Biological Characterization of an International Collection of *Citrus tristeza virus* (CTV) Isolates

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ABSTRACT. Symptoms induced by 266 isolates of *Citrus tristeza virus* (CTV) obtained from 30 countries were evaluated in graft-inoculated Mexican lime, sour orange, Madam vinous sweet orange and Duncan grapefruit seedlings, and sweet orange plants budded on sour orange rootstock. Sequential tests were conducted for 16 yr in a USDA-ARS quarantine facility at Beltsville, MD using consistent protocols. Two standard isolates were included in each test for reference. The isolates tested varied markedly in their ability to induce symptoms in specific indicators as well as in the severity of symptoms. The different patterns of symptoms observed in the five indicators suggested that seedling yellows, the decline syndrome in sweet orange grafted on sour orange, stem pitting in sweet orange, and stem pitting in grapefruit are independent expressions of CTV pathogenicity and may occur in various combinations. Although some general associations between some symptoms and CTV genotypes and reaction to the selective monoclonal antibody MCA13 were observed, absolute correlations were not established.

Index words. Decline, stem pitting, seedling yellows, genotype markers, ELISA, MCA13.

Citrus tristeza virus (CTV) apparently originated in Southeast Asia, which is also the proposed center of origin of many commercially grown citrus species and varieties. CTV has subsequently become widespread in many other citrus growing regions through international movement of infected citrus plants or budwood, which was then subsequently propagated or became a reservoir for natural spread of the virus by aphid vectors (5, 40, 41). Although there is circumstantial evidence that isolates in different countries may have a common origin (1, 41, 42), the precise pathways of CTV distribution are difficult to determine.

Field observations and experimental inoculation of CTV isolates to different citrus varieties have indicated extensive diversity in the types and severity of symptoms induced by different isolates within a single region, as well as between isolates from different citrus growing countries and regions (6, 17, 30, 31, 35, 42). Symptoms that are common in some areas are rare or non existent in others. For example, stem pitting in grapefruit is common in Australia and South Africa, but rarely observed in Florida. However, the comparison of symptoms observed in different regions has been complicated by differences in environment, the indicator plants used and the evaluation process employed.

Isolates that induce a particular symptom in sensitive hosts may go undetected if the host is not present. Most isolates that cause decline in trees propagated on sour orange, severe stem pitting in grapefruit, limes, oranges and pummelos are symptomless in mandarins. Symptoms induced by specific isolates can also be modified by passage of these isolates in certain hosts (6, 31, 47) or when transmitted by aphids (6, 7, 46).

The need to clarify the relationships between the relatively mild CTV isolates found in the US with isolates in many other areas that caused far more severe symptoms was identified in a national review of potential exotic citrus pathogen threats to the US citrus industry in

1984. A project was established to: 1) compare at a single location the relative severity of US isolates of CTV with those reported in other countries, 2) identify isolates with the greatest potential to hamper production of important commercial cultivars in the US, and 3) identify mild isolates with potential to cross protect against severe isolates. Other goals were envisioned, including promotion of national and international cooperative studies on isolates of joint interest, establishing an international collection of CTV isolates that would facilitate evaluating CTV resistance and tolerance in citrus germplasm resources, testing new diagnostic protocols for CTV, and studying the basic properties of CTV. Prior establishment of a research program on citrus canker in the Fruit Laboratory of the USDA, ARS, Beltsville Agricultural Research Center in Beltsville, MD, provided a valuable precedent for conducting research on exotic citrus pathogens in a proactive manner using a national facility well isolated geographically from any commercial citrus plantings (18).

This paper summarizes the results of a 16-yr project to characterize the biological traits of 266 isolates of CTV from 30 countries. During the course of this project a remarkable amount of new information on the molecular properties of CTV has emerged (3, 22, 24, 26, 28). These studies revealed that CTV is a genetically diverse and complex pathogen (19, 21, 27, 43), and attempts were made to associate specific biological properties with serological or molecular properties shared among isolates (2, 23, 37, 38, 39). Some comparisons between symptom patterns, genotype patterns and reactivity to the selective monoclonal antibody MCA13 (39) were made during the course of this study and are included in this report. Information on the initial development of the collection, the development of a standardized biological testing protocol and some preliminary applications has been previously published (11, 12, 14).

MATERIALS AND METHODS

Location. The CTV isolate collection was maintained and all indexing tests were conducted in a quarantine glasshouse at the USDA, ARS Beltsville Agricultural Research Center, at Beltsville, MD, with approval by state and federal regulatory agencies. The potential that a citrus pathogen exotic to the US would escape and become established was minimal since this site is 1,500 km distant from commercial plantings, and winter temperatures do not favor survival of citrus and other known hosts of CTV outdoors. The glass house was equipped with evaporative cooling pads, partially shaded in the summer, and heated by steam in the winter. Temperatures normally ranged from 20-26°C, but maximums approaching or exceeding 30°C were common during summer.

Establishment and maintenance of isolates. All non-US isolates submitted into the collection were received as infected budwood under a permit issued by the USDA Animal and Plant Health Inspection Service (APHIS). This budwood was used to graft-inoculate Madam Vinous sweet orange seedlings to establish a source plant. Once the isolate was selected for testing, a “B” number was assigned to the isolate and recorded in a spreadsheet along with the original coding for the isolate and a summary of its known properties. Investigators from many countries including, Argentina, Australia, Brazil, China, France, India, Israel, Japan, Peru, South Africa, Spain, Taiwan, Turkey and Venezuela generously provided many isolates and valuable background information.

Other isolates were collected by the authors and by colleagues with an interest in the collection while conducting surveys or research projects in different countries. These

isolates were selected because of interesting symptoms observed in the field or experimental studies, to broaden the geographic base of the collection, and to characterize isolates detected in various epidemiological studies (13). Representative isolates from California and Florida were also introduced into the collection for comparative purposes.

Isolates obtained from other collections were generally free of graft-transmissible pathogens other than CTV. The pathogen content of field-collected CTV isolates was less certain and co-infections of tatter leaf, huanglongbing, and several viroids were detected in several instances. Co-infection with tatterleaf and viroids did not impair evaluation of CTV symptoms in the indicators used, but isolates with known or suspected infections of huanglongbing were excluded from CTV biological characterization.

