Comparison of Molecular and Biological Characteristics of Fourteen Moroccan Isolates of *Citrus tristeza virus*

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ABSTRACT. A collection of *Citrus tristeza virus* (CTV) isolates from Morocco has been classified by characteristic symptoms produced on the standard citrus indicator hosts and by using asymmetric RT-PCR, hybridization to specific probes and single strand conformation polymorphism (SSCP) with the coat protein gene (CPG). CPG amplicons were hybridized with CTV group-specific probes in an RT-PCR/ELISA assay. The isolates generally reacted with the expected probes according to their symptom patterns, except CTV-3 CTV-8, CTV-9 and CTV-10. CTV-3 causes severe stem-pitting (SP) on sweet orange and was isolated from Troyer citrange. It reacted only with probe Gr.3b. This is unusual, because most severe SP isolates react with probes Gr.3a or Gr.5 or both Gr.3b and Gr. III. CTV-8, CTV-9 and CTV-10 reacted strongly with probes Gr 2 or Gr M, indicative of mild isolates, but their *in planta* reactions also included quick decline (QD) and SP symptoms. The QD reaction on sour orange rootstock was always found associated with SP isolates. No isolates like T36, a QD isolate from Florida, were found. In SSCP many isolates gave two conspicuous bands, except for isolates CTV-1, CTV-3, CTV-4, CTV-5, CTV-7, CTV-8, CTV-9 and CTV-10 which produced a more complex pattern, indicative of mixtures. These results provide molecular confirmation for the presence of mild, severe SP and QD inducing isolates of CTV in Morocco. Presence of the QD isolates is of special concern because of all the very susceptible sour orange rootstock in wide use in Morocco and the entire Mediterranean region, and especially with the recent establishment of the brown citrus aphid, *Toxoptera citricida*, the most efficient vector of CTV, in Madeira.

Index words. Citrus tristeza, coat protein gene, asymmetric RT-PCR, RT-PCR/ELISA.

Citrus tristeza, caused by *Citrus tristeza virus* (CTV), occurs worldwide (2), and has been found in North Africa wherever sufficient efforts were employed. Thus far, the impact of tristeza remains minimal, with no significant economic losses reported, but with the establishment in Madeira (1) of the brown citrus aphid, *Toxoptera citricida*, the most efficient vector of CTV (11), the whole Mediterranean region is seriously threatened.

CTV usually exists in nature as a mixture of strains (5) causing a wide diversity of symptoms that range from barely noticeable vein clearing on Mexican lime (although the virus titer is high in sap) (12), to decline and death of trees on sour orange rootstock, and poor vigor with stem-pitting regardless of scion and rootstock (6). Typing of prevailing CTV strains is a key element for predicting disease impact and devising appropriate control strategies suitable to specific regions. In Morocco surveys for CTV eradication have been conducted (7, 8). A panel of 14 isolates collected during these surveys was characterized using standard citrus indicator hosts (12). In this paper we report the molecular characterization and classification of the strains composing this same panel of isolates.

MATERIALS AND METHODS

Source of CTV isolates. All 14 isolates (Table 1) collected from previous surveys of citrus germplasm blocks in Morocco were tested (8). These isolates were previously graft transmitted to the standard citrus indicator hosts for biological characterization (12). For this study, fresh bark was collected from CTV-infected Madame Vinous sweet orange test plants kept under glasshouse conditions.

Immunocapture-reverse transcription-asymmetric polymerase chain reaction (IC-RT-PCR)

TABLE 1
ORIGIN AND BIOLOGICAL CHARACTERISTICS OF THE 14 *CITRUS TRISTEZA VIRUS* ISOLATES STUDIED IN MOROCCO

Isolate number	Host of origin	Biological characteristics			
		LR	QD	GSP	OSP
1	Sun of Summer Orange[a]	++	++	++	+
2	Little Leaf Chinese Sour Orange[b]	-	-	-	-
3	Troyer Citrange[a]	++	-	++	++
4	Mautauban Citrange[a]	+	-	-	-
5*	Meyer Lemon[c]	++	++	++	-
6	Foster Orange[b]	+	-	-	-
7	Satsuma Saigon Mandarin[a]	++	-	+	-
8	Pan-American Mandarin[d]	++	++	+	+
9	Pan-American Mandarin[b]	+	++	+	-
10	Large-Leaf Chinese Sour Orange[b]	++	++	+	+
11	Kino Kuni Mandarin[a]	+	-	-	++
12	Ponkan Mandarin[b]	++	-	-	-
13	Rusk Citrange[b]	+	-	-	-
14	Tripoli Orange[a]	-	-	-	-

*Reference isolate; a: El Menzeh Exptl. Station; b: Souihla Exptl. Station; c: INRA Citrus Research Center; d: Aïn Taoujdate Exptl. Station; LR: Lime Reaction; QD: Quick Decline; GSP: Grapefruit Stem Pitting; OSP: Sweet Orange Stem Pitting; -: no reaction; +: moderate reaction; ++: severe reaction.

