arrows above the alignments. CTV isolates VT, H33 major component (H33), H33 minor component (LIC04), T30, T385, SY568, NUagA and T36 are labeled on the left. Note H33 and LIC04 are not complete at the 5' end. After López *et al.*, (1999); Ayllón *et al.*, (2001).

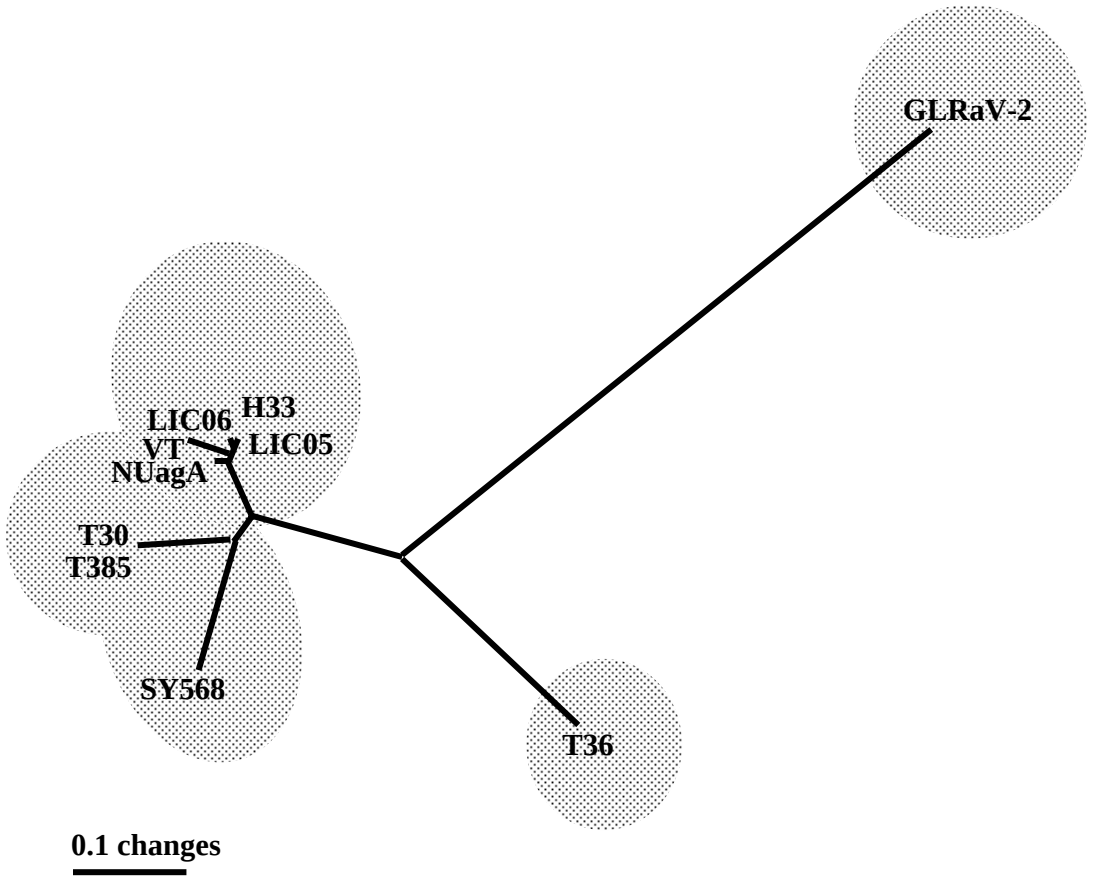

FIG. 4.6. Unrooted phylogram of the amino acid alignments from ORF 1b (RdRp) of *Citrus tristeza* virus. H33; H33 major component, LIC05; H33 minor component, LIC06; H33 minor components, T36, VT, NUagA, SY568, T30, T385 and GLRaV-2 RdRp using neighbor joining methods in ClustalX, PAUP*and maximum likelihood methods in Tree-puzzle (Thompson *et al*., 1997; Swofford, 2000; Strimmer and von Haesler, 1996).

TABLE 4.6

Citrus tristeza virus* isolate H33 minor component deduced amino acid comparisons to the RdRp, p6, HSP70h and p20 from isolates T30, T385, SY568, NUagA, VT, T36, H33 and equivalents in *Grapevine leafroll associated virus-2

CTV H33	Region	Sequence															
		T30		**T385**		**SY586**		**NUagA**		**VT**		**T36**		**H33**		**GLRaV-2**	
		I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
LIC05	RdRp	18	32	18	32	16	27	19	33	13	31	18	30	17	31	54	69
LIC06	RdRp	18	32	18	32	16	27	19	33	13	31	18	30	17	31	54	69
LIC05	p6	31	37	31	37	31	37	31	37	31	37	31	37	31	37	14	28
LIC06	p6	96	96	96	96	96	96	100	100	92	94	92	94	100	100	30	42
LIC05	HSP70h	26	41	26	40	26	40	27	41	31	43	26	40	27	41	15	31
LIC02	p20	93	97	93	97	96	98	96	98	94	96	95	97	87	89	14	36

I; amino acid identity, S; amino acid similarity, all figures are percentages.

H33 major component, individual H33 minor components and T36, VT, T30, T385, SY568, and NUagA genomes. In all trees and models LIC01 and LI03, when compared to the same regions in the seven complete CTV genomes, were in the same clade as VT, which was always relatively distant from the H33 major component (data not shown). LIC04 5'-UTR and 5' part of ORF1a (251 nt) were aligned with the full length genome sequences (284 nt in T36, 279 in VT, 285 nt in T30 and T385, 284 nt in SY568, 283 nt in NUagA, and 266 nt in H33 major component) as in Fig. 4.5. The LI04 sequence was always in the same clade with VT and H33 major component, with a position between VT and H33 major component. LIC05 and LIC06 were compared at the predicted amino acid level with the RdRps of the seven other CTV genomes (Fig 4.6.). RdRp sequences LIC05 and LIC06 clustered with H33 close to the VT and NUagA branches, most distant from T36 branch, with SY568 and T30/T385 branches, respectively, at moderate distance. This coincides with the predicted amino acid similarity comparisons for the alignments in Table 4.6. LI05 and LI06 differ from each other by 2% (similarity) but both differ from H33 by 4% amino acid similarity.

In CTV ORF 3 (GLRaV-2 ORF 2 equivalent, predicted 6-kDa protein, p6) amino acid comparisons, LIC06 was very closely related to T30, T385, SY568, H33, SY568 and NUagA on a branch separate from VT and also T36, whilst LIC05 was very different from the CTV cluster and the GLRV-2 equivalent protein. Phylogenic trees from these sequences all agreed with this observation, and an example is shown in Fig 4.7. HSP70h comparisons (ORF 4 in CTV and ORF 3 in GLRaV-2), using LI06 were observed to be similar to CTV ORF 3, with these sequences being highly conserved

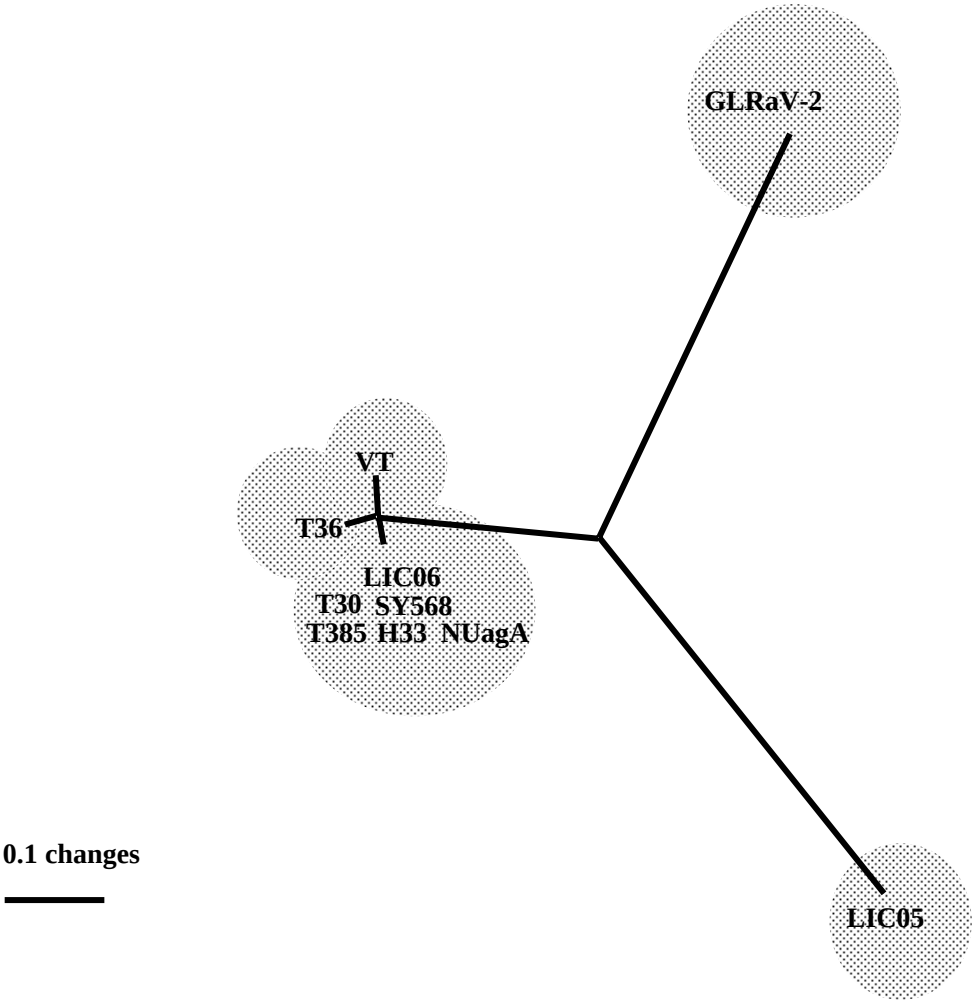

FIG. 4.7. Unrooted phylogram of the amino acid alignments from *Citrus tristeza virus* ORF 3 (p6) of nine CTV sources. H33; H33 major component, LI05; H33 minor component, LI06; H33 minor component, T36, VT, NUagA, SY568, T30, T385 and GLRaV-2 ORF 2 (p6) genome using neighbor joining methods in ClustalX, PAUP* and maximum likelihood methods in Tree-puzzle.

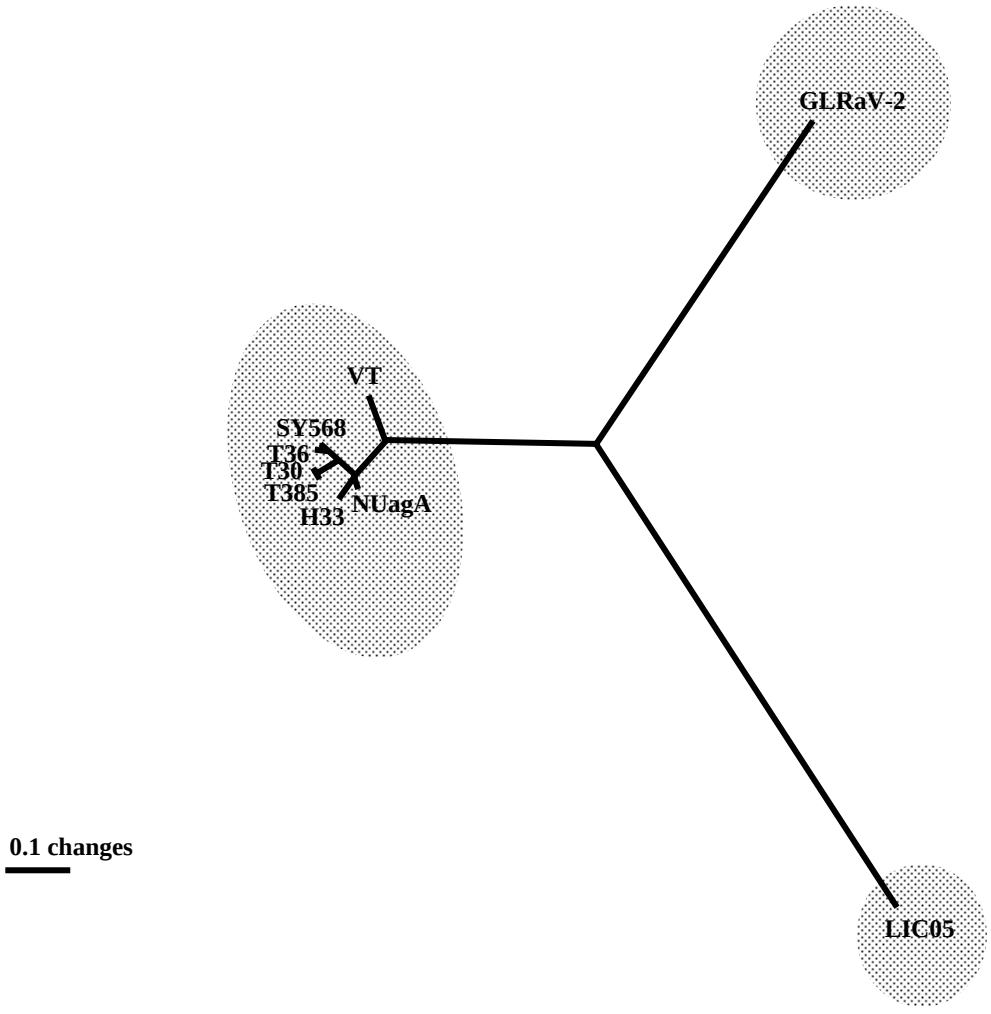

FIG. 4.8. Unrooted phylogram of the amino acid alignments from ORF 4 (HSP70h) of eight *Citrus tristeza virus* isolates. H33; H33 major component, LIC02; H33 minor component, T36, VT, NUagA, SY568, T30, T385 and GLRaV-2 genome using neighbor joining methods in ClustalX, PAUP* and maximum likelihood methods in Tree-puzzle.

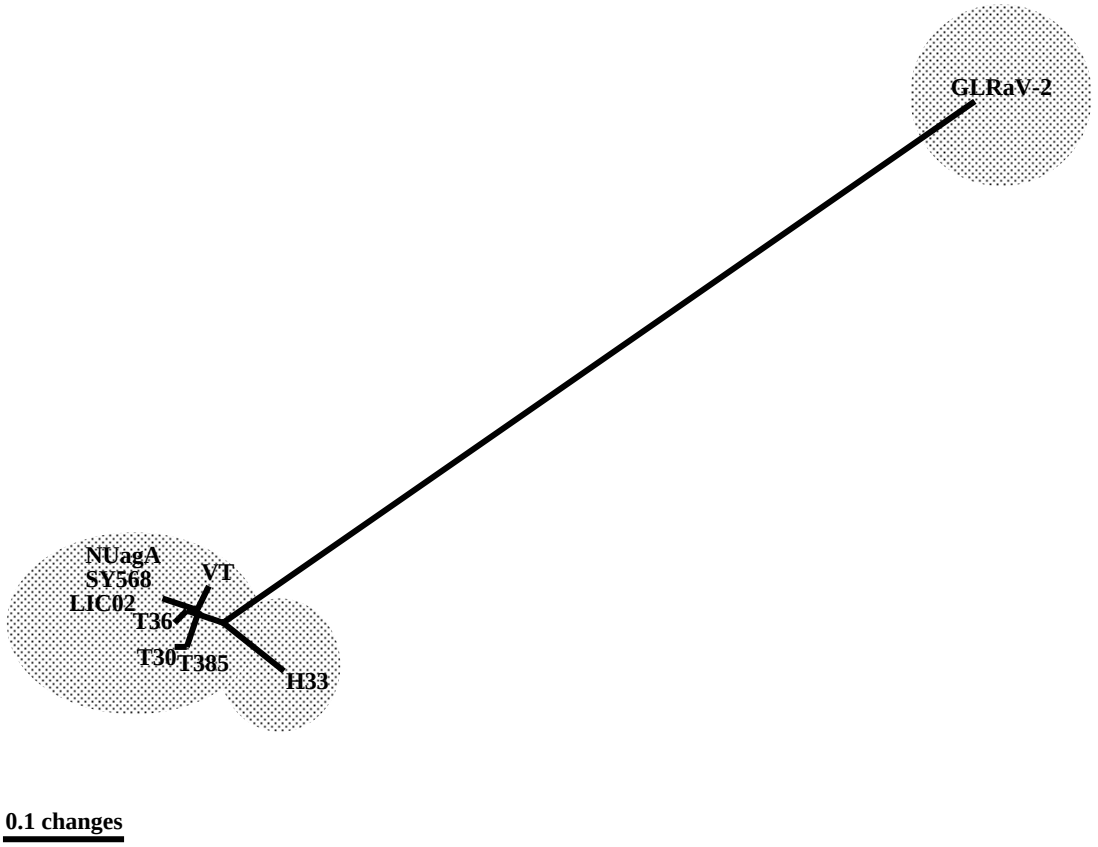

FIG. 4.9. Unrooted phylogram of the *Citrus tristeza virus* ORF 10 (p20) amino acid alignments from eight *Citrus tristeza virus* isolates. H33; H33 major component, LI02; H33 minor component, T36, VT, NUagA, SY568, T30, T385 and GLRaV-2 ORF 8 (p24) using neighbor joining methods in ClustalX, PAUP* and maximum likelihood methods in Tree-puzzle.

