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Title

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Journal

International Organization of Citrus Virologists Conference Proceedings (1957-2010), 13(13)

ISSN

2313-5123

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Publication Date

1996

DOI

10.5070/C502k6053x

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Interference Between Citrus Tristeza Virus (CTV) Isolates Detected by Analysis of Double Stranded RNA (dsRNA)

P. Moreno, J. Guerri, M. R. Albiach, J. F. Ballester-Olmos, and M. E. Martínez

ABSTRACT. Interference between citrus tristeza closterovirus (CTV) isolates was studied by co-inoculating Madam Vinous sweet orange or Citron plants simultaneously or successively with mild and severe isolates, and monitoring plants for symptom expression and double stranded RNA (dsRNA) patterns. Twenty mild and two severe isolates were tested in several combinations. In most of the combinations assayed, three types of situations resulted: 1) the dsRNA pattern of the co-inoculated plants was the addition of the individual patterns, suggesting multiplication of both isolates without detectable interference; 2) the presence of the dsRNA pattern of only one of the isolates in the co-inoculated plant, suggesting exclusion or a drastic titer reduction of the other isolate; 3) new bands never before detected in plants infected by either isolate alone appeared in the co-inoculated plants accompanied by the pattern of one or both isolates. Plants inoculated only with mild isolates remained symptomless; whereas plants inoculated only with a severe isolate, and most of those co-inoculated with a mild and a severe isolate showed various degrees of symptoms. Whenever the dsRNA pattern of the severe isolate was detected in the co-inoculated plants, alone or in combination with the pattern of the mild isolate or with new bands, symptoms of the severe type were observed. When this pattern was not detected, the plants were symptomless. This suggested that symptom expression requires detectable dsRNA levels of the severe strain and that analysis of dsRNA of the co-inoculated plants can be used for rapid screening of mild isolates for cross protection, so long as the dsRNA patterns of the mild and the severe isolate can be differentiated. The strong subgenomic bands detected in some isolates suggest the presence of defective RNAs in the infected plants.

Index words. Cross protection, defective RNAs, graft-inoculation, mild isolates, stem pitting isolates.

Citrus tristeza closterovirus (CTV), the causal agent of one of the most destructive diseases of citrus, has many variants differing in their biological characteristics, particularly in the type and intensity of the symptoms induced in different hosts (3). Severe CTV isolates can cause stunting, stem pitting, low yield, and poor fruit quality in several commercial cultivars (22). Damage caused by these isolates can be partially avoided by cross protection (6, 18, 26). The molecular basis of this technique, which consists of pre-inoculating plants with a mild isolate capable of interfering multiplication of the severe isolate, is not known. Usually, long and costly field experiments are necessary to select the proper mild isolate for each location and cultivar. A procedure to monitor multiplication of the severe isolate in co-inoculated plants would be very helpful for quickly screening the protecting capacity of many mild isolates in the greenhouse. Only those mild isolates which protect in the greenhouse would be assayed under field conditions.

In recent years, procedures used to discriminate CTV isolates have included reaction with monoclonal antibodies (5, 19), analysis of peptide maps of the coat protein (9), hybridization with complementary DNA (cDNA) probes of the CTV genome (1, 24), analysis of restriction fragments of the coat protein gene (8), and double stranded RNA (dsRNA) analysis of infected plants (7, 10, 14, 15, 16, 17). In this study we monitored interference between CTV isolates by dsRNA analysis of co-inoculated plants and by symptom expression.

MATERIALS AND METHODS

CTV isolates. Aphid-transmitted isolates from a collection at IVIA were characterized by symptom expression, serology and dsRNA patterns. The mild isolates included T300 (3), T385 (16), and 18 isolates (T3, T6, T7, T10, T11, T13, T14, T16, T19, T20, T21, T23, T24, T27, T28, T31, T32, and T40) separated from T385 by different transmission methods (14, 15, 16). These isolates induced mild to moderate symptoms in Mexican lime but did not cause seedling yellows or stem pitting on grapefruit or sweet orange. The severe isolates were T388 and T318. T388 was obtained from an early satsuma illegally imported from Japan (4), and T318 was separated from T385 by passage through several hosts (14, 15). Both severe isolates induce a seedling yellows reaction and produce very severe symptoms in Mexican lime as well as stem pitting in grapefruit and sweet orange.