Plant sources and inoculations. Healthy plants for indexing tests were container propagated in glasshouse facilities at the U.S. Horticultural Research Laboratory, Orlando, FL and shipped to Beltsville prior to inoculation. As previously described (14), a standard set of indicator plants (Table 1) was used for most indexing tests. Grafted propagations of Mexican lime source H7 on Alemow rootstock were used to measure the vein clearing (VC), leaf cupping and stem pitting reactions associated with CTV infection in lime plants. Seedlings of sour orange were used to detect the seedling yellows (SY) syndrome induced by certain CTV isolates (10, 47). Seedlings of Duncan grapefruit (Duncan) were used to measure the ability of isolates to cause stem pitting in grapefruit (GSP), and also served as an additional indicator for SY (29). Seedlings of Madam Vinous sweet orange (MV) were used to measure ability of isolates to induce stem pitting in sweet orange (OSP). Plants composed of Hamlin or Valencia sweet orange scions grafted on sour orange seedling rootstocks (sweet/sour) were used to evaluate the ability of isolates to cause stunting and chlorosis that reflects a CTV-induced injury to the phloem at the budunion which is associated with classic tristeza decline (44). Grafted propagations of Duncan on rough lemon seedlings (Duncan/RL) were used in several tests to clarify the development of GSP for certain isolates whose strong SY response stunted Duncan seedlings and precluded accurate evaluation of GSP due to diminished growth. Stem diameters of the test plants were generally 5-8 mm at the time of inoculation.

TABLE 1
VARIATION IN SYMPTOM SEVERITY AMONG AN INTERNATIONAL COLLECTION OF *CITRUS TRISTEZA VIRUS* ISOLATES USING FIVE CITRUS INDICATORS

Indicator	Reaction	Severity (0-3 scale)[y] 0 to 0.5	0.6 to 1.5	>1.6 to 2.5	>2.6	Total # isolates
Mexican lime	Lime (LR)	30[x]	85	73	62	250
Sweet/sour[z]	Decline (DEC)	87	58	40	60	245
Sour sdlg.	Seedling yellows (SY)	139	31	30	57	257
Duncan sdlg.	Grapefruit stem pitting (GSP)	160	42	36	16	254
Madam Vinous sdlg.	Sweet orange stem pitting (OSP)	167	37	21	29	254

[z]Grafted combination of sweet orange scion on sour orange rootstock.
[y]Severity ratings based on average readings from three replicate plants. 0 = symptomless, 1 = mild symptoms, 2 = moderate symptoms and 3 = severe symptoms.
[x]Number of isolates.

Three plants of each indicator were graft-inoculated using two to three blind buds or leaf pieces from systemically infected Beltsville source plants. Budding and pruning equipment was disinfected with diluted household bleach. Viability of the inoculum was visually monitored, and when failures occurred, plants were re-inoculated. Indicator plants were cut back approximately two weeks after inoculation to force new growth which was confined to a single new stem. Plants were fertilized regularly to maintain vigorous growth and sprayed as needed to control insect and mite infestations. Because of space and logistical constraints, two tests per year (Spring and Fall) were initiated with 20 to 25 isolates per test. Uninoculated controls and plants inoculated with Florida mild isolate T30 (B2) and a California severe isolate SY568 (B6) were included in each test as internal references. Plants were labeled with the isolate code and a code letter that identified the host and test replicate.

Evaluation of symptoms. Symptoms were rated on a 0 to 3 scale where 0 indicated an absence of definite symptoms, 1 indicated mild, but definite symptoms, 2 indicated moderate symptoms, and 3 indicated a very severe reaction. Intermediate scores (for example, 0.5) were recorded in some cases to better differentiate readings, especially in the mild range. One evaluator was involved in rating all tests and in making the final scoring, but whenever possible, ratings were reviewed and confirmed by one or more additional evaluators to reduce bias and oversights. Symptoms were recorded for each indicator plant during the course of the test. Mexican lime and sour orange indicators were generally observed for six mo, and sweet/sour, Duncan, and MV plants were observed for 12 mo. Total growth from the point of cutback after inoculation was recorded at the end of the test. Intermediate growth measurements were made if plants were pruned during the test. Stem pitting was determined at the termination of the test or when plants were pruned. Stems were briefly autoclaved to facilitate rapid and clean removal of the bark. All test plants that did not show definite symptoms were assayed by double antibody sandwich indirect (DAS-I) ELISA (see ELISA section) to verify that infection was established. Symptomless test plants which tested negative for CTV were not scored.

At the conclusion of the test a rating for each of the five indicators was calculated based on the average ratings for the three replications per indicator. Whenever possible, and especially when any of the ratings were questionable or highly variable, the index was repeated. The final rating for the isolate was based on information from all tests.

ELISA. A DAS-I ELISA protocol previously described (13) was used for serological assays. Extracts of young bark or leaf mid rib tissue were prepared in 0.05 M Tris or phosphate buffers at a 1/20 dilution using a Kleco tissue pulverizer (Garcia Manufacturing, Visalia, CA). The coating antibody (1 µg/ml) was IgG prepared from rabbit anti-CTV polyclonal antisera. The detecting antibody for routine tests was a mixture of two different broadly reactive monoclonal antibodies (Mabs). The Mab MCA13 (39) was used as the detecting antibody in tests to correlate symptom expression with reactivity to this selective Mab. Commercially prepared goat anti-mouse antibody alkaline phosphatase conjugates were used to detect bound CTV specific Mabs. Assays were considered positive if the OD_{405} values were 0.20 units or more above the values for healthy extracts which were normally between 0.05 and 0.15.

Genotype analysis. Marker based genotype analysis was done using an immunocapture-RT-PCR procedure as previously described

(19, 21, 23). Extracts of fresh or desiccated tissue were prepared using a tissue pulverizer and virions were captured by magnetic beads coated with anti CTV polyclonal antibodies. Genotype assignment was based upon amplification of sequence specific markers as previously described (21, 23).

