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Variations in Pathogenicity and Double-Stranded RNA (dsRNA) Patterns of Citrus Tristeza Virus Isolate Induced by Host Passage

P. Moreno, J. Guerri, J. F. Ballester-Olmos, C. Fuertes-Polo, R. Albiach, and M. Martínez

ABSTRACT. Four citrus tristeza virus (CTV) isolates obtained by natural infection of Mexican lime seedlings kept in the field were graft-inoculated into Smooth Seville orange plants. Five years later, sweet orange and Mexican lime seedlings were graft-inoculated with the original isolates maintained in lime or the sub-isolates kept in Smooth Seville orange. Variations in pathogenicity and/or dsRNA profiles were observed among some of the isolates and the corresponding sub-isolates.

Several sub-isolates, differing by pathogenicity and/or dsRNA profiles could be separated from the field isolate T- 385, which was obtained from a symptomless sweet/sour orange tree. One of these, sub-isolate T-317, induced mild symptoms in Mexican lime, and was symptomless in Etrog citron, rough lemon, sour orange and Eureka lemon, but induced vein clearing and stem pitting in sweet orange. The sub-isolate in sweet orange, T-318, was graft or aphid-transmitted to Mexican lime (sub-isolates T-305 and T-305[a], respectively). These three sub-isolates induced severe vein clearing, vein corking, stem pitting and stunting in Mexican lime, vein clearing and stem pitting in citron, rough lemon and sweet orange, and seedling yellows in sour orange, Eureka lemon or Duncan grapefruit. T-317 and T-318 had similar dsRNA profiles, whereas those of T-305 and T-305[a] were different. When T-317 was graft-transmitted from citron to Mexican lime and then to sweet orange, the dsRNA profile of the new sub-isolate was similar to that of T-305, but the sweet orange plants were symptomless.

The variations in pathogenicity and/or dsRNA observed indicate that many field isolates of CTV are mixtures of different strains, some of which can disappear or be at low titer when passed through certain hosts.

Index words. Aphid-transmission, graft-transmission, indicator plants, separation of strains, Smooth Seville orange.

Citrus tristeza virus (CTV), the causal agent of one of the most important diseases of citrus, is a closterovirus that has numerous strains differing in biological properties (4, 5). Current strains present in a citrus area determine in part the epidemiology of the disease, the importance of the damage caused, and the possible control strategies. Hence there is interest in characterizing virus strains affecting major varieties in different locations. Identification of CTV strains has been done on the basis of biological properties (2, 8, 9), reaction with monoclonal antibodies (19, 20), peptide map analysis of the coat protein (11), differential hybridization with cDNA probes (23), or analysis of double-stranded RNA (dsRNA) in infected plants (6, 7, 12, 18).

The presence of a mixture of strains in CTV field isolates was suggested many years ago (10, 21). Some authors presented evidence for the elimination of certain components in the process of aphid or graft-transmission (15, 22, 24, 25). In some cases, inoculation of severe CTV isolates into certain hosts enabled these authors to recover mild sub-isolates that, frequently, protected against the original isolate (22, 24, 25).

In previous work (17, 18) we obtained evidence of strain separation from a single CTV isolate by dsRNA analysis of different subcultures. Some of the sub-isolates obtained differed from the original isolate by their dsRNA profile and by symptoms induced in certain hosts (16). In this paper we present the variations observed in dsRNA profile and in the pathogenic properties of different CTV isolates when filtered through several hosts.

MATERIALS AND METHODS

Hosts and virus isolates.

Filtration through Smooth Seville orange. Mexican lime seedlings grown in four-liter cans were placed in several citrus plots with high CTV incidence and kept there for 8

months to expose the plants to natural infection. Four of the CTV isolates obtained by this procedure were graft-inoculated to Smooth Seville (Smooth Flat Seville) orange plants and kept in this host for 5 yr. After this period, both the original isolate maintained in lime and the sub-isolate kept in Smooth Seville orange were graft-inoculated onto Pineapple sweet orange seedlings for dsRNA analysis, and onto Mexican lime for symptom evaluation. Symptom intensity was rated on a scale of 0 (no symptoms) to 4 (very severe symptoms).

