Segregation of Sweet Orange Stem Pitting Types and Stunting Factors in Subcultures from the Severe SY568 Strain of *Citrus tristeza virus*

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ABSTRACT. A strain of *Citrus tristeza virus* (CTV SY568) that causes severe stem pitting and stunting and occasional vein corking in sweet orange and grapefruit in experimental infections was inoculated to a grapefruit which grew to a large greenhouse plant and developed these symptoms. Blind bud inoculum from this grapefruit source was used to infect 18 individual sweet orange seedlings. Plant and symptom development was monitored through three cycles of growth and cut back over a 2 yr-period. Individual plants ranged in height from severely stunted to no clear stunting, compared to non-inoculated controls. Vein corking was common on the leaves of the two most severely stunted plants, but was rare or absent on other plants. Stem pitting was present in all plants including the ones with no obvious stunting. At least two distinct types of stem pitting were observed, one being deep pitting (14 plants) and the other being multiple pin hole pits that gave a honeycombing effect (three plants). Only one plant, the most severely stunted, which was more severe than the parental SY568 in sweet orange, clearly showed both types together in the same plant. Total growth varied between each cycle, but the relative symptoms including the types of stem pitting were consistent. Each plant was used as a source of inoculum for a pair of sweet orange seedlings. The differences between the subcultures were also seen in these plants through three periods of re-growth following severe pruning over a 2-yr period. RNase protection assays using probes to different genes in the CTV genome clearly showed the presence of at least two CTV genome variants, one (A) more abundant than the other (B), and often present as a mixture of both types (A and B) or as only one of these types (A). No plant contained only the second variant (B). These results suggest that this very severe form of CTV is a mixture of strains, has different factors for stem pitting and stunting, and has more than one stem pitting factor which may make it difficult to map this trait to specific RNA sequences.

Strains of *Citrus tristeza virus* (CTV) vary widely in their ability to cause symptoms in different *Citrus* species (5, 13). These range from non-existent to mild and severe expression of symptoms such as decline, stunting, chlorosis, seedling yellows, vein clearing, vein corking and stem pitting. It has long been thought that the average CTV infection is made up of a variety of strains (4, 9, 17) or variant populations of a single strain (2, 16) which vary in their virulence ability (14).

In the late 1970s a particularly severe isolate of CTV was obtained from a Minneola tangelo on the field station at University of California-Riverside (UCR) and designated as strain SY568 (aka UCR field 12B isolate, or Beltsville Agricultural Research Center isolate B6) (1). It causes severe stem pitting, stunting, and vein corking in sweet orange and grapefruit. Strain SY568 has been extensively characterized and a complete genomic sequence has been determined (20). Results of that study also gave evidence that SY568 is a mixture of strains. This study looks at the effects of graft transmission on the segregation of some individual components of CTV isolate SY568 using both biological and molecular analyses.

MATERIALS AND METHODS

Virus strains, hosts, and biological evaluations. An isolate of CTV (SY568) has been maintained at UCR for almost 20 yr in a single sweet orange plant. It has remained severely stunted and the stems are deeply pitted. In the mid-1980s this plant was used to inoculate several Duncan grapefruit seedlings, one of which has been maintained in a

greenhouse since then. Eighteen Madam Vinous sweet orange seedlings were trained to a single leader shoot and allowed to grow until the stem diameters were 1.0 cm. Each seedling was cut back to a height of approximately 20 cm prior to inoculation. Three blind bud bark wedges of 2 cm in length were taken from green bark tissue of the Duncan grapefruit plant infected with CTV SY568 (see above) and inserted into the bark of the sweet orange trees. Unrestricted growth was allowed for 2 yr at which time the trees were evaluated for overall growth and appearance of CTV symptoms. After visual evaluation, trees were cut back, bark tissue was removed and the stems were scored for the degree of stem pitting. These 18 trees were also evaluated and cut back after an additional 7 mo growth period and then again 10 mo after that. In order to enhance the appearance of the pitting for photography, a medium brown wood stain was brushed onto the woody twigs being sure to introduce the stain into the recesses of the pits. After 2-3 min, a paper towel was used to remove the excess stain from the surface of the twigs, leaving the pits stained brown, while the surface was the natural color of the wood. Twigs from healthy trees that were not pitted were treated identically. Twigs were allowed to dry overnight and then photographed with black and white negative film.

