Interference of a Non-Seedling Yellows by a Seedling Yellows Strain of Citrus Tristeza Virus in Sweet Orange

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ABSTRACT. When a non-seedling yellows strain (T505S) and a seedling yellows strain (SY560) of citrus tristeza virus (CTV) were co-inoculated into sweet orange seedlings, the co-existence of both strains was indicated by double-stranded ribonucleic acid (dsRNA) analysis. Stable marker dsRNAs specific for each strain (Mr 1.2×10^6 for T505S and Mr 1.7×10^6 for SY560) were detected by polyacrylamide gel electrophoresis of total dsRNA extracted from individual plants. The level of detection of the T505S marker was not as prominent as in singly infected plants, and its detection became more difficult as time after co-inoculation with SY560 increased. This is evidence for interference between strains of CTV, and in this case the seedling yellows strain is the more aggressive. This interference also was noticed when plants were first inoculated with T505S and later inoculated with SY560. Detection of the T505S marker dsRNA could not be enhanced by repeated re-inoculations of SY560 infected plants with T505S. The SY560 marker was prominent in all plants inoculated with SY560 regardless of when or if they also were inoculated with T505S. Neither strain produced any obvious symptoms in sweet orange, a host chosen for its ability to express dsRNAs of CTV in a stable and reproducible manner. These results provide additional evidence that strains of CTV can co-exist in individual plants but that some components may be hard to detect when interference is active.

Viruses that occur in double or other mixed infections predictably will have some interactions, either with each other or with their hosts, that will discriminate the mixed infection from any of the possible single infections. Various examples and the range of possible interaction have been reviewed (9, 10, 12, 15, 19, 22). Effects at the level of symptom expression are the easiest to detect and describe, but additional interactions involving the replication, accumulation, translocation, and transmission of the interacting viruses, which may take place without any direct effect on symptoms of the host, are less easy to monitor. They are especially difficult to assay when the two agents of interest are strains of a single virus.

Cross protection is an example of an interaction between virus strains that has been the subject of numerous studies with citrus tristeza virus (CTV), and the use of this phenomenon to deliberately pre-immunize susceptible varieties as a tool for disease management in the field has been practiced with some success. Varieties that have been protected in this way include Pera sweet orange and limes in Brazil (3, 18), grapefruit in Australia (4, 23, 24) and S. Africa (25), Hassaku pummelo-mandarin in Japan (21), *Citrus hystrix* DC. in Reunion (1) and acid lime in India (2).

The majority of such studies with CTV have relied solely on symptom expression to monitor the effects of the interaction. This reflects a lack of laboratory methods that can be used to discriminate between some strains. Double-stranded RNA (dsRNA) analysis has been shown to discriminate between strains of CTV (6,7) and this paper presents the first attempt to use this technique to investigate virus-virus, as opposed to virus-host, interactions in sweet orange doubly infected with two strains of CTV (T505S and SY560). Previous studies have indicated that the chosen strains can be distinguished from each other by dsRNA analysis (6, 13). In this study sweet orange was chosen as a host because it has proven the most reliable for dsRNA analysis of numerous strains of CTV (6, 7). A similar approach to the study of interference and cross protection has been attempted using cucumoviruses (5, 8) or tobamoviruses (Jarupat and Dodds, unpublished).

MATERIALS AND METHODS

Hosts. Seedlings of Madam Vinous sweet orange, Standard sour orange, Eureka lemon and Duncan grapefruit were used.