Filtration through sweet orange and/or Mexican lime. A CTV isolate obtained from a symptomless sweet/sour orange tree was aphid-transmitted to a Mexican lime plant, and 2 months later two bark pieces from this lime were graft-inoculated to 40 Etrog citron plants. When these sub-isolates in citron were analyzed for dsRNA content, a variety of electrophoretic profiles was observed (17, 18). Some of the sub-isolates also differed in symptom intensity induced in Mexican lime (16). One of them, obtained from citron no. 33, was mild in Mexican lime but induced stem pitting on sweet orange (16). This sub-isolate, named T-317, was used for the following inoculations outlined in Fig.1: i) T-317 from citron was indexed by graft-inoculation in Mexican lime, Etrog citron/rough lemon, sour orange, Eureka lemon, and Pineapple sweet orange; ii) sweet orange inoculated with T-317 from citron was coded T-318. This sub-isolate was indexed by graft-inoculation in Mexican lime, Etrog citron/rough lemon, sour orange, and Pineapple sweet orange plants; iii) Mexican lime plants were graft or aphid-inoculated with T-318, using *Aphis gossypii* in the conditions previously described (13). These sub-isolates were coded T-305 and T-305[a], respectively. T-305 was indexed in Pineapple sweet orange and Duncan grapefruit, and T-305[a] in Etrog citron/rough lemon, sour orange, Eureka lemon, and Pineapple sweet orange. Usually 2-6 plants of each species were inoculated with two bark pieces of the inoculum source plant. All plants used were seedlings except the combination Etrog citron on rough lemon. Plants were grown in a temperature-controlled greenhouse (18-26C), using a standard potting mix and fertilizing system (1).

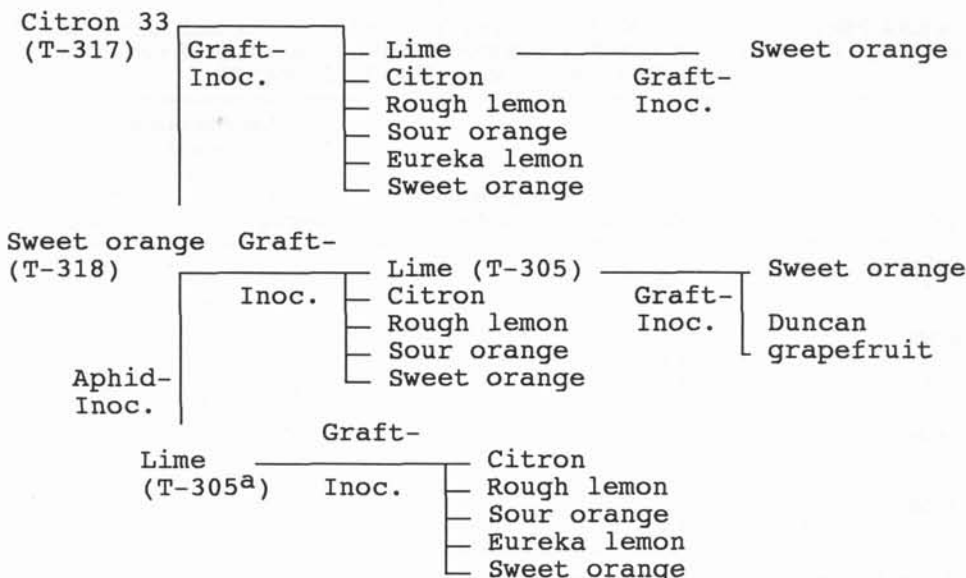

Fig. 1. Outline of the inoculations performed with the CTV isolate T-317.

Double-stranded RNA (dsRNA) analysis.

DsRNA from young bark was analyzed by phenol extraction of nucleic acids, purification by CF-11 cellulose column chromatography and separation by polyacrylamide gel electrophoresis (PAGE), using the procedure of Dodds *et al.* (6) in the conditions previously established (18). DsRNAs were observed by staining with ethidium bromide.