Each of the 18 sweet orange trees from above, designated as parental subcultures, was used to graft inoculate two additional sweet orange seedlings, designated as secondary subcultures A and B (1A, 1B through 18A, 18B). The 36 secondary subcultures were rated for growth and CTV symptoms as before.

Molecular analyses of CTV isolates. Double-stranded (ds) RNA was isolated from 7 g of tissue and purified using CF-11 chromatography (10, 15). DsRNAs were separated using 6% polyacrylamide gel electrophoresis, stained with ethidium bromide, visualized on an ultraviolet transilluminator, and photographed using Polaroid type 57 black and white film.

Total nucleic acid extracts were purified from 300 mg of leaf tissue and used as target RNA for RT-PCR (7). A 619 bp fragment of the coat protein gene was amplified in a single tube RT-PCR protocol using primers 64 (5'-TGACATTAGTAAC TACGACATCATCAGCCC-3', antisense) and F1 (5'-GAAGAACAAAAACAAGGAAAC-3', sense). Reverse transcription was carried out for 45 minutes at 42°C, the samples were denatured for 2 min at 94°C, then amplified using 40 cycles consisting of 94°C/1 min; 50°C/1 min; and 72°C/2 min, followed by final extension at 72°C for 5 min. Products were analyzed on 1% agarose gels, stained and photographed as above.

Ribonuclease protection assays (RPAs) were carried out between denatured dsRNAs from each of the 18 parental subcultures and ^{32}P-labeled RNA transcript probes representing the coat protein gene (CP), heat shock protein (HSP65) gene, and the 5' untranslated region (5' UTR) of CTV isolate SY568 (20). Resulting fingerprints were obtained using 6% polyacrylamide/7M urea gel electrophoresis of the final RPA products and subsequent autoradiography of dried gels.

RESULTS

Biological symptom expression in sweet orange seedlings. The 18 parental subcultures showed great variability in the degree of stunting as well as the amount and type of stem pitting that was present (Table 1). Trees were rated for growth and appearance based on a scale from 1 (healthy or mild) to 5 (severely stunted) (Fig. 1). Based on an average of these ratings for three harvests over a 2 yr-period, tree 11 was the most severely stunted and

TABLE 1
BIOLOGICAL SYMPTOMS OF 18 PARENTAL *CITRUS TRISTEZA VIRUS* SUBCULTURES AND UNINOCULATED CONTROLS

Tree no.	Growth and appearance[a]	Type and degree of stem pitting	Leaf vein symptom
Healthy	1	None	None
1	3	Honeycombing	Severe clearing
2	3	Many deep	Mild clearing
3	2	Many deep	None
4	1	Many deep	None
5	1	Few deep	None
6	2	Many deep	Mild clearing
7	1	Many deep	Mild clearing
8	3	Many deep	Severe clearing
9	4	Many deep	Mild corking
10	3	Few shallow	Mild clearing
11	5	Many deep/honeycombing	Severe corking
12	4	Few deep	Mild clearing
13	3	Few shallow	None
14	2	Honeycombing	Mild clearing
15	2	Honeycombing	None
16	1	Many deep	None
17	2	Few deep	None
18	3	Few shallow	Mild clearing

[a]Scale of 1 (none/mild) to 5 (severe) assessing degree of stunting and overall fitness of plant.

consistently received a rating of 5. Trees 9 and 12 were also severely stunted and were both rated as a 4. Trees 4, 5, 7 and 16 averaged a score of 1, similar to the healthy control. The other 11 trees all averaged ratings of 2 or 3. Leaves from only two sweet orange trees (9 and 11) developed vein corking, which was extensive in the grapefruit tree used as the inoculum source. Leaves on several trees developed mild vein clearing, and trees number 1 and 8 were scored as having severe vein clearing (Table 1).

After the trees were rated for growth, they were pruned back severely, all tissue was collected, leaves and bark were removed, and the intensity of stem pitting was recorded for the woody part of the twigs. Stem pitting was present in all plants, even those that were scored as similar to the healthy control in growth and appearance (Table 1). Two quite different types of pitting were observed, discrete, usually deep pits (ranging from few to many, trees 2-10, 12, 13, and 16-18, Fig. 2, stems A and B) and shallower, but more extensive pinhole sized pits which gave a "honeycombing" effect (trees 1, 14, and 15, Fig. 2, stem C). One plant (No. 11) developed a combination of these 2 types (Fig. 2, stem D). Notably, tree 11 was the most stunted, had both severe types of stem pitting, and produced vein corking on the leaves. All plants were scored identically for stem pitting over each of the three harvest periods.