Virus strains. Two strains of CTV, a non-seedling yellows (T505) and a seedling yellows (SY560) type, were selected for the experiments. The description of these strains has been reported previously (6). Strain T505 gives no reaction in sour orange, grapefruit and lemon and very mild stem pitting in sweet orange. Strain T560 gives a seedling yellows reaction in sour orange, grapefruit and lemon and no reaction in sweet orange. A subculture of non-seedling yellow strain T505 designated as T505S, was derived from a single Etrog citron 861-S1 seedling that had been inoculated with the parental culture of T505 which is maintained in sweet orange. Some plant to plant variability in dsRNA expression encountered with this strain (T505) that was absent for the other strain (SY560) was observed in Etrog citron and has allowed us to select a subculture with a prominent Mr 1.2 x 10^6 dsRNA that is not normally expressed in the parental culture. This marker dsRNA proved stable for this subculture in sweet orange and is not expressed by SY560. SY560 has two distinctive dsRNA markers (Mr 1.7 x 10^6 and Mr 0.5 x 10^6) that are not expressed by T505S.

Inoculations. The four citrus species (listed above) were grown one to two seedlings per pot and trained to a single leading shoot for 12-16 months or until the stem was 7 to 10 mm in diameter. Each individual seedling was cut back to a height of 20 cm above the top of the pot prior to inoculation. The seedlings were singly inoculated or simultaneously co-inoculated with the two CTV strains which were maintained in sweet orange. Two patch grafts (0.5 to 1.0 cm) were taken from young green bark tissue of the source plant and used as inoculum for any given strain when inoculating a single seedling.

New flush growth was trained to a single leading shoot for 6 months then cut back to 10 cm above the previous cut. Bark tissue was removed from individually harvested shoots and 2 g of sample was analyzed for dsRNA. The rest of the bark tissue samples were stored at -20 C. The new growth of individual seedlings was harvested and analyzed twice more, 3 months apart if necessary.

The infected plants also were re-inoculated either once or twice as detailed in Fig. 1, and subsequent growth was used for dsRNA analysis every 3 months after inoculation. A summary of the various inoculation treatments is shown in Fig. 1 and Table 1, and the different treatments are identified by numbers. There were four seedlings per treatment and the entire experiment was replicated three times.

Isolation and analysis of double-stranded RNA. The method described previously (6) was used except bentonite (nuclease inhibitor) was not added (14). All results are for 2.0 g of bark tissue from individual seedlings. In an attempt to detect heterogeneity in the major RF dsRNAs, Mr 13.3 x 10^6, of the two strains, prolonged electrophoresis for 18-21 hr in polyacrylamide gels was used. The electrophoresis buffer was changed every 5-6 hr.

RESULTS

Symptom expression. Strains T505S and SY560 did not cause any obvious symptoms in sweet orange and so symptom expression could not be used as a measure of interference or cross protection for these strains.

DsRNA profiles of CTV strains T505S and SY560 in sweet orange. The marker dsRNAs of T505S (Fig. 2, lane 1, band b) and SY560 (Fig. 2, lane 2, bands a and c) noted in the source plant used to maintain the isolates (Fig. 2, lanes 1 and 2) were consistently observed in stained electrophoresed polyacrylamide gels when bark tissue from singly infected sweet orange used for the experiment was analyzed (Fig.