RESULTS

Filtration through Smooth Seville orange. Table 1 summarizes the variations observed in dsRNA profile and symptoms induced in Mexican lime between several CTV isolates and the corresponding sub-isolates obtained by passage through Smooth Seville orange. Three types of situations were observed: i) RN-15 and its sub-isolate AA-78 did not differ either in dsRNA profile or in symptoms induced in Mexican lime; ii) R-146 and two sub-isolates (AA-67 and AA-70) induced similar symptoms in lime but both sub-isolates differed from the source isolate by their dsRNA profile; iii) Ca-346 and A-154 differed from their sub-isolates (AA-73 and AA-84, respectively) both by symptom intensity and dsRNA profile. As an example, dsRNA profiles of Ca-346 and AA-73 are compared in Fig. 2. Both electrophoretic profiles had a 13.3×10^6 daltons band, corresponding to the full length replicative form, but differed by the number and/or position of the subgenomic bands. These differences were observed in at least 3 different analyses carried out during a 2-yr period

Filtration through sweet orange an/or Mexican lime. Table 2 summarizes the symptoms observed when T-317 and the sub-isolates T-318 and T-305[a] were graft-inoculated onto different indicator plants. Isolate T-317 induced mild vein clearing and stem pitting in Mexican lime, and was symptomless in Eureka lemon, sour orange, Etrog citron and in sprouts produced by rough lemon rootstock, but it induced vein clearing and stem pitting in sweet orange. Nevertheless, when sweet orange plants were inoculated with T-317 passed through Mexican lime (see Fig. 1) they grew vigorously and did not show either vein clearing or stem pitting (Fig. 4). This result was observed in three different experiments.

TABLE 1
DSRNA PROFILE AND INTENSITY OF SYMPTOMS INDUCED ON MEXICAN LIME BY SEVERAL CTV ISOLATES AND THE CORRESPONDING SUB-ISOLATES OBTAINED BY PASSAGE THROUGH SMOOTH SEVILLE ORANGE

			Symptom intensity (0 to 4)[z]	
Source isolate	Sub-isolate	Changes in dsRNA profile[y]	Vein clearing	Stem pitting
RN-15	....	....	2	1
"	AA-78	–	2	1
R-146	....	–	2	2
"	AA-67	+	2	3
"	AA-70	+	2	2
Ca-346	....	....	2	4
"	AA-73	+	1	2
A-154	....	....	3	3
"	AA-84	+	1	1

[z]0 = no symptoms; 4 = very severe symptoms
[y] + = dsRNA profiles of the original isolate and the sub-isolate were different; - = No difference between dsRNA profiles.

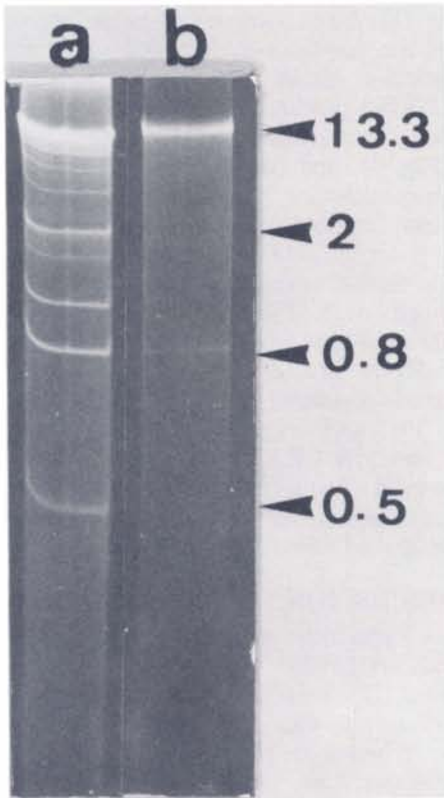

Fig. 2. DsRNA profiles obtained from Pineapple sweet orange plants inoculated with CTV isolate Ca-346 maintained in the original host (Mexican lime) (a) or passed through Smooth Seville orange (b). DsRNA in each lane was purified from ca. 5 g bark tissue, separated by polyacrylamide gel electrophoresis (5% acrylamide), and stained with ethidium bromide.

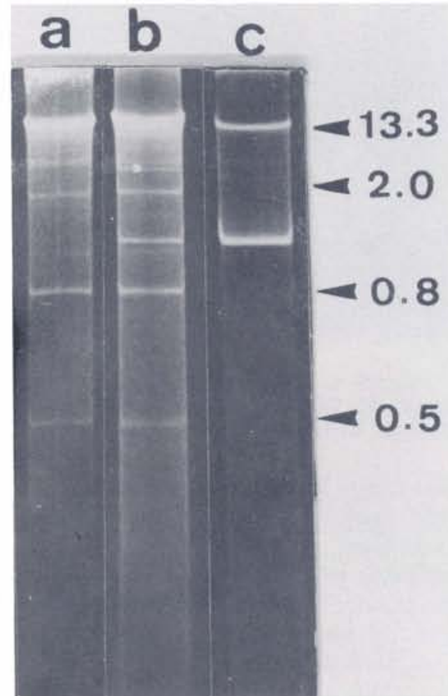

Fig. 3. DsRNA profiles obtained from Pineapple sweet orange plants inoculated with sub-isolates T-305[a] (a) or T-305 (b) or Etrog citron plant inoculated with CTV isolate T-317 (c). Sub-isolates T-305 and T-305[a] were obtained by graft or aphid-transmission, respectively, of the sub-isolate T-318 to Mexican lime (see Fig. 1). DsRNA was purified, separated, and stained as in Figure 2.