RESULTS

Twenty eight complete and six partial biological characterization experiments were conducted between 1984 and 1999, and involved the production, inoculation and evaluation of over 8,400 individual plants. Two hundred and sixty-six isolates were evaluated, including 223 primary isolates and 43 subisolates obtained by multiple or single aphid transmissions from 27 primary sources. One hundred and sixty seven isolates were indexed at least twice with emphasis on isolates with unusual properties, or where uncertainty existed about one or more readings in the primary test of the isolate.

Although test condition varied somewhat over the 16 yr testing span, symptom expression in plants inoculated with standard mild and severe controls was relatively consistent. Occasionally, a plant inoculated with the B6 severe control produced milder symptoms than expected, which probably is a reflection that it likely contains a mixture of strains (34). In general, reactions were relatively consistent among the three replicate plants per host that were inoculated with each isolate, and even moderate differences in symptom severity between isolates were readily apparent.

Mexican lime reactions. Mexican lime plants were observed for vein clearing (Fig. 1a), leaf cupping, stunting and stem pitting (Fig. 2). The composite score for the lime reaction (LR) reflected all the components observed, although particular weight was given to stunting and SP. Symptoms in Mexican lime showed a broad range of severity. Four isolates caused no visible canopy or SP symptoms although infection was confirmed by ELISA (Table 2). Lime plants infected with some isolates grew vigorously even though they showed vivid vein clearing and abundant, but discrete stem pits. Some isolates induced strong stunting, and these plants often showed a diffuse vein clearing involving most of the leaf area. Strongly stunted plants often had thickened bark, and when the bark was peeled from the stem, areas of porous wood pitting (Fig. 2e) were observed along with brownish gum-like deposits (8). This reaction was similar to the TB-PWP syndrome described for Duncan plants (see Grapefruit reaction section). Overall, 30 isolates induced reactions rated at 0.5 or less, while 85 isolates showed ratings between 0.6 and 1.5, and 135 isolates showed ratings of 1.6 or greater. Of the latter, 62 had ratings of 2.6 or higher (Table 1).

Sweet/sour reactions. Sweet/sour plants were evaluated for stunting (Fig. 1d) and leaf chlorosis, especially chlorosis along the major veins (Fig. 1c), that indicated a girdling effect expected from CTV-induced phloem necrosis at the bud union. Precocious flowering and fruit set were also regarded as indicators of phloem dysfunction. A number of isolates produced visual reactions in the sweet/sour indicators, but did not induce SY in sour orange seedlings. Other isolates induced stunting and general chlorosis in sweet/sour plants and also induced prominent SY in sour orange seedlings. In the latter case the symptoms in the sweet/sour plants may have reflected SY effects as well as the bud union associated phloem necrosis, but we were unable to partition the relative contribution of SY in the overall visual rating for sweet/sour plants. Sweet/sour plants were also examined for SP at the completion of the test. Not surpris-

Fig. 1. Typical symptoms of *Citrus tristeza virus* (CTV) infection in different indicators. A) Vein clearing in Mexican lime. B) Seedling yellows (SY) symptoms in sour orange (note chlorosis and reduction in leaf size). C) Mid-vein chlorosis in leaves of plants of sweet orange grafted on sour orange and infected with a decline-inducing isolate of CTV. D) Different levels of stunting in plants of sweet orange grafted on sour orange six mo after inoculation with five different isolates of *Citrus tristeza virus* (CTV). Uninoculated control is at right.

ingly, SP in the sweet orange scion was frequently observed in plants infected with isolates that induced marked SP in MV seedlings. In some cases, this SP may also have contributed to the stunting observed in the sweet/sour plants.

As with Mexican lime, severity of symptoms varied markedly in the sweet/sour plants. Ratings for 87 isolates were less than 0.5 while 58 isolates had ratings from 0.6 to 1.5, and 100 isolates were given ratings of 1.6 or greater (Table 1). In gen-

