Development of a Worldwide Collection of Citrus Tristeza Virus Isolates

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ABSTRACT. A collection of citrus tristeza virus (CTV) isolates from different citrus-growing regions of the world was started in 1984 in a quarantine facility at Beltsville, MD. Isolates with diverse biological properties have been received through cooperation with scientists in Argentina, Australia, Brazil, California, China, Colombia, Costa Rica, Florida, France, Hawaii, India, Israel, Japan, Peru, the Philippines, Reunion, South Africa, Spain, Taiwan, Tanzania, and Venezuela. To date, 175 CTV isolates have been established and are being maintained. Symptom expression on five different indicator plants has been evaluated or is under evaluation for 114 isolates. Transmission of 28 isolates by *Aphis gossypii* has been achieved, and 22 aphid transmitted subisolates also have been evaluated for symptomatology. Results confirm great biological diversity among CTV isolates. This diversity in reaction is independent of the effects of host, environment, and evaluation methods. Severity of reaction was not consistent in different hosts for some isolates. Severity of stem pitting in grapefruit and sweet orange was not necessarily correlated to the decline reaction observed in young, grafted sweet orange on sour orange plants. This collection is a valuable source of CTV isolates for research on characterization and identification of specific isolates and for further studies on mild strain cross protection.

Index words. indicator plants, stem pitting, seedling yellows, aphid transmission, ELISA.

Citrus tristeza virus (CTV) is present in most citrus-growing areas of the world (1). It is endemic throughout Southeast Asia, where it apparently originated, and it subsequently has been introduced into most other citrus-growing regions by movement of infected citrus plants or budwood. Natural spread by several different aphid vectors has occurred subsequently in many of these new locations. CTV has caused severe damage to citrus production in many areas and has been the subject of extensive research (1). The biological properties of CTV are remarkably diverse, and many different isolates of CTV have been reported (1, 7, 13, 16).

Two major economic disease effects are associated with CTV infection (1, 13). One is a decline and rapid death of trees grafted on sour orange rootstocks that is associated with a CTV-induced phloem necrosis at the budunion. The second is stem pitting in the trunks, limbs, and twigs of infected trees. While not lethal, severe stem pitting reduces tree vigor, fruit size, and limits lime and grapefruit production in many areas. Some stem pitting isolates also severely damage sweet orange (7). Observations by various workers suggest that different loci in the virus genome are involved in decline and stem pitting syndromes (13, 16, 17).

A yellowing and stunting (seedling yellows) effect in experimentally inoculated sour orange, lemon, or grapefruit seedlings is frequently considered indicative of a severe strain (1). Vein clearing, leaf cupping, stem pitting, and stunting are diagnostic symptoms of CTV infection in Mexican lime (1).

Quantitative differences in severity of all symptoms have been observed. Some CTV isolates cause severe symptoms in nearly every susceptible host, others cause severe symptoms in some hosts and only mild reactions in others, and some are benign in all hosts. Symptom severity in Mexican lime, the universal indicator host of CTV, is not predictive of severity in other hosts.

While genetic diversity among isolates is evident, other factors influence symptoms. For example, high temperatures reduce symptom severity and may influence ratings made in different climate areas (21). Coinfection with two different isolates of CTV or with other pathogens in the same plant occurs frequently, and has contributed to the confusion about CTV symptoms, especially in those reports based only on field observations. For example, in

many areas of Southeast Asia coinfection with citrus greening and CTV occurs, and it is difficult to determine the specific effects of each of these phloem-limited pathogens unless they are separated and evaluated individually (1). Much of the information on the severity of different CTV isolates is based on subjective evaluations made by different workers without a standard reference and without standardization of conditions, indicator plants, and terminology.

Recently, a more standardized approach to biological characterization of CTV isolates with five different indicators was developed (7).

