Use of the Coat Protein (CP) and Minor CP Intergene Sequence to Discriminate Severe Strains of *Citrus tristeza virus* (CTV) in three U.S. CTV Isolate Collections

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ABSTRACT. A rapid assay is needed to distinguish potentially mild vs. severe strains of *Citrus tristeza virus* (CTV). Multiple alignment performed on the coat protein (CP) and the minor coat protein (CPm) intergene sequences (~91-99bp) from different CTV isolates revealed that severe strains generally associated with orange stem pitting (OSP), grapefruit stem pitting (GSP) and seedling yellows (SY) share a conserved sequence which is absent in mild (T30-like genotype) and decline isolates (T36-like genotype). Two assays were developed to differentiate such isolates: i) multiplex one-step real-time RT-PCR; ii) restriction fragment length polymorphism (RFLP) analysis. The real-time assay consisted of broad spectrum detection using a universal primers/Taqman probe (Cy5-labeled) and a primers/MGB-TaqMan probe (FAM-labeled) specific for VT and T3 genotypes of CTV. The multiplex real-time assay simultaneously detected of all CTV isolates and differentiated VT and T3 genotypes in our tests. The RFLP assay is based on primers which amplified a target sequence containing unique restriction D*de*I or S*sp*I sites. The D*de*I restriction site was conserved among VT and T3 genotypes and absent in T30 and T36 genotypes. In contrast, the S*sp*I restriction site was conserved in mild isolates and absent in the severe strains. Both assays were validated with a panel of local and international CTV isolates from different CTV collections and using natural or artificial combined infections. Both new assays differentiated between genetically and biologically different CTV strains.

Index words. Detection, stem pitting, seedling yellows, multiplex qPCR, RFLP, enzyme restriction sites

Citrus tristeza virus (CTV), the causal agent of tristeza disease, is known to have great genetic and biological diversity among strains (2,10,17,18,30). Mild isolates cause only mild or no symptoms in sensitive citrus hosts and usually result in no economic damage. Decline isolates cause a stock/scion incompatibility on sour orange rootstock and is readily overcome by use of tolerant or resistant rootstocks. Severe isolates cause stem pitting (SP) and seedling yellows (SY) but symptoms vary in intensity with hosts and isolates.

CTV-SP is debilitating disease that affects grapefruit and sweet orange causing abnormalities in phloem development which result in tree stunting, reduced vigor, and losses in fruit size, quality, and yield (23). Seedling yellows (SY) reaction is generally associated with isolates which induce yellowing and stunting in sour orange, lemon and grapefruit seedlings. Although SY is a reaction obtained in a biological index, it can be presumptive evidence for the presence of severe isolates. The impact on growth is likely complicated by interactions of different strains. For example, Garnsey et al. (10) reported that some severely stunted grapefruit plants infected with CTV-SY isolates show two different thickened bark syndromes (TBS): woody bristle (TBS-WB) and porous wood pitting (TBS-PWP). The latter was considered a severe form of SP and that a strong SY reaction may mask stem pitting symptoms (10).

Bioindexing using grapefruit and Madam Vinous sweet orange seedling is commonly used to detect grapefruit (GSP) and sweet orange (OSP) isolates, while SY-inducing isolates are commonly identified using sour orange, grapefruit and lemon seedlings (10). Biological characterization is the accepted standard procedure used to define biological activity of CTV isolates but is laborious, time consuming and expensive.

Several approaches have been developed for specific identification of severe CTV strains (19): (i) ELISA tests using monoclonal antibodies (MAbs) for the detection of decline or stem-pitting isolates (20,21); (ii) reverse transcription (RT)-polymerase chain reaction (PCR) assay with selective primers (9,14,16); (iii) hybridization with specific probes (8,12).

Since most severe isolates react to MAb MCA13 (21), it has been used to detect severe CTV isolates in Florida, Caribbean Basin and Costa Rica (11). However, MCA13 does not distinguish between isolates that induce stem pitting or only decline. Furthermore, failure to detect some severe isolates or finding no correlation between MCA13 reactivity and quick decline symptoms has been reported (22). A need exists, therefore, for more selective probes (29).

The purpose of this research was to develop a simple, sensitive and reliable test for simultaneous detection and discrimination of potentially SP and SY CTV isolates. Based on the multiple alignment of the nucleotide sequence of the gRNA portion including the minor coat protein (CPm) and the major coat protein (CP), the intergene sequence between these regions were used to develop two independent assays to detect known CTV isolates and to differentiate those inducing seedling yellows or stem-pitting reactions: (i) a multiplex real-time RT-PCR assay; and (ii) restriction fragment length polymorphism analysis (RFLP).

MATERIAL AND METHODS

Virus isolates. Madam Vinous seedlings infected with CTV isolates SY568, P81 (AY995567), T36, T3, T68, B192at (7), T318 (1) were used to develop and optimize the assays. The assays were validated against a panel of isolates maintained at USDA, ARS, Parlier, CA (P); Central California Tristeza Eradication Agency (CCTEA), Tulare, CA; and the Exotic Citrus Pathogen Collection (ECPC), USDA, ARS – Beltsville, MA (B) (Table 1). ECPC isolates were from 19 countries. Samples consisted of desiccated (ECPC samples) or fresh leaf petioles (Parlier samples) or bark tissue (CCTEA samples).

