A New Viroid is the Causal Agent of the Citrus Cachexia Disease

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ABSTRACT. Citron tissue infected with severe isolates of cachexia (xyloporosis), known to be free of all other citrus diseases and to induce a strong reaction in Parson's special mandarin, contained a new species of viroid RNA. Symptomless tip tissue of plants infected with cachexia (Ca) 902 or 908 (UCR virus collection) was phenol extracted and processed by LiCl partitioning and Cf-11 cellulose chromatography. These nucleic acid extracts, when analyzed by sequential polyacrylamide gel electrophoresis in native and denaturing conditions, displayed an RNA of about 300 nucleotides not observed in healthy extracts. Both circular and linear forms of a viroid RNA, designated as the putative citrus cachexia viroid (CCaV), were detected. No homology to either citrus exocortis viroid (CEV) or citron variable viroid (CVaV) was observed with hybridization of CCaV to cDNA made to CEV and CVaV.

When other cachexia sources (Ca 903, 904, 907), known to comprise mixtures of the cachexia agent and mild forms of the "exocortis disease" were similarly analyzed, both a common CCaV component and additional viroid RNAs were detected.

The circular form of CCaV was isolated from denaturing gels by electroelution and transmitted to healthy citron. Secondary inoculations to Parson's Special mandarin from these citron sources have been made and the induced host reactions have confirmed the identity of CCaV as the cachexia disease agent. Nucleic acid extracts from cachexia sources were also inoculated to other hosts for viroids, such as *Gynura aurantiaca*, *Lycopersicon esculentum* cv. Rutgers, and *Cucumis sativus* cv. Suyo. Although all inoculated plants remained symptomless, the CCaV could be recovered only from cucumber after 30-45 days incubation.

These properties suggest that the cachexia disease source material contains a previously undescribed viroid which is the causal agent of the disease.

"Cachexia" is a term introduced by Childs (2) to describe a condition of Orlando tangelo characterized by gumming and browning of phloem tissues, wood pitting, and bark cankers. The disease was shown to be graft transmitted (3) and to also affect mandarins, mandarin hybrids, tangelos, kumquats and *Citrus macrophylla*. Although the cachexia disease can be found in most citrus growing regions of the world, many citrus varieties, including grapefruit, sweet orange and lemon are symptomless carriers of the agent. Mild, moderate and severe isolates of the disease exist. Severe isolates inducing severe leaf chlorosis, stunting, and dieback can be lethal to citrus on susceptible rootstocks.

Comparison of symptoms and wood specimens (3) suggested a possible relationship between the cachexia disease and the xyloporosis disease affecting Palestine sweet lime in Palestine reported earlier by Reichert and Perlberger (9). However, a definitive comparative study of both diseases has never been reported (11).

The widespread occurrence of cachexia coupled with the lengthy and laborious bioassay, originally requiring incubation periods of 2-7 yr in Orlando tangelo and improved to 6 months to 1 yr in Parson's Special mandarin (12), has stimulated a search for the causal agent in order to improve detection and indexing procedures.

The viroid hypothesis was introduced by Roistacher *et al.* (13) because of the similarity in transmission properties between the cachexia agent and the citrus exocortis viroid (CEV). Additional data supporting this include: 1) the absence of any evidence of vector transmission; 2) the ineffectiveness of thermotherapy (1); and 3) the ease of elimination of the agent by shoot tip grafting (14).

This report presents evidence for the isolation and purification of a small, transmissible, viroid-like RNA

from cachexia disease sources which reproduced the disease symptoms on inoculated index plants. We propose the name *Citrus Cachexia Viroid* (CCaV) for the causal agent of the cachexia disease.

MATERIALS AND METHODS

Source, transmission and bioassay hosts for the cachexia disease. The principal cachexia disease isolate used in these studies was derived from an old line navel orange tree in 1963 from the University of California-Riverside (UCR) collection and designated as Ca 902. This isolate has been maintained in sweet orange and consistently bioassayed in Parson's Special mandarin as a severe cachexia isolate with a rating of 8 on a scale where 10 is most severe. The Valencia orange host indexed negative for all other known citrus diseases including severe and mild forms of the exocortis disease.