Biochemical approaches to characterization of CTV isolates include peptide analysis of the coat protein (10), dsRNA analysis (6, 18,) nucleic acid hybridization analysis with cDNA (22), analysis of viral inclusions (5), and serological differentiation with monoclonal antibodies (8, 11, 19, 20).

We are assembling a collection of CTV isolates from different citrus-growing regions of the world to reflect the full range of CTV symptom types. The goals of this project are to create a library of different CTV isolates with well-characterized biological properties which will be useful references for citrus virologists worldwide, to identify mild isolates with potential for cross protection, to identify the most significant severe isolates to control, to develop rapid means for identifying specific isolates of CTV, and to characterize properties such as aphid transmissibility, dsRNA components, inclusion body morphology and composition, and serological reaction.

The current status of this CTV collection is reported in this paper.

ISOLATE INTRODUCTION

Selection criteria. Selection of isolates for the collection has been based on several criteria. One was to include isolates from different citrus-growing areas which are well characterized and have been studied extensively. Direct comparison of these standard isolates is needed to evaluate and compare relative severity of isolates from different citrus areas. Numerous cooperators have responded willingly to requests for CTV isolates and information. In some cases, isolates were entered from uncharacterized field sources if they possessed unusual properties. A second goal was to obtain isolates that would represent the entire range of symptoms and symptom severity exhibited by CTV. Special interest has been placed on isolates that induce severe host reactions, that induce very mild reactions, and those that reportedly induce unique host responses. A third consideration was isolates with potential for mild strain cross protection. As many isolates as possible with reported or suggested protective effects are being included.

Submission methods. All isolates are received under permits issued by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS). The CTV-infected budwood or leaf tissue is received by APHIS personnel and then released to a USDA quarantine greenhouse at the Beltsville Agricultural Research Center (BARC) at Beltsville, Maryland, for propagation. This site is approximately 1500 km from the nearest commercial citrus planting. Each isolate is graft-inoculated to Madam Vinous sweet orange and, if possible, to one or more diagnostic indicators. After inoculum survival is verified, the receptor plant is cut back to force new growth. The second flush of new growth is assayed by double antibody sandwich ELISA (DAS ELISA) (2) to confirm that infection has been established. The isolate is then assigned a code number and pertinent data are entered into database and spread sheet files. As of March 1990, 175 isolates have been established. The origin of these is shown in Table 1.

APHID TRANSMISSION

Many of the isolates received have been transmitted experimentally by aphids at their point of origin prior to their deposition into the collection. As time permits, additional isolates, espe-

TABLE 1
SUMMARY OF EXOTIC CITRUS TRISTEZA VIRUS COLLECTION AT BELTSVILLE, MARYLAND, AS OF MARCH 1990

State or country of origin	No. of samples	Reported severity at origin		
		Mild/Mod[z]	Severe[y]	Unknown
Argentina	1	–	–	1
Australia	4	2	2	–
Brazil	9	3	5	1
California	16	8	8	–
China	16	1	3	12
Colombia	10	3	5	2
Costa Rica	4	–	–	4
Florida	9	5	4	–
France	1	1	–	–
Hawaii	13	3	4	6
India	16	3	12	1
Israel	12	3	9	–
Japan	11	5	4	2
Peru	4	–	4	–
Philippines	8	–	2	6
Reunion	1	–	1	–
South Africa	11	4	5	2
Spain	12	3	4	5
Taiwan	13	4	2	7
Tanzania	3	–	–	3
Venezuela	2	–	–	–
TOTAL	175	49	74	52

[z]Isolates that cause symptoms primarily in Mexican lime. Symptoms in other hosts are mild (if present) or not recorded.
[y]Isolates that cause strong SY and/or strong stunting in young sweet orange on sour orange and isolates that cause strong stem pitting in sweet orange or grapefruit or other CTV-tolerant varieties.

cially those which were field collected and may be contaminated with other graft-transmissible pathogens, are aphid transmitted. To date, we have obtained 76 aphid-transmitted isolates which represent 29 different original sources. All transmissions were by Florida colonies of *Aphis gossypii*.