Isolation of total RNA. The extractions of total RNA from CTV-infected samples and healthy controls were performed using 100-150 mg of tissues as reported by Saponari et al. (27).

Multiplex real-time RT-PCR assay using TaqMan probes. *Primers and probe design***.** For broad spectrum CTV detection the primers and the Cy5-labeled-probe were used as previously described (27). A primer set and a 6- carboxyfluorescein (FAM)-labeled minor groove binding (MGB) TaqMan probe (CPi-VT3) selective for VT and T3 genotypes, generally associated with CTV-SP and -SY isolates, were also designed. These primers and probes were designed using Primer Express 3 software (Applied Biosystem) and manually adjusted based on the multiple alignment of the genomic sequences comprised between the minor and the major coat proteins (CPm and CP)

TABLE 1
ORIGIN AND DESCRIPTION OF CTV ISOLATES USED IN THE TESTS

Isolate	Description	Origin
	Exotic citrus pathogen collection, USDA, ARS, Beltsville, MD	
B3	Quick decline isolate, also mild seedling yellows (SY)	Florida
B7	Nartia isolate, relatively mild. Used for cross protection, contains a mixture of T30 and VT genotypes (16)	South Africa
B8	Severe grapefruit step pitting (GSP) isolate	
B46	GSP isolate	
B58	Sweet orange stem pitting (OSP) isolate	
B22	Strong reaction in Mexican lime, no SY or decline reaction	Israel
B23	SY isolate	
B24	SY isolate	
B25	Strong reaction on Mexican lime, no SY	
B79	VT genotype (16) used for cross protection trials	
B199	Severe decline isolate, strong Mexican lime reaction and SY	
B29	Mild cross-protective isolate	Japan
B30	Severe SY isolate	
B31	Severe GSP isolate	
B185	Strong reaction in Mexican lime and SY on sour orange and grapefruit	
B188	Moderate GSP reaction	
B119	T30 genotype Natural CTV isolate in cross-protection experiment	Hawaii
B131	OSP isolate	Colombia
B165	Severe in Mexican lime, SY in lemon, VT genotype. Complete genome sequence: GenBank accession n. EU076703.	India
B166	SY in sour orange	
B195	Stem-pitting isolate in Coorg and Cleopatra mandarin	
B219	Aphid transmitted isolate inducing strong SY in grapefruit	
B37	Severe decline and SY isolate (Garnsey, unpublished data)	China
B71	OSP isolate	
B83	T36-like genotype (16), mild in biocharacterization assay (Garnsey, unpublished data)	
B65	Field source has stem pitting in trunk	
B211	Collected from vigorous tree. T3 genotype (16) and SY in biocharacterization (Garnsey, unpublished data).	
B252	Mild isolate from Ponkan tree	
B270	Mandarin stem pitting isolate	
B316	Collected as apparently mild isolate but causes SY, OSP and GSP in biocharacterization assay (Garnsey, unpublished data)	
B248	Strong Mexican lime reaction, moderate SY (Garnsey, unpublished data)	Venezuela
B255	Severe OSP isolate	Indonesia
B32	Typical Spanish mild isolate	Spain
B158	From a decline tree, mild in biocharacterization assay (Garnsey, unpublished data)	
B405	T318, OSP and SY isolate	
B271	T30 genotype (16)	Costa Rica
B277	Mild isolate from Valencia on sour orange rootstock	Belize
B305	Mild isolate in biocharacterization assay (Garnsey, unpublished data)	Cuba
B314	Mild GSP isolate	
B391	From declining tree on sour orange rootstock	Puerto Rico
B192	Mild isolate with no reaction in Mexican lime	Corsica
B337	Mild isolate	Turkey
B340	Severe strain from Tahiti lime	Trinidad
B339	Mild isolate	
B77	Stem pitting isolate	Brazil
B152	Californian SY (SY 576). VT genotype (16)	California
B334	Field tree in early stage of quick decline	

	Central California Tristeza Eradication Agency (CCTEA), Tulare, CA	
32 CCTEA isolates	**(See table 4)**	Central California
	USDA, ARS, Parlier, CA	
P1, P51, P100, P21, P25, P28, P35, P36, P43	Mild isolates on sweet orange	California
P108-35at	Mild GSP isolate	
P108-39at	SP isolate with VT genotype	
P108-1Bat, P108-7at, P109-2at	Severe SY isolate	
P108	OSP isolate	
P109	OSP and SY isolate	
SY553	Meyer lemon isolate	