The cachexia agent was transmitted by bud inoculation to the symptomless host, citron, which on the basis of transmission studies, appears to maintain a high titre of the agent. Nucleic acid extractions and viroid purification were made from citron.

Extracted preparations suspected of containing the cachexia agent were inoculated by razor slashing the stems of citron seedlings. After 6-12 weeks incubation, the plants were monitored for positive transmission by both extraction and detection of the putative cachexia viroid and by bioassay on Parson's Special mandarin.

Secondary inoculations to herbaceous hosts were made by stem slashing *Gynura aurantiaca* or needle puncture through an inoculum drop on the hypocotyl of young cucumber (*Cucumis sativus* cv. 'Suyo') plants as the first true leaves were emerging. After 3 weeks, cucumber plants were trimmed to 2-3 nodes and all foliage removed. The regrowth was collected and extracted after an additional 3 weeks.

Ultimately, all nucleic acid fractions or purified viroid preparations to be tested for the cachexia agent were bioassayed on Parson's Special mandarin. This procedure involved mechanical transmission to healthy citron seedlings and subsequent bud inoculation from these sources to Parson's Special mandarin forced on rough lemon rootstock. Plants were first observed for the browning reaction at the budunion after 6-7 months and subsequently at irregular intervals up to one year after inoculation.

Nucleic acid extraction and viroid detection. Actively-growing tip tissue of symptomless carrier citron was collected and either extracted immediately or frozen and powdered in liquid nitrogen and stored at –20 C for use later. Tissue extraction and nucleic acid isolation were as previously reported (4, 19). This included homogenization in the presence of phenol, Tris-HCl buffer, SDS, EDTA, and mercaptoethanol followed by concentration by ethanol precipitation and 2M LiCl salt partitioning. The 2M LiCl soluble nucleic acid fraction was subjected to CF-11 cellulose chromatography and selective elution to enhance the concentration of specific viroid RNA (16).

Viroid detection was accomplished by sequential polyacrylamide gel electrophoresis (PAGE) as described by Semancik and Harper (18) and modified by the application of the low pH denaturing gel system (10). These procedures followed by silver staining (2) provided for detection of circular viroid molecules to a sensitivity of about 100 pg.

Infectious viroid molecules were recovered from denaturing gels by electroelution in an IBI Model UEA unidirectional electroelutor after gels were stained with ethidium bromide, visualized on a UV transilluminator and the gel piece containing the viroid RNA excised.

Electrotransfer hybridization. Viroid molecules were transferred directly from denaturing polyacrylamide gels containing 8M urea onto

nylon-based membranes (Nytran) using an LKB Transphor apparatus. Membranes were hybridized with complementary DNA (cDNA) probes made to the major citrus viroids, citrus exocortis viroid (CEV), the CV-Ib component of the "citron variable viroid" (CVaV) isolate, and the citrus cachexia viroid (CCaV). Random-primed cDNA probes were made essentially by the procedure as reported by Maniatis *et al.* (8) using the cloned Moloney Murine Leukemia virus reverse transcriptase enzyme (Bethesda Research Laboratories). Conditions of hybridization were as presented by Garger *et al.* (6).

RESULTS

Transmission of the cachexia agent with nucleic acid extracts. When citron tissues containing the cachexia agent were subjected to the nucleic acid extraction procedures which have been effective in the isolation of viroid RNA, transmission of the cachexia agent to Parson's Special mandarin was accomplished (table 1). The 2M LiCl soluble fraction known to concentrate small RNA molecules and viroids contained significantly greater levels of the cachexia agent than the insoluble fraction in which the high molecular weight viral nucleic acid would be concentrated. Since salt mediated partitioning of nucleic acid species is not absolute and the cachexia bioassay procedure involves a lengthy systemic reaction, it is not surprising that at least some traces of infectivity are recovered in both salt fractions.