Twenty-two aphid-transmitted isolates are being tested for symptom expression. In some cases, the sub-isolates have differed in symptom expression from the parent. However, the full syndrome of some severe isolates of CTV, such as the Capao Bonito isolate, was readily transmitted (23).

A colony of *Toxoptera citricidus* has also been established in a strict quarantine facility at Frederick, Maryland, to allow comparative studies on transmission of selected CTV isolates by *T. citricidus* and *A. gossypii* (23).

BIOLOGICAL CHARACTERIZATION

Biological characterization of CTV isolates in the BARC collection is done on Mexican lime, sour orange, Duncan grapefruit, Madam Vinous sweet orange, and a grafted combination of sweet orange on sour orange rootstock (Swt/So) as previously described (7) and summarized in Table 2. Three plants of each indicator are graft inoculated (2-3 buds or leaf pieces per plant) with each isolate to be tested. Normally 15 to 20 isolates are evaluated at one time. One severe isolate (B6) and one mild isolate (B2) are included as standards in each test. The B6 isolate is a severe CTV source from California (SY 568) which produces a severe reaction in Mexican lime, severe seedling yellows (SY) in sour orange and grape-

TABLE 2
BIOLOGICAL CHARACTERIZATION PARAMETERS FOR CITRUS TRISTEZA VIRUS

Host[z]	Symptoms[y]	Evaluation period[x] (months post-inoculation) 2	4	6	12
Mexican lime	VC, LC,	X	X	X	
Mexican lime	SP			X	X
Sweet/Sour	Chlor,. fl. stunt		X	X	X
Sour Orange	Chlor., stunt	X	X	X	
Duncan Grapefruit	Chlor., stunt	X	X	X	
Duncan Grapefruit	SP				X
MV sweet orange	SP, stunt				X

[z]Mexican lime is a clonal propagation on alemow, sweet/sour is Hamlin or Valencia sweet orange grafted on sour orange, and MV sweet orange is Madam Vinous sweet orange seedlings. All plants graft inoculated when stem diameter was 5-7 mm.
[y]VC = vein clearing, LC = leaf cupping, SP = stem pitting, Chlor. = chlorosis, fl = flowering.
[x]Approximate reading times. Plants cut back after inoculation and foliar symptoms read during successive flushes. Stem pitting reading made by peeling bark from main stem and branches.

fruit seedlings, severe stunting in Swt/So combinations, and a severe stem pitting (SP) in Duncan grapefruit and Madam Vinous sweet orange (7). The B2 isolate is a mild Florida source (T30a) which produces mild symptoms in Mexican lime and no discernible reaction in the other indicators (7). All plants are incubated on adjacent benches in a single glasshouse. Close regulation of temperatures in this facility, has not been possible, especially in summer, and temperatures frequently exceed 30 C for extended periods and may exceed 35 C for short periods during some summer days. From fall through early summer, however, temperatures are generally between 20 and 30 C. The house is partially shaded in summer and unshaded in the winter. Although warmer than an ideal environment (21), good symptoms of all types are reproduced by standard CTV isolates under these conditions. The use of consistent mild and severe standards provides continuity in evaluation between tests. Plants are cut back following inoculation, and new flush growth is evaluated for symptoms as indicated in Table 1. Indicator plants that remain symptomless are indexed by DAS ELISA to verify that they are infected, but symptomless. Tests are repeated at least once where possible, and whenever results are variable or uncertain. Symptom severity is rated visually on a 0 to 3 scale.