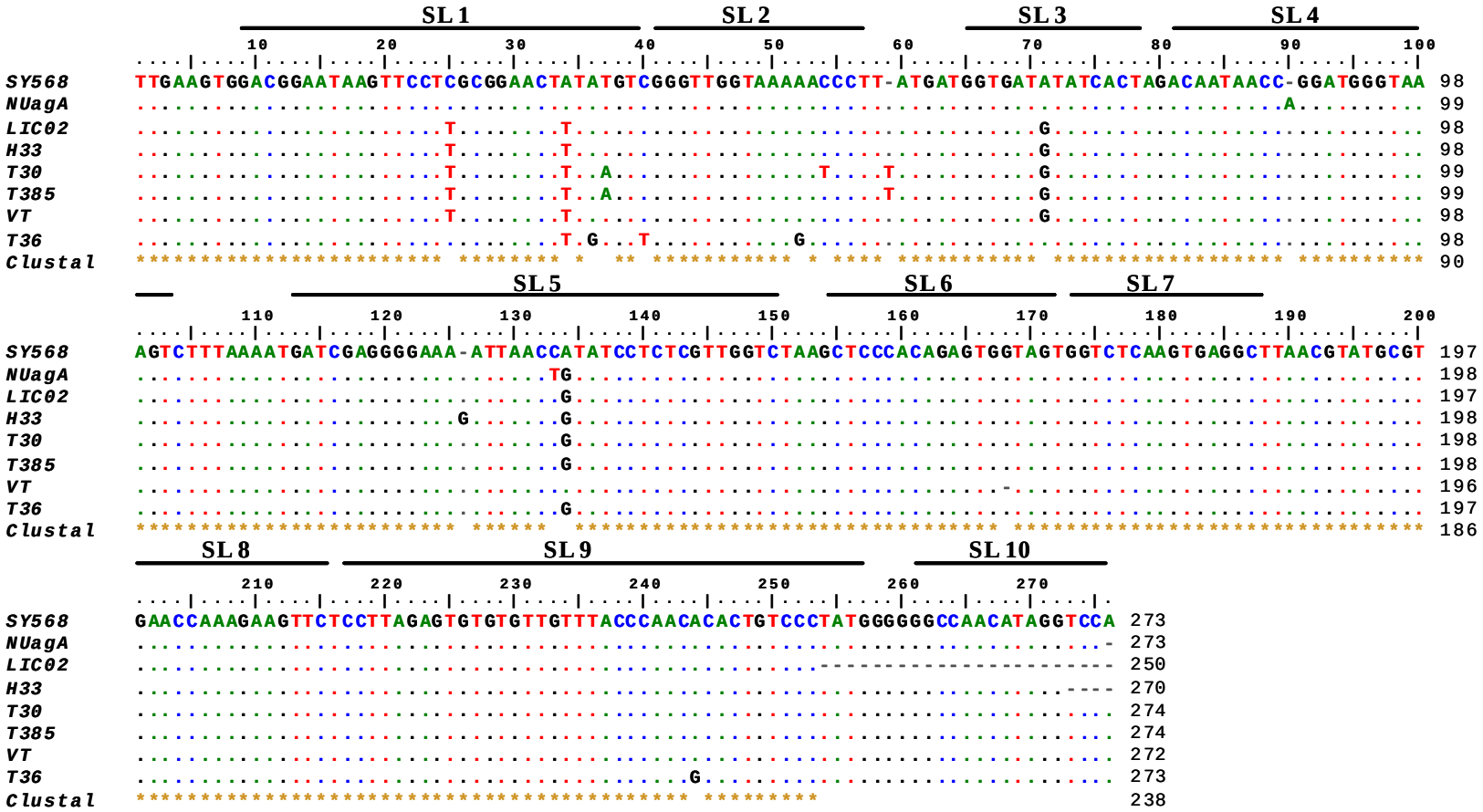

FIG. 4.10. Alignment of *Citrus tristeza virus* (CTV) 3'-UTR showing the position of ten predicted stem loop structures. SL; stem loop. CTV isolates SY568, NUagA, LIC02 (H33 minor component), H33 (H33 major component), T30, T385, VT and T36 labels are on the left. Note CTV LIC02 and H33 are incomplete at the 3' end.

within the CTV group (very small differences), and GLRaV-2 was appropriately positioned away from this group, with a third line of major divergence being LIC05 (Fig 4.8.). LIC05 did not align very well with the other CTV p6 or HSP70h sequences. The amino acid similarities between all CTV p6s and LIC06 were between 94-100% similarity and for LIC05 were 37%. Between LIC05 and all HSP70hs the similarity was between 40-41%. Amino acid similarities to GLRaV-2 p6 and HSP70h analogues was always lower.

LIC02 ORF 10 amino acid comparisons with CTV ORF 10 (predicted 20-kDa protein, p20) and GLRaV-2 ORF 8 equivalent predicted protein (24-kDa, p24), consistently gave a clustering of the CTV sequences, with the outgroup being highly divergent from this (Fig. 4.9.). Within the CTV clustered sequences, H33 major component was always the sole member of one branch and LIC02 was always relatively distant from this in a clade with T36.LIC02 3'-UTR comparisons (Fig. 4.10.) revealed very few nucleotide differences between all the CTV isolates, but LIC02 was always in a clade with the H33 major component (data not shown), and the sequence alignments indicated the type II amplicon, indicative that VT-like characteristics could be generated, which was similar to the H33 major component 5'-UTR (Ayllón *et al*., 2001).

4.5 DISCUSSION

H33 is a severe CTV isolate with a cumulative bioindex of 26 and biotype X under Texas conditions. All the three severe components are present; sweet orange or grapefruit on sour orange decline, seedling yellows on sour orange, grapefruit and lemon

seedlings and stem pitting on sweet orange and grapefruit scions. CTV-like flexuous filaments have been purified and visualized under TEM. Purified viral fractions from H33 react with CTV CP and CTV p20 antibodies, indicating these proteins are structural components to the virion. Extracted leaf sap from H33 reacts with CTV CP polyclonal antibodies by ELISA and with MCA-13 antibodies in tissue blot analyses, which indicate that CTV is present and CTV can cause severe sweet orange on sour orange decline symptoms.

4.5.1 H33 major component sequence analyses

The 5'-UTR of H33 was 89 nt with an estimated 17-20 nucleotides missing compared to the other full-length CTV genomes sequenced so far. Pairwise comparisons of the CTV 5'-UTRs placed H33 major component most similar to VT (90% identity) and least similar to T36 (71% identity). Further to this, the alignments could predict an amplicon being produced using

Type II primers (VT) in a CTV strain differentiating procedure based upon the amplification of four products within the 5'-UTR and part of ORF1a, corresponding to 284 nt of isolate T36, as alignments show in Fig. 4.5. (López *et al.,* 1998; Ayllón *et al*., 2001). A system developed by Hilf *et al.* (1999) differentiates between CTV isolates based upon the VT, T30 and T36 CTV genomes. Amplicons VT-5', VTPOL, VTK17, T30-5', T30POL, T30K17, T36K17 and T36CP regions have been obtained for H33, but not for T36-5' or T36POL regions (R. Brlansky, pers.com.), confirming a VT-like

component is present. Using the 5'-UTR sequence alone in comparisons, H33 major component is in the Type II (VT) Hilf grouping (Hilf *et al*., 1999), confirming this data.

The 5'-proximal ORF1a encodes a 349-kDa putative polyprotein containing four functional domains; two leader papain-like proteases (L1 and L2PRO), a methyltransferase (MTR), and a helicase (HEL) (Karasev *et al*., 1995). The N-terminal portion contains the putative L1 and L2PRO domains which contain the predicted catalytic cysteine residues at positions 404 and 889 and histidines at positions 565 and 949, respectively. The MTR domain contains all the conserved motifs typical of positive-strand RNA viral type I MTRs. The HEL domain has all seven conserved motifs of the type I helicases (Agranovsky *et al*., 1994).

RdRp, ORF 2 and ORF 3

ORF 1b possesses a characteristic conserved RdRp motif (Dolja *et al*., 1991). As with the other CTV isolates, the 'rare' codon for Arginine (CGG) is present at the '+1' frameshift location, predicting a stalling function to the ribosome (Dolja *et al*., 1994). ORF 2, a 303 amino acid ORF of predicted molecular mass of 33-kDa has an amino acid similarity of between 89-95% with the other CTV sequences (Table 4.5). The function of this protein is unknown and it does not align with any other protein in the databases. ORF 3 is a 51 amino acid putative protein (predicted to be a hydrophobic 6-kDa molecular mass protein).

HSPH70h and ORF 5

ORF 4 encodes a protein (65-kDa) which has significant sequence homology to cellular heat-shock protein-70 (HSP70), and has been predicted to have a role in virion movement (Alzhanova *et al*., 2002). ORF 5 encodes 536 amino acids (a predicted 61-kDa protein) which is reported to have a very distant homology to HSP90, and is postulated to be involved in virion movement (Alzhanova *et al*., 2001). Conservation at the amino-acid level of the predicted 65-kDa and 61-kDa proteins is high (with amino acid similarity being at least 92%).

CP and CPm

ORF6 and ORF 7 encode 240 and 223 amino acid proteins with predicted molecular masses of 27 and 25-kDa, respectively. The predicted 27-kDa protein is the diverged copy of the coat protein (CPm) and 25-kDa, the coat protein (CP). There is at least 91% amino acid similarity between these two proteins and their respective counterparts in the other CTV sequences.

3' distal features

ORF 8 (167 amino acid protein), ORF 9 (119 amino acid protein) encoding putative proteins of unknown function, have amino acid similarities ranging from 86-96%. ORF 10, a 182 amino acid protein encoding a putative product of 20-kDa (p20) is found in cellular viral inclusions (Gowda *et al.,* 2000) and in a transient assay system in *Nicotiana benthamiana*, has been found to have activity which suppresses post-

transcriptional silencing (Reed *et al*., 2003), and is highly conserved (at least 90% amino acid similarity across all CTV isolates). ORF 12 (209 amino acids) encodes a predicted protein of 23-kDa (p23). This has the conserved RNA binding motive which is delimited by arginine (base 51) followed by a charged amino acid not well conserved by all CTV isolates (serine, base 54), then overlapping with this is a zinc-finger domain, which has conserved basic amino acids at residues 68, 71, 75, and 85 (cysteine or histidine). Amino acids 46-180 are absolutely required for the asymmetrical accumulation of viral positive and negative-stranded RNAs (López *et al*., 1998; Satyanarayana *et al*., 2002a). This ORF has the most conserved amino acid sequence across all CTV isolates (at least 96%), and is a suppressor of post-transcriptional gene silencing.

The H33 3'-UTR is 270 nucleotides in length, and an estimated 3-4 nucleotides are missing, for the alignment see Fig. 4.10. The 10 stem loop (SL) structures predicted using the MFOLD program (Satyanarayana *et al*., 2002a) were confirmed to be present under the same thermodynamics (data not shown). The one nucleotide difference between the H33 sequence and any of the other CTV sequences is in SL5, where it does not affect the stem structure (found to be important for viral replication). The 3'-UTR has the highest conservation of nucleotide identity across all genomes relative to any other component (minimum of 97% identity).

4.5.1 Phylogenic comparisons of the H33 components to the other CTV isolates

Phylogenetic analyses of H33 major component to the other full length CTV sequences published indicates that H33 fits into the general evolutionary progression of isolates from VT (severe CTV) through to T30 and T385 (mild CTV), and not the T36 evolutionary branch (Fig. 4.4.). H33 major component RdRp trees continue this theme with H33 clustering in a group with VT and NUagA, which is separate from the T36 branch and the SY568, T30 and T385 branch (Fig. 4.4.). Closterovirus HSP70h are known to be highly conserved (for a review see Karasev, 2000) H33 major component HSP70h is in a clade with all the CTV isolates apart from VT (Fig.4.8.). CTV p6 comparisons indicate a trichotomy, with H33 major component in a group separate from both VT and T36 (Fig. 4.7.). The CTV p20 sequences have very few intraspecies differences compared to any of the other amino acid sequences analyzed (Fig. 4.9.), but the H33 major component p20 sequence provides another branch of slight diversity here.

Six other H33 components were found in this study and all have diversity to the H33 major component sequences, but it is unknown whether they represent full-length components, recombination events, D-RNAs or other sub-viral components. In analyses of LIC05 and LIC06 RdRp amino acid sequences clustered around the H33 RdRp compared to the other CTV RdRps. LI06 sequences (p6) were divergent from H33 major component within a clade only, whereas LI05 were divergent from CTV as much as GLRaV-2, which was unexpected. The LIC05 HSP70h comparisons also gave this trend, suggesting a possible intraspecies recombination event. The p6 and HSP70h sequences for LIC05 are more divergent than that generally recognized for CTV isolates

by other workers (Karasev, 2000). For such a conserved CTV protein, the LIC02 p20 and H33 p20 sequences provided relatively high diversity compared to the other CTV p20s (Fig. 4.9.).

From this approach H33 CTV has been found to be composed of at least one full-length genome and variants which cluster around this primary. One sequence was found which was in part highly divergent from CTV, which could represent a different closterovirus species (see CHAPTER VI). These data are contrary to those reported for two other CTV sequences, namely T30 and T385 CTV genomes and three other CTV isolates from different geographic regions collected at different times (Albiach-Martí *et al.,* 2000c). T30 and T385 had an estimated 0.5% nucleotide variability when analyzed. This was surprising considering the lack of proofreading ability for viral RNA polymerases is thought to be the source of the inherent generation of mutant genomes which constitute the viral quasispecies (Domingo *et al*., 1995), but convergent evolution was not ruled out. T30, T385 and the other CTV isolates used in the study all have mild phenotypes. Perhaps CTV isolates which cause damage to citrus have far greater genetic diversity. CTV D-RNAs are known to be associated and encapsidated with the helper CTV genome, and most have been characterized from severe CTV isolates (Mawassi *et al*., 1995b).

CTV D-RNAs have been found which are composed of sequences from the 5' proximal region fused to the 3' distal region of the gRNA; usually they are 2.0 to 5.0 kb size range (Mawassi *et al*., 1995b; Karasev *et al*., 1997; Yang *et al*., 1997). Two D-RNAs have also been characterized (LMT1 and LMT2) which are composed of a 5’

genome equivalent of 0.7 kb, and both are more abundant than the gRNA in CTV infected cells. Another characterized D-RNA (LaMT) contains CTV ORF1a and 1b and terminates before the ORF 2 promoter, it is approximately 11 kb in size, and is found in lower proportion than gRNA in infected citrus protoplasts. (Che *et al*., 2001). Large D-RNA species (~12 kb) constructed with intact ORF1a and 1b genes fused to variable length 3'distal region portions, are easily slash-transmitted to citrus plants, and also readily infect *N. tabacum* protoplasts (Che *et al*., 2002). No interspecific RNA viral associations have been documented with CTV although it is common to find CTV in combination with other graft transmissible RNA infective agents (Roistacher, 1991). One indication that leads to the importance of such sub-viral RNA components is that there are variable specifities of different CTV isolate replication complexes found in experiments with synthetically composed CTV D-RNA components in a *N. benthamiana* protoplast system (Mawassi *et al.,* 2000). Until the 'shot-gun' approach is used to sequence phenotypically different CTV isolates, however and these are analyzed for diversity in population structure and recombination likelihood, differences between sampling error might lead to various conclusions about CTV population structure within one plant.