TABLE 1
TRANSMISSION OF THE CACHEXIA DISEASE WITH NUCLEIC ACID EXTRACTS

Sample	No. infected/ Total	Bioassay rating[z]
2M LiCl fractions		
insoluble	1/2	0,3
soluble	2/2	6,6
CF-11 cellulose eluants		
35% ethanol-buffer	0/2	0,0
25% ethanol-buffer	2/2	2,2
0% ethanol-buffer	1/2	7,0
Electroeluted CCaV		
circular form	7/7	1,7,2,6, 6,2,6

[z]Rating indicates the severity (1-10) of the browning reaction on individual Parson's Special mandarin buds forced on rough lemon rootstock 7-8 months after bud inoculation from citron seedlings which had previously been inoculated with nucleic acid extracts.

Cellulose chromatography of the 2M salt soluble fraction further supported a viroid-like structure for the cachexia agent. Step elution with 35%, 25%, and 0% ethanol solutions demonstrated that the bulk of the infectivity eluted in the 0% ethanol or buffer fraction (table 1) as has been observed for CEV (19). Cachexia infectivity was completely excluded from the DNA-rich (35%) eluant. However, a greater level of the total infectivity was recovered in the 25% fraction than would be expected for extracts containing CEV. This observation suggested a possible subtle difference in the basic structure of the cachexia agent as compared with CEV. This property was exploited in later steps to obtain highly purified cachexia agent.

Detection of a new small transmissible RNA associated with cachexia sources. Samples enriched for the cachexia agent by CF-11 cellulose chromatography were subjected to sequential PAGE. The "viroid zone" of a native 5% gel, as defined by Rivera-Bustamante *et al.* (10), contains potentially all possible viroids reported to date on the basis of the molecular size range. This region, delimited by CEV and avocado sunblotch viroid (ASV), was excised and subjected to denaturing PAGE (dPAGE) in the presence of 8M urea. A distinct band (fig. 1B) which was not observed in comparable preparations from healthy seedling citrons (fig. 1A) could be defined only after silver staining. When samples containing the new band were slash inoculated into healthy citron seedlings, the cachexia component could be de-

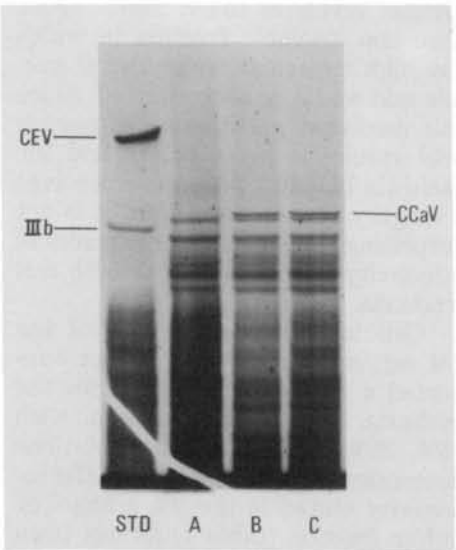

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of nucleic acid extracts under low pH, denaturing conditions (8M urea) and silver staining following electrophoresis in 5% gels as described for sequential PAGE. Samples were from healthy citron (A), citron which contained Ca 902 isolate (B), and healthy citron after inoculation with nucleic acid extract of Ca 902 citron isolate (C). Standard channel (STD) contains citrus exocortis viroid (CEV) and citrus viroid (CV) IIIb as markers.

tected in extracts analyzed after 6-12 weeks incubation (fig. 1C). After treatment with pancreatic RNAse, no evidence of this new component was detected in cachexia disease extracts.

It is important to note in fig. 1 that this new viroid-like band can be masked by the high concentration of silver stained substances in the healthy citron extract background. Also, the concentration of the cachexia-related PAGE band implied by the staining intensity is much lower than that expected for CEV recovered from similar tissue extracts.

These results, nevertheless, establish the presence of a transmissible, small RNA associated with cachexia disease sources. Estimation of molecular size by the relative migration in dPAGE (fig. 1) indicates that the new RNA species is considerably smaller than CEV but slightly larger than the citrus viroid, (CV), IIIb (5), thus conforming to a group II viroid.