We have completed at least a partial biological evaluation on 135 isolates. All isolates have infected Mexican lime. Nine of them induced very mild symptoms similar to B2. A majority have produced typical vein clearing and leaf cupping with moderate stunting of the canopy. Twenty-one isolates have produced very severe stunting, leaf chlorosis and/or vein corking. Affected plants have thickened bark and large areas of striated fine pitting in the wood, which also is very brittle. Fifty-eight isolates have caused moderate to strong reactions in Swt/So indicators, and 66 caused moderate to strong SY reactions in sour orange (not all SY sources have been tested on Swt/So indicators). Fifty-two isolates caused moderate to strong SP in grapefruit, and 33 isolates caused moderate to strong SP in Madam Vinous sweet orange. In general, our observations of host specificity and severity have paralleled those reported by the donor or previous workers. That is, isolates reported to be mild in various localities have been mild, whereas isolates reported to induce severe SY or SP symptoms also did so in our tests. We have also confirmed prior observations that some isolates of CTV which induce severe SP in grapefruit do not induce

TABLE 3
SYMPTOM SEVERITY INDUCED BY SELECTED CITRUS TRISTEZA VIRUS ISOLATES IN FIVE INDICATORS

Isolate	Symptom severity[z]				
	Mex. Lime	Sweet/Sour	Sour	Duncan	MV
B2	0.5	0	0	0	0
B22	2.0	0	0	0	0
B162	2.0	2.5	0	1.0	0
B47	2.0	1.5	0	3.0	0
B31	2.0	0	0	3.0	1.5
B13a	1.0	3.0	2.0	1.5	1.5
B14	3.0	3.0	3.0	3.0	3.0

[z]Symptoms rated on a 0 to 3 scale from no reaction to severe. Indicators and evaluation period as indicated in Table 2.

a noticeable SY response in sour orange or an appreciable decline reaction in Swt/So (16). This variability in response is illustrated in Table 3.

ANTIGEN LIBRARY

Epitope diversity in the coat protein of different CTV isolates has been recently recognized (19). At least nine distinct epitopes have been recognized by testing various isolates with different monoclonal antibodies (Mab) (8, 11; Garnsey *et al.*, unpublished). To facilitate studies on serological properties of CTV and the development of strain specific serological probes, we are preparing freeze-dried extracts of tissue infected with the different isolates in the collection to create a library of antigens. Bark and/or leaf petiole and midrib tissue from young growth of systemically infected Madam Vinous sweet orange plants is collected, powdered in liquid nitrogen, extracted with a dispersion homogenizer in four volumes of 0.05 M Tris buffer containing 50 mg sucrose per ml, filtered and freeze dried in 1 ml aliquots. Extracts have been prepared for 185 isolates and 181 isolates have been tested against panels of different CTV Mabs. Preliminary results show that none of the Mabs tested to date react to all isolates. Some epitopes in the coat protein are highly conserved among most isolates while others are less common. The Mab CTVMCA13, which discriminates mild and decline-inducing isolates from Florida with high efficiency (20), gives a complex reaction pattern to isolates of different biological reactivity. In addition to decline-inducing isolates, CTVMCA13 reacts to some isolates which do not produce distinct effects in Swt/So, but do cause stem pitting in other hosts (for example B31 in Table 3). Aphid passage has also affected serological reactivity (12; Yokomi *et al.*, unpublished).

IN VITRO STORAGE-INFECTIOUS PREPARATIONS

Attempts have been initiated to preserve *in vitro* infectious preparations of the CTV isolates in the collection. The procedure used is similar to that previously described (9). Young tissue is collected during periods of mild temperatures, and processed as for antigen extracts, except that the tissue is not ground with a dispersion homogenizer to avoid particle shearing. Six 2.5 ml aliquots are usually prepared and freeze dried. A small aliquot is frozen for observation by SSEM. Each preparation is evaluated for infectivity by stem slash inoculation to Etrog citron or Mexican lime indicators (9) after rehydration in water.

To date, extracts of 111 isolates have been prepared. Assay for infectivity is now in progress and infectivity has been confirmed for 10 isolates. The maximum length of storage will be determined by periodic assay, but is expected to be 10 yr or more.