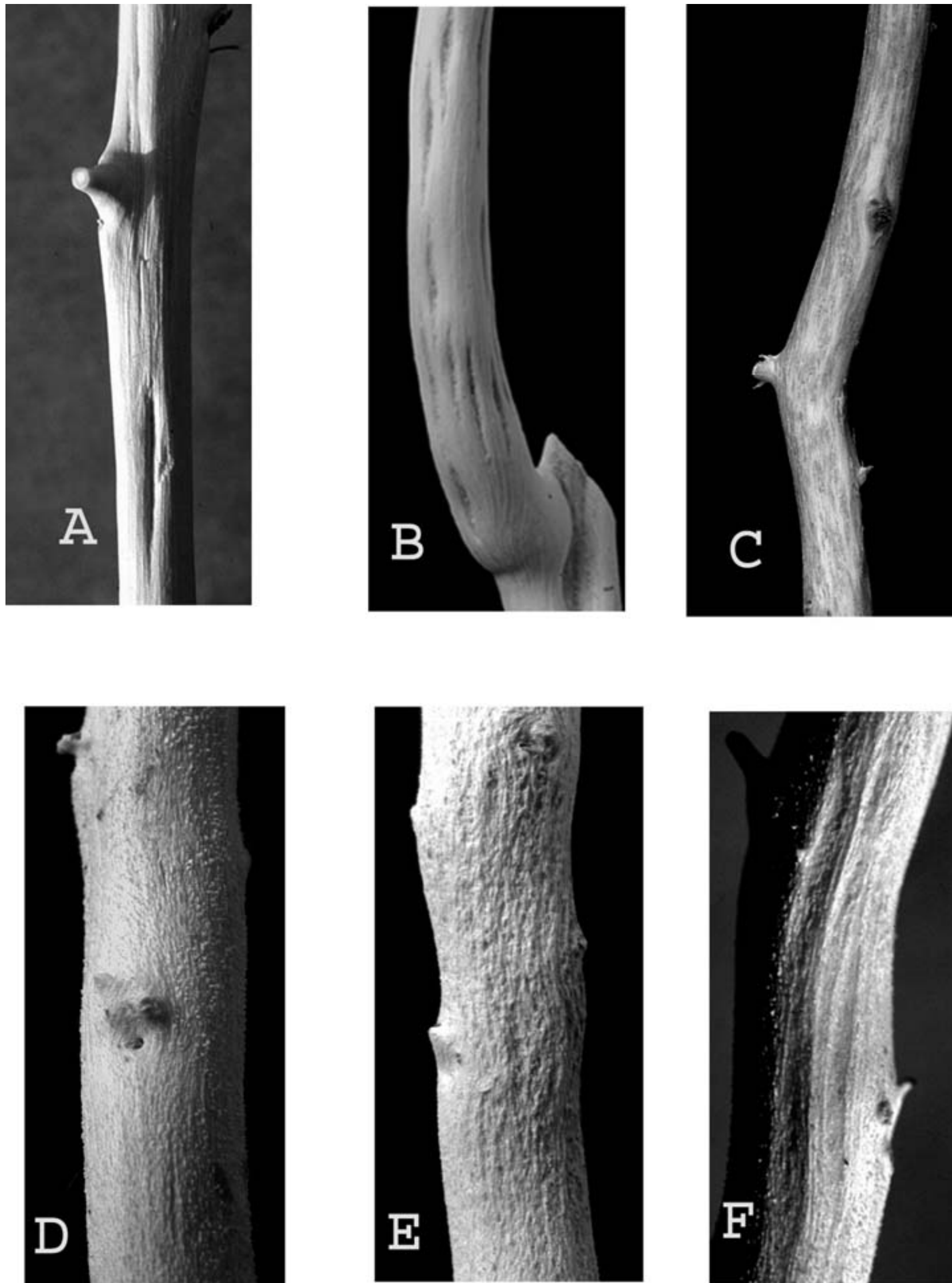


Fig. 2. Stem pitting and other wood symptoms associated with *Citrus tristeza virus* (CTV) infections. A) Mild stem pitting with scattered discrete pits, B) Moderate stem pitting with numerous well defined pits, C) Severe stem pitting with numerous pits, often coalesced, and frequently associated with presence of gum. D) Stem showing fine wood bristles (WB) frequently observed in stunted plants of sour orange and Duncan grapefruit with a thickened bark syndrome (TBS) and showing a severe seedling yellows (SY) reaction, E) Stem showing a porous wood pitting (PWP) that was observed in stunted plants of Mexican lime, Duncan and Madam vinous sweet orange with TBS, and F) a Duncan stem showing both WB and PWP. Stem pitting ratings were made six mo post- inoculation for Mexican lime and sour orange, and 12 mo post-inoculation for plants of sweet orange grafted on sour orange, Duncan grapefruit, and Madam Vinous sweet orange.

TABLE 2
SYMPTOM PATTERNS AND MEAN AND RANGE OF SYMPTOM SEVERITY IN FIVE CITRUS INDICATORS USED FOR BIOLOGICAL CHARACTERIZATION OF AN INTERNATIONAL COLLECTION OF *CITRUS TRISTEZA VIRUS* ISOLATES

Symptom pattern[y]	No. isolates	Mexican lime (LR)	Sweet/sour (DEC)	Sour orange (SY)	Duncan grapefruit (GSP)	Madam Vinous sweet orange (OSP)
0	4	0[z]	0	0	0	0
1	32	0.9 (0.5-2.0)	0.04 (0-0.5)	0.03 (0-0.5)	0.02 (0-0.5)	0
2	20	0.9 (0.5-1.5)	1.0 (0.5-2.0)	0.03 (0-0.1)	0.04 (0-0.5)	0
3	36	1.6 (0.5-2.8)	1.7 (0.5-3.0)	1.7 (0.5-3.0)	0.02 (0-0.3)	0.01 (0-0.3)
4	16	2.0 (0.8-3.0)	1.4 (0.5-2.5)	1.0 (0.5 -2.5)	1.6 (0.5-2.7)	0.1 (0-0.3)
5	44	2.5 (1.5-3.0)	2.1 (0.5-3.0)	1.9 (0.5-3.0)	1.5 (0.5-3.0)	1.9 (0.5-3.0)
6	42	2.4 (1.0-3.0)	2.2 (1.0-3.0)	2.4 (1.0-3.0)	0.1 (0-0.5)	1.8 (0.5-3.0)
7	6	2.5 (2.0-3.0)	1.4 (0.5-2.5)	0	2.5 (2.0-3.0)	2.2 (1.5-2.7)
8	9	2.2 (1.5-2.5)	0.2 (0-0.5)	0.1 (0-05)	2.2 (1.0-3.0)	1.3 (0.5-2.4)
9	23	1.6 (1.0-2.2)	1.3 (0.5-2.5)	0.03 (0-0.1)	1.7 (0.5-2.8)	0
10	21	1.5 (1.0-2.8)	0.1 (0-0.5)	0	1.6 (1.0-2.2)	0

[y]Number codes for different patterns of symptom expression observed in the different indicators as illustrated in Fig. 5.
[z]Symptoms rated on 0 to 3 scale where 0 = symptomless, 1 = mild, 2 = moderate, and 3 = severe. Values over 0.05 rounded off to nearest tenth.

eral, the highest decline reaction ratings were associated with isolates that also induced significant SY responses. However, some isolates that did not induce SY in sour orange seedlings did induce moderate to strong symptoms in the sweet/sour plants (Table 2, pattern 9).

Sour orange reactions. Leaf chlorosis, reduction in leaf size, changes in leaf texture and stunting were considered in making an overall rating for SY in sour orange seedlings (Fig. 1b). In some cases, plants were cut back a second time during the test period to force an additional flush of new growth. In general, seedlings with strong leaf chlorosis were markedly stunted. Initially, sour orange seedlings were not examined for SP, but in later tests stems were also peeled and observed for SP. Some severely stunted plants had a thickened bark which was originally designated as a cheesy bark reaction. When the bark was removed, fine bristle-like pegs were observed on the wood (Fig. 2d). This response is defined here as TBS-WB (thick bark syndrome with wood bristles) and is associated with SY (29). It is distinct from the porous wood pitting syndrome (see Grapefruit reactions section) where the bark may also be abnormally thickened. In addition to the TBS-WB syndrome a few isolates induced a classic SP response in sour orange seedlings. These plants generally also showed vein clearing, but did not show SY. Overall, 139 isolates gave negligible SY readings (0.5 or less) while 57 gave readings of 2.6 or greater (Table 1). The majority of isolates that failed to induce SY in sour orange seedlings were readily detected by ELISA. However, some isolates failed to replicate in sour orange to levels detectable by ELISA, even though the graft inoculations were apparently successful, and the other four indicators became infected by inoculation from the same source. Some plants with obvious SY symptoms also had low ELISA readings. However, this may have reflected the absence of young tissue suitable for ELISA rather than poor replication.