CHAPTER V

EVALUATION OF PATHOGEN-DERIVED RESISTANCE IN TRANSGENIC RIO RED GRAPEFRUIT PLANTS WITH AN UNTRANSLATABLE *CITRUS TRISTEZA VIRUS* COAT PROTEIN GENE

5.1 SUMMARY

An untranslatable p25 coat protein (CP) gene of *Citrus tristeza virus* (CTV) was genetically transformed into the genome of the Texas commercial Rio Red grapefruit (*Citrus paradisi* Macf.) variety, and 52 independent transgenic lines were produced. When plants propagated in duplicate from each transgenic line were graft-inoculated with Texas CTV isolate H18, there were several types of response to the viral challenge. Individual plants could be identified which had low virus titers by ELISA detection, had a temporal decrease in virus titer, or a delay in virus titer accumulation. Comparisons of all non-transgenic to all the transgenic plants over every assessment revealed significant decreases in virus titer in the transgenic lines compared to that of the non-transgenic lines.

5.2 INTRODUCTION

Citrus is the most economically significant fruit crop in the world with 90 million metric tonnes produced globally, of estimated $12 billion value (FAO, 2002). A major constraint to production are insect-vectored, graft-transmissible pathogens, of which *Citrus tristeza virus* (CTV; Family: *Closteroviridae*; genus: *Closterovirus*) is the most important (Bar-Joseph *et al.*, 1989). CTV causes the decline and death of trees on sour orange, *Citrus aurantium* L. rootstock, and some strains also reduce fruit size and production of scions regardless of rootstock (Garnsey and Lee 1988; Bar-Joseph *et al.* 1989). In commercial nurseries, CTV can also cause drastic foliar chlorosis and reduction of growth in seedlings of sour orange, lemon (*C. limon* L.) and grapefruit (*C. paradisi* Macf.), called seedling yellows (Fraser, 1952). CTV isolates can also vary greatly in the severity of disease symptoms induced in citrus, depending on the scion/rootstock combination (Garnsey *et al.* 1987b; Bar-Joseph *et al.* 1981; Roistacher and Moreno, 1992).

CTV has flexuous filamentous virions ca. 2,000 nm in length, which contain a positive-sense, single-stranded RNA (ssRNA) monopartite genome of approximately 20 kb. The genome has 12 open reading frames (ORFs) potentially encoding at least 19 proteins (Pappu *et al*., 1995). The 5' half of the genome contains the 'Sindbis-type' replication block, whereas the 3' half contains the 'closterovirus hallmark' block (Karasev *et al*., 1997). The closterovirus hallmark array contains 5 ORFs which have analogues in the genomes of all closteroviruses thus far studied. In CTV these are (from 5' to 3' in the genome), the small hydrophobic protein (6-kDa; p6), the heat-shock-70

homologue (p65; HSP70h), the 61-kDa (p61) protein, the duplicate or minor coat protein (CPm; predicted molecular mass 27-kDa, p27) and the major coat protein (CP; predicted molecular mass 25-kDa, p25). The possession of two capsids is a unique feature to filamentous plant viruses, the CPm also forms a characteristic closterovirus structure or 'rattlesnake' feature on the end of virions (Agranovsky *et al.,* 1995; Febres *et al.,* 1996). Thus far, most of the functions associated with the 3' ORFs are structural, with 5-6 of the putative ORF products being associated with intact virions, and several of the ORF products being associated with non-structural functions, such as cell-to-cell movement. The CPm analogue in BYV and *Lettuce infectious yellows virus* (LIYV: Family; *Closterovirus*, genus *Crinivirus*) has been implicated in aiding insect vector transmission (Tian *et al.*, 1999). There are so far no functions described for the CTV ORF 2, 5, 8 or 9 predicted proteins. The putative protein from ORF 10 (20-kDa, p20), is a viral inclusion protein with demonstrated post-transcriptional gene silencing (PTGS) suppressor activity in a heterologous *Agrobacterium tumefaciens* inoculated assay (Gowda *et al.,* 2000; Reed *et al.,* 2003). The CTV ORF 11, a product with a predicted molecular mass of 23-kDa (p23), has an RNA binding and zinc-finger type domains, is thought to be involved in the initiation of minus-strand accumulation, down regulates subgenomic RNA (sgRNA) accumulation of the other the 3' ORFs (Dolja *et al.*, 1994; Satyanarayana *et al*., 2002a), and has demonstrated PTGS-suppressor activity (Lu *et al*., 2002). When the p23 gene was used to transform the CTV susceptible Mexican lime (*C. aurantifolia* [Christm.] Swing.), typical CTV leaf symptoms developed in the transformants (Ghorbel *et al.*, 2001).

CTV is transmitted in a semipersistent manner by several citrus aphids, of which the brown citrus aphid, (BrCA), *Toxoptera citricida* Kirkaldy is the most efficient vector. The virus is not seed transmitted and is recalcitrant to mechanical transmission. CTV is usually spread by using infected tissue during plant propagation (Bar-Joseph and Lee, 1989).

Genetic crossing methods to incorporate CTV resistance genes into citrus cultivars whilst retaining the desirable characteristics of yield and quality have proved to be very lengthy and difficult. Genetic resistance to CTV has been found in citrus relatives *Severinia buxifolia* Poir. (Chinese box-orange), *Swinglea glutinosa* (Blanco) Merr. (the tabog or swinglea) and *Poncirus trifoliata* L. (Raf.), (the trifoliate orange) (Garnsey *et al.*, 1987a; Bar-Joseph *et al.*, 1989). The trifoliate orange is the only one of these species to be sexually compatible with citrus. Nine genera within the *Aurantioideae* (orange sub-family) contain species where CTV introduction by inoculation through aphids and grafting techniques has been unsuccessful (Williams, 1992).

The resistance gene from *P. trifoliata*, *Ctv*, is reported to be a single dominant locus which has been finely mapped to a region of approximately 300 Kbp (Yang *et al.*, 2001; Yang *et al*., 2003). This region has been found to contain 22 predicted genes, including a recognizable plant resistance gene cluster (Deng *et al*., 2000; Deng *et al*., 2001; Yang *et al.*, 2003). The *Ctv* locus product or products are thought to interact at the level of systemic movement of the virus within the plant; therefore they might be expected to interact with one or more movement proteins of the virus or other mobile

signaling molecules, since systemic movement must take place by the vascular tissues. As most of the important citrus varieties are complex hybrids, introgression of the resistance gene into a citrus variety and retaining the desirable qualities via traditional sexual crosses will be extremely difficult. Resistance will have to be in both the scion and rootstock varieties to be efficient. However, isolation of *Ctv* from *P. trifoliata* and insertion of the gene, for instance, into the sweet orange genome via genetic engineering would maintain all the desirable qualities of the fruit and add resistance to CTV symptoms.

Classical cross protection strategies have been used with success against stem pitting CTV strains on grapefruit in Brazil (Costa and Müller, 1980), Australia (Broadbent *et al*., 1991), South Africa (van Vuuren *et al*., 1991), and with decline-inducing CTV [sweet orange (*Citrus sinensis* L.) on sour orange (*C. aurantium* L.)] in Florida and Venezuela (Lee and Rocha Peña, 1992; Ochoa *et al*., 1993). There are the geographic areas are where severe CTV and the BrCA coexist. Cross protection is the use of a mild CTV strain to protect against economic damage by severe CTV strains (Gonslaves and Garnsey, 1989). In the South African citrus clean-stock program, all virus-free citrus propagative material is infected with a 'mild' CTV isolate (pre-immunized) before release to growers. Grapefruit production in South Africa would be uneconomic without this protection (von Broembsen and Lee, 1988; van Vuuren *et al.*, 1993; van Vuuren and da Graça, 2000).

Engineered pathogen-derived resistance (PDR; Grumet *at al.*, 1987), would impart resistance in citrus plants to CTV without losing quality and yield aspects. Protection is conferred by viral nucleic acid sequences (mainly defective or antisense) which are introduced into the plant genome through genetic engineering (Powell-Abel *et al.*, 1986; Beachy *et al*., 1990; Lomonossoff, 1995; Scholthof *et al.*, 1993). Transgenic plants developed by this approach are likely to be protected against infections by the virus from which the transgene is derived, and also closely related strains or viruses (homology-dependent resistance). Citrus plants transformed with CTV coat protein (CP) genes have been produced and are being evaluated by different scientists (Bond and Roose, 1988; Gutiérrez E. *et al.*, 1992; Moore *et al.*, 1992; Luth and Moore, 1999; Domínguez *et al.*, 2001; Ghorbel *et al*., 2001; Febres *et al*., 2003). So far few evaluations have been published, however, or they have been performed on citrus varieties with restricted commercial value in the USA (Mexican lime or Duncan grapefruit). There is one report of transgenic resistance generated to a closterovirus in a woody plant, grapevine (Gonsalves, 2000). This study uses transformants from a commercial red grapefruit variety derived from Texas, Rio Red. The object of this study was to evaluate different Rio red grapefruit transgenic lines for resistance to CTV.

5.3 MATERIALS AND METHODS

5.3.1 Transgene constructs and plant transformation

The untranslatable CTV CP gene was derived from pTEMCP, a clone containing the CP gene of CTV isolate SY568 (Yang *et al*., 1999; Genbank accession number

AF160023). The CP gene fragment was amplified using a sense primer designed with an integrated double stop codon corresponding to 13 nucleotides downstream from the CP initiation codon, and a reverse primer corresponding to the end of the CP gene (Yang *et al*., 2000), henceforth the amplified fragment is termed the *uncp* gene. All plasmids and cloning steps in the procedure are detailed by Yang and co-workers (2000), to construct the binary vector, pBIN34SGUS/*uncp* (Fig. 5.1.). Briefly the binary vector contains three genes between the T-DNA borders. Close to the left T-DNA border there is the neomycin phosphotransferase II gene (*npt*II) gene under the control of the nopaline synthase (*nos)* promoter and terminator. Near the right T-DNA border is an intron-inserted β-glucuronidase gene (*uid*A), under the control of a *Figwort mosaic virus* (FMV) 34S promoter and a *Cauliflower mosaic virus* (CaMV) 35S terminator. The *uncp* gene was placed in between the *uid*A and *npt*II genes, and was under the control of a double 35S promoter with a *Tobacco etch virus* (TEV) 5'-UTR and the 35S terminator. This vector was used to transform epicotyl segments of Rio Red grapefruit using the detailed coculture, selection and regeneration procedures as described elsewhere (Yang *et al.*, 2000). In summary, transformed citrus shoots were selected on DBA3 medium (Deng e*t al.,* 1992) containing kanamycin (100 mg/l), carbencillin (400 mg/l) and cefotaxime (100 mg/l), and stem sections were tested for GUS activity by histochemical assay using X-GLUC and the Jefferson *et al.* (1987) method. GUS-positive shoots were cleft grafted onto greenhouse-grown, 3 month old sour orange seedlings propagated in Conetainers (Steuwe and Sons, Corvallis, OR).

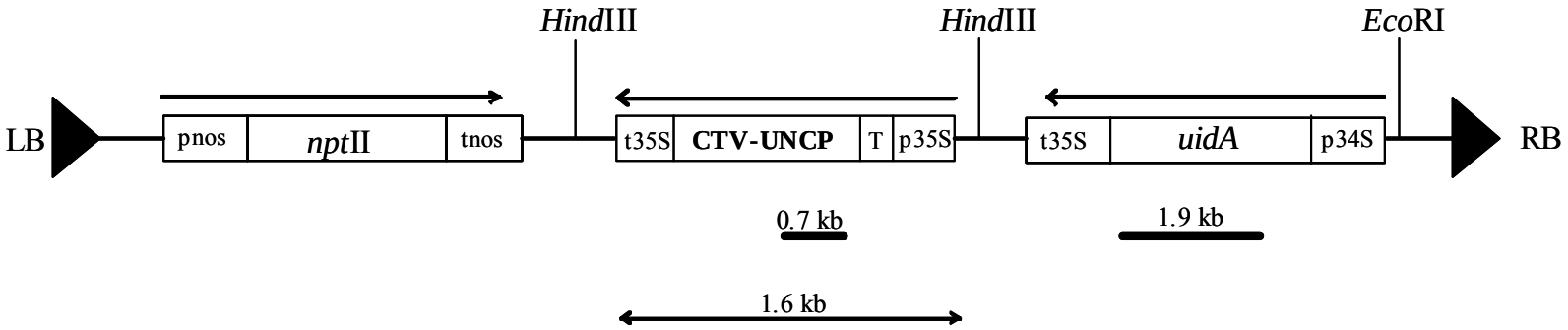

FIG. 5.1. Linear map of the *Agrobacterium* binary vector, pBIN34SGUS/*uncp.* T-DNA borders are represented by the block arrows and marked LB and RB, for left or right border, respectively. Rectangles represent genes; *npt*II; neomycin phosphotransferase II gene, *uidA;* intron inserted β-glucuronidase gene, CTV-UNCP; untranslatable *Citrus tristeza virus* coat protein gene, nos; nopaline synthetase, p35S; dual 35S *Cauliflower mosaic virus*, T; *Tobacco etch virus* 5'-UTR, 34S; *Figwort mosaic virus*, p suffix; promoter, t suffix; terminator. Arrows above the genes indicate orientation of transcription. Heavy lines below the map indicate the position and size of the probes used during analyses, with the estimated size of the *uncp* transcript represented by the double-headed arrow. Not to scale.

5.3.2 DNA and RNA blot assays

At least 3 months after grafting, 0.5-3.0 g of scion leaf issue was used for DNA and RNA analyses. DNA was extracted as described (Chee *et al.*, 1991). DNA (20 μg) was digested with appropriate enzymes and separated by 0.8% agarose gel electrophoresis (Sambrook *et al.*, 1989). After electrophoresis, gels were treated with 0.25 M HCl for 10 min then washed with 0.4 M NaOH for 10 min. Nucleic acids were transferred by the alkaline downward method (Koetsier *et al*., 1993) to nylon membranes (Hybond N^+, Amersham.) for 5 to 12 h. RNA was extracted and transferred to positively-charged nylon membranes using methods as described by Jones *et al.* (1985).

Probes were made with ^{32}P-dCTP generated against the *uncp* or *uid*A genes, using a random primer labeling kit (Gibco-BRL). For *uncp*, a *Sac*I/*Xba*I (0.7 kb) fragment was digested from pRL22/*uncp*, a plasmid made during cloning the *uncp* into pBIN34GUS (Yang *et al*., 2000), and this was used to probe both DNA and RNA blots. For the *uid*A probe A *Sal*I/*Bam*HI (1.9 kb) fragment was excised from pUbiGUS (Ingelbrecht, unpublished).

5.3.3 Virus resistance assay

Seeds of Pineapple sweet orange (*C. sinensis* L.) were obtained fresh from the Texas A&M University-Kingsville Citrus Center variety collection and were sown in sterilized wooden flats (65.0 x 34.5 x 15.0 cm internal dimensions) in sterilized TAMUKCC potting mix No. III (Skaria and Solís-Gracia, unpublished). Plants were transplanted into 1 gallon (3.785 l) pots when they were approximately 15-25 cm tall,

three plants per pot with fresh potting mix, as above. Plants were fertilized, pruned and kept free from diseases and pests according to methods described (Roistacher, 1991). All plants were grown in a shaded greenhouse house cooled by water evaporation and thermostatically controlled air fans.

At approximately 6 months after planting, buds from the *uncp* scions were propagated in duplicate per transgenic event by the "T" graft method (Roistacher, 1991) onto the Pineapple sweet orange seedlings. Each pot consisted of two *uncp* scions and one non-transgenic Rio Red grapefruit scion. Virus-free non-transgenic Rio Red grapefruit buds from the Texas certification program, (Kahlke *et al*., 2000), were used for controls, and propagated onto the sweet orange seedlings at the same time as the transgenic scions. Buds deriving from tissue which had been though the same regeneration as the transgenic scions were used as additional controls.