Each pair of secondary subculture sweet orange trees produced similar growth and appearance when compared to the parental source trees from which they were derived. The type of stem pitting that developed in each secondary subculture was also the same as that of the parent.

Molecular analyses of CTV isolate SY568 parental subcultures. DsRNA patterns of each of the 18 parental subcultures were compared to each other and to the original sweet orange tree infected with SY568. Each pattern was simi-

Fig. 1. Diversity in overall growth and appearance of sweet orange parental subcultures inoculated with CTV SY568 from grapefruit. Most severely stunted (severe, e.g. tree #11), average tree in experiment (moderate, e.g. tree #2), a mild to non-affected tree (mild, e.g. tree #5), and a non-inoculated control (healthy).

lar and included expected bands representing the genomic length replicative form and the subgenomic dsRNA for the coat protein gene (Fig. 3). No distinct differences were observed among any of the samples. The dsRNA profile of the parental grapefruit used as the inoculum source for the 18 trees was not determined due to the poor overall fitness of the tree which did not allow for the removal of the 7 g of bark tissue required for analysis.

Complementary DNA clones of SY568 were used to produce minus-sense RNA transcript probes for the 5' UTR, coat protein (CP), and heat shock protein (HSP, p65) for fingerprinting of the 18 parental subcultures by RNase protection assays (RPA). Previous research has shown that the 5' UTR and coat protein cDNA clones used to produce the RNA transcripts are homologous to the most abundant strain of CTV ("A") within the SY568 isolate (20). The p65 clone however is probably homologous to another strain within SY568 ("B") and fully protects that portion of the RNA population, while the RNA of the more abundant strain is degraded into a recognizable pattern of lower molecular weight fragments.

The 5'UTR probe fully protected a major component in all 18 parental subcultures with a few smaller sized fragments visible in each although the relative amount of these cleavage products varied between samples (Fig. 4). This pattern was identical to that obtained using CTV dsRNA from the SY568 parental sweet orange plant from which the cDNA clones were derived. The CP probe also fully protected a major portion of

