Additional Factors Affecting dsRNA Analysis of Citrus Tristeza Virus

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ABSTRACT. We have investigated factors that may have an effect on dsRNA analysis of citrus tristeza virus (CTV). Storage of bark tissue and purified dsRNA at -20 C for up to at least 4 yr and repeated freezing and thawing of each of them appeared to have no detrimental effect on the quality of dsRNA detected by polyacrylamide gel eletrophoresis. The aqueous phase following phenol extraction could be stored at room temperature or at 4 C with or without ethanol (adjusted to 16.5%) for at least 2 weeks. Buffer saturated phenol which has been kept at room temperature for 2.5 yr was still useful for CTV ds RNA extraction. DsRNA extraction could be done without adding nuclease inhibitors, i.e. bentonite or macaloid. Precipitated dsRNA could be recovered by centrifugation immediately after addition of ethanol and sodium acetate. Hosts which yielded good dsRNAs were Pineapple, Navel, and Valencia sweet orange and the scions of Madam Vinous sweet orange on sour orange rootstock. Shoots from the sour orange rootstock gave poor results. Green bark tissue from the older part of the current shoot usually gave a better result than tissue from the young tender shoot tip and the old brown stem from previous flushes. DsRNA results were least acceptable in the summer from various well infected screenhouse grown citrus species.

Citrus virologists have attempted to develop several techniques for the identification of strains of citrus tristeza virus (CTV) and these may now be used to complement established methods which rely on biological indexing in seedlings of *Citrus* spp. (9). The newer methods include the use of monoclonal antibodies (10, 15, 20), nucleic acid hybridization probes (6, 7, 16, 17, 18), and double-stranded (ds) RNA analysis (3, 4, 5, 6, 8, 13). DsRNA analysis has been used to discriminate between multiple strains since each is often clearly distinguished from all others. The other techniques are better suited for grouping strains into broad categories based on their reactivity or not with a single reagent. Correlations with biological properties have been obtained with all methods, but much more work will be needed before they can be used with confidence to type any strain for virulence without resort to biological indexing.

Dodds *et al.* (6, 8) and Lee (13) have demonstrated the value of dsRNA analysis for CTV detection, diagnosis and for identification of strains. In subsequent studies, the value of the technique for investigating host-virus interactions including host passage effects, seasonal variation, and field surveys has been demonstrated (5, 7, 11). In the course of doing these various experiments we have gained additional insight into factors that need or do not need critical attention in order to obtain reliable results. We have also had numerous requests to provide information and share our experiences in order to solve problems with dsRNA analysis.

This article summarizes several experiments which were designed to answer questions about dsRNA analysis of CTV using cellulose chromatography purification and polyacrylamide gel electrophoresis for analysis. Some of the experiments (PAGE) selected for inclusion in this report will be of special interest to those with limited resources who would like to adopt the technique or simplify the process while reducing overall costs without sacrificing the quality of the results. In addition, experiments which are extensions or continuations of previous studies (more strains, additional hosts, other growth conditions, etc.) are also described.

MATERIALS AND METHODS

Hosts for CTV. Seedling of Madam Vinous, Pineapple, Navel, and Valencia sweet orange, Standard and Brazilian sour orange, Mexican lime, Eureka

lemon, Duncan, Ruby Red and White Marsh grapefruit, Etrog citron and *Citrus excelsa* were used.

Strains of CTV. Four strains were designated, A, B, C and D for this study, and are T505 (A), 19V (B), SY560 (C) and 565V (D). Details of these strains have been reported (5). Seven additional isolates were used, five of which were known seedling yellows type isolates, one which was a known non-seedling yellows type and one of uncertain biology.

Inoculation and sampling. Experimental plants (12-16 month-old healthy seedlings of various *Citrus* spp.) were inoculated with each CTV strain by chip or bud-grafting. The sources of inocula were generally maintained in sweet orange. One set of the same number of non-inoculated seedlings was always included in each experiment.