Scions were forced, according to standard horticultural practices, and once each scion reached 15 cm in length, the rootstock was graft-inoculated approximately 15 cm below the bud union with four lateral meristems, stem sections or leaf midribs from a CTV isolate H18. The forced scion and graft inoculated plant is represented in Fig. 5.2. Each tree was inoculated at least three times (depending on the growth of the scion). Preliminary experiments had used a restricted number of uncp Rio Red lines to determine the length of time needed after CTV inoculation in order to detect CTV by ELISA (data not shown). After inoculation, the scions were allowed to grow without pruning, and the inoculum was removed after 8 weeks. Second and subsequent inocula

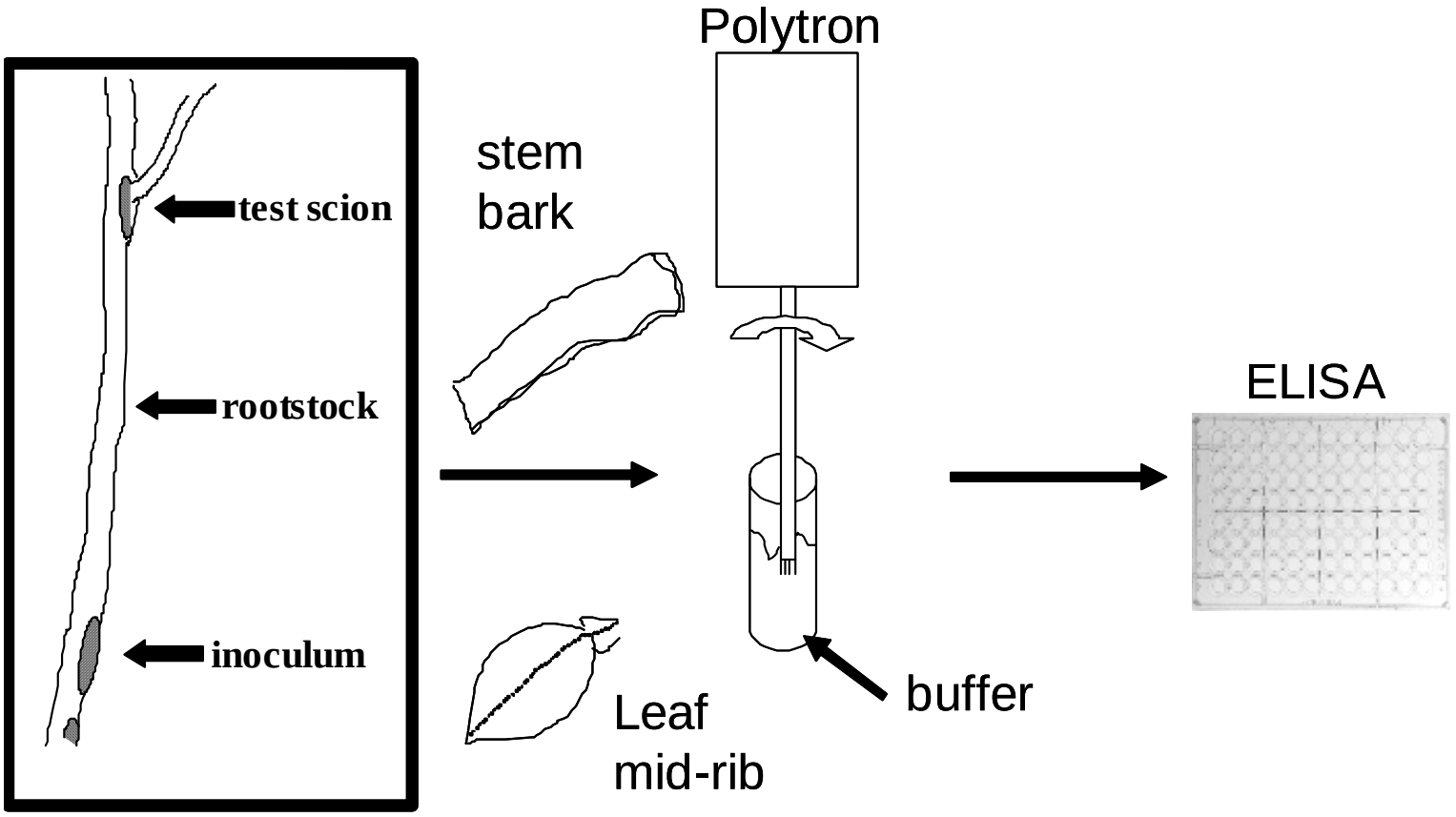

FIG. 5.2. The *Citrus tristeza virus* (CTV) challenge experiment system. Left (inset) is a cartoon of a plant showing the CTV inoculum on the rootstock with the grafted scion above the inoculum. Test tissue can be taken from the rootstock, test scion or inoculum (leaf mid-rib or stem bark), homogenized in buffer and tested by ELISA for a semi-quantitative estimation of CTV concentration.

were left in place. Six-weeks after inoculation, rootstock and scion tissues, where available, were tested for presence of CTV by ELISA (Fig. 5.2.), and then tested at monthly intervals thereafter (23rd January, 19th February and 20th March, 2003).

5.3.4 ELISA

Double antibody sandwich-indirect (DAS-I) ELISA was performed using two CTV polyclonal antisera using the methods outlined by Garnsey and Cambra, (1991) and Rocha-Peña and Lee, (1991). Sterile polystyrene flat bottom (Immulon) 96-well microtiter plates were incubated with CTV IgG CREC 28 (1µg/ml) in carbonate coating buffer (0.05 M sodium carbonate at pH 9.6) for 4 h at 37˚C or overnight at 4˚C. Between each incubation step, plates were washed three times with phosphate-buffered saline with Tween 20 (PBST; 0.02 M phosphate, 0.14 M sodium chloride at pH 4.4, 0.1% Tween 20 [v/v]). From each scion, four freshly collected near-mature leaf mid-veins were transversely cut into 1mm sections and approximately 0.5 g from each test sample was pulverized in 5ml extraction buffer (PBST with 2% [w/v] polyvinylpyrrolidone-40) for 1 min using a tissue homogenizer. The resultant sap for each sample was added to duplicate test wells on the antibody coated microtiter plates. Incubation for antigens was at 4˚C overnight. The secondary antibody, G604-10 in conjugate buffer (PBST plus 2% [w/v] polyvinylpyrrolidone-40, and 0.2% [w/v] ovalbumin) at a dilution of 1:30,000, was added and incubated at 37˚C for 4 h or overnight at 4˚C. Antigoat antibody conjugate with alkaline phosphatase (Sigma A-4187) at 1:30,000 dilution in conjugate buffer was added and incubated under the same

conditions. Substrate (1mg/ml; ρ-nitrophenyl phosphate in 10% [v/v] triethanolamine, pH 9.8) was added and the hydrolyzed enzyme substrate extinction values were collected at A_{405} during the reaction, using a V_{max} plate reader (Molecular Devices, Sunnyvale, CA). The data represent three separate duplicated experiments with uninoculated, CTV infected citrus controls and extraction buffer controls included in each plate.

5.3.5 Statistical analyses

The experiment was designed as a replicated repeated measures test (Zar *et al*., 1999). Leaf samples from one pot were placed on the same microtiter plate for the ELISA, at every test and samples were randomized within the plate. Up to six readings were taken for each ELISA plate at each time point, and these data were observed for development of the reaction of the control samples. One plate reading was used for each group of samples at one time point in the analyses, and this was always the plate where there were maximum differences between the virus-free Rio Red grapefruit wells and the CTV H18 reference isolate wells. Means for each test sample were generated and viewed by eye for standard errors (between wells), none of the measurements had errors over 0.05 units. To standardize plate differences, each sample was compared to the positive control on each plate as a percentage score. Optical density readings were analyzed using SAS (Cary, NC) general linear models procedures (GLM) comparing all non-transgenic scions to all transgenic scions over the three time points.

5.4 RESULTS

5.4.1 Confirmation of *uncp* transformed Rio Red grapefruit plants

After selection, regeneration, GUS-histochemical assay, 52 *uncp* transgenic lines were produced. This was confirmed by Southern hybridizations, and in some cases northern analyses (Table 5.1). Southern analyses with the *Eco*R1-digested genomic DNA (Fig. 5.3.A. gives an example) indicated that there were multiple hybridizations of relatively high molecular weight, indicating multiple sites (0 to 5) which hybridized to the *uid*A gene. Since this gene is located on the T-DNA in close proximity to *uncp*, this implies multiple copies of the *uncp* gene have most likely also been integrated. The *Hind*III digested gDNA blots probed with the *uncp* gene show hybridization to a single band of the predicted size for *uncp*, confirming the presence of *uncp* (data not shown but documented in Table 5.1). Total plant RNA blots (Fig. 5.3.B.) also confirmed in some of the samples that transcripts of the *uncp* gene were produced *in vivo*.

5.4.2 Challenge tests

A preliminary test confirmed that CTV H18 could be detected ca. 30 cm from the point of inoculation in a Pineapple sweet orange seedling using ELISA 4 weeks after graft inoculation (data not shown). The first tests were performed on the scions 6 weeks after the first inoculation, and these tests indicated very few non-transgenic or transgenic scions were CTV-infected.

TABLE 5.1

Rio Red grapefruit lines transformed with an untranslatable coat protein gene from *Citrus tristeza virus* used in the experiments; reporter gene, DNA and RNA analyses

Transgenic line	GUS	DNA *Eco*R1	DNA *Hind*III	RNA
N	0	0	no	0
N	0	0	no	0
EM2	3	4	yes	2
EM2	2	4	yes	2
Y1	3	1	yes	3
Y2	3	1	yes	2
Y3	3	2	yes	2
Y4	1	2	yes	1
Y5	3	1	yes	3
Y6	3	2	yes	3
Y7	0	0	no	0
CH1-1	1	5	yes	nt
CH1-2	2	5	yes	nt
CH1-3	3	5	yes	nt
CH1-4	1	5	yes	nt
CH2-1	3	2	yes	3
CH2-2	3	2	yes	nt
CH3	3	3	yes	3
ZN1	2	1	yes	1
ZN2	1	4	yes	1
ZN3	2	1	yes	2
ZN4	1	4	yes	nt
ZN5	1	4	yes	2
ZN6	3	2	yes	nt
ZN7	3	2	yes	nt
ZN8	3	1	yes	nt
ZN9	3	1	yes	3
ZN10	3	1	yes	2
ZN11	3	1	yes	2

TABLE 5.1 Continued

Transgenic line	GUS	DNA *Eco*R1	DNA *Hind*III	RNA
ZN12	3	2	yes	3
ZN13	3	3	yes	3
ZN14	0	0	no	nt
ZN15	3	4	yes	3
ZN16	2	3	yes	2
ZN17	3	2	yes	3
ZN18	3	2	yes	nt
ZN19	3	1	yes	nt
ZN26B	3	5	yes	nt
ZN21	3	1	yes	2
ZN22	0	0	no	nt
ZN23	2	0	no?	1
ZN24	1	1	yes	nt
ZN25	1	2	yes	3
ZN26A	1	1	yes	2
ZN27	3	1	yes	3
ZN28	1	1	yes	2
ZN29	2	3	yes	nt
ZN30	3	1	yes	3
ZN31	3	1	yes	3
ZN32	3	1	yes	nt
ZN33	2	2	yes	3
ZN34	3	1	yes	3
ZN35	1	1	yes	nt
ZN36	1	1	yes	nt
ZN37	2	1	yes	3
ZN38	3	1	yes	nt
ZN39	3	2	yes	nt
ZN40	3	2	yes	3

GUS; visual assessment of the color reaction in a GUS assay, 0, no color to 3, strong color development, DNA *Eco*R1; Southern analysis using *Eco*R1 digested genomic DNA and a probe made to the *uid*A gene, numerals indicate the number or hybridization bands distinguished, DNA *Hind*III; Southern analysis using *Hind*III digested plant genomic DNA and a probe made to *uncp*, yes indicates a band of the expected size (*ca*. 0.7 Kbp) was detected, RNA; intensity of the ca. 0.7 Kbp hybridization band using total plant RNA probed with *uncp*, on a 0 (no hybridization) to 3 (intense hybridization) scale , nt; not tested.

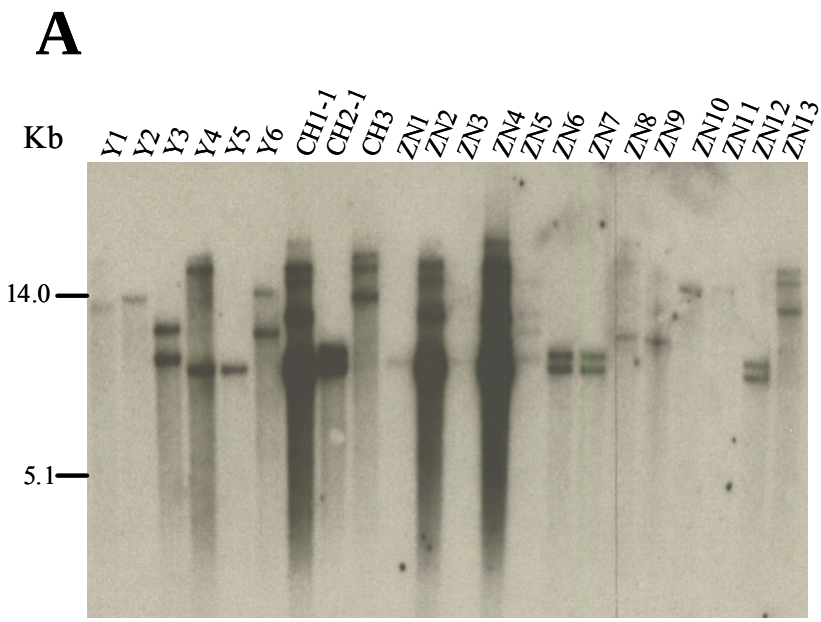

FIG. 5. 3. An example of the Southern and northern blots derived from extracts of the Rio Red grapefruit transformed with an untranslatable coat protein gene from *Citrus tristeza virus* (CTV). A. Southern blot from genomic DNA extracted from the *uncp* Rio Red grapefruit plants. B. Northern blot from total plant RNA extracted from the *uncp* Rio Red grapefruit plants. Genomic DNA (A) or total plant RNA (B) were restriction-enzyme digested (A-*Eco*RI, B-*Hind*III) and electrophoretically separated , then transferred to nylon membranes and probed (A-1.9 kb part of the *uid*A, B-0.7 kb part of the *uncp*). On B, the 1.6 kb RNA species size (right) is estimated from the migration of an RNA marker (left). Y1, Y2, Y3, Y4, Y5, Y6, ZN1, ZN2, ZN3, ZN4, ZN5, ZN6, ZN7, ZN8, ZN9, ZN10, ZN11, ZN12, ZN13,CH1-1, CH2-1, CH2-2, CH3; extracts from *uncp* lines, WT; extracts from a non-transformed Rio Red grapefruit (included on the blot in A but not shown in the photograph).