Grapefruit reactions. Duncan seedlings were evaluated for canopy symptoms, including leaf chlorosis,

vein clearing and corking, as well as for stunting, and SP. Foliar symptoms in grapefruit were associated with both SY and SP. When an isolate induced chlorosis and stunting in Duncan, but no SP, and also induced SY in sour orange, the foliar symptoms in Duncan were considered an SY response. When an isolate did not induce SY in sour seedlings, but caused significant SP in Duncan, the stunting and foliar symptoms in Duncan were considered part of the GSP effect. When SY (as determined in sour orange) and GSP components were both present in the same isolate it was difficult to differentiate the relative degree of foliar effects in Duncan associated with each component. Stunted Duncan plants often had an abnormally thickened bark that indicated an abnormal differentiation of phloem and xylem tissues. Some severely stunted plants infected with SY-inducing isolates had fine bristle-like pegs on the wood surface when the thickened bark was separated from the stem. These were apparently identical to the TBS-WB syndrome described previously for sour orange (Fig. 2d). Removing the thickened bark from the stems of other stunted Duncan plants revealed extensive areas of fine porous pitting in the wood (Fig. 2e), often also associated with some brown gum. This was designated a thick bark syndrome with porous wood pitting (TBS-PWP) and was scored as a severe form of SP. In some cases, TBS-WB and TBS-PWP reactions were observed in different areas of the same Duncan stem (Fig. 2f). TBS-PWP was also observed in Mexican lime and MV plants infected with certain isolates, but not in association with TBS-WB.

In some cases, Duncan seedlings were so stunted that there was very little stem to evaluate for SP. To resolve this issue, inoculations were made to vigorous bud propagations of Duncan/RL. These plants grew vigorously enough after inoculation to produce stems adequate for SP evaluation. Twenty-six of 62 isolates tested on Duncan/RL produced detectable GSP, although in many cases the reaction was relatively mild. Interestingly, 14 of these 62 isolates produced SP in the rough lemon rootstock, but six of the 14 did not induce GSP in the Duncan scion. All but one of the 14 caused OSP in MV. In a few cases, foliar symptoms were observed in Duncan infected with isolates that did not induce GSP and did not cause a SY reaction in sour orange. The ratings assigned to grapefruit were based on SP associated effects. Overall, 160 isolates induced negligible GSP (0-0.5) while 16 induced GSP ratings of 2.6 or more (Table 1).

Sweet orange reactions. MV seedlings were evaluated for vein clearing in young flush leaves, foliar chlorosis, stunting and SP. Readings for SP were made approximately 12 mo post inoculation. The presence of TBS-PWP, as described for Duncan and Mexican lime plants, was considered a severe reaction. One hundred and sixty seven of the 254 isolates tested did not cause OSP in MV while 50 isolates produced ratings of 1.6 or greater, and 29 induced ratings of 2.6 or greater (Table 1). Significant stunting was observed only for isolates that induced severe OSP. Isolates that induced SP frequently induced vein clearing in young MV leaves, but in several cases vein clearing was observed in plants that subsequently did not show significant SP. Vein clearing was most commonly observed in vigorous new growth flushes during relatively warm weather.

Reaction patterns. As data accumulated for individual isolates, it was noted that multiple isolates shared similar patterns of symptom expression. The most common reaction patterns are summarized in Figs. 3 and 4. Patterns are numbered for convenience, and may not match exactly profile numbers previously assigned in other reports (12, 23, 25). Some isolates produced

Pattern No.	LR	DEC	SY	GSP	OSP	No. Isolates
0	o	o	o	o	o	4
1	x	o	o	o	o	32
2	x	x	o	o	o	20
3	x	x	x	o	o	36
4	x	x	x	x	o	16
5	x	x	x	x	x	44
6	x	x	x	o	x	42
7	x	x	o	x	x	6
8	x	o	o	x	x	9
9	x	x	o	x	o	23
10	x	o	o	x	o	21

Fig. 3. Schematic view of 11 different symptom patterns observed consistently while characterizing a collection of *Citrus tristeza virus* (CTV) isolates. LR indicates foliar and stem pitting symptoms in Mexican lime, DEC indicates chlorosis and stunting in the grafted combination of sweet orange on sour orange, SY indicates a seedling yellows reaction in sour orange seedlings, GSP indicates stem pitting in Duncan grapefruit seedlings and OSP indicates stem pitting in Madam Vinous sweet orange seedlings.

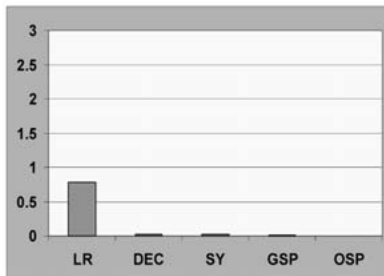
symptoms only in Mexican lime (Figs. 3 and 4, pattern 1), while some induced significant symptoms in all indicators (Figs. 3 and 4, pattern 5). Others showed variable independent expression of symptoms in different indicators (Figs. 3 and 4, patterns 3, 4, 6 and 8). The severity of reaction in specific hosts varied to some extent among isolates with the same general pattern. Mean reaction ratings and ranges are summarized in Table 2.