The inoculated seedlings were cut back and the new flush growth was trained to a single shoot per plant. New shoots were harvested 3 - 6 months after inoculation. The green bark tissue from each shoot was removed, cut into small pieces, mixed, used immediately or stored individually, usually at -20 C. The largest sample stored was a pool of bark of Madam Vinous sweet orange infected with strain C. Unless otherwise mentioned, this was the tissue used for the following experiments.

Isolation and analysis of dsRNA. The method used was a modification of a general scheme (1, 12, 14, 19) adapted for use with *Citrus* spp. infected with CTV (5). Some small changes from the previously described method (5) were adopted. Ethanol concentration for binding dsRNA to CF-11 powder was 16.5%. DsRNA purified from 2.0 g of tissue was resuspended in 20 - 40 µl of eletrophoresis buffer for storage and subsequent analysis by electrophoresis. Electrophoresis was at a constant voltage (100 V) at 45 - 50 mA for 3 hr and gels were stained in ethidium bromide (500 ng/ml). Unless otherwise stated, the dsRNA analyzed on a gel lane represents the amount that was isolated from 1 g of tissue. All other conditions were as previously described (5).

Quality and quantity of reagents used for CTV dsRNA purification

Quality of phenol. Phenol saturated in double-strength STE buffer [single strength STE is 0.1M sodium chloride, 50 mM tris (hydroxymethyl) amino-methane (Tris), 1mM ethylenediamine-tetracetic acid (EDTA), pH 6.8] was collected and stored at room temperature for as long as 33 months. Aged or newly prepared phenol was used as a denaturant for extraction of dsRNAs from buffered extracts from citrus bark.

Exclusion of nuclease inhibitors. DsRNA is a stable molecule which is less readily digested by RNAse than ssRNA. DsRNA was purified from bark with or without the nuclease inhibitors bentonite or macaloid (0.2 ml at 25 mg/ml per 4 ml of nucleic acid extraction buffer) in the tissue extraction buffer.

Effect of storage on quality of purified CTV dsRNA

Storage of aqueous nucleic acid solutions. The aqueous phases obtained after phenol treatment and centrifugation of bark extracts were pooled and divided into five equal samples. The samples were then subjected to the following treatments: 1) regular dsRNA extraction as a positive control; 2) adding ethanol to 16.5% before storage at 2-8 C for 14 days; 3) the same as treatment 2 but stored at 20-25 C; 4) adding ethanol to 16.5% after storage at 2-8 C for 14 days and; 5) the same as treatment 4 but stored at 20-25 C. The samples receiving treatments 2 through 4 were subsequently purified by CF11 column chromatography as usual.

Storage and precipitation of dsRNA with ethanol. The duration of ethanol precipitation conditions on recovery of dsRNAs were evaluated. Two volumes of cold 95% ethanol and 0.1 vol of 3M sodium acetate, pH 5.5

was added to purified dsRNAs. Samples were collected by centrifugation either immediately or 20 min after adding ethanol, or after overnight incubation.

Storage of bark issue. Bark tissue was processed immediately or was stored as a single sample at - 20 C for up to 4 yr. No attempt was made to minimize the effects of thawing each time the stored sample was removed from the freezer in order to obtain a sample for dsRNA purification.

Effect of source of bark tissue on dsRNA result

Injected sweet orange seedlings were cut back and re-trained to a single shoot on 4 occasions, 3-6 months apart. Bark tissue from three sections of individual stems was collected separately as follows; young tender shoot tip 10-15 cm long from the growing point, green older bark from the current shoot, and the old brown stem from previous flushes. The bark samples were then used for dsRNA analysis.

Seasonal effects of dsRNA in experimentally infected Citrus spp.

Plants were inoculated with CTV strains, A, B, C or D in 1984. Three seedlings were used for each of seven species for each strain. Single shoots were harvested and analyzed for dsRNA 6 months after inoculation and then at regular intervals for subsequent experiments. Infected plants were cut back on March 21, 1988 and moved from a greenhouse to a screenhouse. Each new flush was trained to a single shoot which was cut back on June 21, 1988. A new set of single shoots was allowed to grow and these were cut back on September 21, 1988. At each cut back, shoots were peeled of their bark tissue which was stored for dsRNA analysis.