Tissue was also taken from the inoculated rootstocks or graft tissue in place on the test plants, where available, was also tested and in all cases was confirmed positive for CTV. Overall, relative virus titer levels were between individual plants, both non-transgenic and transgenic lines, and varied over time. By time point three, however, there were more trees meeting the threshold level at which a sample is considered CTV positive in this assay system (twice the mean optical density for the virus-free Rio Red grapefruit). The mean differences in optical density per sample were compared to the positive control on each plate as a percentage and are represented as bar charts in Figs. 5.4., 5.5., and 5.6. Patterns of relative virus titer over time for each pot could be classified roughly into two types. In some cases as in ZN29, ZN14, Y2 and Y7 (Fig. 5.4.), non-transgenic scions had detectible CTV at all three data points. ZN29 replicates had very similar virus levels at each time point. Line ZN14, a transformational escape, (Table 5.1), had consistently higher levels compared to the non-transgenic, whereas the other replicate had lower or non-transgenic comparable optical densities. For Y2, one plant had high optical densities in tests 1 and 3, whereas the duplicate plant had very low readings (ELISA 1 and 2). Line Y7 duplicate scions reading were always lower than that of the non-transgenic, with relative virus titer decreasing from the first test point to the last for all scions, even though this line appears to be a transformational escape (Table 5.1). In another group non-transgenic scion optical density readings were low in ELISA 1, but high by ELISA 3 (Fig. 5.5.). One ZN23 scion gave a higher reading than

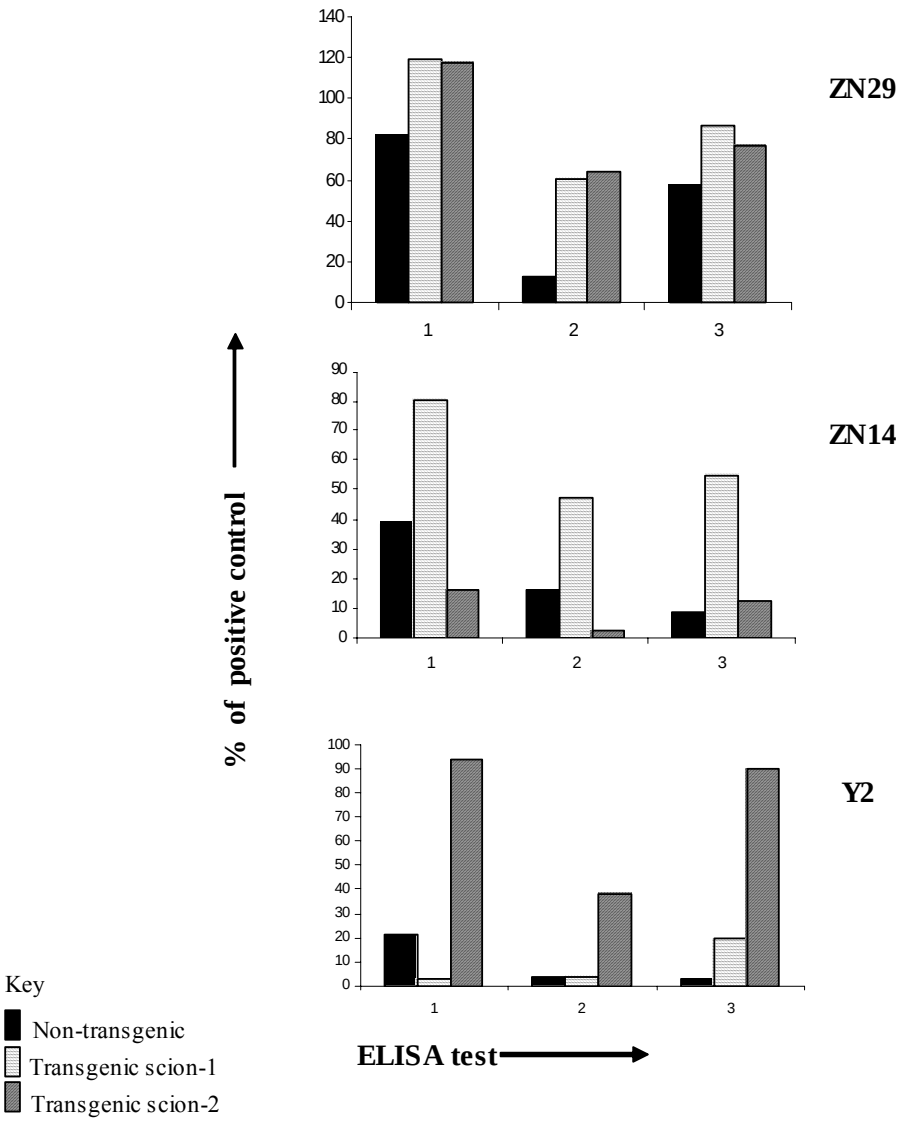

FIG. 5.4. Infection of four transgenic lines with *Citrus tristeza virus* over time assessed by ELISA. ZN29, ZN14, Y2 and Y7; untranslatable CTV coat protein gene lines, % positive control; all readings are the means of the optical density readings (405 nm) in duplicated wells compared to the positive plate control. All standard errors are 0.05 units or less.

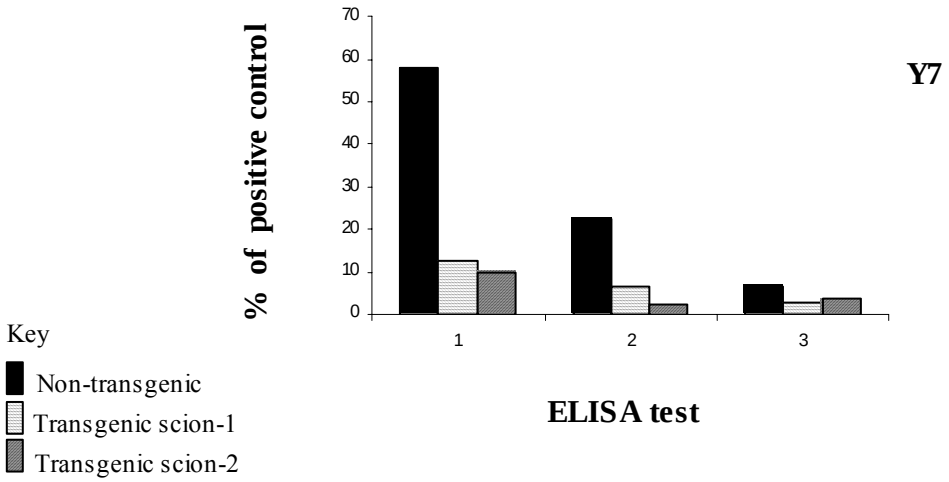

FIG. 5.4. Continued

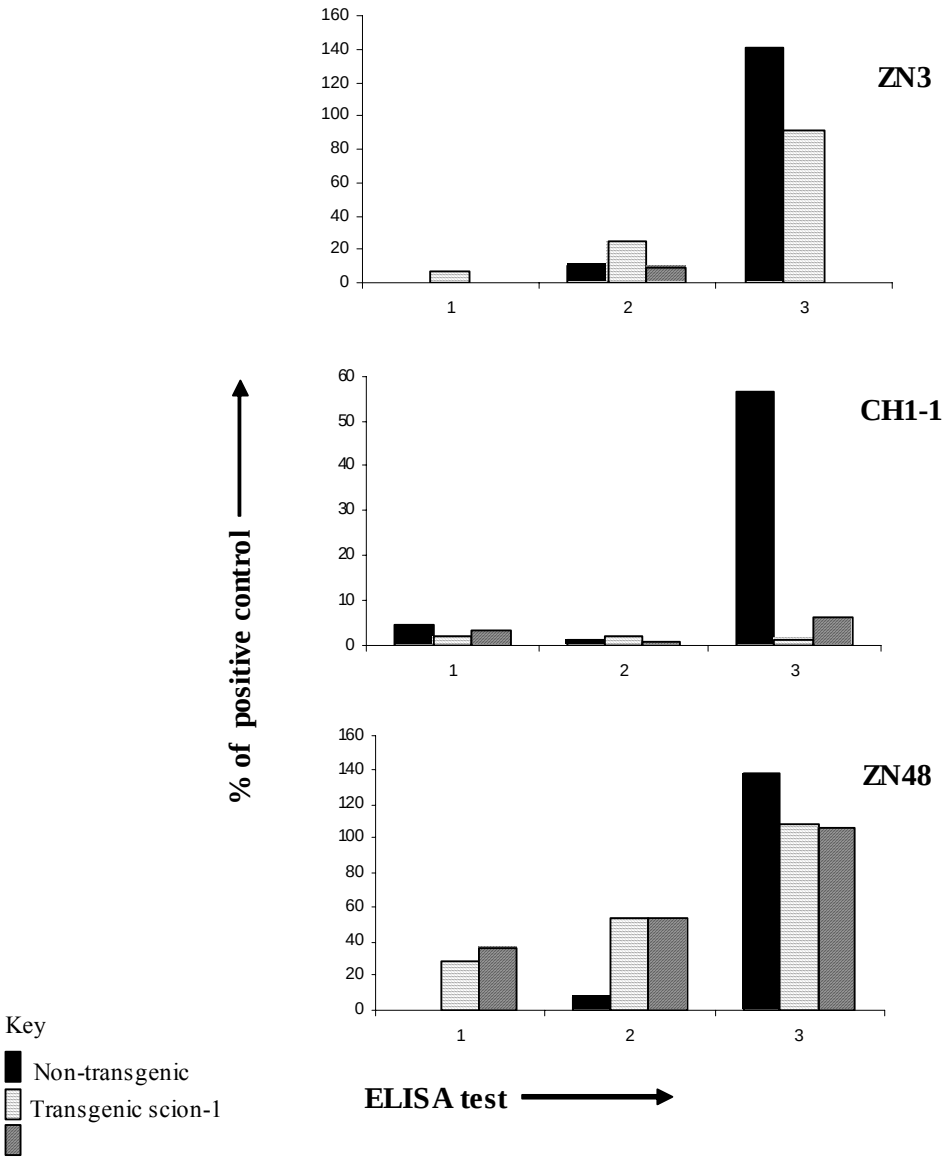

FIG. 5.5. Infection of six transgenic lines with *Citrus tristeza virus* over time assessed by ELISA. ZN3, ZN15, CH1-1, CH2-2, ZN48, ZN23; untranslatable CTV coat protein gene lines, % positive control; all readings are the means of the optical density readings 405 nm) in duplicated wells compared to the positive plate control. All standard errors are 0.05 units or less.

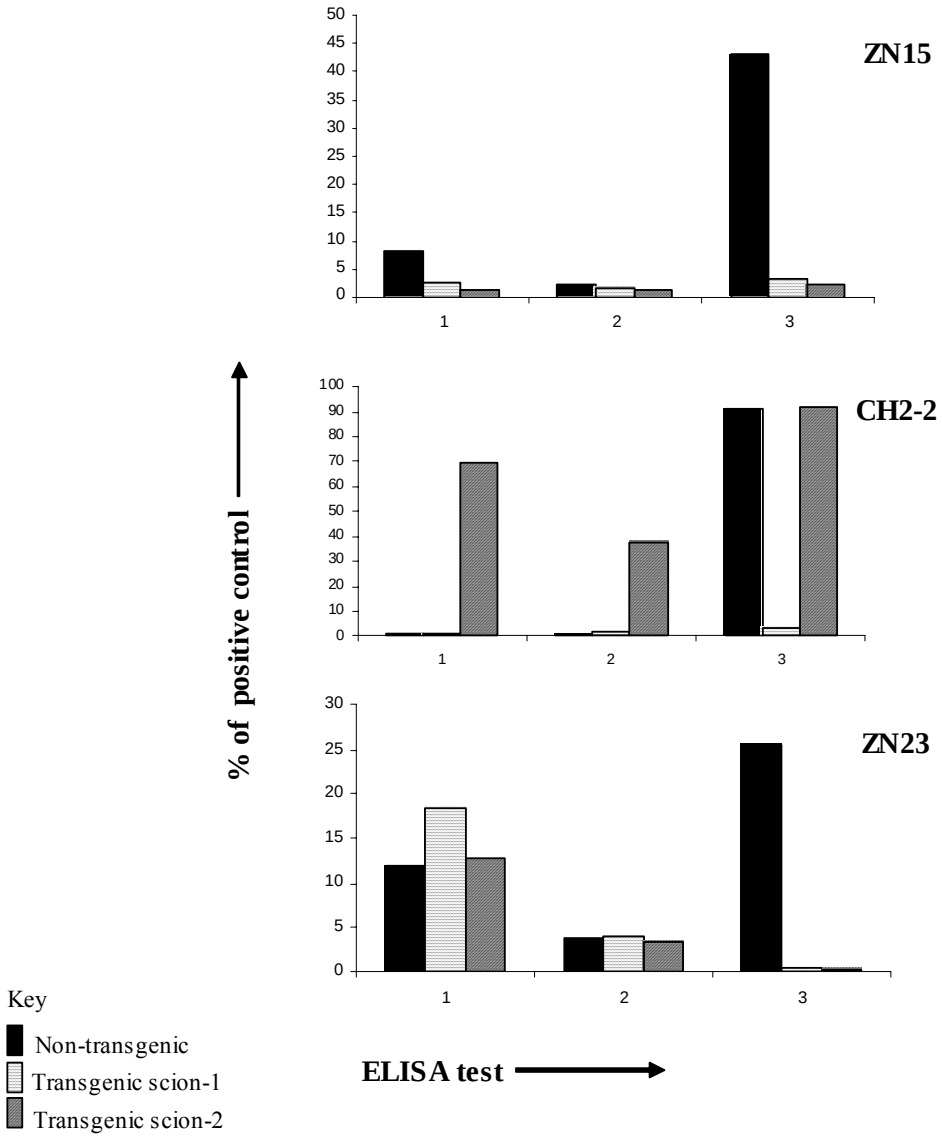

FIG.5.5. Continued

the non-transgenic in ELISA 1 but by ELISA 3 both ZN23 duplicates were far lower than the non-transgenic. With line ZN48, the non-transgenic reading increased over time, whereas the transgenic scions were initially higher than non-transgenic then by ELISA 3, were lower. ZN15, CH1-1 duplicated scions had very low relative virus levels compared to their respective non-transgenic controls, respectively, by ELISA 3. With ZN3 and CH2-2 this same progression was seen with one transgenic scion only. On a similar theme, a third category (represented by EM1, ZN8, ZN35) have super infection of CTV in one or duplicated plants within each transgenic line in ELISA 1, whereas by ELISA 3, the non-transgenic readings are higher, with the transgenic scion readings having decreased (Fig. 5.6.).

In the statistical analyses comparing the all transgenic plants to all the non-transgenic plants over the three assessments by regression analysis there was significant interaction ($p=\geq 0.001$) between the ELISA optical densities and the type of scion (non-transgenic or transgenic). This suggests that overall there are significant decreases in virus titer between the transgenic plants compared to the non-transgenic.

5.5 DISCUSSION

PDR has been documented as providing virus disease control in many plant-virus systems (Baulcombe, 1996; Beachy 1997; Ingelbrecht *et al.*, 1999). Most of these reports have been on herbaceous plants with relatively few PDR reports from woody fruit trees (Ravelonandro *et al.*, 2000). A commercial grapefruit variety, Rio Red was used to develop PDR against CTV. Fifty-two transgenic lines carrying an untranslatable

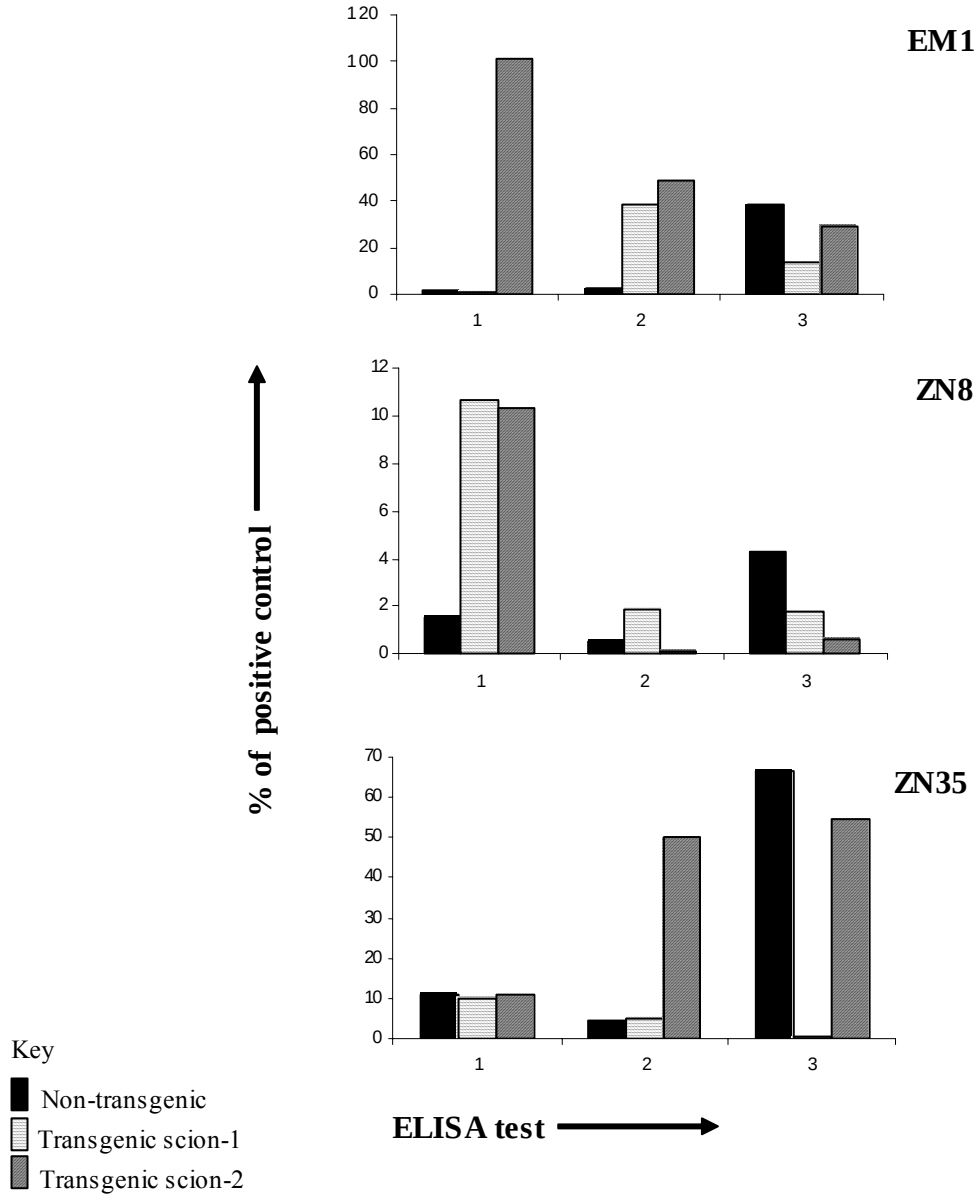

FIG. 5.6. Infection of three transgenic lines with *Citrus tristeza virus* over time assessed by ELISA. EM1, ZN8 and ZN35; untranslatable CTV coat protein gene lines, % positive control; all readings are the means of the optical density readings (405 nm) in duplicated wells compared to the positive plate control. All standard errors are 0.05 units or less.

coat protein gene derived from the severe seedling yellows CTV isolate from California (SY568) were inoculated with a Texas CTV isolate H18, known to cause a moderate and slow seedling yellows symptoms in grapefruit plants, and sweet orange stem pitting. Some individual transgenic plants showed protection against CTV, characterized by a delay in CTV accumulation, or a decrease in CTV accumulation over time, and overall the resistance seen under these testing conditions was statistically improved upon the non-transgenic Rio Red grapefruit plants. Previous preliminary tests on a restricted number of the *uncp* Rio Red lines using different CTV challenge isolates in Texas and South Africa provided similar conclusions (Herron *et al*., 2002).