The distribution of symptoms in the 11 patterns shown in Fig. 3 indicated that syndromes such as decline, SY, GSP and OSP are independent manifestations of pathogenicity. Comparison of pattern 3 to pattern 8 (Fig. 4) clearly shows that the SY and decline syndrome symptoms can be expressed independently of either GSP or OSP. While some isolates induce SP in both grapefruit and sweet orange (Figs. 3 and. 4, patterns 5 and 8) others induced symptoms in only one host (Figs. 3 and. 4, patterns 4 and 6). While nearly all isolates that induced substantial SY in sour orange also induced symptoms in sweet/sour plants, some isolates induced the decline syndrome on sweet/ sour plants, but no SY, indicating that there is not an absolute association between these syndromes. Two of the four patterns with an OSP component also included a SY component while SY was present in two of six patterns that had a GSP component (Fig. 3).

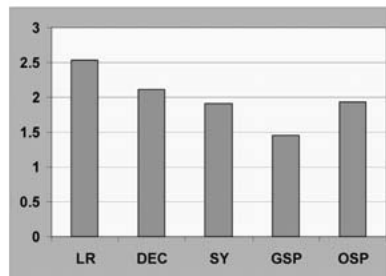
In addition to isolates that could be grouped based on common patterns, 13 isolates expressed symptom patterns that were unique or shared with only several other isolates and did not fit any of the 11 common patterns.

Comparison of aphid-transmitted sub isolates to the parent source. Forty-three aphid-transmitted sub-isolates originating from 27 different primary sources were tested. Some of the aphid-transmitted isolates were obtained by single aphid inoculations while others were obtained using multiple aphids per receptor plant. Nineteen aphid-transmitted sub-isolates produced symptoms in all five indicators that were similar to the parent isolate. Seventeen sub-isolates showed milder symptoms than the parent source in one or more indicators, and seven sub-isolates showed symptoms in one or more indicators that were markedly more severe than those observed in the parent source. These included six aphid-transmitted isolates from B192, an isolate that is symptomless, even in Mexican lime (7), but does react positively to MCA13.

Correlation of biological reaction to isolate genotype. Marker analysis for genotype has been performed on 144 isolates which were biologically characterized. As shown in Table 3, symptom patterns 0 and 1 were predominantly associated with the T30 genotype while symptom patterns 4, 5, 6 and 8 were most frequently associated with the T3 and VT genotypes. However, all four marker profiles



Pattern 1



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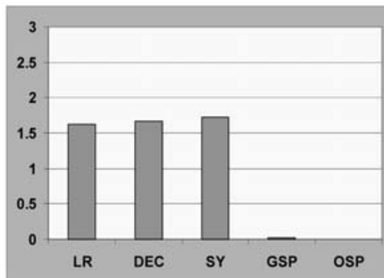

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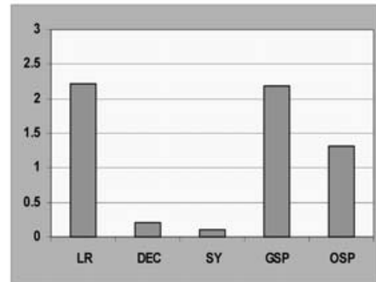

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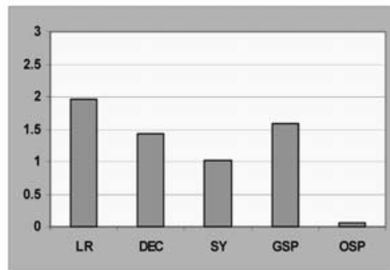
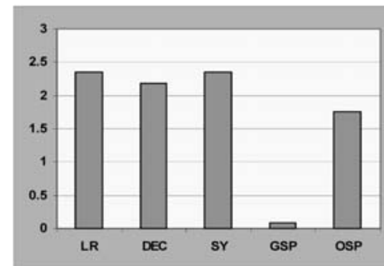


Pattern 4



Pattern 6

Fig. 4. Graphic depiction of symptom severity in indicator plants for six of the different CTV symptom patterns shown in Fig. 3. LR is reaction in Mexican lime, DEC indicates symptoms in the grafted combination of sweet orange on sour orange, SY indicates seedling yellows response in sour orange seedlings, GSP indicates stem pitting in Duncan grapefruit seedlings and OSP indicates stem pitting in Madam Vinous sweet orange seedlings. 0 = symptomless and 3 = severe reaction. Values shown are means for multiple isolates as indicated in Table 2.

were associated with at least six different symptom patterns. The VT genotype was associated with the most symptom patterns.

Association between genotype and symptoms in specific indicators was examined by Hilf, et al (23) and was also variable. In general, SY was not associated with the T30 genotype, and there was a close association between OSP and the T3 genotype. Decline and GSP were associated with several genotypes.

Correlation of symptom profile to MCA13 reactivity. The selective monoclonal antibody MCA13 has been used for rapid presumptive differentiation of "mild" and "severe" isolates of CTV based on observations that it reacted with most isolates that cause decline, SY, GSP and OSP alone or in combination, and did not react to isolates that failed to cause those symptoms (39). During the course of this project, most of the isolates which

TABLE 3
CORRELATION OF SYMPTOM PATTERNS AND *CITRUS TRISTEZA VIRUS* GENOTYPE AS DETERMINED BY MARKER-BASED ASSAY[y]

Symptom pattern[z]	Biocharacterized Isolates	Marker tested Isolates	Marker-based genotype			
			T3	T30	T36	VT
0	4	3	—	3	—	—
1	32	16	—	13	—	3
2	20	15	—	11	2	2
3	36	18	3	2	4	9
4	16	10	6	1	1	5
5	44	22	16	1	—	5
6	42	23	13	1	—	9
7	6	1	—	—	—	1
8	9	5	5	—	—	1
9	23	11	—	5	—	6
10	21	11	—	9	—	2
Not rated	13	7	1	2	2	2

[y]Genotype determined by pattern of amplification products produced using immunocapture RT-PCR and 11 sets of primers (23).
[z]Symptom patterns as shown in Fig 5.

were biologically characterized were also tested for reactivity to MCA13. A comparison of symptom patterns and MCA13 reactivity is summarized in Table 4. All isolates with symptom patterns 3 through 8 which were tested reacted to MCA13. This included all isolates that induce SY and all that induce OSP. Interestingly, some isolates that were symptomless (pattern 0) or reacted only in Mexican lime (pattern 1) reacted to MCA13 while some isolates with a GSP component (patterns 9 and 10) did not react with MCA13.