CTV ds RNA in grafted plants of sweet orange scions on sour orange rootstocks

Brazilian sour orange seedlings were inoculated with CTV strains A, B, C or D, 4 seedlings per strain. All of the test plants were trained, harvested and analyzed for dsRNA. Immediately after harvest, two buds of healthy Madam Vinous sweet orange budded on to each individual test plant. Sweet orange shoots were trained to a single shoot per plant. The shoot was harvested and analyzed for dsRNA.

In a separate experiment, strains C and D [low and high CTV dsRNA accumulation in sour orange respectively (5)] were inoculated into either the sweet orange or the sour orange part of a grafted plant. Four plants were used for each treatment. The test plants were trained to two shoots per plant, one from the sweet orange scion and the other from the sour orange rootstock. The shoots were harvested and analyzed for dsRNA.

Comparison of CTV dsRNA in different cultivars of some Citrus spp.

Emphasis was given to cultivars of commercial interest to the California citrus industry. Strains A, B,C or D were inoculated to Pineapple sweet orange seedlings, four seedlings per strain. Another seven California CTV isolates were separately inoculated into Navel and Valencia sweet orange seedlings, or Ruby Red and White Marsh grapefruit, seven seedlings per cultivar per isolate. All of the plants were trained to multiple shoots, harvested and analyzed for dsRNA.

RESULTS

Quality and quantity of reagents used for CTV dsRNA purification. Results of CTV dsRNA analysis were similar regardless of which solutions of buffer saturated phenol were used. These included freshly prepared solutions as well as those that were between 1 and 33 months (approximately 2.5 yr) old. The older solutions were colored black. The results obtained using these solutions are shown in Fig. 1A, lanes 1 and 2.

Results of CTV dsRNA analysis were similar with (Fig. 1A, lane 1) or without (Fig. 1A, lane 3) the addition

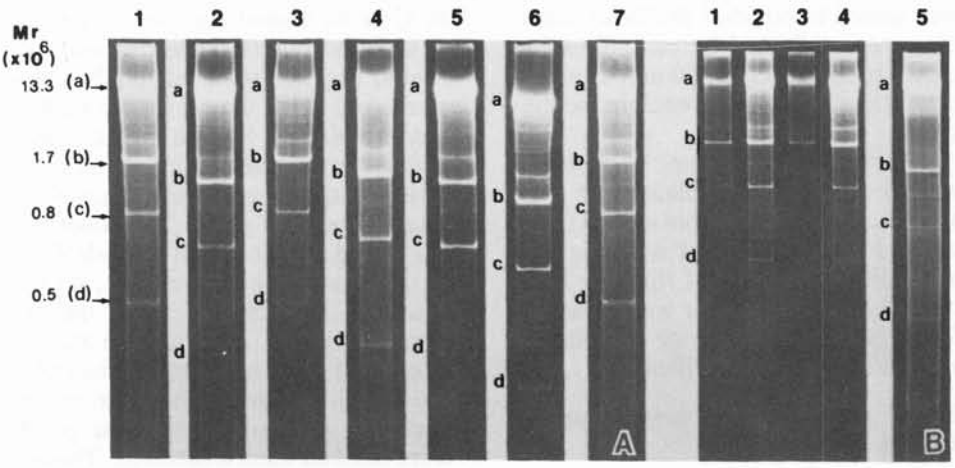

Fig. 1. Modifications to standard dsRNA purification method. DsRNAs of CTV strain C (SY560) stained with ethidium bromide after electrophoresis in 6.0% polyacrylamide gels. DsRNAs from approximately 1 g of Madam Vinous sweet orange tissue was analyzed on each gel lane. DsRNA patterns are from different gels, unless otherwise noted. Labels for specific dsRNA bands show their relative mobilities in the different gels.