assays. Sample extracts were made as for standard ELISA by grinding tissue in a mortar with carborundum and 5 volumes (W/V) of extraction buffer (PBS-Tween + 2% polyvinyl pyrrolidone + 1% ovalbumin) and collecting the supernatant after centrifugation. The procedure for IC-RT-PCR of the coat protein gene (CPG) was as described by Nolasco et al. (10). Briefly, assays were done as a single step protocol. Fifty microliters of each sample extract were added to antibody-coated microtiter plates and incubated overnight at 6°C. After washing, each well was filled with 50 µl of a mix containing 10 mM Tris (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, 4 mM $MgCl_2$, 3U RNA guard, 7.5U reverse transcriptase (RT), 1U Taq polymerase, 0.2 mM each (dATP, dGTP and dCTP), 0.19 mM dTTP, 0.01 mM Digoxigenin-11-dUTP, and 20 nM forward primer (CTV1) and 200 nM reverse primer (CTV10). The RT reactions were incubated at 38°C for 45 min followed by a denaturation step of 94°C for 2 min and 50 cycles of PCR amplification (92°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec) then a final incubation at 72°C for 5 min.

Analysis of CPG amplicons by PCR/ELISA using CTV group-specific probes. Amplification products were analyzed by agarose gel electrophoresis to ascertain that amplicons of the expected size were obtained. The PCR/ELISA assays (3, 10) were performed in microtiter plates using 100 µl reaction volumes. The wells were coated with streptavidin (10 µg/ml) in 50 mM sodium carbonate buffer (pH 9.6) at 37°C for 1.5 h. After washing with PBS-Tween buffer three times, the biotinylated CTV group-specific capture probe was added (Table 2) at a concentration of 20 pmole per well in hybridization buffer (5xSSPE, 0.5M NaCl, 0.1% n-lauroylsarcosine) and incubated at 37°C for 30 min. After washing, 35 µl of DIG-labelled PCR products + 65µl hybridization buffer were added to each well and hybridized with the probes at 37°C for 90 min. After washing, 100 µl/well of an anti-DIG $F(ab')_2$-alkaline phosphatase conjugate (Roche Molecular Biochemicals), diluted 1/1000 in PBS-Tween,

TABLE 2
GROUP-SPECIFIC PROBES FOR *CITRUS TRISTEZA VIRUS* AND THEIR SOURCE ISOLATES

Group type	Isolate identity and biological characteristics
Gr 1	T 36 — A QD inducing strain
Gr 2	Mediterranean mild strain
Gr 3a	Madeira strain — Causes severe GSP and OSP
Gr 3b	B 7 — Causes moderate QD and severe GSP
Gr 4	T 3 — Causes severe QD and OSP
Gr 5	B 249 — Causes severe OSP and possibly GSP
Gr M	T 30 — A mild protective strain

2% PVP-40, 0.2% BSA) was added, and plates were incubated at 37°C for 1.5 h. After washing, the enzyme substrate (p-nitrophenyl phosphate) at 0.75 mg/ml in substrate buffer (9.7% diethanolamine, pH 9.8) was added.

The colorimetric reaction was allowed to develop at room temperature for 1.5 h and monitored at 6 min intervals using a conventional absorbance reader at 405 nm. The probe reactions were determined by measuring their reaction rates during the period when the reaction was developing exponentially. This was compared in the same plate with the concurrent reactions of the probes with a standard panel of known isolates and strains. The types of strains comprising an unknown isolate and their relative amounts (as percentages) were determined by fitting of the data points to the values that would be expected using mixtures of certain amounts of the strains of the control panel. The "Solver add-in" function of the Excel Spreadsheet, was used in an iterative way to determine the values of the proportions of strains that would minimize the sum of squares of deviations to the expected values.

The sequences of the group specific probes were similar to those described by Cevik (4) and Niblett et al. (9) and were utilized in PCR ELISA as described by Nolasco et al. (10). The sequences of the probes are being patented jointly by the University of Florida and Universidade do Algarve.

Single strand conformation polymorphism (SSCP) analysis. The SSCP analysis was done directly from IC-RT-PCR products. The amplified products (2.5 µl) were each mixed with 10 µl of denaturing buffer (19.6 mM EDTA pH 8 in formamide containing bromophenol blue) and denatured for 5 min at 90°C before loading on a non-denaturing 8% polyacrylamide gel. After running 3 h at 200V at 4°C, the gels were silver stained.

RESULTS AND DISCUSSION

Amplification products of IC-asymmetric RT-PCR from all isolates migrated in agarose gels at the expected position for the CPG of 672 bp (data not shown). Results of hybridizations of DIG-labeled amplified CPGs with group-specific probes and their subsequent detection with anti-DIG-F(ab')2 conjugated to alkaline phosphatase are presented in Table 3.

Our results with PCR/ELISA demonstrate a great diversity in strain composition among the 14 isolates tested. Isolates 6, 9, 12 and 14 were classified as "pure" mild T-30-like strains because no other strains were detected in those isolates. Isolates 2, 4, 8 and 13 were classified as pure Mediterranean mild-like strains.

