# CITRUS TRISTEZA VIRUS

# Discrimination of Stem-Pitting from Other Isolates of *Citrus tristeza virus*

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ABSTRACT. Stem-pitting isolates of *Citrus tristeza virus* (CTV) are not thought to be widely distributed in commercial citrus in Florida, so prevention of their introduction and their detection is a regulatory priority. A test was needed as a supplement to the MCA13 monoclonal antibody test which could rapidly discriminate MCA13 reactive stem-pitting (SP) isolates from other MCA13 reactive, non-stem-pitting CTV isolates in field trees, and which also could replace or supplement biological indexing for stem-pitting symptoms. Three nucleic acid based and one serological technique were evaluated as diagnostic tools using isolates from the Florida and the USDA-ARS international CTV isolate collections that caused stem-pitting symptoms in citrus indicators. Sequence specific primers for amplifying CTV genome fragment PM33 and RF137 (from type II isolates of CTV) and genome fragment VT-1, and oligonucleotide probes (ONP) III, IV and V for hybridization studies gave positive results with many isolates that caused stem pitting in sweet orange, grapefruit or both. Enzyme linked immunosorbent assay (ELISA) designed for specific detection of sweet orange stem-pitting CTV and ONP II gave inconsistent results and were not tested further. For nucleic acid based assays, cDNA synthesized from total RNA extracts gave spurious results, whereas cDNA from immunocaptured virions produced clear, reproducible results. No one nucleic acid based technique was superior to the others and none could be used as a stand alone test. Therefore, test results from primers for Type II and VT-1 genome markers and probes II, IV, and V were used together to obtain stem-pitting profiles for isolates testing positive. False negatives were more common than false positives, and new primers are needed to detect isolates not identified by the current tests.

*Index words. Citrus tristeza virus*, detection, RT-PCR, hybridization, stem pitting.

*Citrus tristeza virus* (CTV) first became a concern to Florida citrus growers in the early 1950s and the State Plant Board took active steps to reduce its impact (4). By the 1970s the incidence of CTV in field trees had increased from lower levels to 100% in certain monitored groves, but this did not coincide with an increase in decline disease on sour orange rootstock, which was the economically damaging disease caused by "severe" isolates. Prior to the development of the monoclonal antibody MCA13, only bioindexing on citrus indicators could distinguish "mild" isolates from "severe" isolates (15). The detection of the brown citrus aphid (*Toxoptera citricida*) in Florida in 1995 (9) raised concerns of increased spread and severity of CTV strains as reported elsewhere (16). By 1998, increased loss of trees on sour orange rootstock to decline strains of CTV was documented in southwest Florida (18), and attributed to increased spread of severe CTV by the brown citrus aphid.

A Florida CTV collection has been assembled over the past 40 yr by Grant, Cohen and Garnsey with contributions from surveys of commercial and dooryard citrus taken throughout the state from 1965 to 1998 (12). This collection contained mainly "mild" (T30), and "severe" isolates comprised mostly of T36 with smaller percentages of T3 and VT, and was considered to be repre-

sentative of isolates occurring in the field (12). Many budwood source trees are grown in unprotected, outdoor plots and are likely to become infected with CTV. Although stem-pitting strains of CTV (SP-CTV) are thought not to be widespread in Florida, their introduction and dissemination are a threat to these trees. Budwood sources in Florida are screened with MCA13 monoclonal antibody (15) and trees testing positive with MCA13 cannot be propagated. MCA13 identifies the majority of the stem-pitting isolates of CTV, but does not distinguish isolates that cause only decline from isolates that also may cause stem-pitting, creating a need for more selective probes. The current goal of the Florida Citrus Budwood Registration Program is to restrict the spread of stem-pitting isolates of CTV in budwood source trees with effective detection methods and with the long term goal of placing CTV-free budwood source trees under protective screening.