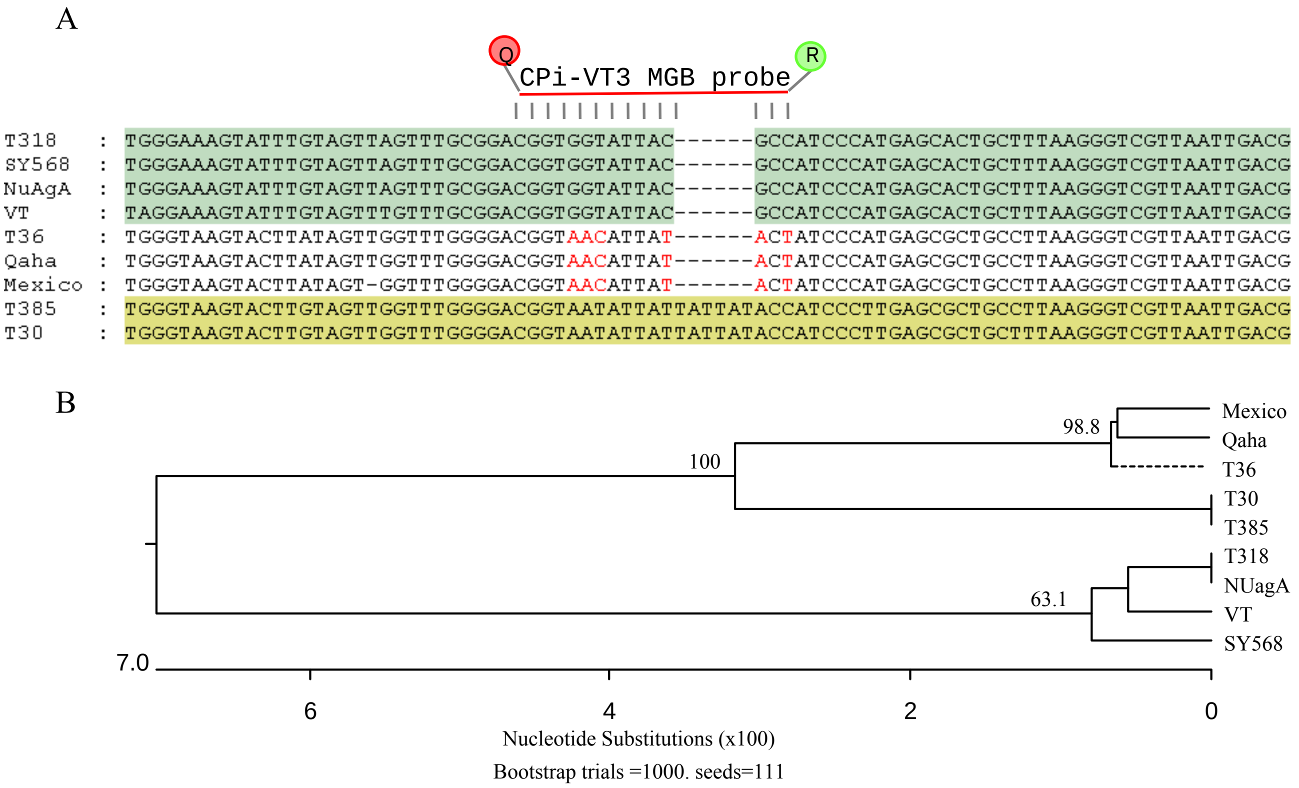

Fig. 1. Minor coat protein (CPm) and CP intergene sequences. (A) Multiple alignment showing the location of the CPi-VT3 MGB-probe used for the selective detection of VT and T3 genotypes, and relative dendogram (B) constructed using MegAlign software (Lasergene 7, DNASTAR).

TABLE 2
PRIMERS USED FOR THE REAL TIME RT-PCR ASSAY AND FOR THE AMPLIFICATION OF THE FRAGMENT SUBJECTED TO RESTRICTION FRAGMENT ANALYSIS (RFLP)

Primer	Sequence (5'-3')	Position[1]	Expected amplicon size[1]
Multiplex real-time RT-PCR			
P27R	GACCCTTAAAGCAGTGCTCA	16,049 -16,068	78
P27F	TACGYGATTTGGGWAAGTAYTTDTA	15,990 -16,014	
FAM-S	6-FAM-ACG GKG RTA TTR CGC -NFQ	16,028-16,042	-
CP25R	TCRGTCCAAAGTTTGTCAGA	16,449-16,468	101
CP25F	AGCRGTTAAGAGTTCATCATTRC	16,367-16,389	
CY5-CP25	CY5-CRCCACGGGYATAACGTACACTCGG-BHQ-2	16,405-16,427	-
RFLP analysis			
SSPI-F	GACTCCRCATTTAAAGGGCTA	15,839 - 15,859	825
SSPI-R	YCCRGTCAAGAAATCYGCACA	16,643-16,663	
DDE-F	ATGTTAGCTAGACGTCAAGGT	15,941-15,961	235
DDE-R	GCAACAACATYGTCSCCTTCTTT	16,152-16,174	

[1] Nucleotide positions and expected amplicon size referred to the GenBank accession no. AF001623. For primer SSPI-F and SSPI-R nucleotide positions and expected amplicon size referred to the GenBank accession no. AF260651.

(Fig. 1A). Forward primer (P27F) was located between the 3' end of the CPm gene and the 5' of the CPm-CP intergene sequence. The reverse primer and the MGB-probe were located in the CPm-CP intergene sequence (Table 2).

*Multiplex real-time RT-PCR assay***.** A one-step multiplex RT-PCR protocol was developed and optimized which consisted of 1x iQ supermix for probes (Bio-Rad Laboratories, Hercules, CA) containing the following primers/probes combination: 160nM CP25F, 320nM CP25R, 80nM Cy5-CP25, 320nM P27F, 320nM P27R, 160nM CPi-VT3. The amplification profile were one cycle at 55°C for 2 min and 5 min at 95°C followed by 45 cycles at 95°C for 15 s and 57°C for 40 s. A dilution series of the CTV-SY568 *in vitro* transcript was prepared as previously reported (27) to generate standard curves and determine the efficiency of the multiplex assay. Each sample was run in duplicate.