A. Effect of variations in purification method. Quality of dsRNAs after using fresh phenol and bentonite (1), using 33-month-old phenol and bentonite (2), using fresh phenol and no bentonite (3), storage of aqueous nucleic acid solution for 14 days at room temperature before addition of ethanol to 16.5% (4), storage of bark tissue for 4 yr at - 20 C (5), and storage of purified dsRNAs in electrophoresis buffer for 4 yr at -20 C (6), ethanol precipitation of dsRNAs for 20 min at -20 C (7).

B. Effect of bark age. Recovery of dsRNAs from old brown stem from a previous growth flush (1), green bark from the older part of the present shoot (2), young tender shoot tip tissue (3), and young tender shoot tip with terminal leaves (4 and 5). Lanes 1-4 depict results from the same gel.

of either bentonite or macaloid to the extraction buffer.

Effect of storage on quality of purified CTV dsRNA. The aqueous phase of buffered bark extracts could be stored on the bench top (20-25 C) or in the refrigerator (2-8 C) with or without the addition of ethanol for up to at least 14 days without any effect on dsRNA detection by PAGE (Figure 1A, lane 4). CTV infected bark tissue samples could be stored at -20 C for up to at least 4 yr without any effect on the dsRNA recovery (Figure 1A, lane 5). The final purified dsRNA could be stored at -20 C in electrophoresis buffer containing 20% glycerol for up to at least 4 yr (Fig. 1A, lane 6). Purified, air dried dsRNA could be stored for 2 weeks at room temperature without effects on recovery; however, it was undetectable after 4 yr of storage. Repeated freezing and thawing of both infected bark tissue and purified dsRNA stored in electrophoresis buffer with glycerol appeared to have no serious detrimental effect on the quality of recoverable CTV dsRNA. The quantity and quality of CTV dsRNA recovered by centrifugation after 20 min (Fig. 1A, lane 7) or overnight storage in ethanol, or when samples were centrifuged immediately after the addition of cold ethanol was similar.

Effect of source of bark tissue on dsRNA result. Green bark from the older part of the current shoot usually was a better source of dsRNA than was tissue from the young tender shoot tip, or the old brown stem from the previous flushes (Fig. 1B, lanes 1-3). The quality of the dsRNA was worse from young tips with terminal leaves than from older green bark. Overall background fluorescence in stained gels was higher and this masked detection of minor dsRNAs. In addition, a band known to be DNA from other studies was more prominent in samples from young tips (Fig. 1B, lanes 4 and 5).