Several techniques have been described as specific for identification of stem-pitting isolates of CTV, including 1) ELISA for selective detection of isolates causing stem pitting in sweet orange (14); 2) RT-PCR for Type II sequence isolates causing stem pitting in sweet orange and/or grapefruit (1); 3) Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) with sequence specific primers for isolates with the VT (12) and T3 genotypes; and 4) RT-PCR amplification of the coat protein gene (11) followed by hybridization with oligonucleotide probes (10). These techniques have not been evaluated sufficiently to be used in a regulatory program. The objectives of this study were to evaluate these techniques for their ability to discriminate stem-pitting isolates from other isolates of CTV and their suitability for use as diagnostic tools for field trees. These techniques were evaluated initially with a standardized panel of CTV isolates, and promising techniques were then used to evaluate a larger and more diverse panel of isolates from the United States Department of Agriculture, Agricultural Research Service (USDA, ARS) International and the Florida CTV isolate collections.

## MATERIALS AND METHODS

**Virus isolates and tissue collection.** The Florida CTV isolate collection was maintained in citrus plants in a greenhouse at the Citrus Budwood Registration Bureau in Winter Haven, Florida. The International CTV collection was maintained at the Citrus Quarantine Facility in Beltsville, MD (7). Young bark tissue was stripped and cut into 2 mm lengths and approximately 1 g placed in a "00 White Wove" coin envelope. Twenty envelopes were placed into a quart plastic freezer bag with the bottom third filled with 8 mesh Drierite (Hammond Drierite Company, Xenia, OH, USA). Tissue was desiccated at 4°C and stored dry at -20°C until assayed.

**Orange stem-pitting ELISA.** Orange stem-pitting ELISA (OSP ELISA) was performed as previously published (14). Four attempts were made to get the technique to work. The first time, the dilution of secondary antibodies (R109) used was 1:15,000, tissue was hydrated in buffer at 4°C for 48 hr instead of 12-16 hr prior to extraction, and was ground for 5 min in the Mini-beadbeater-8 (BioSpec Products, Inc., Bartlesville, OK), instead of a non-specified time in the Kleco Tissue Pulverizer (Kinetic Laboratory Equipment Company, Visalia, CA). Goat anti-rabbit mAb-conjugate with alkaline phosphatase, Sigma A2306, was substituted for Sigma A3687 (Sigma-Aldrich, Inc., St. Louis, MO, USA) which was no longer available. The second time, the secondary antibody (R109) concentration was increased to 1:5,000. The third time, the original grinding

technique in the Kleco Tissue Pulverizer (40 sec) was compared to 5 min grinding in the Mini-beadbeater-8. The fourth time, Kleco extraction was used, with no other changes except to increase the number of isolates tested.

**Nucleic acid extraction and virion immunocapture for RT-PCR.** For RNA extraction, Qiagen RNeasy Plant Kits (Qiagen, Inc., Valencia, CA, USA) were used. Approximately 0.1 g of plant tissue was homogenized in the Mini-beadbeater-8 (Bio-Spec Products) in 1.5 ml of Guanidinium Thiocyanate Buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% *beta*-mercaptoethanol). Following homogenization, 0.5 ml of plant sap was transferred to a QIA shredder column and RNA was subsequently extracted according to the manufacturer's protocol (Qiagen, Inc.).

Immunocapture-RT-PCR (IC-RT-PCR) was performed by homogenizing 0.1 g of dried plant tissue in 1 ml (or 0.5 g fresh tissue in 5 ml) of PBS-5% Sucrose Buffer (0.15 M sodium phosphate buffer, pH 7.4) for 45 sec in the Mini-beadbeater-96 (BioSpec Products, Inc., Bartlesville OK, USA) (13). Following homogenization, plant tissue was hydrated at 4°C for 20 min and then mixed prior to an additional 45 sec homogenization. Samples were then centrifuged at 10,000 rpm for 1 min. An additional 0.5 ml of PBS-5% Sucrose Buffer was then added to each tube and shaken vigorously. Plant sap was centrifuged at 10,000 rpm for 5 min to pellet plant debris, and 0.5 ml of the resulting supernatant was then transferred to a new 1.5 ml tube for immunocapture. Immunocapture was performed as previously described (13) using magnetic beads coated with polyclonal antiserum UF 1052.

**RT-PCR.** Initial testing with Qiagen extracts utilized a two-step RT-PCR method according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). In negative samples extracted with Qiagen reagents, VT-1 and Type II primers yielded extraneous bands and faint bands of a molecular weight similar to those bands obtained from positive samples. With Type II primers the extraneous bands were eliminated by optimizing $MgCl_2$ concentration (2.0 mM) and increasing annealing temperature by 5°C to 60°C. With VT-1 primers, changing the $MgCl_2$ concentration and/or annealing temperature had no impact. The problem of the lack of specificity was eliminated for all primers by using immunocaptured product instead of a Qiagen extraction. Extraneous bands were eliminated from the products of T3-2 by reducing the number of cycles from 35 to 25 cycles.