Inoculations. The indicator plants (Pineapple or Madam Vinous sweet orange seedlings or budlings on sour orange, or Etrog citron Arizona 861-S-1 propagated on rough lemon) were grown in an artificial potting mix (peat moss:sand 1:1), fertilized as described elsewhere (2), graft-inoculated with two bark pieces of each isolate, and maintained in a temperature-controlled greenhouse (18-26 C). After the first inoculation (or co-inoculation), infection of the plants was always confirmed by ELISA using monoclonal antibody 3DF1 (27), which recognizes all the isolates tested in these experiments. The specific isolates and inoculation sequence used in each experiment were as follows:

- Experiment 1. Sets of two citron plants were inoculated with T24 or T27, or simultaneously co-inoculated with T13+T31 or T27+T24, and pruned to induce new flush (Table 1). Five months later the plants were cut back, analyzed for dsRNA pattern, and re-inoculated as indicated in Table 1. A similar operation was repeated 12 months after the first inoculation (Table 1). DsRNA was periodically analyzed during the year after the last inoculation. The isolates used in this experiment are mild and do not induce detectable symptoms in citron.
- Experiment 2. Sets of two Madam Vinous sweet orange plants were simultaneously co- inoculated with any of the 15 mild isolates indicated in Table 2 and the severe isolate T388. The plants were pruned, trained to two branches, periodically sampled for dsRNA analysis and observed for symptoms. One year later each plant was re-inoculated with the mild isolate used in the first inoculation, treated as before, and observed for symptoms and analyzed for dsRNA pattern over 3 yr.

TABLE 1
DSRNA PATTERNS OF CITRON PLANTS CO-INOCULATED WITH SEVERAL MILD CTV ISOLATES

1st inoculation		2nd inoculation (5 months)		3rd inoculation (12 months)	
CTV isolates	DsRNA pattern	CTV isolates	DsRNA pattern	CTV isolates	DsRNA pattern
T13+T31	T13+T31 +NB[a]	T14+T32	T32+T31	—	—
T27+T24	T27+T24	T20	T27+T24	T20	T27+T24
T14	T14	T32	T32 + NB[a]	T14	T32 + NB[a]
T27	T27	T20	T27	T20	T27

[a]NB = New bands not observed in co-inoculated isolates.

TABLE 2
SYMPTOMS AND DSRNA PATTERN IN MADAM VINOUS SWEET ORANGE PLANTS CO-INOCULATED WITH DIFFERENT MILD CTV ISOLATES AND THE SEVERE ISOLATE T388

	Co-inoculated plants		Plants re-inoculated with the mild isolate		
CTV isolate combination	DsRNA profile	SP	DsRNA profile	VC	SP
X[z] + T388	T388	+	T388	+	+
X[z] alone	X[z]	-	—	—	—
T25 + T388	T25 + T388	+	T25 + T388	+	+
T25 alone	T25	-	—	—	—
Y[y] + T388	Y[y] + T388	+	Y[y] + T388 + NB[x]	+	+
Y[y] alone	Y[y]	-	—	—	—
T32 + T388	NB[x]	-	T32	-	-
T32 alone	T32	-	—	—	—
T388 alone	T388	+	—	—	—
Healthy control	—	-	—	—	—

[z]X = Isolates T3, T6, T7, T10, T14, T19, T20, T23, T24, T27, T28 and T31
[y]Y = Isolates T13 and T21
[x]NB = New dsRNA bands not observed in co-inoculated isolates