Restriction fragment length analysis. *Selection of restriction enzymes and target sequences***.** Based on the multiple alignments and the predicted restriction maps of the nucleotide sequences of the CP gene and the CPm-CP intergene sequences of some reference isolates, S*sp*I and D*de*I restriction enzymes (New England Biolabs, Ipswich, MA) were selected for this assay. A first primer set was designed to amplify a sequence of ~235nt (amplicon A) containing a unique D*de*I site in VT and T3 CTV genotypes and no D*de*I restriction site in T30- and T36-like genotypes. A second primer set was selected to amplify a sequence of ~ 825nt (amplicon B) which contained a unique S*sp*I restriction site in T30-like genotypes which was absent in VT-, T3- or T36-like genotypes (Table 2).

*Amplification of the target sequences***.** Three to 5 µl of total RNA were heat denatured at 95°C for 5 min in presence of 0.5µg of random hexanucleotides (Roche Diagnostics, Indianapolis, IN). RNA was then reverse-transcribed by incubation at 42°C for 45 min in a reaction mix (20 µl) containing 1× M-MLV RT reaction buffer (Promega Madison, WI), 500 µM of each dNTP, 200U of M-MLV reverse transcriptase (Promega), 20U of RNasin ribonuclease inhibitor (Promega). Amplification of both fragments was obtained in two separate reactions. An aliquot (2.5µl) of cDNA was PCR amplified in 25µl reaction mix containing a 1× concentration of GoTaq reaction buffer (Promega), 200µM dNTPs, 0.2µM of each primer and 1U of GoTaq DNA polymerase (Promega). Amplification profiles were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s (fragment A)/60 s (fragment B) and 72°C for 30 s/60 s. The reactions were incubated for an additional 10 min at 72°C.

Enzymatic digestion and electrophoresis. Two µl of the amplification products were subjected to enzymatic digestion using D*de*I or S*sp*I restriction enzyme according to the manufacturer's instructions. After 2 h incubation at 37°C, products were analyzed by electrophoresis in 5% polyacrylamide gels. Gels were stained with ethidium bromide.

RESULTS

Multiplex real-time RT-PCR assay using TaqMan probes. *Primer and probes design.* Multiple alignments of the CPm-CP intergene sequences showed difference in nucleotide content and length among the different reference CTV strains analyzed. The resultant phylogenetic relationships (Fig. 1B) were concordant with their biological activities as well as to those obtained using the full length CP gene, confirming the suitability of this genome region for CTV strain differentiation. This was used to select a specific probe which hybridizes with CTV sequences associated with VT or T3 genotypes.

Multiplex real-time RT-PCR assay. All CTV isolates tested were detected by the Cy5-CP25 TaqMan probe. Concomitantly, the selective CPi-VT3 MGB-TaqMan probe reacted with severe CTV isolates included in the test. Regarding the reference isolates (Table 3), the FAM-S MGB–TaqMan probe clearly reacted with the VT, T3, T68, SY568 and T318 genotypes (Ct values were between 15.27 and 20.99) in single infection or in mixture (Table 3). No fluorescence was detected when the CPi-VT3 probe was tested against the T36 and P81 strains, confirming specificity for VT and T3 genotypes. This data on the reference isolates were further corroborated by the results shown in Table 4 which validated the efficiency and the specificity of the assay on a wide panel of isolates. The results were in good agreement with the genotype of the isolate and with the biological data. All isolates with VT or T3 genotype and all isolates that showed SP or SY reaction in a biological index were differentiated by the multiplex assay.

TABLE 3
SPECIFICITY OF REAL-TIME MULTIPLEX ASSAY USING SOME SELECTED REFERENCE ISOLATES

Source[1]	Cycle threshold (Ct) values of multiplex real-time PCR assay		Isolate info
	CY5-P25 probe	CPi-VT3 MGB-probe	
B3	21.07	0	T36 -Quick decline
B148	22.17	20.99	T3 – Decline/stem pitting (SP) / seedling yellow (SY)
B28	17.88	20.18	T68 – Decline/SP
B192AT106	15.04	15.27	Aphid transmitted sub-isolate of B192,– severe SY and SP
B405	17.88	20.00	T318 – containing a mixture of SP and mild isolates
P81	19.22	0	T30 genotype – Mild
SY568	19.78	19.04	Severe SY and SP
[2]SY568+P81	19.62	19.43	---
[2]SY568+B3	19.88	20.48	---

[1] Isolates B3, B148, B28, B192AT, B405 were from the Exotic Citrus Pathogen Collection (ECPC), USDA, ARS – Beltsville, MA (B). Isolates P81 and SY568 were from USDA, ARS, Parlier, CA.

[2]Total RNA was mixed at the ratio of 1:1.

However, SY reactions could also be associated with isolates containing a T36 genotype not detected by the CPi-VT3 probe. To overcome this, a new TaqMan probe was designed and tested for specific detection of these isolates (data not shown). Furthermore, a severe CTV strain was detected in isolates B7 and B192 that produced no severe symptoms on biological indicators. B7 has been reported as a mixture of different genotypes (5,15) and aphid transmission of the B192 (7) isolate revealed the presence of a severe stem-pitting sub-isolate in the parental isolate which, in the mixture, produced no symptoms in Mexican lime (6). Positive reaction was also obtained with two other biologically mild isolates: B25 and B340. B25 has a VT genotype and is associated with strong symptoms in lime; while isolate B340 has a non-standard genotype and was originally recovered from a severely affected Tahiti lime in Trinidad. Studies on the population structure of these isolates have not been conducted but would clarify their status as potentially virulent/severe sources. The severe isolates detected in the USDA, Parlier collection consisted of the two field sources and the aphid transmitted progenies of a virulent isolate found in central California on Valencia top worked to the variety Dekopon (30).