DISCUSSION

The results described here confirm that CTV symptom expression is diverse and complex, and that CTV isolates differ markedly in the severity of symptoms they induce in a single host, and also in their ability to cause symptoms in different hosts. These tests confirmed that CTV isolates commonly found in the USA are relatively mild when compared to many isolates from other locations, and encompass only a portion of the total biological diversity of CTV.

The 11 distinct general reaction patterns recognized using five indicators and a screening method designed to measure only fairly large differences in symptoms do not fully reflect the complexity of CTV symptom responses. For example, the vein clearing and stem pitting in sour orange induced by some isolates was not factored as a separate pattern. Although there were indications that vein clearing and SP in Mexican lime and MV are not always linked, these were not separately scored. Additional patterns would undoubtedly appear if additional indicators were used. For example, the additional indexing for GSP using Duncan/RL suggested that rough lemon could be used also to differentiate isolates since some isolates caused SP in the rough lemon rootstock, but did not cause SP in the Duncan scion while others that caused GSP did not affect the rough lemon rootstock.

The repetition of symptom patterns by multiple isolates indicated that the patterns are real and reproducible and not artifacts of individual tests conducted at different times or readings based on limited numbers of plants. Analysis of symptom patterns

TABLE 4
CORRELATION OF CTV SYMPTOM PATTERNS AND REACTIVITY TO THE SELECTIVE MONOCLONAL ANTIBODY MCA 13

Symptom profile[y]	Biologically characterized Isolates	Isolates tested with MCA13	Reaction[z]		
			Negative	Weak	Strong
0	4	4	2	0	2
1	32	30	17	4	9
2	20	18	9	3	6
3	36	36	0	0	36
4	16	15	0	0	15
5	44	42	0	0	42
6	42	40	0	3	37
7	6	5	0	1	4
8	9	9	0	0	8
9	23	23	6	2	15
10	21	16	9	1	5
Not rated	13	12	3	0	9

[y]Symptom patterns as shown in Fig 5.
[z]Negative = OD_{405} values less than 0.2 greater than healthy control. Weak = >25% of maximum reaction in test. Strong = 25% or greater of maximum reaction in test.

was useful for showing that the decline syndrome, GSP and OSP must have separate virus–related induction mechanisms and for tracking similarities between isolates from the same or from different locations.

It may be convenient to use a symptom pattern or biological group designation for quick characterization of CTV isolates, but these should be constructed and defined to fit each specific application. There was a range of symptom severity for each indicator (Table 2), and grouping isolates by pattern involves decisions on what threshold limits should be employed for the symptoms in each indicator. This threshold may vary with the application. For example, a 0.5 rating threshold for SP in grapefruit may be desirable for studies of virus pathogenicity determinants, but a higher threshold may be more appropriate to correlate GSP to economically significant effects on production.

The most accurate biological definition of specific isolates is still a profile listing the specific symptom ratings for each host. It was not readily possible to show those details for all isolates studied in this paper. However, arrangements for access to these readings will be indicated on the IOCV web site and users can construct isolate groupings for their specific applications.

Several chronic problems were encountered while making symptom ratings. While severe SY reactions are readily recognized, mild forms of SY are harder to recognize, especially if growth conditions for the sour orange plants are not perfect. Another issue is whether to base SY ratings solely on readings in sour orange. Some isolates induced mild SY-like symptoms in Duncan grapefruit seedlings even though they did not cause symptoms in sour orange. In some cases, these symptomless sour orange plants did not index positively by ELISA, even though the inoculation grafts remained viable, and the other indicators were infected using the same inoculum. In this situation the failure of the isolate to infect sour orange and trigger the SY response in that host (4) precludes detection of SY if sour orange is the only indicator used.

Isolates which caused definite SY in sour orange consistently induced symptoms in Duncan seedlings, but

using Duncan seedlings as a primary visual indicator for SY is complicated when a GSP component is present (31). A number of isolates which did not induce SY in sour orange caused strong stem pitting reactions in grapefruit and also caused some stunting and chlorosis. We assumed that the latter symptoms were due to stem pitting and not a grapefruit-specific SY, but, in fact, that assumption is difficult to prove.

Conversely, the presence of a severe SY component often caused such severe stunting that it was not possible to evaluate SP in Duncan seedlings. This was overcome by budding Duncan on rough lemon, a CTV tolerant rootstock, to form indicators that would grow well enough to allow a reading for stem pitting.

Determination of the decline-inducing potential of CTV isolates based on visual symptoms in young grafted plants of sweet orange on sour orange is less direct than the classic histological analysis of budunion pathology (44) which was not feasible in this study. It was assumed that the canopy symptoms observed reflect the effects of phloem necrosis at the budunion. Stunting, chlorosis of leaf mid ribs similar to that induced by girdling, and precocious flowering and fruiting all are symptoms that suggest an interruption in normal phloem transport expected with a CTV-induced budunion incompatibility. However, some of the canopy symptoms observed in sweet/sour plants may actually have reflected SY effects in the root system in addition to those associated with phloem necrosis at the budunion. For example, attempts to screen for budunion effects using plants which were formed with a sour orange interstock between a sweet orange scion and a CTV-resistant Carrizo citrange rootstock failed to produce the same level of symptoms observed in sweet/sour plants as would be predicted if the effects were solely associated with a sweet/sour bud union pathology. In other experiments, plants with a CTV-resistant scion grafted on sour orange showed some stunting and chlorosis when infected with isolates inducing decline and SY, although no virus replication was detected by ELISA in the scions which carried the Ctv-r gene for CTV resistance (32). Absence of infection in the resistant scions should have precluded development of budunion pathology, and indicated that the symptoms observed in the scion may have resulted from SY effects in the root system.

Glass house conditions which favor continual cambial activity and generation of new phloem tissue are also less ideal to expose effects of phloem necrosis than field conditions with periods of winter dormancy. While the severe stunting induced by many isolates was obvious, mild reductions in growth may easily have been masked or under estimated since conducting tests in containers of limited size and pruning the most vigorous plants periodically may constrain the healthier plants from achieving their maximum potential size.