- Experiment 3. Groups of 7-8 Pineapple sweet orange seedlings and 7-8 budlings propagated on sour orange were given one of the following treatments: 1) pre-inoculation with T300 or T385 and challenge inoculated with T388 1 yr later with the challenge inoculum removed after 1 mo.; 2) pre-inoculation and challenge inoculation as in 1) but without removal of the challenge inoculum; 3) pre-inoculation with T300 or T385, with no challenge inoculation; 4) and 5) as in 1) and 2), respectively, but without pre-inoculation; 6) self-inoculated healthy control. The plants were periodically sampled for dsRNA analysis and observed for symptoms for 2 yr after challenge inoculation.
- Experiment 4. Groups of six Madam Vinous sweet orange plants were pre-inoculated with anyone of the 13 mild isolates indicated in Table 3. One year later the plants were divided into three groups: One was left as control, and the other two were challenge inoculated with T318 (Table 3) or with T388 (Table 4). Similar non pre-inoculated groups were inoculated with T318, T388, or self-inoculated, as control. The plants were observed for symptoms and periodically sampled for dsRNA analysis over 3 yr.

DsRNA analysis. DsRNA analysis was performed in the conditions previously established (7, 17). This procedure included pulverization of young bark with liquid nitrogen, extraction of nucleic acids with phenol-detergent, purification of dsRNA by CF-11 cellulose column chromatography, concentration by ethanol precipitation at -20°C, and analysis of dsRNA by 5% polyacrylamide gel electrophoresis. The dsRNA bands were stained with ethidium bromide or silver nitrate.

RESULTS

Experiment 1. The results of this experiment are summarized in Table 1 and Fig. 1. Plants co-inoculated with T27 + T24 yielded a dsRNA pattern that seemed to result from the addition of the indi-

TABLE 3
SYMPTOMS AND DSRNA PATTERN IN PINEAPPLE SWEET ORANGE PLANTS PRE-INOCULATED WITH MILD CTV ISOLATES T300 OR T385 AND CHALLENGE-INOCULATED WITH THE SEVERE ISOLATE T388

Isolate combination	DsRNA pattern	Symptoms	
		Vein clearing	Stem pitting
T300 + T388[z] (a or b)[y]	T300 + T388	+	+
T300 alone	T300	-	-
T385 + T388[z] (a or b)[y]	T388 + NB[x]	+	+
T385 alone	T385 + NB[x]	-	-
T388[z] (a or b)[y] alone	T388	+	+
Healthy control	—	-	-

[z]Severe isolate T388 inoculation by graft
[y]Challenge condition: a = inoculum removed after 1mo.; b = challenge inoculum left in place
[x]NB = New bands not observed in co-inoculated isolates

vidual patterns of these isolates. When these plants were re-inoculated with the isolate T20, the strong band characteristic of T20 did not appear in the triply inoculated plants. This band was transiently observed in plants pre-inoculated with T27 and then inoculated with T20, but it later disappeared and was never observed again even after repeated inoculation with T20 (Fig. 1). A new band not present in T27 or T20 was transiently observed in plants co-inoculated with these two isolates, but after 2 months it disappeared and the final pattern of co-inoculated plants was that which corresponded to T27 (Fig. 1).

Plants pre-inoculated with T14 and then inoculated with T32 yielded a dsRNA profile containing a strong band characteristic of T32 and a new band not detected in any of the individual isolates, but not the high molecular weight band characteristic of T14 (Fig. 1). This band, and two more characteristic of T13, were similarly excluded in plants

TABLE 4
SYMPTOMS AND DSRNA PATTERN OBSERVED IN MADAM VINOUS SWEET ORANGE PLANTS PRE-INOCULATED WITH DIFFERENT MILD CTV ISOLATES AND CHALLENGE-INOCULATED WITH THE SEVERE ISOLATE T318

Isolate combination	DsRNA pattern	Symptoms	
		Vein clearing	Stem pitting
X[z] + T318	X[z]	-	-
X[z] alone	X[z]	-	-
Y[y] + T318	Y[y] + T318	+	+
Y[y] alone	Y[y]	-	-
T318 alone	T318	+	+
Healthy control	—	-	-

[z]X = Isolates T3, T7, T13, T19, T20, T23, T32 and T40
[y]Y = Isolates T10, T17, T24, T25 and T28