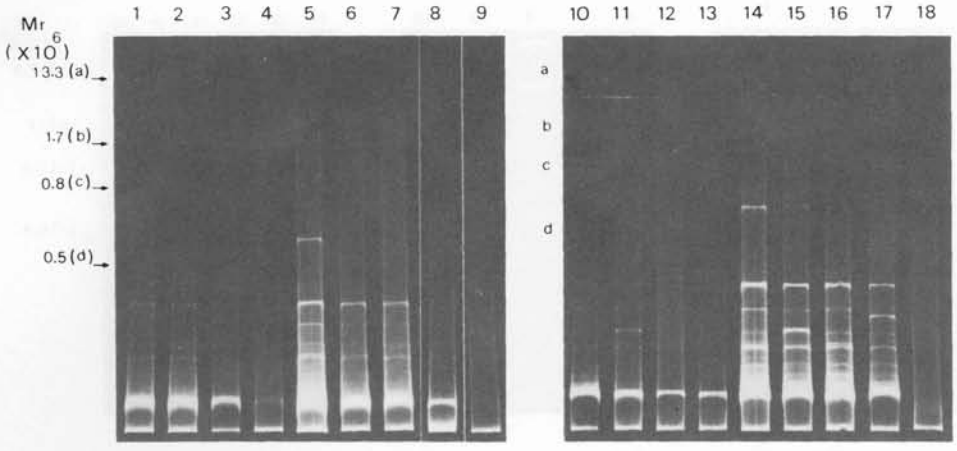

Fig. 2. Seasonal effects on CTV dsRNA recovery from two hosts. DsRNAs of CTV strains A, B, C, and D stained with ethidium bromide after electrophoresis in 6.0% polyacrylamide gels. DsRNAs from approximately 1 g of Madam Vinous sweet orange (lanes 1 - 9) or Standard sour orange (lanes 10 -18) tissue were analyzed on each gel lane. The spring (March 22 to June 21, lanes 1-5 and 10-14) and summer (June 22 - September 21, lanes 6-9 and 15-18) flushes of growth were compared. Non-inoculated controls were also analyzed (lanes 1 and 10).

Seasonal effects on dsRNA in experimentally infected Citrus spp. The amount of dsRNA recovered and the intensity of minor dsRNAs was highest and lowest in extracts from sweet orange and sour orange, respectively, when bark was collected in June after a spring flush (Fig. 2, lanes 2-5 and 11-14). The previously reported (5) host effect, whereby specific dsRNAs detected in sweet orange are repressed in sour orange (for example, compare results for strain C in lane 13 and lane 4) was observed in this experiment. Results for other citrus species (*C. excelsa*, citron, grapefruit, lemon and Mexican lime) fell between these extremes (data not shown) and resembled results from a previous greenhouse study (5). The amount and quality of dsRNA was not as good when isolated from the same plants in the next flush (September), by which time new growth had been exposed to summer temperatures in a screenhouse. The CTV major dsRNA (Mr 13.3 x 10^6 Mr) was reliably detected in all of the samples but accumulation of minor dsRNAs was repressed (Figure 2, lanes 6-9 and 15-18).

CTV dsRNA in grafted plants of sweet orange scions on sour orange rootstocks. CTV dsRNA recovery was poor from Brazilian sour orange seedlings (Fig. 3, lanes 2-5), except for strain D, which was previously reported to yield high levels of CTV dsRNAs (5). Despite this, the yield of dsRNAs from Madam Vinous sweet orange was high when this variety was grafted as a non-infected scion onto these infected sour orange seedlings (Fig. 3, lanes 7-10). Host effects described in the previous section were apparent. DsRNA yield was also high in sweet orange and low in sour orange when non-infected grafted combinations were inoculated with CTV. The overall results were the same regardless of whether inoculation was to the sweet orange scion or the sour orange rootstock (Fig. 3, lanes 13-16).

Comparison of CTV dsRNA in different cultivars of some Citrus spp. The quantity and quality of dsRNAs isolated from Pineapple, Navel, and Valencia sweet orange were similar to that from Madam Vinous, which had been used as a host for CTV in most previous studies of CTV dsRNAs (Fig. 4). All gave reliably high yields of dsRNAs with excellent resolution of minor dsRNAs. Grapefruit cultivars Duncan, Ruby Red and White Marsh gave poorer results than sweet orange (data not shown). In addition to the