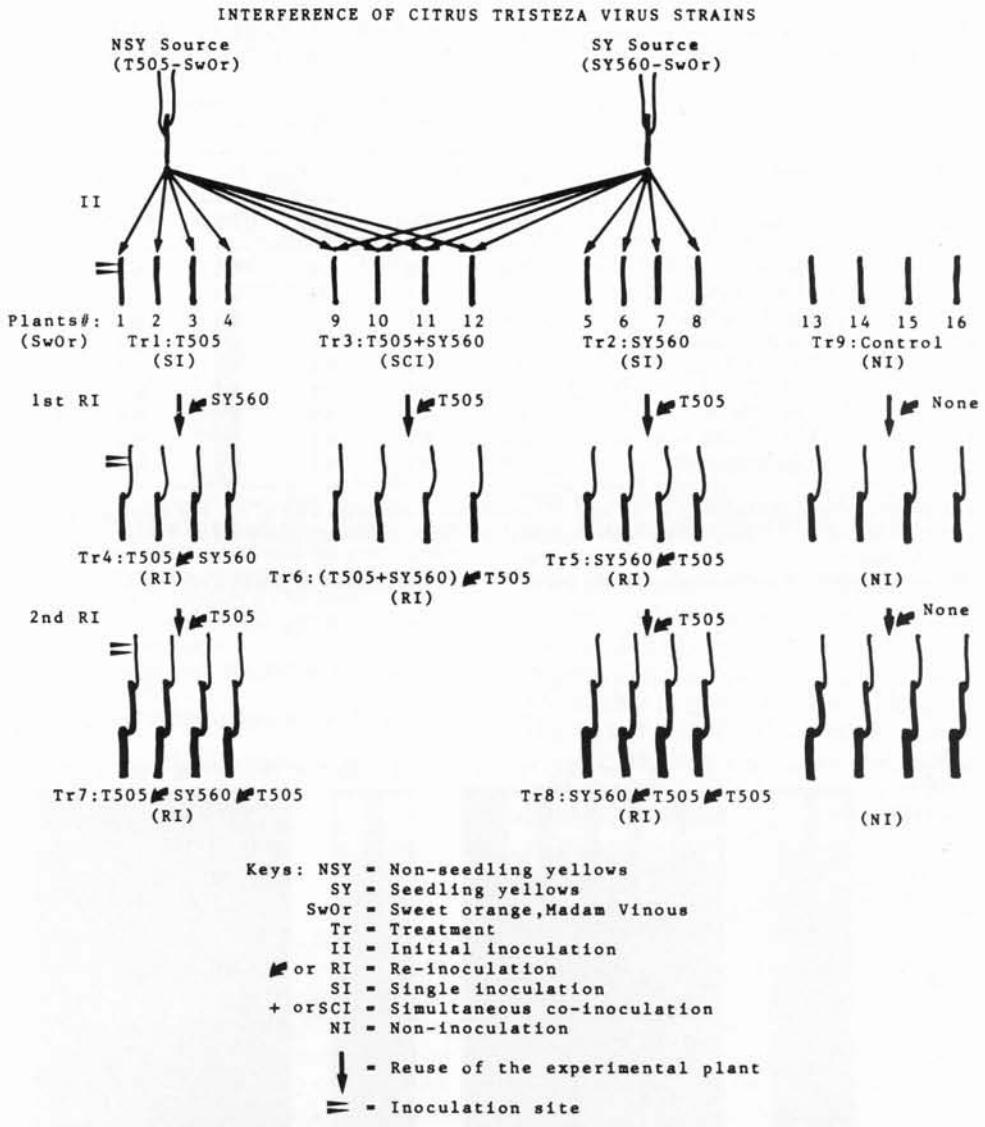

Fig. 1. Schematic of the path of inoculations used in attempts to establish co-infections and examine interactions between two strains of CTV, T505S and SY560.

2, lanes 3 and 4 and Table 1). All three marker dsRNAs were detected in a single gel lane when a physical mixture of the dsRNAs of T505S and SY560 purified from singly infected plants was analyzed (Fig. 2, lane 7). The samples mixed are those illustrated in Fig. 2, lanes 3 and 4.

DsRNAs in single and double inoculated sweet orange. A result similar to that for a physical mixture of the dsRNAs of T505S and SY560, where all three marker dsRNAs could be detected, was observed in 8 to 42% of the seedlings receiving treatments 3, 4 and 7 (Table 1). The intensity of the marker dsRNA (band b) of T505S was not as prominent in mixed infections as it was in single infections (Fig. 2, lanes 5 and 3 respectively). A typical level for the intensity of band b in mixed infection is shown in Fig. 2, lane 6 where it is barely detectable. The treatments in which mixed infections were most noticeable were those where T505S infected plants were co-inoculated or re-