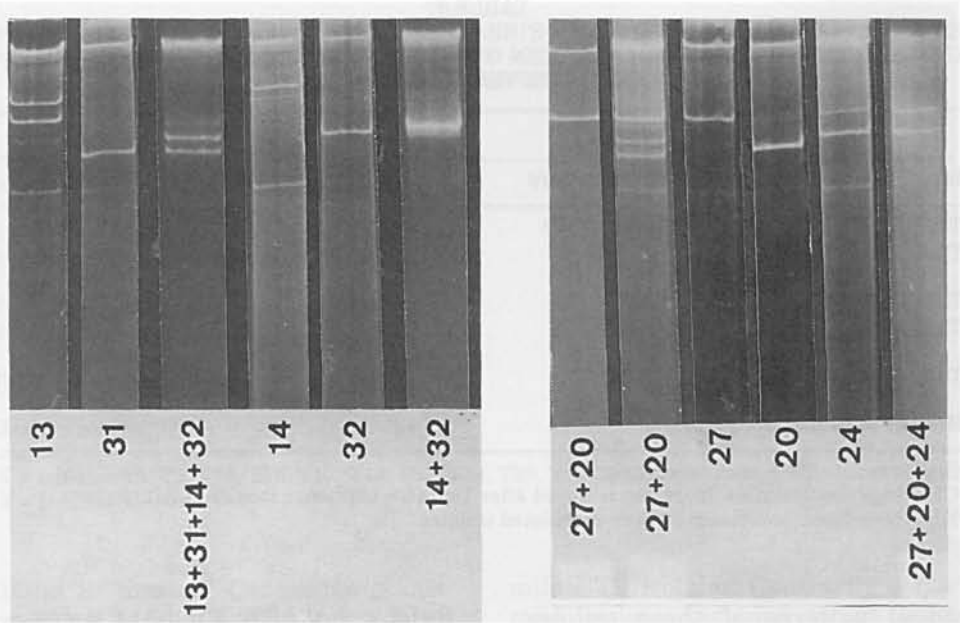

Fig.1. DsRNA pattern obtained from citron plants co-inoculated simultaneously or successively with different mild isolates as indicated in Table 1 (Refer to experiment 1). DsRNAs were separated by polyacrylamide gel electrophoresis (5% acrylamide) and stained with ethidium bromide.

pre-inoculated with T13 + T31 and later inoculated with T14 + T32 (Table 1, Fig. 1). Before inoculation with T14 + T32, these plants showed a dsRNA pattern that contained the bands of T13 and T31, plus a new band not detected in any of the co-inoculated isolates. After the second inoculation, a stable profile contained only the bands characteristic of T31 and T32.

Experiment 2. The results of simultaneously co-inoculating a mild and a severe isolate in sweet orange plants are summarized in Table 2. Three type of situations were observed:

1. Some of the co-inoculated plants yielded a dsRNA pattern containing only the bands characteristic of T388, even after re-inoculation with the mild isolate. Twelve mild isolates behaved in this way. These co-inoculated plants showed vein clearing and stem pitting symptoms, as did control plants inoculated only with T388. The control plants inoculated only with the mild isolate had the dsRNA pattern characteristic of each isolate and were asymptomatic.
2. A second group of co-inoculated plants had a dsRNA pattern that was the addition of the individual patterns of T388 and the corresponding mild isolate. These plants also showed vein clearing and stem pitting. In two cases (T13 and T21), some new bands not detected in the individual dsRNA patterns appeared after re-inoculation with the mild isolate (Table 2).
3. Plants co-inoculated with T32 and T388 showed a transient pattern containing some new bands not present in the individual dsRNA patterns, but after re-inoculation with T32, only the pattern of this mild isolate could be detected. These plants remained symptomless.

Experiment 3. The sweet orange seedlings or budlings pre-inoculated with T300 and challenge inoculated with T388 had a dsRNA

pattern that was the addition of those characteristic of the individual isolates (Table 3 and Fig. 2). These plants showed vein clearing and stem pitting. No difference was observed between plants with permanent challenge inoculum and those in which the inoculum of T388 was removed after 1 month. The control plants inoculated only with T388 or with T300 had the dsRNA pattern characteristic of these isolates (Fig. 2). Those inoculated with T388 showed vein clearing and stem pitting, whereas those inoculated with T300 did not. The self-inoculated control plants did not show symptoms and contained no detectable dsRNA.