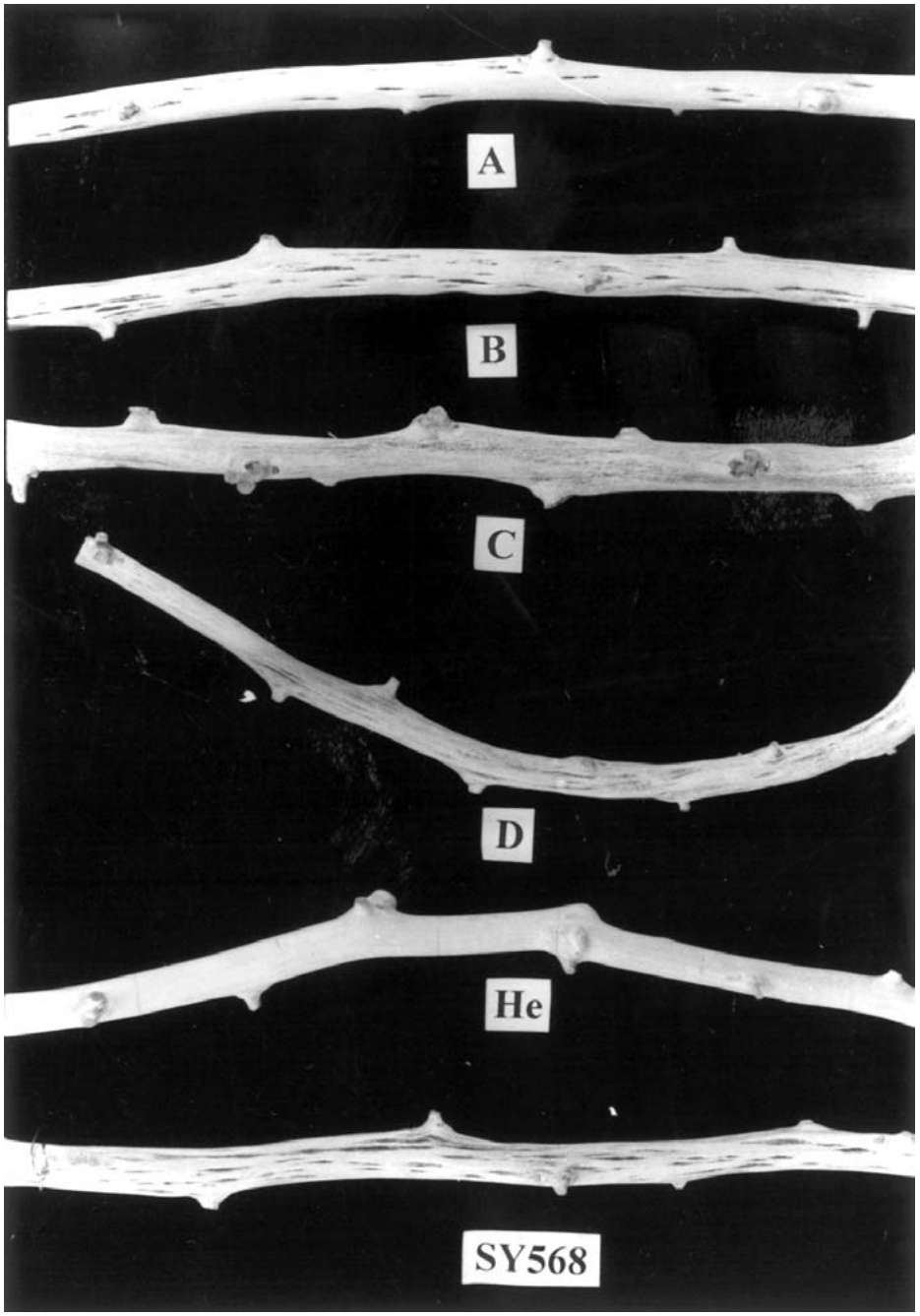

Fig. 2. Representation of 4 typical stem pitting symptoms observed in sweet orange parental subcultures inoculated with CTV SY568 from grapefruit. Few deep (A, e.g. tree #5), many deep (B, e.g. tree #16), honeycombing (C, e.g. tree #14), and honeycombing with deep pits (D, e.g. tree #11). An uninoculated twig (He) and original sweet orange twig infected with SY568 (SY568) are included for comparison.

the RNA population and fingerprints were consistent among all 18 trees and SY568 from the original sweet orange tree (Fig. 5). The only degradation products in the experimental samples were also present in the

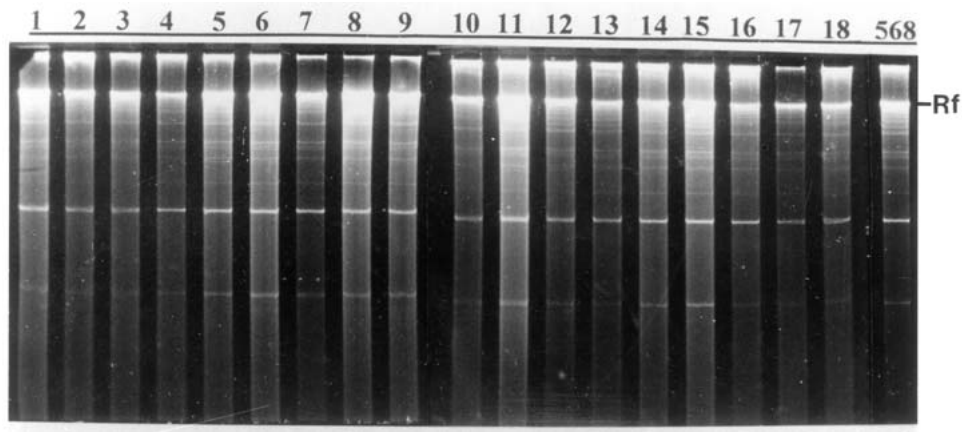

Fig. 3. DsRNAs extracted from sweet orange citrus bark infected with CTV SY568 and electrophoresed through a 6% polyacrylamide gel. Trees are the 18 parental subcultures (1-18) and the original sweet orange SY568 source tree. The position of the replicative form (Rf, 13.3 × 10^6 Da, 19,249 nt) is shown on the right hand margin of the figure.

plus-sense transcript complement control reaction, thus they can be regarded as background in the assay.