The sub-isolate T-318 induced symptoms in all the hosts assayed (Table 2). Symptoms in Mexican lime were severe and included strong vein clearing and stem pitting, vein corking, yellowing, and severe stunting. Cit-

TABLE 2
SYMPTOMS INDUCED ON SEVERAL HOSTS BY CTV SUB-ISOLATES OBTAINED BY HOST-FILTRATION (see Fig. 1).

	T-317			T-318			T-305[a]		
Hosts	VC[z]	SP[y]	SY[x]	VC	SP	SY	VC	SP	SY
Mexican lime	1	1	...	4	4	...	4	4	...
Etrog citron	0	0	...	2	2	...	2	3	...
Rough lemon	0	0	...	2	2	...	3	3	...
Sweet orange	1	2	...	3	2	...	3	2	...
Eureka lemon	0	0	–	ND[v]	ND	ND	1	1	YES
Sour orange	0	0	–	0	0	YES	0	0	YES

[z]VC = Vein clearing (rated on a 0 to 4 scale, whereby 0 = no symptom, 4 = very intense symptom).
[y]SP = Stem pitting (rated on a 0 to 4 scale).
[x]SY = Seedling yellows reaction.
[v]Not done.

Figure 4. Pineapple sweet orange inoculated with T-305 (right) or with T-317 passed through Mexican lime (left).

ron/rough lemon showed vein clearing and stem pitting in both scion and rootstock, and sour orange showed seedling yellows. When sweet orange plants were inoculated with T-318 they usually showed stronger symptoms than the original sweet orange used as inoculum source.

T-318 passed through Mexican lime by graft-transmission (sub-isolate T-305, Fig. 1), induced stunting, vein clearing and stem pitting in sweet orange, and seedling yellows in grapefruit. Mexican lime plants aphid-inoculated with T-318 (sub-isolate T-305[a], Fig.1) were stunted, and showed vein clearing, vein corking, and stem pitting as severe as graft-inoculated lime plants (Table 2). This sub-isolate induced seedling yellows in sour orange and Eureka lemon, and mild vein clearing and stem pitting in Eureka lemon. Vein clearing and stem pitting induced by T-305[a] in Etrog citron, rough lemon, and sweet orange, were similar to, or stronger than, those induced by T-318 (Table 2).

DsRNA profiles induced by T-317, T-318, T-305, and T-305[a], all presented a 13.3×10^6 daltons band, corresponding to the full length replicative form of the genome, but showed differences in the subgenomic bands. Profile of T-317 had a prominent band at the 1.3×10^6 position, that was characteristic of this isolate (Fig. 3, lane c). DsRNA profile associated with T-318 in sweet orange or citron had the same bands as T-317, but sometimes, minor bands at the positions 2, 1.2, and 0.5×10^6 daltons could also be observed. DsRNA profiles of sweet orange or citron plants graft-inoculated with T-305[a] (Fig. 1), had readily visible bands at the positions 2, 1.2, 0.8 and 0.5×10^6 daltons, but not the 1.3×10^6 band characteristic of T-317 and T-318 (Fig. 3, lane a). Sweet orange plants graft-inoculated with T-305 or T-317 passed through lime (Fig. 1) usually showed a dsRNA profile that contained the bands observed for T-305[a] and the 1.3×10^6 band (Fig. 3, land b). In some cases, the 1.3×10^6 band was not observed during the initial months after inoculation, but this band eventually appeared in further analyses.

DISCUSSION

Variations in pathogenicity and dsRNA profile were observed when some CTV isolates were passed through different hosts.