The lack of initial virus accumulation in the scions at a time when the virus could be detected in sweet orange could be attributed to two main factors, a species effect or a temperature effect. CTV may not move as fast in grapefruit, compared to sweet orange. Some workers have reported that grapefruit does not tend to decline in areas of total sweet orange CTV decline (Bar-Joseph *et al.*, 1989). Pigmented grapefruit varieties present problems with CTV cross protection due in part to the slow distribution of protecting CTV isolates throughout the plant (Broadbent *et al.,* 1995). Red grapefruit are also more sensitive to stem pitting symptoms (Marais and Breytenbach, 1996), and also have been shown to influence the strain composition of CTV isolates (van Vuuren and van der Vyver, 2000).

The CTV inoculum was tested before and during the tests and CTV could be detected in sweet orange 4 weeks after graft inoculation with H18 tissue. Additionally, all the rootstock and inoculum tissues *in situ* which were tested harbored the virus, thus

CTV in sweet orange and the inocula could be detected whilst there was little or no detection in Rio Red grapefruit. Temperature sensitivity for the movement and detection of CTV is a known phenomenon (Roistacher *et al.*, 1974; Mathews *et al.*, 1997). Ambient temperatures above 30˚C are considered curative for CTV in citrus (Roistacher *et al*., 1974; Mathews *et al*., 1997). The temperature effect can be ruled out of these tests since the experiments were conducted during the coolest period of the year (below 30˚C throughout testing period).

PDR in Rio Red grapefruit may be developmentally regulated which may account for the initial CTV 'super infection' by some transgenic scions giving way to a drop in virus titer within three months. This effect could be variable depending upon the transgene and the position of the transgene in the genome. The testing tissue type (young or mature leaves) may be important for observing these effects. In this study, samples were taken from the same type of tissue (near mature leaves), and plants were not pruned during the test. Detection of CTV can also be misleading if not performed over a time period since CTV has been reported to be passively transported through phloem tissues in CTV resistant genotypes (Mestre *et al*., 1997). CTV was also detected by RT-PCR from CTV CP transformed plants graft inoculated with CTV (before inocula were removed) which subsequently were defined as resistant (Domínguez *et al.*, 2000).

The untranslatable CTV CP gene inserted into the Rio Red genome could be predicted to confer resistance to CTV though a post transcriptional gene silencing (PTGS) mechanism. The untranslatable transgene could transcribe RNA corresponding to the viral sgRNA for the non-transgenic CP, and provide transcripts which would

interfere with CP translation upon CTV challenge, or be the targets for specific degradation by PTGS, and thus eventually the lifecycle of the virus will be broken in the plant. CTV is known to possess two proteins which have been demonstrated in heterologous systems to be PTGS suppressors; the p20 and p23 proteins (Reed *et al.*, 2003; Lu *et al*., 2003). The p23 gene has an RNA-binding domain which may be a general requirement for a class of PTGS suppressors (Lichner *et al*., 2003). Very rapidly upon CTV challenge, such suppressor molecules might interact with components of the cell's machinery which are required to accumulate the untranslatable transcript. This action might be similar to the process whereby silenced marker transgenes can be active in expression after virus inoculation (Voinnet, 2001).

Two groups have evaluated transgenic citrus plants for resistance to CTV. Domínguez *et al.* (2000), used a translatable CTV CP transgene in Mexican lime and found approximately up to a third of transformants did not show CTV symptoms when graft or aphid inoculated with different isolates of CTV. The majority of transformed plants showed a significant delay in virus accumulation and CTV symptom onset. Febres *et al.* (2003), challenge tested Duncan grapefruit transformed with translatable CTV CP genes and an RdRp gene. All plants were susceptible to CTV by ELISA, with a number of individual plants having lower titers of CTV compared to non-transformed controls.

In this study we have identified plants which gave low CTV titers throughout the experimentation, showed a temporal delay in virus titer or became CTV infected and then showed a reduced virus titer over time. In citrus growing regions where severe

CTV isolates are indigenous, cross protection is the only method so far which has offered a chance to continue citrus production by delaying the onset of CTV symptoms (Costa and Müller, 1980; von Broembsen and Lee, 1988; van Vuuren *et al.*, 1993; van Vuuren and da Graça, 2000). PDR now offers an alternative strategy to meet the same ends, only that PDR should be longer lasting, as cross-protection eventually is overcome.

CHAPTER VI

A NON-GRAFT TRANSMISSIBLE RNA ENTITY IN A *CITRUS TRISTEZA VIRUS*-INFECTED MEXICAN LIME PLANT WITH SIMILARITIES TO MARAFIVIRUSES

6.1 SUMMARY

A cDNA with high similarity to part of the *Oat blue dwarf virus* (OBDV; family *Tymoviridae*, genus, *Marafivirus*) genome was obtained from RT-PCR amplification of dsRNA extracts from a *Citrus tristeza virus*-infected plant. Northern analyses using total RNA extracted from the source plant and RNA extracted from virus purification fractions revealed an RNA species of *ca*. 7.5 kb, when probed with the OBDV-like cDNA. Antibodies raised against OBDV coat protein (CP) reacted weakly with protein extracts from the source plant and produce a specific band of approximately 28-kDa in immunoblots. *Grapevine fleck virus* (GFkV; family *Tymoviridae,* genus*, Maculavirus*) has many features common to marafiviruses including a 7.5 kb genomic RNA. GFkV CP antibodies do not react with protein extracts from the source plant by ELISA. The entity appears to be non-graft transmissible from citrus, and a host range study using herbaceous plants concluded the entity could not be mechanically transmitted.

6.2 INTRODUCTION

Citrus is host to approximately 20 graft-transmissible disorders of which approximately less then half have been formally classified as being caused by viruses or virus-like, but so far there have been no reports of any tymo-like viruses (Roistacher, 1991). The family *Tymoviridae* consists of three genera; *Tymovirus*, *Marafivirus* and *Maculavirus* (Martelli *et al*., 2002). Tymovirus family members compose a group of isometric viruses with relatively small, positive-sense, single-stranded RNA genomes. The type species for the tymoviruses is *Turnip yellow mosaic virus* (TYMV; Morch *et al.,* 1988), for the marafiviruses, *Maize rayado-fino virus* (MRFV; Gámez, 1969; Hammond and Ramirez, 2001), and for the maculaviruses is *Grapevine fleck virus* (GFkV; Boscia *et al*., 1991). Until recently, marafiviruses and tymoviruses were separated mainly by biological differences. Currently characterized marafiviruses are MRFV, *Bermuda grass etched-line virus* (BELV) and *Oat blue dwarf virus* (OBDV), with *Poinsettia mosaic virus* (PnMV; Bradel *et al*., 2000) being a candidate species to be assigned to the genus. MRFV and BELV have narrow host ranges confined to the Gramineae (Brunt *et al*., 1996); PnMV is not known to have a monocot host whilst OBDV has a wide host range which includes both monocotyledonous and dicotyledonous plants (Westdal, 1968; Brunt *et al.,* 1996). OBDV and many of the characterized marafiviruses are transmitted by leafhoppers. OBDV and BELV, in particular, are transmitted by the aster leafhopper, *Macrosteles quadrilineatus* Forbes (Homoptera: Cicadellidae), which is known to be polyphytophagous (Banttari and Zeyen, 1970; Lockhart *et al*., 1985). All three viruses replicate in their leafhopper

vectors and are not mechanically transmitted (Banttari and Zeyen, 1970; Brunt *et al.*, 1996). Tymoviruses are mechanically transmitted in a nonpropagative manner by their natural beetle vectors (Gibbs, 1994). GFkV was recently molecularly characterized (Sabanadzovic *et al*., 2001), and has no known insect vector but is graft transmissible to grapevine plants and has several herbaceous hosts (Martelli, 1993).

A *Citrus tristeza virus* (CTV) infected plant was being analyzed for CTV sequences and symptomology. This plant was graft-inoculated with Texas CTV isolate H33 in 1984 (P. L. W. Timmer, pers. comm.) and has been housed in the same greenhouse since then. A sequence was identified which was marafivirus-like and since no known marafivirus had been documented as infecting citrus, further tests were performed to identify if the sequence represented a possible new virus or a new host range for an already characterized virus.

6.3 MATERIALS AND METHODS

6.3.1 Virus purification

Freshly collected young and mature stem bark (18.0 g) and leaf midribs (13.2 g) were collected from a Mexican lime (*Citrus aurantifolia* Christm [Swingle]) plant known to be infected with CTV isolate H33. An OBDV purification method was used (Edwards *et al.,* 1997) since the entity had sequence identity to the marafivirus, and was carried out as described (D'Arcy *et al.,* 1983). After the final centrifugation on a 10-40% sucrose gradient, 22 500 µl fractions were drawn off with a syringe from the top of the gradient.

6.3.2 RNA analyses

Total RNA was extracted from the virus purification rate zonal fractions (using 100 µl for slot blot and 50 µl for the size estimations) by a double phenol: chloroform extraction followed by aqueous layer ethanol precipitation. Additionally, total RNA was extracted from citrus leaf tissue using a double phenol:chloroform extraction followed by the nucleic acids in the aqueous layer being ethanol precipitated, and then being subjected to a lithium chloride treatment. Final pellets from all sources were washed with 70% ethanol, resuspended in nuclease-free ultra pure water, UV spectrophotometrically quantified, then electrophoretically qualified and stored at -80˚C until use. Formamide and heat denatured RNA (10 µg) was separated by electrophoresis in formaldehyde agarose (1.6%), using a 0.24-9.5 kb RNA ladder (Gibco BRL; 3 µg) as the size marker. For RNA extracted from the viral purification fractions and used for size analyses, the final resuspension volume (13 µl) was treated in a similar manner. After electrophoresis the gels were incubated with 2X SSC for 30 min prior to downward capillary transfer of nucleic acids to Hybond-N+ nylon membranes (Amersham, Little Chalfont, Bucks., UK) using 20X SSC. Hybridization was performed at 65°C according to Church and Gilbert (1984) with a randomly primed cDNA labeled with ^{32}P dCTP probe (Gibco BRL Life Technologies, MD). For slot blot analyses of the RNA from the rate zonal viral purification fractions, the entire sample was denatured using sodium hydroxide, and then vacuum blotted directly onto a nylon membrane.

6.3.3 Indexing on herbaceous plants

Inoculum consisted of citrus source leaf tissue titurated in a pre-chilled mortar and pestle using sterile 0.05 M potassium phosphate buffer, pH 7, at 4˚C. A tissue: buffer ratio of 1:10 (w/v) was used. The resultant sap was then strained through two layers of sterile cheesecloth. Primary leaves near full expansion were dusted with carborundum and then gently rubbed with a gloved finger dipped into the inoculum. Test plants were then rinsed gently with tap water and shaded for 12 h. Plant species tested of cotton (*Gossypium herbaceum*), *Nicotiana benthamiana,* tobacco (*N. tabacum*), *Chenopodium quinoa*, *C. amaranticolor*, maize, cowpea (Vigna), melon, and spinach were raised from seed in a cooled greenhouse and kept pest and disease free. Four seedlings per species were used, with three plants being inoculated and one being inoculated with buffer only. Plants were visually assessed for foliar symptoms daily for 4 weeks after inoculation.

6.3.4 Indexing to grapevine plants

Buds from the Mexican lime (*Citrus aurantifolia* [Christm.] Swingle) source tree were grafted to two virus-free St. George grapevine (*Vitis rupestris* L.) seedlings. This cultivar is a susceptible indicator for GFkV. One St. George grapevine seedling was left uninoculated as a control. Plants were kept under cooled greenhouse conditions (not exceeding 30˚C). The indicator plant foliage growth and stem after grafting was observed for symptoms.

6.3.5 Immunoblots

Rate zonal fractions (10 µl) or titurated leaf tissue (0.5 g) were combined with 2X extraction buffer (Læmmli, 1970), at equal weight per volume. Samples were then placed at 100˚C for 5 min, and 20 µl were separated by 15 or 16% SDS-PAGE with 4.5% stacking gels. One gel was stained with Coomassie brilliant blue R-250 (Fisher Scientific, BP 101-25), whilst proteins from another gel were electrotransferred to nitrocellulose membranes. Each nitrocellulose membrane was blocked with 3% gelatin in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl). OBDV CP polyclonal antibodies (M. C. Edwards, USDA, North Dakota), at 1:10,000 dilution, were used to probe the blots 1% gelatin/TBS. Secondary antibody was goat anti-rabbit IgG-alkaline phosphatase (Sigma A4187) used at 1:2000 in 1% gelatin/TBS. Specific proteins were visualized using bromochloroindolyl-nitro blue tetrazolium substrate in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM magnesium chloride). A broad range pre-stained standard protein marker (BioRad 161-0314), and similarly prepared samples from a virus-free Mexican lime virus fractions or extracted sap were used as controls.

6.3.5 ELISA

A GFkV indirect double antibody sandwich indirect ELISA was used on extracts from all the citrus plants which were grafted from the source tree with the unknown entity (Boscia *et al.*, 1995). CTV ELISA had previously been performed on all of these plants to confirm transmission of CTV. Antibodies were supplied in a kit form

(Agritest, Valenzano, Italy) with positive and negative grapevine controls. Uninoculated and source tree graft inoculated St. George grapevine tissues were also included as samples on the microtiter plates, as well as buffer-only controls. The hydrolyzed enzyme substrate extinction values were read at A_{405} nm.

6.4 RESULTS

6.4.1 BLASTN search and alignments

During a 'shotgun' method sequencing project using dsRNA extracted from a Mexican lime plant infected with Texas CTV isolate H33, a sequence was found with similarity to tymoviruses and marafiviruses, with greatest identity (68%) to OBDV. This clone, hereafter referred to as OL-1 corresponded to nt 2818 to 3975 of OBDV genome using BLASTN (Altschul *et al.*, 1997). The alignment with the OBDV genome indicated OL-1 was not co-linear with the OBDV sequence, however; bases 4 to 216 of OL-1 are similar to OBDV at bases 3030 to 2818, while bases 208 to 1289 of OL-1 are similar to nt 3975 to 2902 of the OBDV genome (Fig. 6.1.).