No significant changes in Ct values were obtained when the CPi-VT3 probe was used in single or multiplex real-time RT-PCR (Fig. 2), demonstrating that the amplification of the two amplicons was balanced and only a minimal competition occurred. Broad spectrum detection by the CY5-CP25 probe in the multiplex assay was comparable to that previously obtained (27). The high efficiency of both assays (R^2 = 0.9976 and R^2 = 0.9963) suggests that it can be used in a quantitative multiplex assay

TABLE 4

DIFFERENTIATION OF *CITRUS TRISTEZA VIRUS* STRAINS USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS (RFLP) AND REAL-TIME RT-PCR WITH CPi-VT3 MGB-TAQMAN PROBE

Symptoms [1]	**Sample**	**RFLP analysis**				**Real time RT-PCR**	**MCA13 reactivity**
		Enzymatic digestion		**RFLP Group III (VT and T3 genotypes)**	**RFLP Group II (T30-like genotypes)**	**CPi-VT3 reactivity**	
		SspI	**DdeI**				
		Exotic citrus pathogen collection, USDA, ARS, Beltsville, MD					
Moderate to strong: SY, OSP, GSP, WB, PWP	B6, B8, B23, B25, B29 ,B31, B46, B58, B79, B131, B148, B152, B188, B192AT, B199, B211	NEG	POS	Yes	No	POS	POS
Mild or symptomless	B32, B83, B305, B337, B339, B340	POS	NEG	No	Yes	NEG[2]	NEG[3]
Symptomless in ML	B192	POS	POS	Yes	Yes	POS	POS
Strong SY, OSP, GSP	B28, B31, B 71, B405, B316						
Decline T36	B3	NEG	NEG	No	No	NEG	
		Central California Tristeza Eradication Agency (CCTEA), Tulare, CA					
Mild or symptomless	CCTEA, 116, 117, 118, 120, 127, 128, 137, 139, 141, 146, 150, 154, 155, 160, 161, 162, 163, 165, 170, 172, 175, 180, 183, 192, 197, 193, 199	POS	NEG	No	Yes	NEG	NEG
Mild or symptomless	CCTEA115	POS	NEG	No	Yes	NEG	POS
Mild or symptomless	CCTEA108, 114	NEG	NEG	No	No	NEG	
Strong SY, OSP, WB	CCTEA106, 107	NEG	POS	Yes	No	POS	
		USDA, ARS, Parlier, CA					
Mild or symptomless	P1, P35, P43, P21, P28, P81, P51, P25, P36, P100, UCD 81	POS	NEG	No	Yes	NEG	NEG
Mild or symptomless	P108-35at, PN	NEG	NEG	No	No	NEG	
Moderate to strong: SY, OSP, GSP, WB, PWP	SY553, P108, P109, P108-1Bat, P108-7at, P109-2at, P108-39at	NEG	POS	Yes	No	POS	POS

[1] Garnsey unpublished data; Yokomi unpublished data. Symptoms evaluated by grafting on a standard panel of 5 indicators. GSP = Grapefruit stem pitting; OSP= orange stem pitting; WB= woody bristle on grapefruit and/or sour orange; PWP= porous wood pitting in grapefruit and/or sour orange; SY= seedling yellows.

[2] B340 was positive; B337 not tested.

[3] B83, B337 and B340 were positive. Therefore the MCA13 epitope in this group not correlated to their biology.