Preparative recovery of a new viroid. Using large quantities (100-500 g) of tissue processed as above with an added second chromatography on CF-11 cellulose, the cachexia-related viroid-like RNA could be recovered as a more highly purified preparation (16). Much of the healthy background material observed after PAGE analysis (fig. 1A) could be removed by loading the cellulose with the sample contained in 35% ethanol-buffer, washing it extensively with 30% ethanol-buffer, and collecting only that fraction which eluted with 25% ethanol-buffer (16). Samples concentrated in this manner and processed by sequential PAGE and dPAGE, provided excellent gels from which the pure cachexia RNA component could be electroeluted and bulked.

When analyzed by native PAGE, contents of this preparation migrated as a single electrophoretic component (fig. 2A) with a relative migration rate similar to the host 7S RNA (fig. 2B). With excision from the gel, as represented in figure 2, and analysis under denaturing conditions (dPAGE), the single band was resolved into the circular and linear

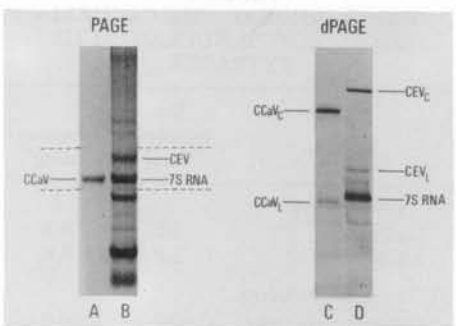

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) of purified CCaV (A), and a standard citrus exocortis viroid (CEV) preparation (B), followed by sequential denaturing gel electrophoresis (dPAGE) of the indicated segment (=====) and silver staining indicating separation of circular ($CCaV_C$) and linear ($CCaV_L$) forms of CCaV (C), and circular (CEV_C) and linear (CEV_L) forms of CEV (D). Host 7S RNA is indicated as an internal marker in partially-purified preparations.

molecular forms (fig. 2C) characteristic of viroids. The very similar migration of the linear form to that of the 7S host species suggests a comparable molecular size. This situation exemplifies how viroid-like RNAs can be masked within host RNA bands in native PAGE and that sequential dPAGE is essential to expose the presence of all viroid-like species.

Cachexia disease symptoms induced by the purified viroid RNA. The circular form of the cachexia-related viroid, designated as $CCaV_C$ in figure 2C, was recovered in sufficient quantities to serve as an inoculum source. It had already been established that the viroid associated with cachexia disease tissue could be transmitted to citron seedlings as part of complex nucleic acid extracts. (fig. 1). Nevertheless, the independent transmission of the cachexia-related viroid as well as its direct implication as the etiological agent of the cachexia disease had not been demonstrated.

Aliquots of the electroeluted $CCaV_C$ were inoculated into healthy citron seedlings by stem slashing. The plants were topped and tip tissue collected 6-12 weeks post inoculation. Transmission of the viroid was confirmed by extraction and analysis as presented in figure 1. Buds from the above infected citrons were then grafted as inoculum sources to rough lemon rootstocks budded with Parson's Special mandarin. The Parson's Special mandarin scion bud was forced and plants were maintained either under warm (22-38 C) greenhouse conditions in Riverside or at the Lindcove Experiment Station greenhouse under warmer conditions. After 6 months incubation, bark patches were removed at the budunion to monitor for the appearance of the browning reaction. A weak reaction (1-2) was observed after 6-7 months and became strong (6-7) on the same bioassay plants 7-8 months postinoculation (fig. 3). Production of the classical cachexia disease symptoms was observed on 7/7 bioassay plants with 4/7 plants displaying a strong reaction (table 1).

Fig. 3. Browning reaction of cachexia disease in Parson's Special mandarin (see arrows) on rough lemon rootstock 8 months following bud inoculation with citron which was previously inoculated with purified $CCaV_C$ as shown in Fig. 2C.

Since the intensity of the bioassay reaction increased so dramatically over a one-month period, it could be anticipated that even those plants showing only the weak browning reaction will display a strong reaction with completion of the standard incubation period of 1 yr.

These results clearly demonstrate that the cachexia disease is caused by a small, transmissible RNA with viroid properties which we propose to be designated as the *Citrus Cachexia Viroid* (CCaV).

Analyses of cachexia disease isolates in California and Spain. Identification of the CCaV was made using extracts from Ca 902, a disease isolate which apparently contains only cachexia. Other cachexia isolates, some of which are known to contain