Using the p65 HSP probe, two major patterns were observed as before. One pattern had a relatively weak, fully protected fragment near the top of the gel that co-migrated with the complement control band indicating high sequence similarity

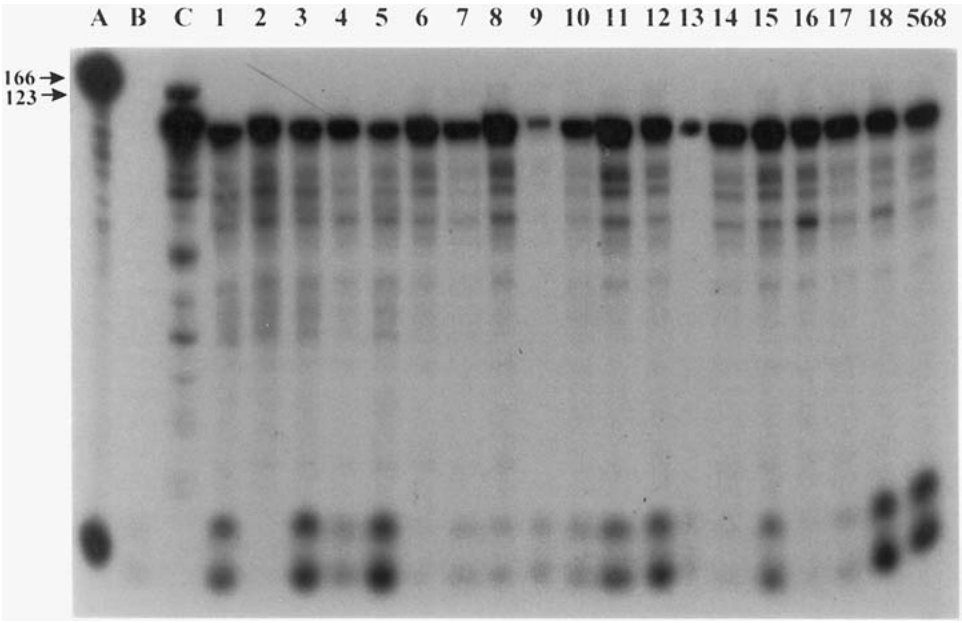

Fig. 4. Autoradiograph of RNase protection assay using the 5' UTR probe against no target, no RNase (A), no target, with RNase (B), +-sense complement target, with RNase (C), denatured dsRNA from 18 sweet orange parental subcultures inoculated with SY568 from grapefruit, with RNase (1-18), denatured SY568 dsRNA from original sweet orange tree, with RNase (568). Sizes in nucleotides of full-length probe including some non-viral plasmid sequences (166) and of the section of probe containing viral sequences only (123) are shown on left margin.