**IC-RT-PCR with Type II primers.** First strand cDNA was synthesized from immunocaptured virions as previously described (12). Amplification was in a 25 µl reaction containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 U of Taq polymerase (Applied Biosystems) and 0.6 µM of each primer (Table 1) to which was added 5 µl of cDNA template from IC-RT. The PCR cycling profile was an initial denaturation at 94°C for 1 min, 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension of 2 min at 72°C. PCR products were analyzed in 1.5% agarose gels in 0.5× TBE buffer.

**IC-RT-PCR with Sequence Specific primers.** First strand cDNA was prepared from immunocaptured virions as previously described (13). Products were amplified in a 25 µl reaction using the same concentrations listed above. PCR parameters for VT-1 (Table 1) were 35 cycles of 30 sec at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR cycling profile for T3-2 (Table 1) is the same as for VT-1 except for a reduction in the number of cycles to 25.

**Hybridization with oligonucleotide probes (ONP).** Oligonu-

TABLE 1
PRIMER SEQUENCES FOR T36 COAT PROTEIN, TYPE II, VT-1 AND T3-2

| Sequence Specific Primers | | | |
|---|---|---|---|
| Primer | Strand | Sequence | Expected product size |
| VT-1 (12)[1] | (+) | gta ccc tcc gga aat cac g | 564 bp |
| | (-) | ggt agg gtc tac tcg ttt cat | |
| T3-2 | (+) | gtg ttg agg tcc cga gcg tc | 652 bp |
| | (-) | gat cga gac ggt tta gag atg | |
| T36CP (11) | (+) | atg gac gac gaa aca aag aaa tg | 672 bp |
| | (-) | tca acg tgt gtt gaa ttt ccc a | |
| Type II Primers | | | |
| PM33 (1) | (+) | ccc gta ccc tcc gga aat cac g | 266 bp |
| RF137 (1) | (-) | ccg tar agg gac lat cgg c | 266 bp |

[1]Numbers in parentheses refer to indicated literature citation.

cleotide probes I through VI (10) were labeled with digoxygenin (DIG) with the 3' End Labeling Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. CTV coat protein genes amplified with T36CP primers (Table 1) were targets for hybridization with the oligonucleotide probes. cDNA template from IC-RT reactions was amplified in a 100 µl reaction containing 1× reaction buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of Taq polymerase and 0.2 µM T36CP primers (Promega Corp., Madison, WI, USA). Products were amplified according to the same parameters for VT-1 listed above. Five microliters of each product and 5 µl of a DNA ladder molecular weight marker (Promega Corp., Madison WI) were run on 1.5% agarose gels in 0.5× TBE Buffer, and the resulting bands were analyzed with the Spot Denso function of the Alpha Imager 5500 (Alpha Innotech Corp., San Leandro, CA, USA) to approximate the concentration of final products. If the concentration was greater than 60 ng in 15 µl of PCR product, the 15 µl of product was mixed with 30 µl of 10× SSC and 5 µl of 0.05% Bromophenol Blue and loaded onto Zeta Probe GT membranes using a BioDot Apparatus (Biorad Laboratories, Hercules CA, USA). After samples were loaded, DNA was denatured in 100 ml 0.4M NaOH for 10 min followed by two five min washes in distilled water. The membrane was neutralized in 100 ml of neutralization buffer (0.2 M Tris-HCl, pH 8.0, 1× SSC, 1% SDS) for 10 min. DNA was fixed to the membrane with the Stratalinker (Stratagene, La Jolla, CA, USA) using the auto-crosslink function. Membranes were air-dried and stored at room temperature prior to hybridization.