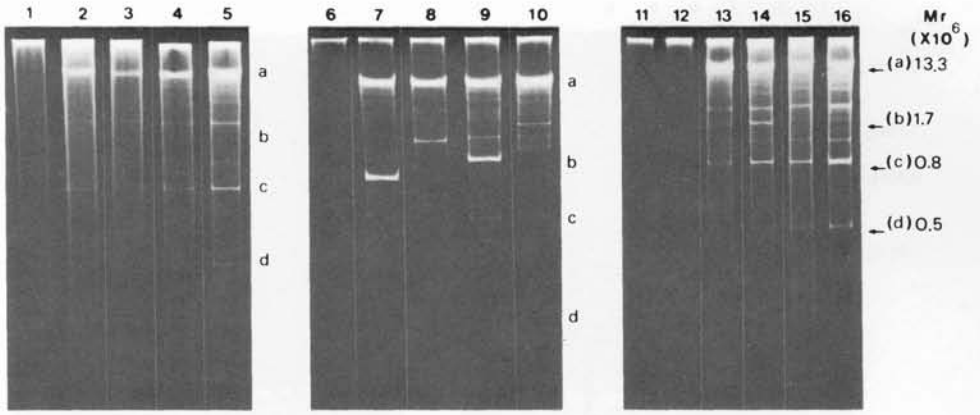

Fig. 3. CTV dsRNAs in grafted trees with sweet orange scions and sour orange rootstocks. Accumulation of dsRNAs in CTV-infected sour orange seedlings prior to being grafted with non-infected sweet orange (lane 1, non-inoculated and lanes 2-5, strains A, B, C and D respectively). Accumulation of dsRNAs in initially non-infected sweet orange scions after grafting to CTV-infected sour orange rootstocks (lanes 6, non-inoculated and lanes 7-10 strains A, B, C and D respectively). Accumulation of dsRNA in sour orange shoots from rootstocks (lanes 13 and 15) or sweet orange scions (lanes 14 and 16) inoculated in the rootstock with strain C (lanes 13 and 14) or strain D (lanes 15 and 16). A non-inoculated plant with a sour orange rootstock (lane 11) and a sweet orange scion (lane 12) was also tested.

commercial citrus tested, sour orange cultivars Standard and Brazilian gave poorer results in other experiments than sweet orange with most strains (Fig. 2, lanes 11-14 and Fig. 3, lanes 2-5).

DISCUSSION

It appears that the dsRNA purification method is a flexible one and can be expected to give good results even when some steps are treated with less attention to detail than is normally recommended for nucleic acid purification. Comments and suggestions for CTV-dsRNA analysis are summarized in Table 1. There is no need to prepare fresh phenol at regular intervals if dsRNA extractions are being initiated only occasionally. Efforts to prevent ribonuclease activity by the addition of bentonite or macaloid also seem to be unnecessary. Phenol, which is a good nuclease inhibitor, appears to be satisfactory with the buffer, solutions and tissues used. This knowledge should result in savings in the cost of phenol for dsRNA analysis and in the time

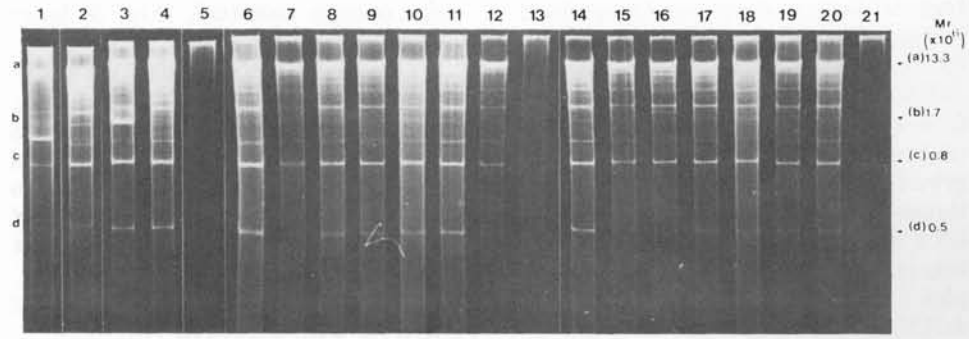

Fig. 4. CTV dsRNAs in three sweet orange cultivars. Pineapple sweet orange inoculated with strains A, B, C and D (lanes 1-4 respectively) and non-inoculated (lane 5). Navel (lanes 6-12) and Valencia (lanes 14-20) sweet orange inoculated with seven additional, different isolates of CTV and non-inoculated (lanes 13 and 21).