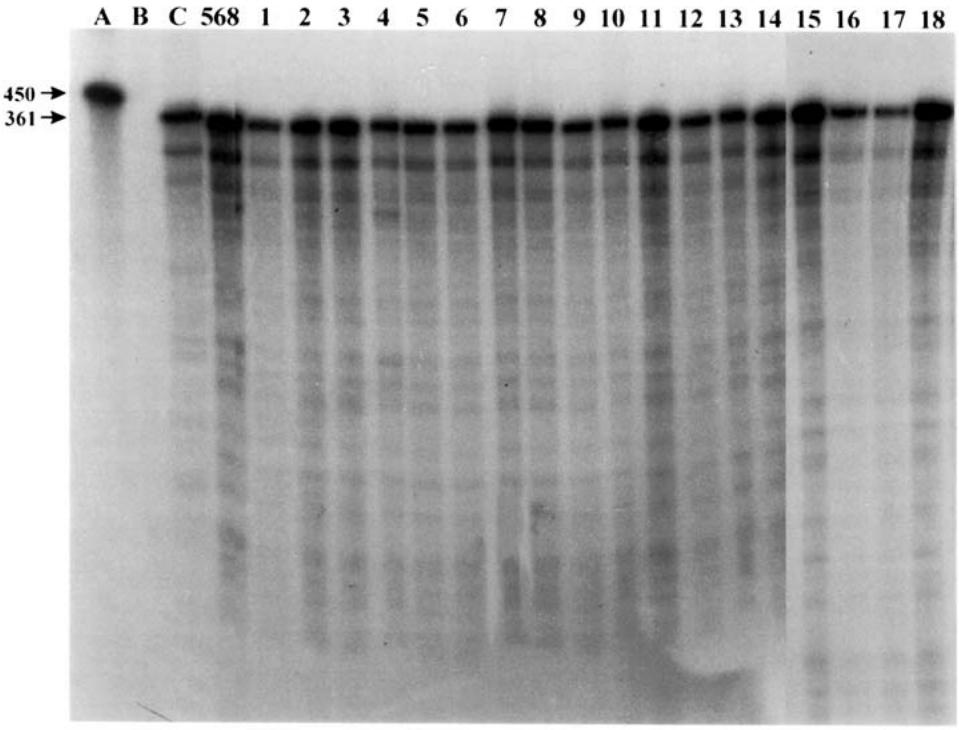

Fig. 5. Autoradiograph of RNase protection assay using the coat protein gene probe against no target, no RNase (A), no target, with RNase (B), +-sense complement target, with RNase (C), denatured dsRNA from 18 sweet orange parental subcultures inoculated with SY568 from grapefruit, with RNase (1-18), denatured SY568 dsRNA from original sweet orange tree, with RNase (568). Sizes in nucleotides of full-length probe including some non-viral plasmid sequences (450) and of section of probe containing viral sequences only (361) are shown on left margin. Note that this figure is a composite made from several different autoradiographs.

with the probe. Additionally, a large amount of lower molecular weight bands formed a pattern which was consistent between samples (Fig. 6). The second pattern was composed of only the lower molecular weight fragmentation pattern with no fully protected band visible. No samples produced a pattern of the fully protected band alone. This result confirms the presence of at least two different strains.

DISCUSSION

The biological observations made on the 18 parental subculture sweet orange trees indicated that there are multiple populations of SY568 that can segregate and subsequently vary in their degree of severity in the plant. This was indicated previously by Roistacher and co-workers when mild subcultures of SY568 were obtained following aphid and graft transmission of the severe parent isolate through *Passiflora* spp. (11, 12). Since all 18 grafted trees were of the same age and size when inoculated and the 36 secondary subcultures produced symptoms identical to their respective parental subculture, it can be assumed that the differences between trees was due to the influence of the CTV population and not to genetic differences within the trees.

Variation in the severity and type of stem pitting found in each of the trees could be segregated into at least four different categories (few deep or shallow, many deep, honey-