Membranes were prehybridized at 37°C for 1 hr in 10 ml of prehybridization solution (5× SSC, 5× Denhardt's solution, 0.01 M $NaPO_4$, pH 6.8, 1 mM EDTA, 1% SDS, 0.1 mM ATP and Salmon Sperm DNA at 0.2 mg/ml). Probes were added to the prehybridization solution at a final concentration of 100 ng/ml and hybridized at 37°C for 1 hr. Following hybridization, membranes were washed at room temperature in 60 ml of 6× SSC for 10 min. For probes I, III, IV and VI, membranes were washed twice in 15 ml of 4× SSC, 1% SDS for 10 min at 45°C. The wash stringency was increased for probe V by washing membranes once in 15 ml of 4× SDS, 1% SDS at 50°C for 10 min and once at 55°C for 10 min. Immediately following washes, membranes were visualized using Chemiglow West Chemiluminescent Substrate (Alpha Innotech, San Leandro, CA, USA) according to the

manufacture's specifications. Images were recorded with the Alpha Fluorochem Imager 5500 CCD camera using movie mode to optimize exposure time. Membranes were used for a single hybridization.

## RESULTS

**Orange Stem-Pitting ELISA.** After four attempts, we were unable to obtain reproducible results with designated positive controls using the published or slightly modified protocols, so this procedure was not considered further.

**Characterization of isolates with amplified markers and hybridization probes.** All probes hybridized to and gave clear positive results for the source isolates from which they were developed (5). The T36CP primers were used to produce the cDNA for blotting, since these primers performed consistently and produced higher amounts of product compared to the CN 119, 120 primer pair (data not shown). This eliminated the need to repeat amplifications.

Molecular profiles were obtained for 245 isolates from the International CTV collection (Table 2) and

TABLE 2
MOLECULAR MARKER PROFILES, MCA13 STATUS AND STEM-PITTING SYMPTOMS FOR INTERNATIONAL CTV ISOLATES

| Profile | VT-1[a] | Type II[b] | T3-2[c] | ONP III[d] | ONP IV[e] | ONP V[f] | MCA13 | Isolates w/profile | OSP only | GSP only | OSP+ GSP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | + | - | - | - | - | - | 7 | 0 (7) | 7 (7) | 0 (7) |
| 1 | - | + | - | - | - | - | + | 1 | 0 (1) | 0 (1) | 1 (1) |
| 2 | + | + | - | + | - | - | + | 42 | 15 (42) | 14 (42) | 13 (42) |
| 3 | - | + | - | - | - | + | + | 2 | 0 (2) | 1 (2) | 1 (2) |
| 4 | - | + | - | + | - | - | + | 4 | 1 (4) | 2 (4) | 1 (4) |
| 5 | + | + | - | + | - | + | + | 9 | 4 (9) | 3 (9) | 2 (9) |
| 6 | - | + | - | + | + | - | + | 1 | 0 (1) | 0 (1) | 1 (1) |
| 8 | - | - | - | - | - | + | + | 6 | 0 (6) | 6 (6) | 0 (6) |
| 9 | + | + | + | + | + | - | + | 1 | 1 (1) | 0 (1) | 0 (1) |
| 10 | + | + | - | - | - | - | + | 2 | 0 (2) | 0 (2) | 2 (2) |
| 11 | + | + | - | - | + | + | + | 1 | 0 (1) | 1 (1) | 0 (1) |
| 12 | + | + | - | - | + | - | + | 6 | 2 (6) | 2 (6) | 2 (6) |
| 14 | + | + | - | + | + | + | + | 6 | 3 (6) | 0 (6) | 3 (6) |
| 15 | - | - | - | + | - | - | + | 5 | 1 (5) | 2 (5) | 2 (5) |
| 16 | + | + | - | + | + | - | + | 4 | 1 (4) | 0 (4) | 3 (4) |
| 17 | + | - | - | + | - | + | + | 2 | 1 (2) | 0 (2) | 1 (2) |
| 18 | + | + | + | + | + | + | + | 2 | 0 (2) | 0 (2) | 2 (2) |
| 19 | + | - | - | + | - | - | + | 11 | 2 (11) | 0 (11) | 9 (11) |
| 20 | + | + | - | - | - | + | + | 5 | 0 (5) | 3 (5) | 2 (5) |
| 21 | + | + | + | + | - | - | + | 1 | 1 (1) | 0 (1) | 0 (1) |
| 22 | - | + | - | + | + | + | + | 1 | 0 (1) | 0 (1) | 1 (1) |
| 23 | - | - | - | - | + | + | + | 2 | 2 (2) | 0 (2) | 0 (2) |
| 24 | + | - | - | + | + | + | + | 4 | 1 (4) | 0 (4) | 3 (4) |
| 25 | - | + | - | - | + | - | + | 2 | 0 (2) | 0 (2) | 2 (2) |