TABLE 1
COMMENTS AND SUGGESTIONS REGARDING SOME STEPS INVOLVED IN THE SIMPLIFICATION OF dsRNA ANALYSIS OF CTV AS DESCRIBED BY DODDS ET AL. (5)

Steps	Comments and suggestions
Tissue selection	
Variety	Most commercial sweet orange varieties are good. Sour orange rootstock does not affect dsRNA result from sweet orange scion.
Type	Older green bark tissue is preferable.
Collection	Following a period of moderate weather is best.
Tissue storage	
Temperature	Freeze at -20 C.
Duration	Up to at least 4 yr even with thawing and freezing cycles.
Nucleic acid extraction	
Phenol	Need not be freshly made. Storage at room temperature is fine.
Additional RNase inhibitors	Not necessary
Concentration of dsRNA	Centrifugation can be done immediately after addition of ethanol/acetate.
Stability of dsRNA	DsRNA in phenol extracted buffered sap is not easily degraded at room temperature. Final dsRNA sample in water or buffer stays good for years when stored at -20 C.

that need not be spent preparing additional nuclease inhibitors. Infected bark tissue can be stored for several years and still be used as a source of good quality dsRNAs of CTV. Partially purified dsRNAs of CTV can be left in STE buffer at room temperature for at least 2 weeks before cellulose chromatography is started. After chromatography, there is no need to incubate partially purified dsRNA in ethanol for any length of time in order to precipitate and concentrate it, and so it is realistic to proceed with this purification step immediately after the addition of ethanol. Once purified and concentrated, CTV dsRNAs stand up very well to storage frozen in buffer. These observations can be helpful when designing experiments since it is now known that times for tissue storage, and the steps for dsRNA purification and analysis can be interrupted at various points. They are also helpful in determining how many samples to process at a given time, and tend to make it more feasible to handle larger numbers. In this study it was not uncommon to handle 64 samples at a time. No doubt other modifications could be made to the method. It was not the purpose of this study to investigate all possible short cuts, but to point out that most laboratories, including many from developing countries that have contacted us in recent years, should be able to adapt this technique for dsRNA detection.

Attention has been paid to the interpretation of dsRNAs other than the major 13.3 x 10^6 dsRNA for strain discrimination (5, 7, 13). Experiments involving hosts and seasonal influences reported in this paper and in a previous study (5) indicate that attention to detail is needed if comparisons are to be made, especially if it is known that samples to be compared have different histories. Particular attention needs to be given to the growth conditions before samples are collected. Results of this and another study (5) indicate that minor dsRNAs appear to accumulate best when temperature conditions

were moderate rather than extreme and when the host was sweet orange. In this study, SY560 seemed to be the least affected by high temperatures. The observation that strains of CTV respond selectively to high temperatures has been previously demonstrated (2). In addition to the need to optimize harvest times for detection of maximum strain differences, choice of sampling date will be an important consideration for surveys of field collected tissues.

Previous studies have emphasized the effects of host species on the quantity and quality of dsRNA for a given strain (5, 11). The results presented here for grafted plants (sweet orange scions on sour orange rootstocks) confirm and extend these observations. One host could not alter the levels and quality of dsRNAs expected for the other, regardless of whether inoculation was before or after the grafting and, if after, at what site. The consequence of this was that dsRNA yields for some strains of CTV were high or low in different parts of the same plant (scion and rootstock).

A previous study (5) indicated that dsRNA results, especially those for minor dsRNAs, were better in sweet orange than in grapefruit for most CTV strains. Sufficient analysis of several commercial cultivars of sweet orange and grapefruit have now been completed and the results support the original conclusions. This strengthens the belief that dsRNA analysis can be used to survey commercial sweet orange groves for the incidence and type of CTV in individual trees. Surveys of grapefruit would be better done by first obtaining an isolate by inoculation to sweet orange seedlings. Care should be taken to take into account the possible effects of host passage through grapefruit, including the suppression of specific dsRNA components (11). Similar measures and care would be needed if the focus of dsRNA analysis was on rootstocks such as sour orange.

The modifications to the dsRNA extraction methods mentioned in this application also work well for plants infected with other RNA plant viruses such as tobacco mosaic (tobamovirus), cucumber mosaic (cucumovirus) and virus-like agents of avocado (unpublished data).

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