Plants pre-inoculated with T385 and challenge inoculated with T388 (with or without inoculum removal after 1 month) showed vein clearing and stem pitting, and had a dsRNA pattern containing new bands not detected in the individual isolates (Table 3 and Fig. 2). Control plants inoculated only with T385 were symptomless. Variations in the dsRNA pattern which depended on the sampling time, were observed in plants inoculated with T385 as well as in those challenge inoculated with T388.

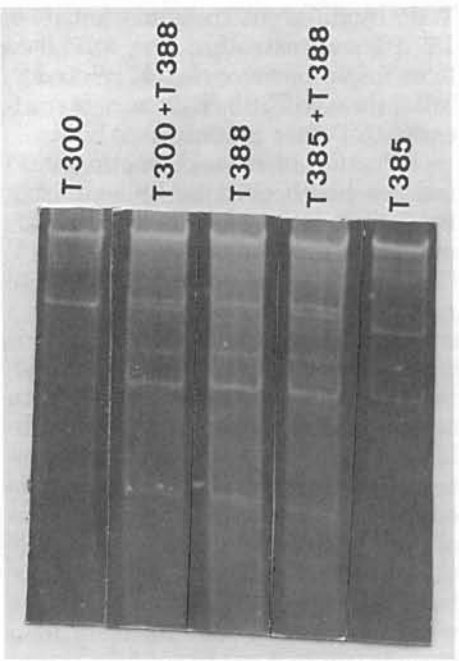

Fig. 2. DsRNA pattern obtained from sweet orange plants pre-inoculated with T300 or T385 and challenge inoculated with the severe isolate T388 (Refer to experiment 3). DsRNAs were separated and stained as in Fig. 1.

Experiment 4. Results obtained with plants challenge inoculated with T318 are shown in Table 4. Two situations were observed: 1) The challenge inoculated plants did not develop symptoms and had the same dsRNA pattern as their corresponding control that was only pre-inoculated. This was observed with eight of the mild isolates. 2) The challenge inoculated plants had a dsRNA pattern that was the addition of the individual patterns of the mild and the severe isolates. These co-inoculated plants showed vein clearing and stem pitting whereas the corresponding controls without challenge inoculation did not. This situation was observed with five of the mild isolates. The control plants inoculated only with T318 showed symptoms whereas the self-inoculated controls were symptomless and did not contain detectable dsRNA.

All the plants pre-inoculated with any of the 13 mild isolates and challenge inoculated with T388 showed vein clearing and stem pitting, as did the control inoculated only with T388, whereas the control plants without challenge inoculation were symptomless (Table 5). Most of the challenge inoculated plants had a dsRNA pattern that was the addition of the individual patterns of the mild and the severe isolates, but in two cases (T20 and T25) only the dsRNA pattern of T388 could be detected.

DISCUSSION

In these experiments, evidence for and against interference between mild and severe isolates was obtained. Three situations were

TABLE 5
SYMPTOMS AND DSRNA PATTERN OBSERVED IN MADAM VINOUS SWEET ORANGE PLANTS PRE-INOCULATED WITH DIFFERENT MILD CTV ISOLATES AND CHALLENGE-INOCULATED WITH THE SEVERE ISOLATE T388

Isolate combination	DsRNA pattern	Symptoms	
		Vein clearing	Stem pitting
X[z] + T388	X[z] + T388	+	+
X[z] alone	X[z]	-	-
Y[y] + T388	T388	+	+
Y[y] alone	Y[y]	-	-
T388 alone	T388	+	+
Healthy control	—	-	-

[z]X = Isolates T3, T7, T10, T13, T17, T19, T23, T24, T28, T32 and T40
[y]Y = Isolates T20 and T25

found in most of the combinations assayed: 1) The dsRNA pattern of the co-inoculated plants was the addition of the individual patterns, suggesting multiplication of both isolates without detectable interference. 2) The dsRNA pattern of one of the isolates could not be detected in the co-inoculated plants, suggesting exclusion or a drastic titer reduction of this isolate in the co-inoculated plants. 3) New bands not detectable in any of the isolates appeared in the co-inoculated plants accompanying the pattern of one or both isolates. Whenever the dsRNA pattern of the severe isolate was detected in the co-inoculated plants, alone or in combination with the pattern of the mild isolate or with new bands, symptoms were observed. Vice versa, when the pattern of the severe isolate was not detectable, the plants remained symptomless. This seems to indicate that, independently of the mixture of strains in the co-inoculated plants, symptom expression requires a detectable multiplication of the severe strain.