6.4.2 RNA analyses of the viral preparation fractions and H33 RNA

The RNA blot from the electrophoresed RNA extracted from the rate zonal sucrose gradient fractions (Fig. 6.2.A.) showed strong hybridization with fractions 14 and 17 to ^{32}P labeled OL-1, with slight hybridization to fraction 11 (note fraction 12 was not loaded out of error), as observed in Fig. 6.2.A. The lower fraction, 17, gave a very

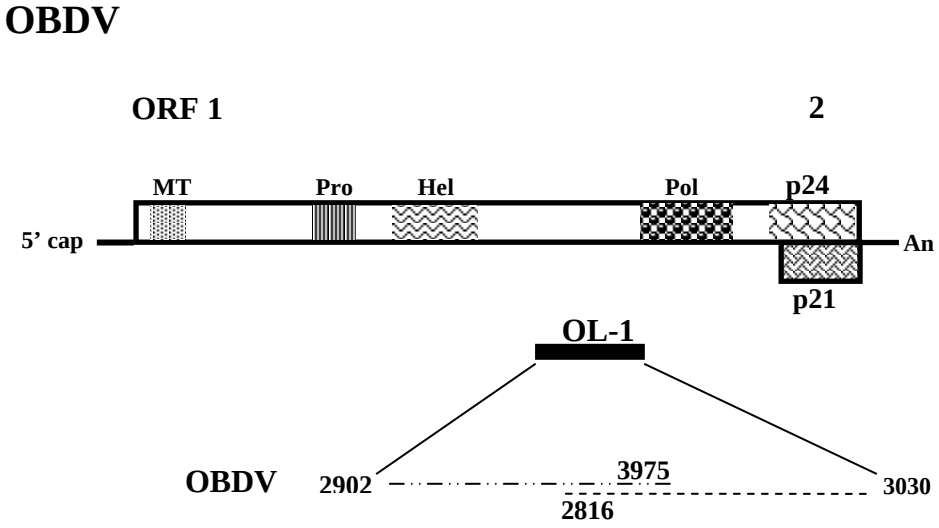

FIG. 6.1. The genome structure of *Oat blue dwarf virus* (OBDV) and the approximate position of the cDNA, OL-1. OBDV; genome structure and annotations, ORF 1 includes MT; methyltransferase domain, Pro; putative protease, Hel; helicase, Pol; polymerase; ORF 2 has two overlapping reading frames, p24; putative 24-kDa protein gene, p21; predicted 21-kDa protein gene. Below, OL-1, approximate position of sequence OL-1 when aligned with the OBDV genome; the dashed lines represents the non co-linearity of the sequence; numbers represent how the two parts of OBDV match up to OL-1.

distinct band at *ca.* 7.5 kb, below which dark general hybridization was observed. The same *ca.* 7.5 kb band was found in H33 source plant RNA extracts when hybridized in a similar manner (see positive control on the gel in Fig. 6.2.A. as an example). The slot blot showed hybridization reactions with the OL-1 probe for all the RNA extracted from the sucrose rate zonal gradient fractions (Fig. 6.2.B.). The reactions for the upper ten sucrose gradient fractions were weak and the strongest reactions were observed to be with fractions 11, 12, 14, 15 and 17. Two more Northern blots were done using RNA from sucrose rate zonal gradient fractions 11, 12, 14, 16 and 17 with electrophoretic separation on agarose gels to confirm which fractions strongly hybridized to OL-1. Hybridizations to fractions 12, 14 and 17 were the strongest (data not shown). The northern blot of RNA extracted from citrus plants which had been grafted from H33 source plant over a period of 5 years (Fig. 6.3.) showed hybridization to the positive control only, therefore the entity is not graft-transmissible.

Immunoblots of proteins extracted from the sucrose rate zonal gradient fractions using OBDV antibodies (Fig. 6.4.A.) showed a very weak cross reaction with a protein of *ca.* 28-kDa, which is dissimilar in size to that of OBDV coat proteins. This suggests a weak similarity between the coat protein of this unidentified virus and that of OBDV. The immunoblot of source plant graft-inoculated plant protein extracts using OBDV antibodies also had a very weak association at *ca.* 28-kDa (Fig. 6.4.B.). The ELISA tests revealed no cross reaction to GFkV antibodies (data not shown) with any of the sap

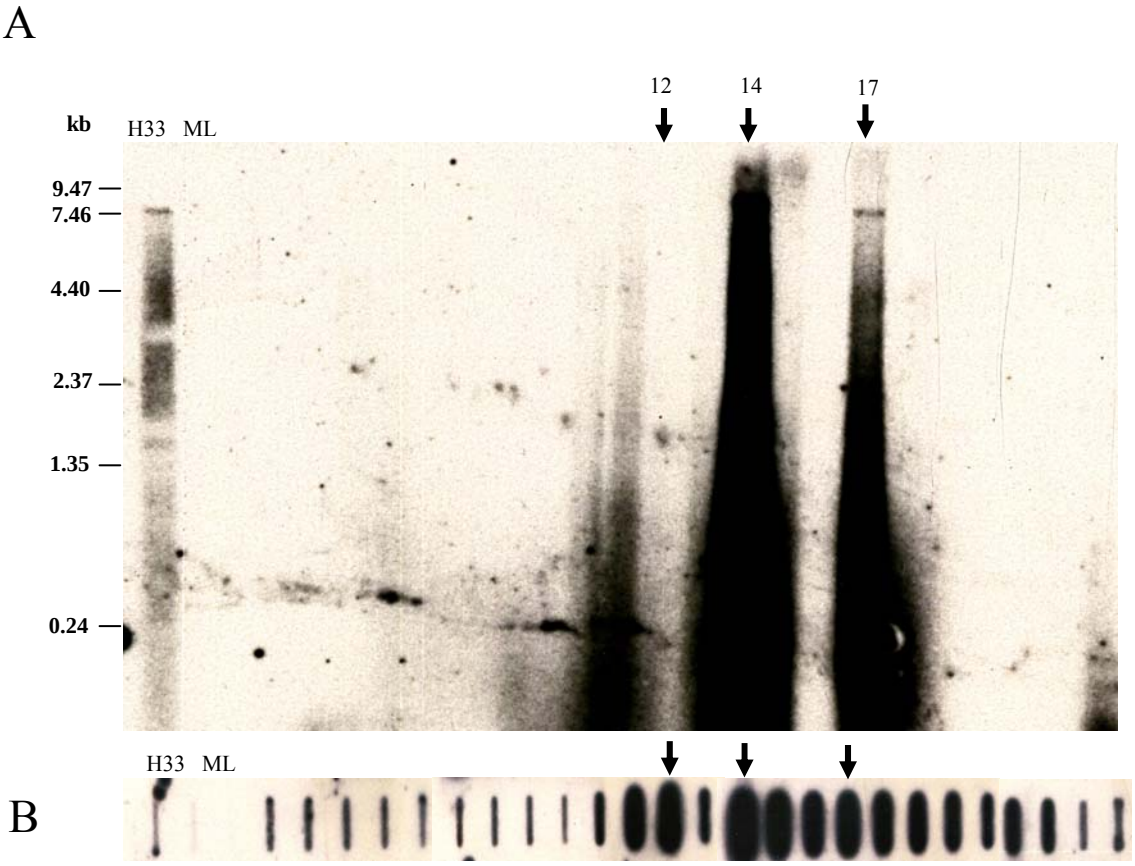

FIG. 6.2. Northern hybridizations from the sucrose rate zonal fractions. A. RNA separated by electrophoresis. B. RNA slot-blot, with slots aligned with A for comparison. In both blots, the fractions were loaded after the controls (H33 and ML), left to right on the figure, starting with fraction 1 (first fraction taken from the top of the tube), ending with 25 (last fraction from the bottom of the tube), with only fractions 12, 14 and 17 marked. H33; plant total RNA extracts from H33, ML; plant total RNA extracts from a virus-free Mexican lime plant. Note in A fraction 12 was not loaded.

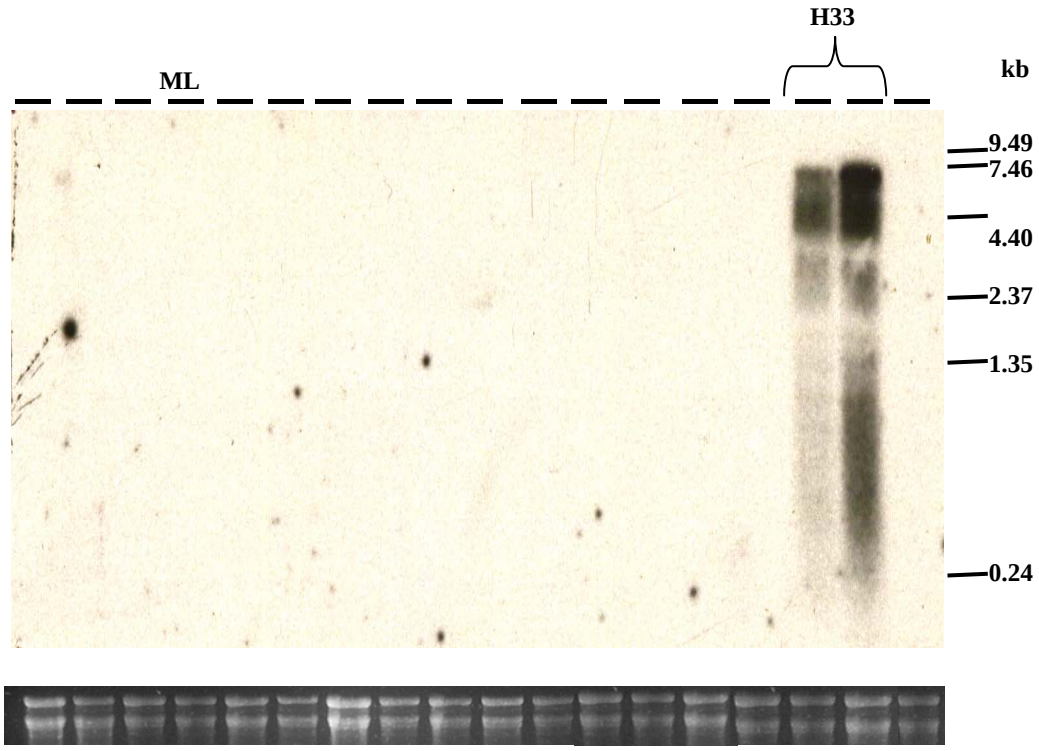

FIG. 6.3. Northern blot of total plant RNA extracts using a ^{32}P labeled OL-1 probe. Above, 15 total RNA test samples were tested (wells not marked). H33; total RNA extracts from H33, ML; total RNA extracts from a virus-free Mexican lime plant. Below the blot is a sample of cellular RNAs stained with ethidium bromide and visualized over UV illumination from the gel before blotting to show the RNA loading.

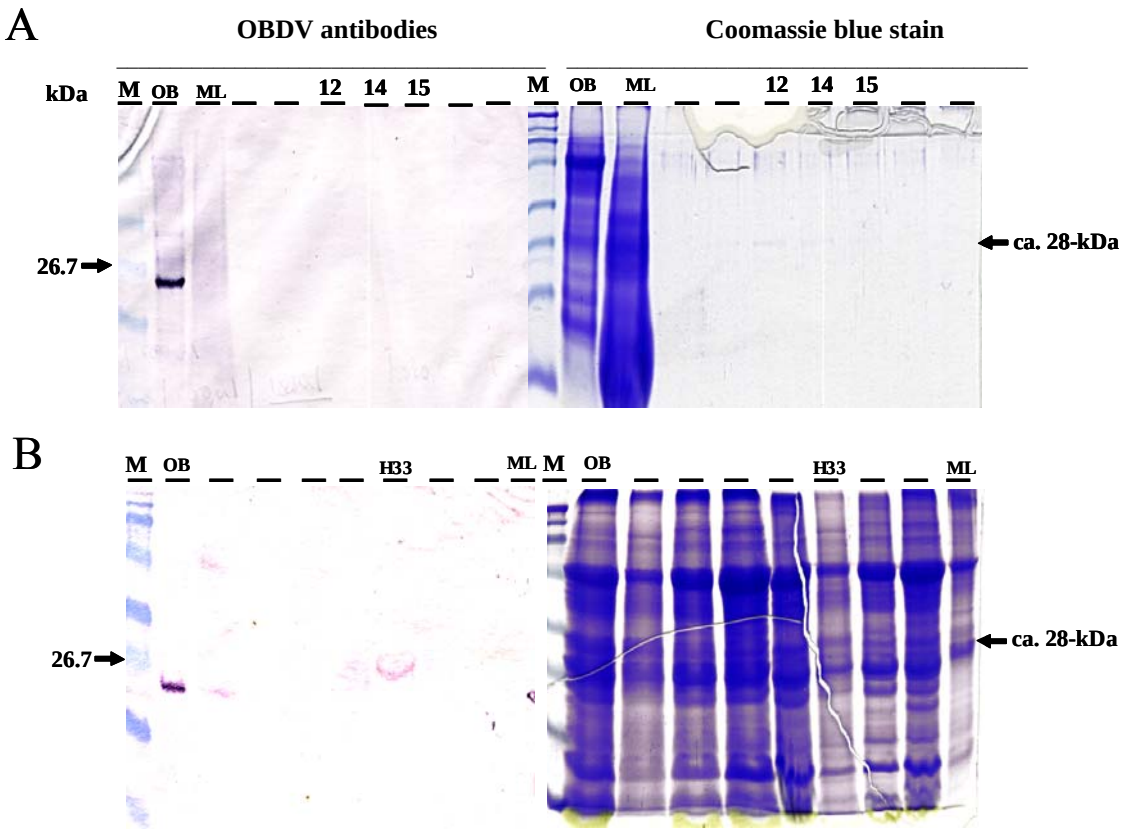

FIG. 6.4. Analyses of total proteins extracted from the sucrose rate zonal fractions and *Citrus tristeza virus* isolate H33-grafted plants. A. Sucrose rate zonal fractions B. Coomassie blue stained gels (right) and immunoblots (left) using OBDV antibodies, 12, 14 and 15; sucrose rate zonal gradient fractions for which specific reactions were observed, M; broad spectrum protein marker (only 26.7-kDa is indicated). Extracts from H33 graft-inoculated plants, OB; total proteins extracted from OBDV infected oat, ML; total proteins extracted from a virus-free Mexican lime plant, H33; total proteins extracted from H33 (the only plant sample to give a specific reaction at *ca*. 28-kDa).

extracted from the citrus plants grafted plants, or the virus-free grapevine control supplied with the kit. The GFkV positive grapevine control supplied with the kit did give a good reaction, indicating the tests were valid.

None of the species mechanically inoculated with sap from the source plant were observed with any foliar symptoms different from the mock inoculated controls. Graft inoculation of source tissue to St. George grapevine did not reveal any foliar symptoms from the source plant, H33 graft- inoculated or non-inoculated indicator St. George grapevine characteristic of Grapevine fleck disease; that is, peripheral leaf vein clearing and/or wrinkling of the leaves at least six months after infection. Essentially the plants looked the same as the non-inoculated St. George grapevine seedling.

6.5 DISCUSSION

The OL-1 sequence appears to be a rearrangement of part of the OBDV genome which could have resulted from a cloning artifact, a defective RNA of possible viral origin or a sequence related to OBDV by mutation. The wide-spread positive reaction with the OL-1 probe to RNA extracted from sucrose rate zonal gradient fractions using an OBDV purification procedure suggests that the entity was disrupted by the procedure. The *ca*. 7.5 kb RNA hybridization band in one fraction (17) was also identical in size to that obtained in northern blots of total RNA samples extracted from H33. This indicates the entity is most likely an ssRNA virus. Most of the viral RNA was degraded so that,

rather than separating into a narrow band in the sucrose gradient, it was dispersed throughout. Analyses of the viral purification procedure products suggest that the majority of putative viral particles did not remain intact during the extraction and purification process.

GFkV was purified from 100-150 g of young root tissue or leaf main veins and petioles and an estimated 0.2 mg of virus was recovered (Boulila *et al.*, 1990), with lower virus titers obtained from the leaf tissues. Thus an additional problem might be the low titer of virus in certain citrus tissues, if for instance the virus is phloem limited. GFkV was purified by isopycnic density gradient centrifugation of grapevine extracts containing GFkV (Sabanadzovic *et al*., 2001), and an upper component containing subgenomic RNA and a lower component containing the genomic RNA were reported. Although no opalescent bands were visualized after the purification procedure used in this study, the highest 'peaks' of hybridization to the unknown entity were in rate zonal fractions 14 and 17, with 17 being lower in the centrifugation tube and containing the putative gRNA of *ca.* 7.5 kb. Greater amounts of infected tissue from the single Mexican lime plant infected could be obtained by propagating rooted cuttings so that virus purification conditions and different tissue types can be tested for viral yields.

The weak reaction between the OBDV antibody and the proteins extracted from sucrose rate zonal gradient fractions which gave strong hybridization to OL-1 in the Northern hybridizations, suggests there is some weak protein antigenic similarity between OBDV and the unknown entity. The OBDV genome is approximately 6.5 kb in length and three capsid proteins have been identified - the most abundant being a protein

of 22.2-kDa and the other two proteins and are of predicted molecular weights of 25.5-kDa and 26.6-kDa, respectively (Edwards *et al.*, 1997). The OBDV 26.6-kDa protein is very clear on the positive OBDV samples in Fig. 6.4. OBDV cannot be transmitted by mechanical means, only by *M. quadillineatus* leaf hoppers within which the virus replicates. The OBDV capsids are similar in size to those reported for the capsid proteins for two other marafiviruses; MRFV and BELV, and both cross-reacted to OBDV antibodies in immunodiffusion tests (Lockhart *et al*., 1985; Izadpanah *et al.*, 2002).