Development of a rapid assay that would provide a direct quantitative measure of phloem necrosis would be highly valuable for measuring this economically important disease syndrome.

In general, the symptoms and symptom severity observed in these tests were consistent with the available prior field or experimental observations. In some cases, observed discrepancies could have been due to a mixed infection with a change in the ratio of component mixtures over time. Isolates were often maintained for extended periods in Madam Vinous source plants before characterization was done. There were practical advantages to maintaining the collection in a single propagating host, but isolates collected from other hosts and graft-transmitted to MV may have been subjected to a host selection pressure that affected the genotype or haplotype population. For example,

SY-inducing strain may become suppressed in sour orange and grapefruit after a long infection (6, 31), but when the isolate is transferred back to a permissive host, such as sweet orange, it may reappear. In the future, it may be desirable to maintain primary isolates in the original host where possible.

Variation in symptoms induced by primary isolates and sub-isolates from those sources that were derived by aphid transmission also indicated that the diversity of biological properties may not always be recognized by analysis of the primary isolate. Sub isolates that express more severe symptoms than those observed in the parent source are especially interesting in this regard. Presence of "hidden" components has been previously reported (6, 7, 33, 46) and this suggests that a complete understanding of disease expression may involve not only identification of the specific viral determinants for each symptom, but also how virus and host factors interact to affect symptom expression and severity.

While there were some interesting general associations between marker-based genotypes and symptom expression, such as the association of OSP with the T3 genotype, we could not show that specific symptoms or symptom profiles were unique to a particular genotype. In a study on CTV genotype marker profiles that also encompassed additional isolates which have not been biologically characterized (23), some isolates did not form amplification products with any genotype-specific primers. This suggests that additional genotypes may exist. If so, these may also be present in mixtures with the currently defined genotypes in various isolates and would obviously complicate existing efforts to correlate genotypes and biological properties. Isolates with recombinant genomes could also complicate correlation of symptoms to genotype (23). The marker characterization of B270-1 as a T30 genotype was not consistent with its symptom pattern 5, and is a notable example of an isolate that needs further evaluation.

Some of the aphid transmitted sub-isolates that were biologically characterized in this study have not been assigned a genotype by marker profiling and these results may help resolve the noted discrepancies. While further definition of CTV genotypes may help clarify correlations between symptoms and genotype, it seems probable that symptom expression is a result of complex interactions not easily predicted only by marker-based genotyping.

Evaluation with MCA13 of an array of CTV isolates with diverse symptom patterns and genotypes indicated close positive correlations between a positive MCA13 response and presence of SY and OSP reactions. However, the association between MCA13 reactivity and decline and GSP symptoms was less well defined and multiple exceptions were observed in both directions. It is unlikely that the specific nucleotide sequence encoding the MCA13 epitope in the CTV coat protein is directly involved in any of the CTV pathogenicity reactions.

The international collection of CTV isolates has been valuable for understanding the range and diversity of symptoms induced by CTV and for evaluating the relative severity of different isolates. The current collection, however, cannot be considered completely comprehensive. A full range of isolates has not been included from many of the 30 plus citrus growing countries or areas that are currently represented in the collection. The relative frequency of symptom types or genotypes among isolates in the collection may not reflect the relative frequency of these in the country of origin because collections were made to look for differences among isolates, and not their relative distribution.

Many citrus growing countries and areas are not represented at all, including a number of areas in

southeast Asia which is the presumed origin of CTV and which may contain the greatest diversity of primary CTV genotypes. Isolates with new properties also continue to be discovered, such as the recently reported trifoliate resistance breaking isolate from New Zealand (9).

The presence of multiple isolates with the same symptom patterns and genotypes also indicates that considerable redundancy exists in the current collection. This is not surprising since some accessions from field sources were obtained by sampling several trees in the same general location which may have been infected with identical isolates (20). Similarly, isolates that were obtained from geographically distinct areas may have a common origin and be essentially identical (1).

Long term maintenance of a large collection of CTV isolates *in planta* requires an extensive commitment of greenhouse space and technical support. Over time it will be necessary to eliminate redundant isolates, and also improve *in vitro* preservation and retrieval methods so infectious CTV virions of isolates can be preserved *in vitro* and reestablished *in planta* as needed in the future. The potential to preserve CTV *in vitro* as lyophilized, concentrated extracts from infected plants has been demonstrated (15), but is not highly efficient and the maximum time isolates can be preserved has not been established.

In addition to serving as resource for biological and molecular characterization of CTV (23, 34), the collection has been used for evaluation of diagnostic protocols for CTV detection (38, 45), and to evaluate CTV host resistance (16).

The development and characterization of the collection of CTV isolates at Beltsville can be viewed as a useful model for conducting research of importance to a globally distributed agricultural commodity (18). The US has an important citrus industry, and the research facilities located a safe distance from citrus producing areas has fostered a number of international cooperative research efforts on CTV which have contributed to the rapid increase of information about this pathogen (1, 2, 7, 12, 18, 23, 28, 34, 36).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of many colleagues world wide, including, M. Bar-Joseph, P. Barkley, K. Bederski, A. Dodds, T. Gottwald, M. Koizumi, K. Manjunath, L. Marais, J. Moll, P. Moreno, G. Müller, C. Niblett, C. Roistacher, H. Su, and M. Zemzami for contribution of isolates to the collection and valuable information on isolate properties and sources. The project could not have been conducted without the excellent technical assistance of many people including, J. Bash, Univ. of California, Riverside, C. Bierman, J. Bittle, and C. Halliday, ARS, USDA, Orlando, and M. Civerolo, G. Ingineri, and C. Paul, ARS, USDA, Beltsville. The administrative support of the late M. Faust, Leader of the Fruit Laboratory, ARS, USDA, Beltsville and H. J. Brooks, National Program Staff, ARS, USDA was critical for establishment and early support of the project. D. J. Gumpf passed away February, 2003, but his interest, enthusiasm and dedicated participation will always be remembered.

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