TABLE 1
DETECTION OF TWO STRAINS OF CTV, T505S AND SY560, IN SWEET ORANGE AFTER CO-INOCULATION OR RE-INOCULATIONS WITH THE SECOND STRAIN

Treatments	Strains and type of inoculations[a]	Number of plants expressing strain specific dsRNA markers[b]					
		Expt. 1		Expt. 2		Expt. 3	
		T505S	SY560	T505S	SY560	T505S	SY560
1	T505S	4/4	0/4	4/4	0/4	4/4	0/4
2	SY560	0/4	4/4	0/4	4/4	0/4	4/4
3	T505S + SY560	0/4	4/4	1/4	4/4	3/4	4/4
4	T505S < SY560	3/4	4/4	0/4	4/4	2/4	4/4
5	SY560 < T505S	0/4	4/4	0/4	4/4	0/4	4/4
6	Tr3 < T505S	0/4	4/4	0/4	4/4	0/4	4/4
7	Tr4 < T505S	1/4	4/4	0/4	4/4	0/4	4/4
8	Tr5 < T505S	0/4	4/4	0/4	4/4	0/4	4/4
9	Non-inoculated	0/4	0/4	0/4	0/4	0/4	0/4

[a]Co-inoculation is indicated with a "+" and re-inoculation is indicated with a "<". For example, plants already infected with T505S which were re-inoculated with SY560 are indicated as T505S < SY560. Tr = treatment.
[b]Number of plants showing strain specific marker(s) over the number of plants inoculated.

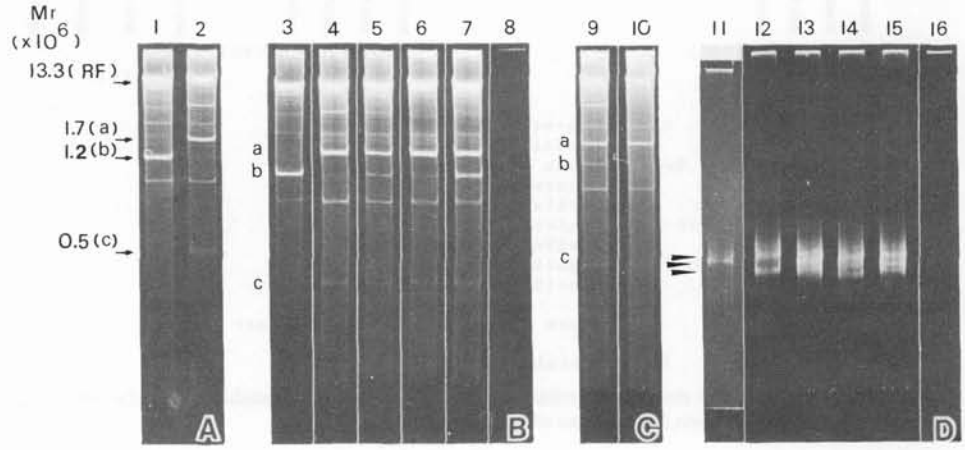

Fig. 2. DsRNAs of two strains of CTV, T505S and SY560, detected in singly or mixedly inoculated plants. Polyacrylamide gels (6.0%) were electrophoresed for 3 hr (Panels A, B and C), or 21 hr (Panel D) and stained with ethidium bromide. Lanes illustrate T505S (1) and SY560 (2) in the sweet orange plants used as a source of inoculum to initiate the experiment. Note the marker dsRNAs that identify T505S (marker "b", Mr 1.2 x 10^6), and SY560 (markers "a", Mr 1.7 x 10^6, and "c", Mr 0.5 x 10^6) in addition to the major RF dsRNA (Mr 13.3 x 10^6, near top of gel) that is common to both strains. Lanes 3 and 4 = single infection of T505S and SY560 (treatments 1 and 2), respectively. Lanes 5 and 6 = co-inoculated plants (treatment 3) showing markers "a" and "c" (both lanes) and "b" (lane 5 readily detectable, lane 6 barely detectable). Lane 7 = a physical mixture of the dsRNAs used for the results shown in lanes 3 and 4. Lane 8 = non-inoculated control. Lanes 9 and 10 = a plant expressing all three marker dsRNAs before re-inoculation with SY560 (lane 9) and only marker "a" and "c" after re-inoculation (lane 10).