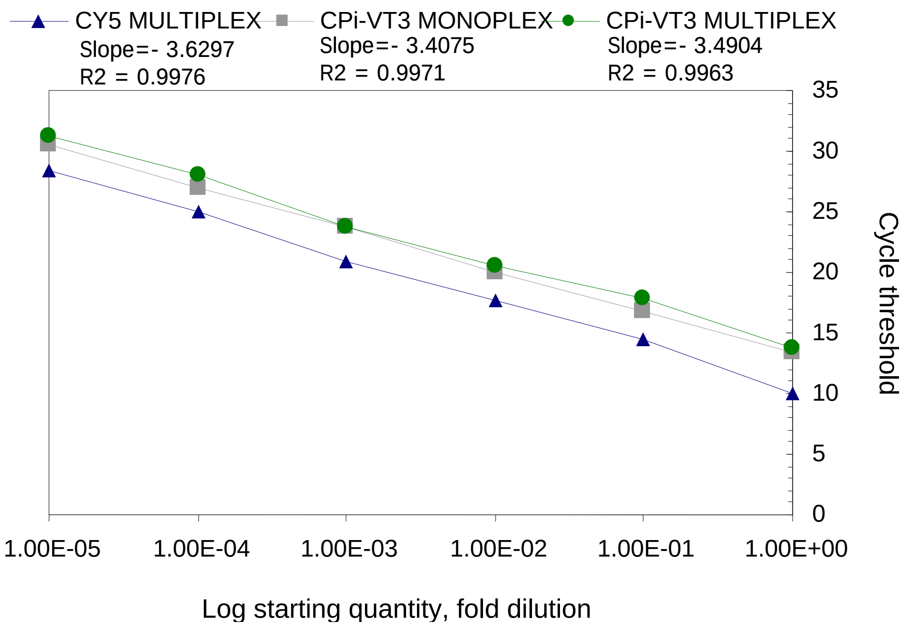

Fig. 2. Standard curves generated using two replicates of a series of 10-fold dilutions of the Citrus tristeza virus-RNA transcript of the isolate SY568. CY5 multiplex and CPi-VT3 multiplex curves are obtained using the universal primer/probe set along the P27F/R primers and the CPi-VT3 MGB-probe in multiplex reaction. CPi-VT3 monoplex curve is the result of the monoplex reactions.

Restriction fragment length analysis. *Selection of the restriction enzymes***.** Phylogenetic analysis of the CP-CPm intergene sequences showed that CTV isolates clustered in three different groups (Fig. 3). Predicted restriction map of each group shows that they could be differentiated by using both selected restriction enzymes as follows: Group I - T36-like isolates in which no digestion occurred for both fragments; Group II -T30-like isolates in which digestion occurred only with S*sp*I (in fragment A); Group III included VT and T3 genotypes in which digestion occurred with D*de*I (in fragment B).

Enzymatic digestion. RT-PCR using the two selected primer sets yielded the amplicons (A and B) of the expected size in all isolates tested, confirming broad spectrum reactivity. When an aliquot of the RT-PCR product obtained from the reference isolates was subjected to enzymatic digestion with D*de*I or S*sp*I, a strong correlation was found with the predicted restriction map (Fig. 4). Digestion of amplicon A with D*de*I produced two DNA fragments (~155 and 79bp) and occurred only in the CTV-SP and CTV-SY isolates. Digestion of amplicon B with S*sp*I occurred in the T30-like isolates and resulted in two DNA fragments of 628 and 197bp.