The data from this study indicate a single protein of *ca.* 28-kDa in translated form associates with the unknown RNA entity, which does not fit the typical marafivirus profile of coat proteins ranging between 22-26.5-kDa. Northern blots suggest the entity genome to be approximately 7.5 kb or larger (above 7.4 kb was more difficult to estimate with the RNA marker used) which is approximately the same size as that reported for BELV, but is distinctly larger than any other members of the genus. In these characteristics the putative virus more closely resembles GFkV. GFkV has a single-stranded, positive-sense RNA genome *ca.* 7.4 kb size, and a coat protein consisting of a single capsid with a molecular mass calculated at approximately 28-kDa. Although GFkV is closely related to marafiviruses and tymoviruses, it has distinct sequence dissimilarity and properties so that it has been delimited into the genus, *Maculovirus* (Mayo, 2002; Sabanadzovic *et al*., 2001).

GFkV is reported to be graft transmissible but not transmissible by mechanical means, and has no known insect vectors. The aster leafhopper is indigenous to Texas (Beirne, 1952; Kwon, 1988); therefore hoppers could potentially have transmitted the entity to the citrus plant. In this mode of transmission, the entity may be more similar to the marafiviruses. The entity caused no symptoms in graft-inoculated citrus, St. George grapevine, a range of herbaceous hosts, and graft-inoculated citrus did not react with OBDV antibodies. Together with the ELISA tests where GFkV antibodies did not react to the infected citrus plant extracts, this indicates the entity is not GFkV.

Members of the tymoviruses and marafiviruses contain the highly conserved 16 nt subgenomic RNA promoter or 'tymobox' sequence near the 3' end of the putative viral replicase sequence (Ding *et al*., 1990; Edwards *et al.,* 1997; Schirawski *et al*., 2000), whereas GFkV does not have this sequence. Primers designed to this sequence in marafiviruses (the 'marafibox', Izadpanah, *et al*., 2002) failed to produce an amplicon by RT-PCR from total RNA isolated from the infected plant (data not shown).

Marafiviruses differ from tymoviruses in that they have a distinct phloem tropism, are transmitted by leafhoppers, have a restricted host range within the Gramineae (except the candidate marafivirus, PnMV), and do not induce chloroplast vesiculation in infected cells. Marafiviruses also have two coat proteins compared to a single coat protein species noted for the tymoviruses, a genomic RNA which is polyadenylated rather than possessing the tymovirus 3' tRNA-like structure, and also lack an analogue for the tymovirus ORF 2 movement protein (Dreher *et al*., 2000; Edwards, 2000; Sabanadzovic *et al*., 2001).

No tymoviruses or marafiviruses have been reported in citrus before, and the effect of the new entity on citrus remains to be determined, although there has been observation of no difference in the severity of CTV symptoms in Mexican lime seedlings inoculated from the original plant. Citrus exocortis viroid and CTV doubly-infected citrus plants have been reported as significantly more stunted than citrus plants infected with either entity in the absence of the other (van Vuuren and da Graça, 2000). A close relative of CTV has been reported as the cause of a viral synergism (Rochow and Ross, 1955; Pruss *et al.*, 1997). *Sweet potato chlorotic stunt virus*, SPCSV (genus, *Crinivirus*, family *Closteroviridae*), and *Sweet potato feathery mottle virus*, SPFMV (genus, *Potyvirus,* family *Potyviridae*), act in synergy within sweet potato (*Ipomoea batata* L.). Only coinfection of SPFMV with SPCSV produces severe leaf symptoms and stunting, called sweet potato severe virus disease. SPCSV is thought to enhance the multiplication of SPFMV in tissues outside the phloem (Karyeiija *et al*., 2000). CTV may act as a helper virus to other virus species, *vice versa* or other interactions may occur.

The presence of an RNA entity in citrus with a genome of *ca.* 7.5 kb, whose proteins have a weak antigenic reaction to OBDV antibodies, and a nucleotide similarity of 68% between a cDNA deriving from plant dsRNA extracts and part of the OBDV genome, indicate a possible new entity has been identified in citrus. Further studies of this entity would involve the propagation of cuttings from the CTV H33 isolate plant in order to provide enough tissue for testing the parameters for extraction and purification (~100 g tissue per extraction). Electron microscope visualization of such purification

procedure fractions would also be used to visualize virus particles (if any); since these could not be confused with CTV if isometric in structure (CTV has filamentous virions). DsRNA extractions from such fractions would be used as a starting point to generate a cDNA library to obtain further sequence data of the entity. Electron microscopy would also be used to study the CTV H33 isolate plant tissues for mitochondrial and/or chloroplast vesicles, known to be induced in infected tissues of the currently identified marafiviruses and tymoviruses, but not maculaviruses.

Tymoviruses possess isometric particles with relatively small, simple genomes and propagate in their plant and vector hosts. Members of this family of viruses have been rapidly delimited through sequence information, and the group is emerging as a diverse group with members of economic agricultural importance. Further study of these viruses, host ranges and their interactions in plants and in their insect vectors is needed.

CHAPTER VII

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Citrus tristeza virus (CTV) can cause extreme economic losses due to death of trees on sour orange rootstocks or in the case of stem pitting of scions, reduction in fruit size regardless of scion type. Currently in the commercial citrus region of the Lower Rio Grande Valley of Texas (LRGV), incidence of CTV is low with natural spread possible via indigenous, relatively inefficient aphid vector species, *Aphis gossypii* Glover, *A. spiraecola* Patch, and *Toxoptera aurantii* Boyer de Fonsclombe. The efficient CTV vector, the brown citrus aphid (*T. citricida* Kirk., BrCA) is now established in Mexico and Florida. The expected entry of this aphid into Texas is imminent. Risk of economic losses due to CTV will become greater, not only in the LRGV but in the adjacent citrus growing states. For CTV management strategies to be effective, information must be known about the types and severity of CTV present in Texas, and long term research studies for developing viral resistance strategies must be put in place based upon these conclusions before the BrCA arrives.

This study was the first to characterize Texas CTV isolates collected from different locations in Texas. The study has supplied more basic information concerning one Texas CTV isolate (a complete genome), and identified a new citrus virus-like agent. The information from the characterization studies complemented the pathogen-

derived resistance (PDR) studies. A study on the BrCA attempted to determine some of the CTV transmission factors necessary for aphid transmission, with the aim to incorporate the findings into future PDR strategies. Rio Red grapefruit with an incorporated untranslatable CTV coat protein (CP) gene were evaluated for resistance to Texas CTV, and potential scions were identified which might provide adequate CTV protection in the future.

Biological and laboratory indexing of Texas CTV (CHAPTER II) so far have established that damaging tristeza types do exist. Molecular methods which could reliably predict CTV severity were compared with biological indexing to characterize CTV isolates collected from the Lower Rio Grande Valley (LRGV) and East Texas. Fifteen CTV sources were indexed on a host range of Mexican lime, sour orange, sweet orange on sour orange, Duncan grapefruit and Madam Vinous sweet orange indicator plants. Some CTV isolates were also indexed on Texas commercial cultivars Rio Red and Star Ruby grapefruit, Marrs and N-33 sweet orange. Severity ratings for CTV isolates were based upon biotype groups (0-10) or cumulative mean relative indices. Molecular characterization was carried out using poly- and monoclonal (MCA-13) antibodies, seven oligonucleotide probes and single-stranded conformational polymorphism (SSCP) all derived from the CTV major coat protein (CP) or gene. All CTV isolates produced vein clearing symptoms on inoculated Mexican lime plants. The

majority of the CTV sources tested were in biotype groups IX and X (caused decline of sweet orange on sour orange, seedling yellows on sour orange and grapefruit seedlings and stem pitting of grapefruit and/or sweet orange), and one isolate was in biotype I (mild). Statistical analyses of the biological versus the molecular data were performed to determine if there was any predictive value of the severity of CTV, using the molecular techniques.

The BrCA transmits CTV in a non-circulative, non-propagative, semipersistent manner, with as yet no identified helper proteins. Aphids were fed on crude tissue preparations (not subjected to long periods of high speed centrifugation) of CTV across artificial membranes and transmitted CTV to virus-free receptor plants at low efficiency, CTV p20, p27 and p25 proteins could be detected in immunoblots from these crude tissue preparations (CHAPTER III). A purified CTV preparation was not transmitted by the BrCA in this manner. In infectivity neutralizations using three CTV-derived antibodies (p25, p27 and p20) fed *in vitro* to aphids, there were no differences in transmission between the majority of treatments and the control samples. In one transmission experiment, the CTV p20 antibodies significantly enhanced CTV transmission compared to buffer only, pre-immune antiserum or no antibody treatments. This suggests the inactivity of CTV p20 could, in fact, aid BrCA transmission of CTV virions.

The most severe CTV isolate characterized, H33, from Meyer lemon (*Citrus meyeri* Tan.), was studied in more detail (CHAPTER IV). Near full length flexuous filaments (2000 nm) were visualized by transmission electron microscopy in

preparations from the infected plant, and the preparations also reacted in immunoblots to CTV coat protein and CTV p20 antibodies. Shot-gun cloning and sequencing methods were used to sequence the entire genome. The majority of sequences (346) were aligned into a consensus H33 major component genome of 19,232 nt. Six other aligned sequences (H33 minor components) were analyzed and phylogenetically compared to the H33 major component, and T36, VT, T30, T385, NUagA, and SY568 CTV genomes at the 5'-UTR, RdRp, p6, p20 and 3'-UTR regions. This study is the first to give an insight into the population structure of a CTV isolate which did not use primer walking strategies. CTV-infected Meyer lemon propagative tissue has been grown in all the major citrus US growing states, therefore this sequence data is valuable information for many scientists when considering pathogen-mediated protection strategies.

An untranslatable CP gene of CTV was incorporated into the Texas commercial Rio Red grapefruit variety, and fifty-one two transgenic lines were produced (CHAPTER V). Duplicated trees from each transgenic line were graft-inoculated with CTV isolate H18 together with wild type and non-transformed controls. There was a wide range of responses to the viral challenge. Individual plants could be identified which had consistently low virus titers by ELISA detection, had a temporal decrease in virus titer, or a delay in virus titer accumulation. Comparing all wild types to all the transgenic plants over every assessment revealed significant decreases in virus titer in the transgenic lines compared to that of the wild type using regression analyses. Other workers using transgenic grapefruit have found similar effects. Duncan grapefruit with an incorporated translatable CP gene were found to delay accumulation of CTV

challenge only (Febres *et al*., 2003). Demonstrated resistance in citrus to CTV has been found in a proportion of Mexican lime plants transformed with an expressed CP (Domínguez *et al*., 2002).

A cDNA with high similarity to part of the *Oat blue dwarf virus* (OBDV; family *Tymoviridae*, genus, *Marafivirus*) genome was obtained from dsRNA extracts of a CTV H33-infected plant (CHAPTER VI). Northern analyses using total RNA extracted from the source plant and from virus purification fractions revealed a genomic RNA of an estimated size of *ca*. 7.5 kb. OBDV CP antibodies reacted weakly with protein extracts from the source plant and produced a specific band of approximately 28-kDa. *Grapevine fleck virus* (GFkV; family *Tymoviridae,* genus*, Maculavirus*) has many features common to marafiviruses and a 7.5 kb genomic RNA. GFkV CP antibodies do not react with protein extracts from the source plant by ELISA. The entity appears non-graft transmissible to citrus, and a host range study to herbaceous plants indicated the entity may not be mechanically transmitted.

Future experiments for the CTV severity testing would be to evaluate the genome based methods, for instance the 5'-UTR analyses (Hilf and Garnsey, 2000; Ayllón *et al*., 2001) and aim to develop further genome- based methods. As a starting point this can be done by analyzing the Genbank full-length CTV sequence data for potential to generate low, medium, and high polymorphisms, and the greater the number of full-length CTV sequences there are to do this, the more robust the analyses will be obtained (Moonan *et al*., 2000; Moonan and Mirkov, 2002). Therefore many more CTV genomes need to be sequenced in order represent the nucleic acid variation within the species.

Improvements to methods in cDNA library generation, 'shotgun' techniques and sequence analysis software make the generation of large amounts of genome data a fairly rapid process. Based upon sampling the areas of the genome identified (RT-PCR, SSCP, RFLP) from various field CTV isolates, sequencing the regions obtained and bioindexing the isolates, a model(s) could be proposed. Verification of the procedure would have to involve testing different CTV isolates from as many different geographic areas as possible, that is, international cooperation between the various CTV research groups.

The *in vitro T. citricida* CTV acquision and transmission method described in CHAPTER II can be further optimized to be a useful tool to test for the possible function of the various CTV proteins. In Florida, CTV extraction conditions may be evaluated and transmissions with the BrCA can be repeated many times to do this. Other methods of feeding aphids could be evaluated at this time, for instance, *in vitro* transmission with CTV-infected citrus protoplasts.

CTV challenge experiments to evaluate transgenic citrus need to continue. The possibility that Agro-inoculation by slashing infected mature citrus plants with a CTV suppressor (Voinnet *et al*., 1999; Smith *et al*., 2000) to halt damaging CTV symptoms must be evaluated in the future. The use of chimeric transgenes to confer multi-virus resistance (Jan *et al.*, 2000) need also be evaluated. Many different parameters of the challenge system need to be tested. For instance, using one CTV isolate or a mix of several CTV isolates, alteration of the different rootstock combinations and the timing of the challenge. The single greatest advance would be made if a simple, non-labor

intensive, inexpensive, relatively rapid, *in vitro* test could be developed using RT-PCR, for instance. One major problem with using RT-PCR to detect CTV is that CTV may be detected passively after inoculation, therefore making evaluation of hundreds of plants expensive as several assessments would be needed over a relatively long period (Mestre *et al*., 1997). The citrus indicator Mexican lime reacts with most CTV isolates to give a leaf vein clearing 2-3 months after inoculation, and CTV is easily detectible in its plant tissues by ELISA. Mexican lime could be either used first in the testing procedure by transforming with the test transgene or with test Agro-inoculation constructs and CTV challenged before transformation of other commercial cultivars. Alternatively, buds of Mexican lime could be grafted onto the present transgenic scions, and the growth out from this could be evaluated for CTV symptoms (that is, each plant would have a rootstock of sweet orange, inter-stock of a transgenic scion, and a scion of Mexican lime). Methods to cultivate, CTV inoculate, and assess CTV symptoms in Mexican lime would need to be investigated in order that smaller plants could be used. One problem with this would be that evaluation would be in juvenile *Citrus*, which may not reflect the activity in mature plants, therefore this would have to be another parameter to test at evaluation.

Future work to determine as to the nature of the unknown entity in citrus would involve propagation of the infected plant by layering, so that enough tissue could be obtained to test extraction procedures under different buffer and preparative conditions. If a good preparation were to be obtained, transmission electron microscopy would be

used to quickly visualize particle structure (if any), and nucleic acids could be extracted to construct a cDNA library and to obtain further sequence data.

In summary, this study was multifaceted and gathered basic information concerning the severity of CTV isolates and evaluation of PDR in transformed Rio Red grapefruit. More detailed information which can be of use to scientists in the future was gathered concerning the genome of a Texas severe CTV isolate, *in vitro* acquisition and transmission of CTV by the BrCA and identification of a possible new citrus infective agent.

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VITA

Caroline Mary Herron
125 High Street Chesterton
Cambridge
UK
Tel. ++44-01223-516189

Education: B.Sc. (Hons) Biology, University of Nottingham, UK, July 1982.
M.Sc. Crop Protection, University of Bristol, UK, September 1996.
Research project title for M.Sc: The isolation and partial characterization of Citrus tatter leaf virus in non-commercial citrus in Texas.

1990-1994: Plant Pathologist, Belize Citrus Growers' Association, Research and Extension Unit, Dangriga, Belize, Central America.

1987-1990: Plant Pathologist, Research and Development Unit, Booker Seeds Limited, Feering, Essex, UK.

1983-1987: Scientific Officer, Cereal Pathology, National Institute of Agricultural Botany, Cambridge, UK.

Recent Publications

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