DsRNAs from single infections of sweet orange with T505S (lane 11) and SY560 (lane 12) and their physical mixtures (lane 13) are compared to dsRNAs from co-inoculated (lane 14, treatment 3) and re-inoculated (lane 15, treatment 7) plants by prolonged electrophoresis of the major dsRNA (Mr 13.3 x 10^6).

inoculated with SY 560 (Table 1, treatments 3 and 4).

Samples from plants that were infected for some time with SY560, either alone or together with T505S and were re-inoculated with T505S (treatments 5 to 8) gave no evidence for the presence of band b (except one plant in treatment 7). The dsRNA pattern in such samples could not be distinguished from the pattern expected for SY560 alone.

In a separate experiment from the one summarized in Fig. 1, plants in which all three marker dsRNAs were detected (Fig. 2, lane 9) were re-inoculated with SY560. New growth in these plants gave no evidence for band b of T505S (Fig. 2, lane 10). Without knowing the history of such plants it would be difficult to conclude that T505S was present, since it is at undetectable levels.

Prolonged electrophoresis of dsRNA from sweet orange. The major RF dsRNA of SY560 was heterogeneous, since it was resolved as two components (Fig. 2, lane 12) after prolonged electrophoresis in polyacrylamide gel, whereas the RF of T505S was homogeneous, since it remained a single component with an electrophoretic mobility intermediate between the values for the two components of SY560 (Fig. 2, lane 11). Three components were resolved when the physical mixture of the two dsRNAs was electrophoresed (Fig. 2, lane 13), as would be expected from the results for the individual strains. Three components were also detected in dsRNAs from some doubly inoculated plants, which is additional evidence for double infection (Fig. 2, lanes 14 and 15). This kind of evidence was obtained for plants that were either co-inoculated (treatment 3) or re-inoculated (treatment 7).

Other hosts. The three dsRNA markers were not expressed adequately in grapefruit, sour orange and lemon, confirming results of previous studies (6). The only exception was the expression of the T505S marker in lemon. It was not possible to draw a conclusion about the status of double infection in doubly inoculated plants of these species. Therefore, symptom expression in these hosts was not examined.

DISCUSSION

Using a technique of dsRNA analysis that has proved useful for detecting and analyzing mixed infection of strains of other viruses, it has been demonstrated that two strains of CTV can co-exist in sweet orange under some circumstances involving either co-inoculation or delayed inoculation of one of the strains.

An interference was observed since the level of detection of the marker dsRNA of T505S was always less than in single infections, and its detection became more difficult as time after co-inoculation increased. One of the interesting results of this study is the conclusion that under some circumstances a strain of CTV (T505S) can co-exist in sweet orange with another strain (SY560), but at levels too low to detect by regular dsRNA analysis. A similar general conclusion was also drawn in a previous study (13) in which grapefruit was shown to be a host in which the specific marker dsRNAs of the two strains used in the present study were repressed to undetectable levels in single infections, but which, at least for SY560, re-appeared upon subculture of the isolate in sweet orange.

These two types of experiments both support the idea that isolates of CTV need to be carefully evaluated to determine to what extent they represent mixtures of strains (11, 16, 17, 20). The previous study (13) led to the conclusion that SY560 is itself a mixture of strains, and results of prolonged electrophoresis of the Mr 13.3×10^6 RF dsRNA of SY560 in the present study also suggest this since, unlike T505S, the RF segregated into at least two components. Evidence for RF dsRNA heterogeneity in SY560 based on shorter electrophoresis times (in tube gel system) was reported previously (7).

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