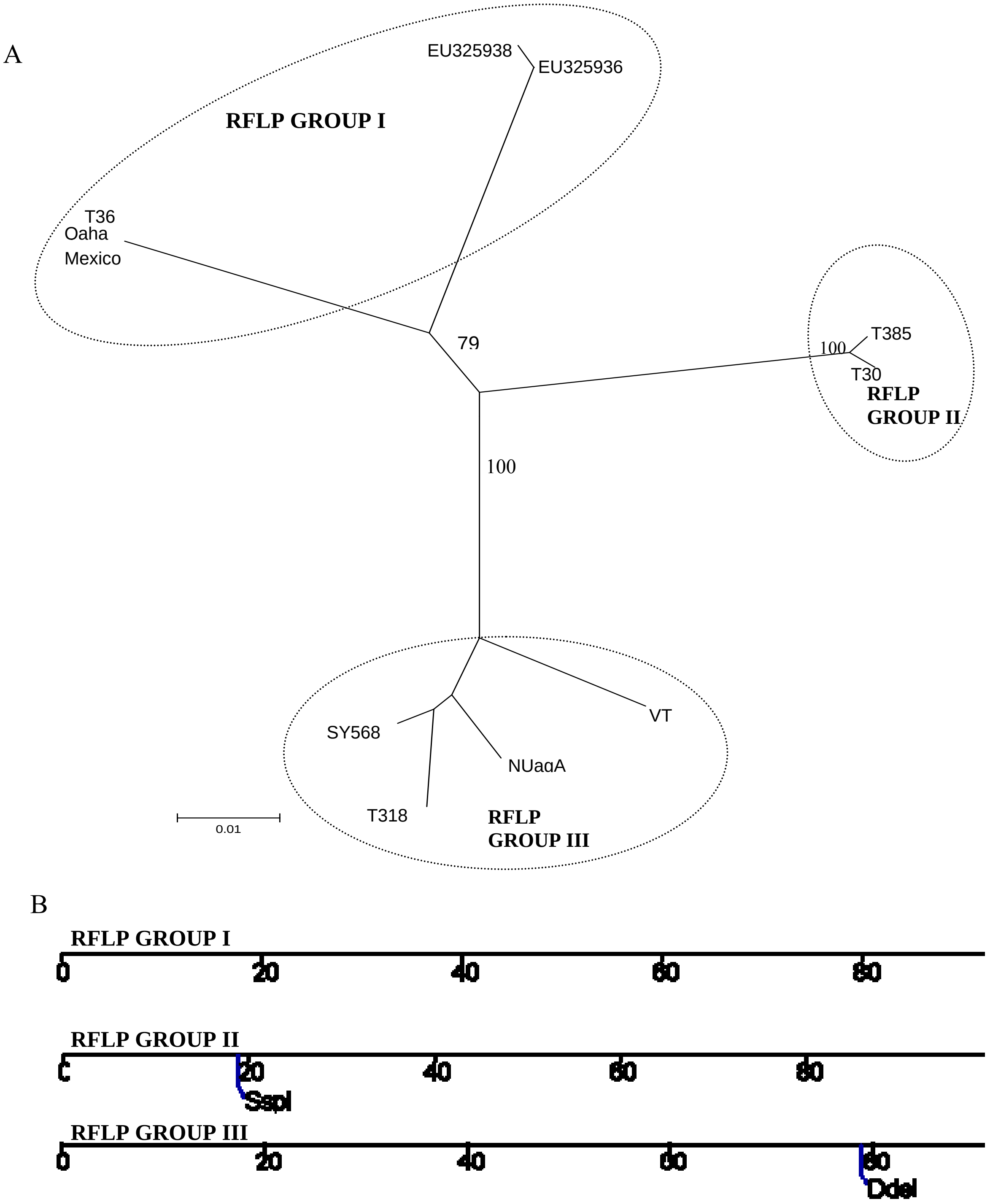

Fig. 3. Phylogenetic relationships and enzymatic restriction maps. (A) Unrooted parsimony tree based on the nucleotide sequences of the coat protein (CP) and CP minor (m) intergene sequence and CP gene. Bootstrap value for 1000 replicates are indicated. Branch length is proportional to number of nucleotide changes.

(B) Predicted restriction maps of the CP and CPm intergene sequences with S*sp*I and D*de*I restriction enzymes, for each restriction fragment length polymorphism (RFLP) group.

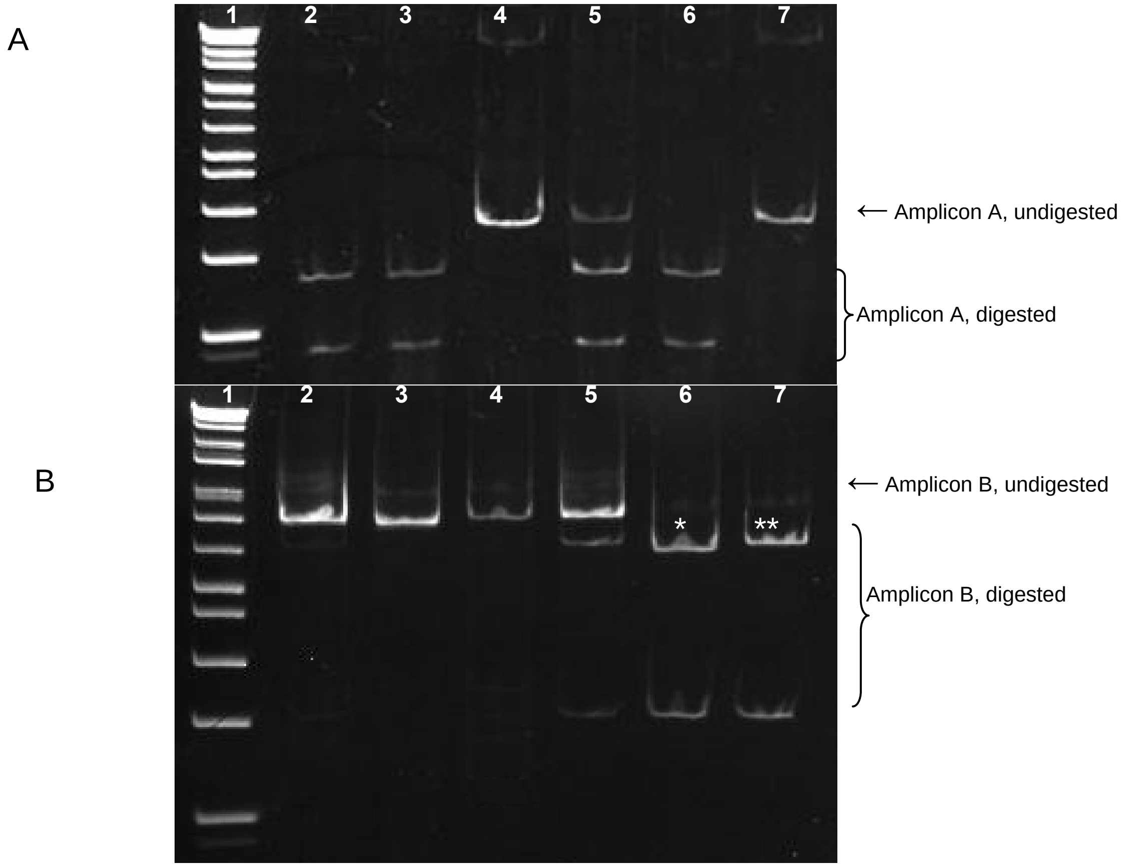

Fig. 4. Electrophoretic analysis in 5% polyacrylamide gel of the amplicons A and B after enzymatic digestion with D*de*I (A) and S*sp*I (B), respectively. Lane 1: 1Kb Plus DNA Ladder (Invitrogen); lane 2: SY568; lane 3: T3; lane 4: T36; lane 5: T318; lane 6: T68; lane 7: P81. * DNA fragment of 620bp; ** DNA fragment of 628bp.