None of the mild isolates assayed impaired detection of the severe isolate T388. Roistacher and Dodds (20) also failed to cross protect against a sweet orange stem pitting isolate by pre-inoculation with 100 different mild CTV isolates. Contrarily, a few mild isolates avoided or at least delayed symptom expression and dsRNA detection of T318 (Experiment 4). The fact that T318 and all the mild isolates tested for cross protection with it were separated from T385 by different transmissions (14, 15, 16) suggests that the mild isolates might be more closely related to T318 than to T388. This may be necessary for cross protection to occur.

Selection of cross-protecting mild isolates has been done by searching for infected trees with good performance in areas devastated by CTV (18). This is the practical approach to rebuilding a citrus industry when severe strains are endemic. But if cross protection has to be implemented before massive effects from severe strains occur, many mild isolates have to be tested for cross-protecting ability. DsRNA analysis enabled us to monitor multiplication of the severe isolate in plants co-inoculated with a mild and a severe isolate. Though co-inoculated plants were maintained for relatively long periods (up to 3 yr) to confirm symptom expression and stability of the dsRNA patterns, multiplication of the severe isolate was usually detected a few months after inoculation. Thus, the procedure allows for

rapid screening of many isolates for cross protecting capacity in the greenhouse, so long as the dsRNA pattern of the mild and the severe isolate can be unequivocally differentiated. Jarupat and Dodds (12) also detected interference between two CTV isolates by dsRNA analysis.

A critical point in the cross protection experiments is how to perform the challenge inoculation. A permanent inoculum grafted on the pre-inoculated plant has been used (21), but it may cause cross protection breakdown even with mild isolates protecting under field conditions (Müller, pers. comm.). Aphid inoculation is probably a milder challenge and it better mimics disease pressure in the field. However, this procedure may be cumbersome when a large number of plants need to be inoculated. In Experiment 3 we compared graft inoculation with or without inoculum removal one month later. Both procedures yielded 100% of the inoculated plants infected. Since removal of the inoculum 1 mo. after inoculation seemed a milder challenge than the permanent inoculum, we adopted the first procedure in further cross-protection experiments.

Detection of new bands in co-inoculated plants not detectable in individual isolates was somewhat puzzling, but recent findings may be helpful to explain these results. CTV produces in infected plants subgenomic RNAs that are 3' co-terminal (11). Replicative forms of these RNAs may account for some of the non-full-length dsRNAs detected in infected plants, but some of the most conspicuous subgenomic bands do not correspond to the size expected for the 3' co-terminal replicative forms of subgenomic RNAs. These bands react with a cDNA probe close to the 3' end of the CTV genome (P. Moreno, unpublished data), thus they have to be transcribed from the CTV genome.

The presence of defective RNAs (D-RNAs) of CTV in infected plants has recently been documented (13). D-RNAs very often reach higher titer than full genome RNA in infected plants. It is, therefore, likely that some of the prominent bands observed in different CTV isolates may be D-RNAs generated during replication of these isolates. Co-inoculation with other isolates might give some replicative advantage to certain D-RNAs, causing the disappearance of others or appearance of new ones not previously detected. The presence of D-RNAs can modulate the symptoms expressed by infected plants (23, 25). From this standpoint, the influence of D-RNAs in symptom expression of co-inoculated plants is presently unknown.

ACKNOWLEDGEMENTS

M. R. Albiach was the recipient of a doctoral fellowship from the Instituto Nacional de Innovación y Tecnología Agraria y Alimentaria (INIA). This work was funded by the INIA projects 8179 and SC93-111.

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