[a]The VT genotype is identified by VT-1 primers.
[b]Type II CTV isolates contain stem-pitting isolates and give a positive reaction with primers PM33 and RF137.
[c]The T3 genotype is identified by T3-2 primers.
[d]ONP III is a hybridization technique using probe Oligonucleotide III to hybridize with product amplified with primers to T36 coat protein gene.
[e]ONP IV is a hybridization technique using probe Oligonucleotide IV to hybridize with product amplified with primers to T36 coat protein gene.
[f]ONP V is a hybridization technique using probe Oligonucleotide V to hybridize with product amplified with primers to T36 coat protein gene.

100 isolates of CTV from the Florida collection (Table 3) for the indicated molecular markers. With ONP II, results were inconsistent as background varied with repeated testing and could not be eliminated with changes in the stringency of the washes. Because of the inconsistency of the results obtained with ONP II on field samples, it was not used in further testing. The other oligonucleotide probes, ONP III, ONP IV and ONP V, gave clear positive and negative results for field as well as greenhouse samples and were used for further testing.

A profile was generated for each isolate by combining the results obtained with each test (Tables 2 and 3). Isolates that tested negative for all the tests were assigned a profile of 0. For Florida isolates with a profile of 0, 30 were also negative for stem-pitting in both sweet orange and Duncan grapefruit indicators (8). There was a consistent pattern for these Florida isolates. If they tested negative for MCA13, they were also positive for ONP VI, indicating the presence of the T30 genotype. If they tested positive for MCA13, they also tested positive for ONP I, indicating the presence of the T36 genotype. This specific correlation did not hold for 28 isolates from the International CTV isolate collection with a profile of 0. Of these, 14 isolates tested MCA13 negative[1] and only B276, B296, and B339 tested positive for T30 by ONP VI. Of the 14 isolates that tested MCA13 positive,[2] only B181, B209, B271, and B359 tested positive for the T36 genotype with ONP I.

The 137 international isolates that tested positive for stem pitting by the laboratory tests (Table 2) also caused stem-pitting symptoms in the Duncan and sweet orange indicators, including a porous wood pitting, a severe form of stem pitting (6). Except for 7 and 13, all profiles were represented in this group.

Nineteen Florida isolates tested positive for stem pitting in laboratory tests and all caused stem pitting in grapefruit with five also causing stem pitting in sweet orange (Table 3). None of these caused stem pitting only in sweet orange. Two tested negative for MCA13 but caused mild stem pitting on grapefruit indicators. Only eight of the 25 profiles (Table 3) found to date are represented in Florida, with profile 5 predominant in the Florida collection. Type II primers amplified a product from 17 of the 18 isolates.

Seventeen CTV isolates from the Florida collection were negative for all stem-pitting markers, but devel-

[1]B5, B35, B190, B213, B276, B296, B336, B339, B349, B354, B356, B366, B402, B403

[2]B4, B151, B158, B159, B160, B181, B209, B271, B294, B301, B337, B344, B359, B364

TABLE 3
MOLECULAR MARKER PROFILES, MCA13 STATUS AND STEM-PITTING SYMPTOMS FOR FLORIDA CTV ISOLATES

| Profile | VT-1 | Type II | T3-2 | ONP III | ONP IV | ONP V | MCA13 | Isolates w/profile | OSP only | GSP only | OSP+ GSP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | + | - | - | - | - | + | 2 | 0 (2) | 2 (2) | 0 (2) |
| 1 | - | + | - | - | - | - | - | 1 | 0 (1) | 1 (1) | 0 (1) |
| 2 | + | + | - | + | - | - | + | 1 | 0 (1) | 0 (1) | 1 (1) |
| 3 | - | + | - | - | - | + | + | 2 | 0 (2) | 2 (2) | 0 (2) |
| 4 | - | + | - | + | - | - | + | 2 | 0 (2) | 1 (2) | 1 (2) |
| 5 | + | + | - | + | - | + | + | 6 | 0 (6) | 5 (6) | 1 (6) |
| 6 | - | + | - | + | + | - | + | 2 | 0 (2) | 0 (2) | 2 (2) |
| 10 | + | + | - | - | - | - | + | 1 | 0 (1) | 1 (1) | 0 (1) |
| 15 | - | - | - | + | - | - | + | 1 | 0 (1) | 1 (1) | 0 (1) |
| 17 | + | - | - | + | - | + | + | 1 | 0 (1) | 1 (1) | 0 (1) |