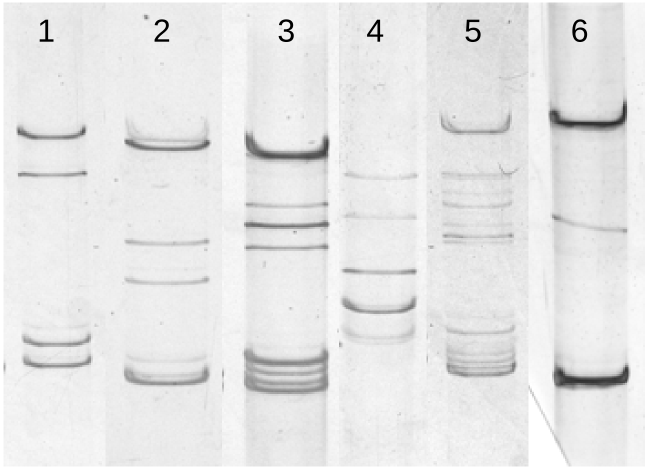

Fig. 5. Single strand conformation polymorphism (SSCP) profiles of the coat protein gene amplified from the isolates: B28 (lane 1), B31 (lane 2), B71 (lane 3), B192 (lane 4), B316 (lane 5), B405 (lane 6). All isolates showed a complex SSCP pattern which supports the RFLP results indicating that a mixture of strains occurs in these isolates.

When mixtures of isolates from group II and III occurred in the sample, digestion occurred for both amplicons and both the expected digested fragments and the undigested amplicon were observed (Fig. 4, lane 5). SSCP analysis of the CP gene was performed to confirm the existence of a mixture of different genotypes (29) (Fig. 5). T68 (B28), an isolate inducing severe SY and SP symptoms was digested by both enzymes and no undigested bands were observed which further suggested that mild components were absent. It was further demonstrated that T68 contains two different CP-sequence variants identified as CP68-1 and CP68-4. CP68-1 is phylogenetically related to the SY568 isolate (98.2% of nucleotide identity), CP68-4 to the T3 isolate (97.8% of nucleotide identity). Both CP variants contained the D*de*I restriction site, confirming association of this restriction site with the severe genotype. In addition, CP68-4 contains (like the T30-like genotypes) the S*sp*I restriction site, which results in a RFLP profile of two fragments of 620 and 197bp instead of 628 and 197bp for the T30-like genotype (Fig 4).

Nevertheless all severe CTV-SP and CTV-SY included in the test were detected by RFLP analysis using D*de*I (Table 4) showing that this enzyme can be used to discriminate isolates associated with VT and T3 genotypes.

DISCUSSION

Differentiation of virulent versus mild CTV isolates is a major concern for the management of CTV. Introduction and spread of the brown citrus aphid, *Toxoptera citricida*, the most efficient CTV vector, and an anticipated increase in the incidence of virulent CTV strains once this vector is established have heightened the need for rapid differentiate of CTV strains, especially those that are associated with OSP and/or GSP.

Sieburth et al. (29) tested some selective primers (VT-1, Type II and T3-2) and oligoprobes (ONP III, ONP IV, ONP V) on a large panel of CTV isolates to discriminate stem-pitting isolates and evaluated the procedure(s) for use as diagnostic tools for field trees. They found that no single marker could be associated with all CTV isolates which induced stem pitting and concluded that biological, serological and molecular assays needs to be combined to best define the profile of a CTV isolate.

Recently, real-time RT-PCR protocols have been developed to detect CTV in citrus and in the aphid vector (4,26,27). In this paper, a multiplex real-time RT-PCR assay was developed to detect CTV and simultaneously identify if it is associated with a VT or T3 genotype. A unique marker (MGB-TaqMan probe) was developed and proved to be specific and properly identified SY- and SP-inducing isolates from an international panel of geographically and biologically different isolates. The CPi-VT3 probe did not react with decline isolates or with mild isolates (T30 genotypes) which react with MCA13, hence, proved more selective for severe isolates than MCA13 (Table 4).

RFLP of the amplified coat protein gene has been described and used by others to categorize CTV isolates with similar properties in different groups (3,13,24). Seven different CTV groups were defined based on the H*nf*I and/or R*sa*I restriction fragments and some correlation among the groups and biological properties of the isolates were found (13). In our RFLP assay using enzyme digestion of CP amplicons with D*de*I or S*sp*I, isolates clustered into three main groups: Group I - T36-like isolates;

Group II - mild T30-like genotype; and Group 3 – VT and t3 genotype isolates. Even if large genetic diversity was encountered among the isolates within each group, the isolates were effectively categorized as potentially mild or severe SP and SY. The RFLP test provided good correlation with the potential virulence of the isolate. Furthermore, the fragment selected for the RFLP analysis has only one restriction site per enzyme which makes the resultant RFLP profile easy to interpret (e.g. digestion occurred or not). The effectiveness of the assay is based on the specific nucleotide sequences of the restriction site, thus, mutation of single nucleotide can cause the failure of the digestion and invalidate the test. In our study, one mild isolate (PN – GenBank acc. n. EU325932) out of 50 mild isolates tested showed single nucleotide mutation in the S*sp*I restriction site (AATATT/AATGTT). Even so, if this is a T30-like isolate, no digestion will occur with SspI. Multiple infection of mild and CTV-SP or CTV-SY isolates can also revealed using these enzymes. Thus, this procedure would be useful in the search for mild cross-protecting isolates or for severe isolates hidden by a mixture of isolates.

In conclusion the CPm-CP intergene sequences allowed the design of two independent assays based on a single marker for differentiation of potentially severe CTV strains. Both assays proved useful to detect the presence of VT or T3 genotypes in infected plants even in a mixture with mild or decline isolates. The results obtained using the real time RT-PCR and RFLP assays were in 98.9% agreement. Because the real-time RT-PCR protocol is more simple, it is a great tool for large scale molecular characterization and differentiation of mild versus potentially virulent CTV isolates.

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