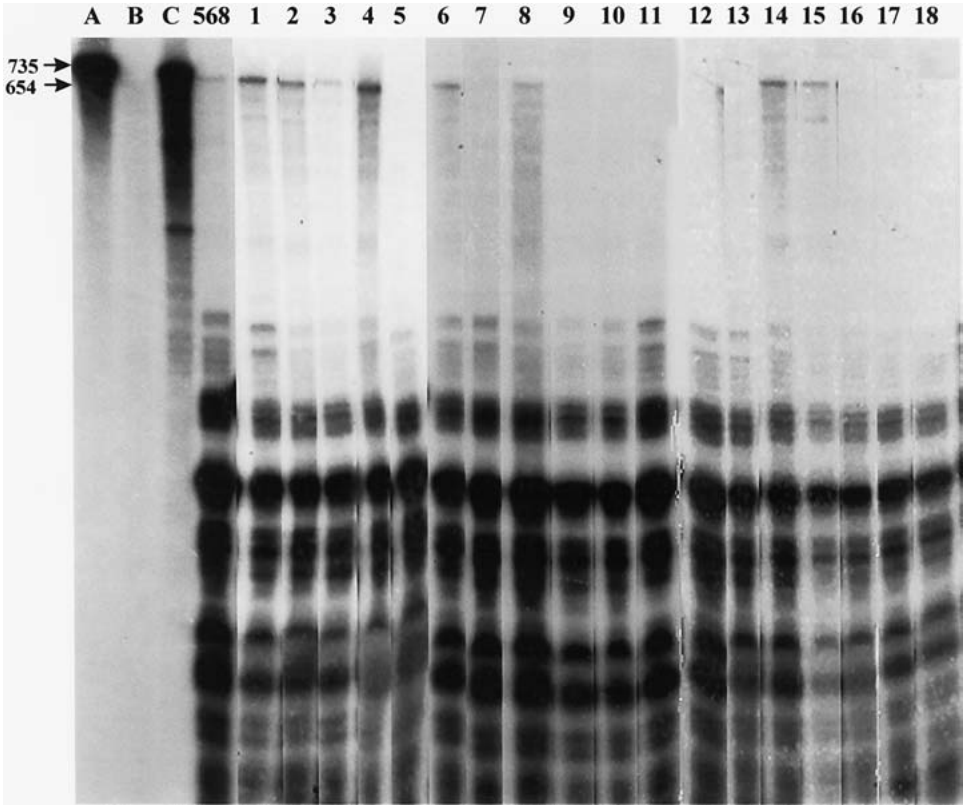

Fig. 6. Autoradiograph of RNase protection assay using the p65 heat shock protein probe against no target, no RNase (A), no target, with RNase (B), +-sense complement target, with RNase (C), denatured dsRNA from 18 sweet orange parental subcultures inoculated with SY568 from grapefruit, with RNase (1-18), denatured SY568 dsRNA from original sweet orange tree, with RNase (568). Sizes in nucleotides of full-length probe including some non-viral plasmid sequences (735) and of section of probe containing viral sequences only (654) are shown on left margin. Note that this figure is a composite made from several different autoradiographs.

combing alone, deep pits and honeycombing together), but did not directly correlate with the severity of growth and appearance of the tree. Some of the trees with large numbers of deep pits grew similarly to the non-inoculated healthy control trees. The most severely stunted tree did contain both types of severe pitting (many deep pits and extensive honeycombing). This combination of the two pitting types may indicate that at least two populations of SY568 interacted together to produce the most severe symptoms in sweet orange. The development of vein corking on the leaves of trees 9 and 11 seemed to have a dramatic effect on the vigor of these trees, as they were the most stunted. This is possibly due to an inability of corked leaves to efficiently make and move nutrients throughout the tree.

DsRNA analysis of the 18 parental subcultures showed no obvious differences between them or in comparison to the SY568 infected parental sweet orange tree. This assay did however demonstrate the uniformity of the overall experiment. Since dsRNA analysis primarily detects the titer of the viral population and the size and number of the molecular components, it was apparent that the overall composition of the SY568 isolate is similar

between the trees and that variation in biology is more likely due to discrete sequence differences within the population. If the strains of CTV found in the 18 trees varied greatly this might have been detected in the number of subgenomic dsRNA segments visible (3) or defective interfering dsRNA segments known to be common in CTV isolates (8, 19). In this and other studies the absence of such additional dsRNAs is a characteristic of SY568 (20). This also supports the suggestion that sequence diversity rather than the effects of accessory RNAs is responsible for biological differences in this system.

While RNase protection assays can be used to map differences within genomes (6, 18), the purpose of using them with these CTV isolates was to determine gross banding patterns for comparison. The 5'UTR and CP probes gave consistent results for each of the trees and no obvious correlation between a given pattern and the biology of that isolate could be made. Based on the prior knowledge that the p65 probe can determine if components A and B of SY568 are present in a sample, the 18 trees contained either both of the components, or only component A. Using visual inspection of the intensity of the bands on the autoradiographs from the RPA analyses, strain A is more abundant than strain B when in mixed infection. By comparing the biological results to the presence of either a mixed infection or only strain A, a slight correlation can be made. No plant that contained both strains A and B had a rating greater than 3 (out of 5) on the growth and appearance scale. The two trees which were most severely affected (most stunted, severe pitting, vein corking, ratings of 4 and 5) were trees 9 and 11, which both contained strain A alone. It is possible that strain B is able to partially attenuate the symptoms of strain A when in mixed infection.

It has been conclusively determined that there are at least two different strains of CTV within isolate SY568. However, it has not been concluded that there are only two strains present. Due to the different types of stem pitting found and the variability in the reactions of trees identified as having one or two different components, it is likely that there is at least one other strain of CTV present that is able to segregate independently of the other two. Additional experiments are underway using multiple and single aphid transmissions from the 18 parental subcultures and further study of the 36 secondary subcultures in order to more fully characterize all of the strains present in isolate SY568.

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