OTHER USES

The CTV collection is used for several other research activities in addition to those mentioned above. The dsRNA profiles associated with different isolates have been determined. A comparison of dsRNA patterns between aphid-transmitted isolates and the parent sources is now in progress. The collection is also a source of material to evaluate the specificity and efficacy of cDNA probes to identify CTV isolates. In addition to being a source of antigen for evaluation of monoclonal antibodies, selected isolates are being increased to be purified as a source of immunogen for the generation of new monoclonal or polyclonal antisera.

Several experiments have been initiated to evaluate the protective ability of different mild isolates now in the collection against the decline syndrome in sour-rooted sweet orange trees and against stem pitting in grapefruit or sweet orange. These tests cannot provide long-term information on cross protection, but the increased availability of strain-specific probes (6, 10, 19, 22) allows more rapid and quantitative evaluation of protection early after challenge inoculation. The diverse array of severe isolates in the collection also is valuable for testing the range of protection conferred by a given mild isolate and for generation of experimentally induced variants.

Several studies have also been initiated to evaluate the resistance of citrus germplasm to CTV infection. A recently released citrus breeding line, US119 (3), which carries the trifoliate orange CTV resistance gene was evaluated for resistance to selected stem-pitting isolates of CTV in the collection in addition to the standard Florida isolates. Additional studies are under way to investigate the selective resistance of some shaddock selections to certain CTV isolates.

DISCUSSION

The collection of CTV isolates assembled at BARC has provided the first opportunity to compare directly the biological properties of diverse isolates of CTV collected worldwide. This has permitted more accurate comparison of isolates from different regions than previously possible and eliminated confusion about the effect of host and environmental variables and mixed infections on symptom expression. The data for the isolates tested can be used as a reference by researchers in the area of origin to evaluate additional isolates on a relative basis. In time, it should be possible to establish the relationships of very severe isolates which have appeared in different citrus-growing areas and to determine their origin.

The collection of isolates and the associated database are constructed so that new information and new isolates can be incorporated. Some comparisons are now possible between biological properties and epitope content in the coat protein. As information becomes available from further tests with additional monoclonal antibodies, from hybridization studies with specific cDNA probes, from analysis of dsRNA species, from sequencing studies and from other methods of characterization, it will be added to the database and increase the usefulness of the collection.

Only a limited number of isolates can be maintained in container-grown plants at one time, and permanent maintenance of an extensive culture collection *in planta* is expensive and labor intensive. Based on preliminary results, it will be possible to preserve a library of CTV isolates *in vitro*. In this way the collection of virus germplasm will be permanent and only those isolates under active investigation will need to be maintained in plants.

Quarantines restrict access to viable CTV cultures for researchers in many citrus-producing areas. However, freeze-dried extracts for use as antigens and for testing other probes can be prepared in noninfectious form which is safe to use in restricted areas. Preparation of multiple aliquots of a freeze-dried preparation provides for

consistent antigen sources which are very convenient and valuable for doing comparative assays over time (such as during screening of monoclonal antibodies).

Emphasis to date has focused on establishing a useful collection, on establishing protocols for its maintenance, and on biological characterization. In the future, emphasis will shift to utilization of specific isolates for research and reference purposes. We anticipate that the collection will be an invaluable asset for new studies on cross protection and for evaluation of plants genetically engineered for coat protein-mediated cross protection (4). It has been difficult to test mild isolates against a full complement of severe isolates in many areas, including Florida.

There is still a great need for rapid and specific probes that can distinguish specific CTV isolates. Access to a diverse and well-characterized collection of CTV isolates is highly important for rapid evaluation of new probes.

We greatly appreciate the isolates and information which have been contributed. Suggestions for improving and augmenting the collection are welcome. The entire current list of isolates in the collection is not shown because it changes frequently, but a brief current catalog of the isolates in the collection will be made available to those requesting it from any of the authors.

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