oped stem pitting in Duncan grapefruit or sweet orange indicators. Two isolates, FL272 and FL 302, were negative for MCA13 but also were negative for both the T30 and T36 genotypes as well. Twelve MCA13 negative isolates were the T30 genotype,[3] while twelve isolates that were MCA13 positive were the T-36 genotype.[4] Twenty-eight CTV isolates from the International CTV Collection were positive for stem pitting in grapefruit indicators, and in some cases also in sweet orange indicators,[5] but were negative for stem-pitting markers.[6]

Isolates positive for stem-pitting markers, but negative for stem pitting in biological indicators were present in both the Florida and International CTV collections (Table 4). Profiles 7 and 13 were represented only among these isolates and isolates with these profiles have the T3 genotype (positive for T3-2 marker). Twenty-four isolates which indexed negative for stem-pitting had profile 1 or 2.

## DISCUSSION

OSP ELISA did not give accurate, repeatable results, and was eliminated from further evaluation after extensive testing. Interpretation of the OSP ELISA results by using a cutoff value of two times the OD reading of the healthy control (14) or ten times the OD reading of

[3]FL149, FL204, FL209, FL217, FL224, FL237, FL278, FL245, FL251, FL267, FL298 and T4

[4]FL86, FL104, FL145, FL154, FL165, FL169, FL173, FL188, FS329, FS577-1, and FS651 (FL184)

[5]B275, B372, B373, and B376

[6]B67, B73, B83, B162, B180, B182, B183, B186, B187, B188 B275, B287, B297, B303, B310, B335, B338, B353, B355, B362, B367, B368, B372, B373, B376, B391, B392, and B399

TABLE 4
PROFILES OF ISOLATES POSITIVE FOR STEM-PITTING IN LABORATORY TESTS, BUT NEGATIVE BY BIOLOGICAL INDEXING IN SWEET ORANGE AND DUNCAN GRAPEFRUIT INDICATORS

| Profile | VT-1 | Type II | T3-2 | ONP III | ONP IV | ONP V | MCA13 | ONP I (T36) | Isolates |
|---|---|---|---|---|---|---|---|---|---|
| 1 | - | + | - | - | - | - | - or ND[a] | - | B50-1, B214, B272, B351, B386, T69-1 |
| 1 | - | + | - | - | - | - | + | - | B29, B30-1, B30-2, B30-2-1 |
| 2 | + | + | - | + | - | - | + | + | FL187, T67-1 |
| 2 | + | + | - | + | - | - | + | - or ND | B22-1, B25, B66, B119, B120, B152, B198-1, B199-1, B200, B204, B211, B229 |
| 4 | - | + | - | + | - | - | + | - | B10-3, B12-1, B65-2, B192 |
| 4 | - | + | - | + | - | - | + | + | FL53 |
| 5 | + | + | - | + | - | + | + | + | FL192, FL207 |
| 5 | + | + | - | + | - | + | + | - | B10, B22, B24, B131 |
| 6 | - | + | - | + | + | - | + | - | B228 |
| 7 | - | + | + | + | + | - | + | - | T3 |
| 10 | + | + | - | - | - | - | + | + | B340 |
| 11 | + | + | - | - | + | + | + | ND | B76 |
| 13 | - | + | + | - | + | - | + | - | B148 |
| 16 | + | + | - | + | + | - | + | - | B422 |
| 25 | - | + | - | - | + | - | + | - | B222 |

[a]ND = No data.

T36 (10) did not allow for differentiation of stem-pitting isolates from other CTV isolates. The inability to discriminate stem pitting from mild and decline CTV isolates may be attributed to not using the antibody combination of R109/3E10 (3E10 was unavailable), which seemed to produce stronger results (14). Using a different conjugate antibody, as the original conjugate antibody used was unavailable, also may have contributed to these results.