Isolate 3 was unusual; based on its biological characterization and its reaction with the single probe Gr.3b, it should be classified as a pure stem-pitting strain of both sweet orange and grapefruit, and of

TABLE 3
RESULTS OF HYBRIDIZATION OF DIG-LABELED AMPLIFIED CPGS OF THE 14 *CITRUS TRISTEZA VIRUS* ISOLATES FROM MOROCCO WITH GROUP-SPECIFIC PROBES IN AN IC-RT-PCR-ASYMMETRIC/ ELISA

Group-specific Probes	Isolates													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gr1	0*	0	0	0	0	0	0	0	0	0	3	0	0	0
Gr2	42	99	9	98	0	0	0	99	0	16	26	0	100	0
Gr3a	48	0	8	0	0	0	11	0	0	0	0	0	0	0
Gr3b	10	0	77	0	45	0	0	0	0	0	4	0	0	0
Gr4	0	0	5	0	0	0	89	0	0	0	6	0	0	0
Gr5	0	0	0	0	52	0	0	0	1	4	15	1	0	0
GrM	0	1	0	2	3	100	0	1	98	80	45	99	0	100

*Results of PCR ELISAs monitored at 405 nm and expressed as percentage of the OD for the corresponding strain within the isolate assayed. Values under 10% are not considered as significant.

all the isolates we have tested from throughout the world, it appears to be unique. Most severe SP isolates react with probes Gr.3a or Gr.5 or with both Gr.3b and Gr.III (not tested in this report). Sequencing of the CPG of this isolate verified that it clusters with the other SP isolates of group 3a (4, 9). Therefore, CTV strains which react only with probe Gr.3b should be considered as a new variant of group 3a. The remaining isolates reacted as complex mixtures of (i) mild (T 30 and Mediterranean mild) with OSP strains (isolates 10 and 11), (ii) Mediterranean mild with QD and GSP/OSP strains (isolate 1), (iii) QD with GSP strains (isolate 7) and (iv) QD with OSP strains (isolate 5).

SSCP analysis of IC-RT-PCR products amplified from the coat protein gene (Fig. 1) showed patterns with two conspicuous bands for isolates 2, 6, 11, 12, 13, 14 and the known control B1. Isolates 1, 3, 4, 5, 7, 8, 9, 10 and the known control B53 showed multiple band profiles identifying them as a mixture of strains. The SSCP patterns for isolates 7 and 10 confirm their reactions with multiple probes. How-

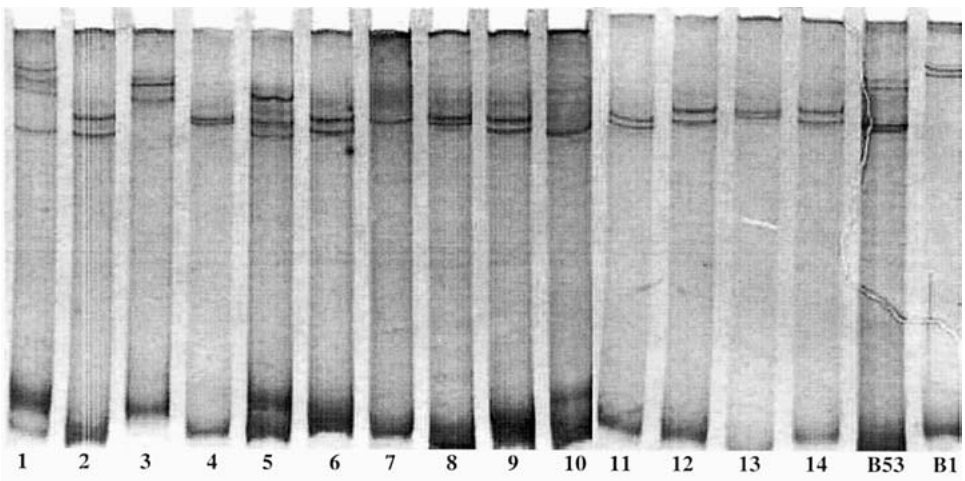

Fig. 1. SSCP migration patterns of the CPGs of 14 *Citrus tristeza virus* isolates from Morocco. Samples were run for 3 h at 200V at 4°C on a non-denaturing 8% polyacrylamide gel, which was then silver stained.

ever, isolate 11, which also reacted with multiple probes gave only two clear bands by SSCP. This result could occur if one strain was highly predominant over the others, which were present at concentrations below SSCP detection levels, or if their bands co-migrate on SSCP gels. This could be resolved by sequencing of the CPGs of multiple clones of the same isolate.

Our results provide molecular confirmation of the considerable biological diversity present in the 14 CTV isolates earlier taken from citrus germplasm blocks in Morocco. We have also detected the presence of one new strain. The presence of QD and severe GSP and OSP strains of CTV in North Africa, where sour orange still remains the dominant rootstock, places the citrus industry of this entire region at high risk of an imminent and destructive outbreak of tristeza, especially with the threat of *T. citricida* invading the Mediterranean Basin both from Yemen in the East and Madeira in the West.

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