When four CTV isolates, kept in Mexican lime, and the corresponding subcultures maintained in Smooth Seville orange for 5-yr, were compared for dsRNA profiles in sweet orange, three of the sub-isolates differed from their corresponding former isolate. These variations suggest that the original isolates contained several CTV strains, but some of them did not replicate in Smooth Seville orange and were filtered out. Jarupat, *et al.* (14) observed reduced intensity or disappearance of one dsRNA band when their isolates T-505 and SY-560 were maintained in grapefruit, and they attributed this change to the presence of more than one CTV strain in the original isolate. Changes undergone by our isolate Ca-346 included disappearance of several dsRNA bands, which might indicate the presence of a more complex mixture in this isolate and/or a more selective filtration by Smooth Seville orange. Alternatively, the bands eliminated could belong to a single strain inducing a more complex dsRNA profile.

Variations in dsRNA profile were accompanied in some cases by changes in the intensity of symptoms induced in

Mexican lime (Table 1). The fact that CTV isolates with different dsRNA profiles may be biologically indistinguishable has been previously observed (18). Several authors have obtained mild CTV isolates when severe tristeza-seedling yellows isolates were passed through grapefruit (22, 24, 25). These mild isolates usually cross-protected against the original severe isolate. None of the attenuated sub-isolates obtained in this work showed cross-protecting ability against T-388, a severe CTV isolate inducing seedling yellows and stem pitting in several hosts (3).

Variations in pathogenicity and/or dsRNA profile were also observed when sub-isolate T-317, obtained from a field isolate mild in sweet/sour orange (T-385) (17, 18), was passed through sweet orange and/or Mexican lime. This sub-isolate induced mild, or no symptoms in several hosts, including citron and Mexican lime, but produced vein clearing and stem pitting in sweet orange (see Fig. 1 and Table 2). The sub-isolate passed through sweet orange (T-318) induced severe symptoms in all the indicators assayed (see Fig. 1 and Table 2). These results suggest that sub-isolate T-317 contained a mixture of CTV strains and that some of them were protecting against severe effects of T-318. These protecting mild strains may be at a lower titer, or be completely eliminated in sweet orange, thus breaking the initial ratio of strains in citron. When T-318 was graft-transmitted to different hosts, including citron, it always induced severe symptoms. This fact seems to favor the hypothesis that some mild strains present in T-317 may be eliminated by sweet orange.

Evidence for the presence of mild protecting strains in the original field isolate (T-385) has been presented (16). In this previous work, sweet orange plants were inoculated with T-318 alone or co-inoculated with 22 mild sub-isolates obtained from T-385. Plants inoculated with the mixture were vigorous and symptomless, whereas those inoculated with T-318 were stunted and pitted.

Though T-317 and T-318 showed dramatic differences in symptom expression, only minor differences could be detected between dsRNA profiles induced by both sub-isolates in citron or sweet orange. This finding confirms previous observations that pathogenicity and dsRNA profile are not necessarily related (18).

When T-318 was graft or aphid-transmitted to Mexican lime (sub-isolates T-305 and T-305[a], respectively), the new sub-isolates showed similar pathogenicity and both of them were at least as severe as T-318 in sweet orange and other hosts.

Sweet orange plants inoculated with T-305 or with T-317 passed through lime (Fig. 1), showed the same dsRNA profile but had a different pathogenic response (Fig. 4). Inoculation with T-305 resulted in stunting, vein clearing, and stem pitting, whereas plants inoculated with T-317 passed through lime were vigorous and symptomless. This is an indication that passage through lime induced some change in the initial mixture present in T-317. Somehow this change blocked release of pathogenicity usually observed when T-317 was directly inoculated onto sweet orange. The fact that passage through lime always induced an intensification of dsRNA bands at the positions 2, 1.2, and 0.5×10^6, is further evidence that lime induces some change in the mixture of CTV strains. Results obtained in this work and others (16, 17) indicate that many CTV isolates are probably a mixture of strains. Composition and/or relative concentrations of individual strains in the mixture may change according to the host and perhaps other conditions, giving rise to the variations in symptom intensity and/or dsRNA observed. The fact that host and climatic conditions may induce changes in CTV isolates (18), highlights the need to classify CTV isolates as mild or severe on specific hosts, indicating environmental factors, and gives some concern as to the wisdom of importing exotic CTV isolates for use in field experiments.

These findings are also important in the evaluation of cross-protection, since results can be affected by host and other factors that could modify the final balance of CTV strains. To obtain reliable results, those experiments should be done with isolates known to be mild in the specific host to be protected, under local conditions.

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