No single stem-pitting marker was associated with all isolates that caused these symptoms. An isolate was considered to be positive for stem-pitting marker(s) if any of those tests were positive. All test results were combined to create profiles identified by specific numbers. ONP III reacted with more stem-pitting isolates than the Type II and VT-1 primers, which also identified a large number stem-pitting isolates. Marker T3-2 was useful to distinguish Florida isolates with the T3 genotype that test positive for stem-pitting markers but do not cause stem pitting in either sweet orange or grapefruit seedling indicators. The oligonucleotide probes were useful for developing the profiles, particularly for profiles 8 and 15, as isolates in these profiles tested negative for both the Type II and VT-1 primers. But a positive reaction with a specific probe does not necessarily indicate an isolate will cause a particular type of host reaction. Previously, ONP III and V were reported as identifying isolates causing stem pitting in both sweet orange and grapefruit, and ONP IV was reported as identifying isolates causing stem pitting only in sweet orange (10). Our results showed that ONP III identified isolates that caused stem pitting in both sweet orange and grapefruit indicators, but also identified isolates which cause stem-pitting in grapefruit indicators only, or caused no stem pitting in the indicators tested. Probe ONP IV detected isolates causing stem pitting in both sweet orange and grapefruit indicators, while ONP V did detect isolates with stem pitting in both indicators, but also identified those causing pitting in grapefruit only or those that were symptomless.

Biological indexing is an essential component of any program to evaluate CTV isolates for their ability to cause stem pitting. In Florida, the main citrus crops are sweet orange and grapefruit, so biological indexing was carried out in Madam Vinous sweet orange and Duncan grapefruit seedling indicators. Where other types of citrus are of economic importance, biological indexing in a sensitive variety of those types should be carried out. Correlation of the stem-pitting reaction in biological indicators with stem-pitting markers was not perfect. Verification of a positive result with biological indexing was considered important to identify stem-pitting isolates. Isolates testing positive for stem pitting markers but which show no stem pitting, may either just be an isolate such as T3 that does not express stem pitting, or the expression of stem pitting may be masked by other isolates (2). Broadbent et al. (3) discovered that sub-isolates obtained from single aphid transmission using field isolates did not always have the same biological characteristics as the original isolates. B192 is an example of an isolate that did not show stem pitting in indicators inoculated with the original field isolate, but which yielded some of the aphid transmitted sub-isolates which did (2). This shows the danger of assuming that an isolate reacting to stem pitting markers, but that does not cause stem pitting in biological indicators, is a single strain of CTV. Mixtures of isolates in the International CTV collection have been documented (20). Through aphid transmission, an isolate of CTV causing stem-pitting was recovered from T66 (19), a Florida isolate that does not cause stem pitting and reacts negatively to the stem-pitting

markers. It would be ideal to know if the isolates that test positive for stem-pitting markers, but do not cause stem pitting in sweet orange and grapefruit indicators, do contain "hidden" isolates with the capability of causing stem pitting.

Also of concern are the isolates that cause stem pitting in biological indicators, yet test negative for the presence of stem-pitting markers. Trees testing MCA13 positive are disqualified from use as a budwood source in the Florida Budwood Registration Program (17). Most of those isolates testing negative for MCA13 were the T30 genotype, but a few were not. It is important to develop markers to identify these isolates that cause stem pitting but are not identified by any of the existing markers. It is also possible that isolates are mixtures and the component causing the stem pitting may not be the component detected by either MCA13 (simultaneous presence of T36 isolates) or by the stem-pitting markers. Newly developed markers should be tested for their ability to detect stem-pitting isolates using a large collection such as the USDA, ARS International CTV collection, as results on a less diverse number of samples may be misleading.

Initially, we had hoped it would be possible to find a single test that could identify isolates as potentially causing stem pitting symptoms. The results indicated that a profile composed of all test results was more reliably associated with potentially harmful symptoms than any single marker. Although we did obtain both false positives and false negatives, 75% of the isolates were correctly identified as causing or not causing stem-pitting with the markers. These stem-pitting markers would not be useful in a budwood program where the obvious solution is to use only screen-protected budwood sources that test negative for all isolates of CTV. However, they have been useful for identifying isolates for further study, particularly those with false positive or false negative results. These techniques will prove valuable as a first step in field survey and diagnosis as many samples can be run quickly.

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