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.

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-

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.
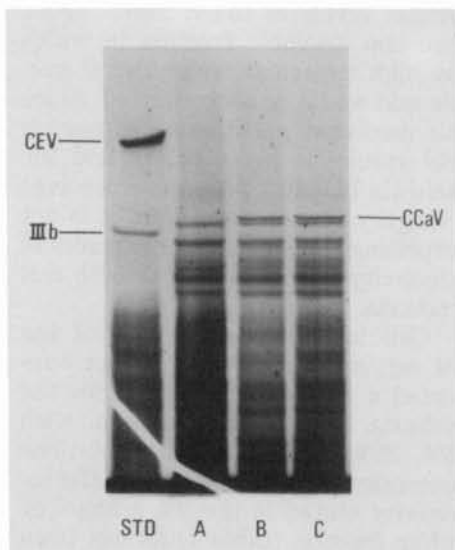
Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of nucleic acid extracts under low pH, denaturing conditions (8M urea) and silver staining following electrophoresis in 5% gels as described for sequential PAGE. Samples were from healthy citron (A), citron which contained Ca 902 isolate (B), and healthy 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
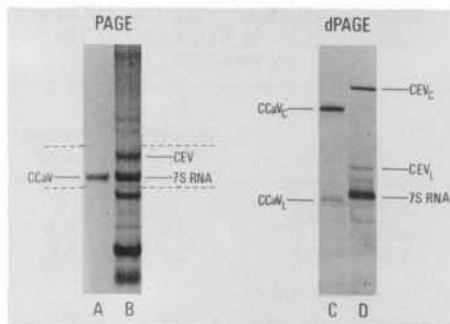

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) of purified CCaV (A), and a standard citrus exocortis viroid (CEV) preparation (B), followed by sequential denaturing gel electrophoresis (dPAGE) of the indicated segment ($=====$) and silver staining indicating separation of circular ($CCaV_C$) and linear ($CCaV_L$) 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 bioas-

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.

say plants with 4/7 plants displaying a strong reaction (table 1).

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
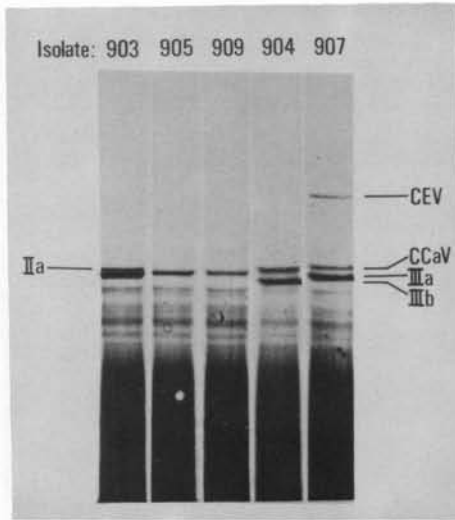
Fig. 4. Polyacrylamide gel electrophoresis as presented in fig. 1 of citron extracts from different cachexia disease isolates containing either pure CCaV (Ca 905, 909) or CCaV (Ca 903, 904, 907) mixed with other citrus viroids (CV-IIa, -IIIa, -IIIb) and CEV.

various forms of the exocortis disease, are also maintained in the UCR collection. In addition, cachexia isolates have been maintained in Moncada (Valencia), Spain as part of the virus collection of the Citrus Variety Improvement Program of Spain. As putative cachexia diseases sources, these isolates should contain the common component identified as CCaV. PAGE analyses confirmed the presence of a common viroid band (CCaV) in all isolates tested (fig. 4). The summary presented in table 2 indicates two groupings of both pure cachexia sources and those which contain other citrus viroids (CV) including CEV (4, 5). Identical results were obtained from independent analyses of the same isolates (Ca 902 and Ca 905 propagated as X 704 and X 707 respectively) which had been maintained in both California and Spain for many years.

TABLE 2
VIROID PROFILE AND SEVERITY OF CACHEXIA DISEASE ISOLATES
FROM CALIFORNIA AND SPAIN

Isolate[z]	Source	Raction on Parson's special mandarin[y, x]	Viroid profile	
			CCaV[x]	Citrus viroid
California:				
Ca 902	Old-line Navel orange	(8)	+	–
Ca 905	Prior Lisbon lemon	(6.5)	+	–
Ca 908	Howell grapefruit	(8)	+	–
Ca 909	Citrus sp. (Japan)	(1-2)	+	–
Ca 903	Willow leaf mandarin	(5.5)	+	IIa
Ca 904	Marsh grapefruit	(2-8)	+	IIIb
Ca 907	Ricote lemon (Spain)	(1-2)	+	CEV,IIIa
E 821	Eureka lemon	(1-2)	+	CV-Ib,IIa,IIIb
Spain:				
X 704	same as Ca 902	+	+	–
X 707	same as Ca 905	+	+	–
X 701	Corsica 114	+	+	–
X 712	Salzara satsuma mandarin	+	+	–
X 710	Wash. Navel precoz	+	+	IIIb,IIIc
X 711	Clementina tardia	+	+	CEV,Ia,IIIb-d
X 713	Mineola tangelo	+	+	IIa,IIIc

[z]Abbreviations: Ca = cachexia; E = exocortis; X = xyloporosis (cachexia); CV = citrus viroid; E 821 = citron variable viroid source.
[y]Browning reaction: 0 = none to 10 = severe.
[x]+ = present.

It is also interesting to note that what was originally believed to be the source of a moderate exocortis isolate (E 821) and subsequently was reported as the "citron variable viroid" (CVaV) (15), is comprised of a mixture of four viroids including a mild form of CCaV which could be detected by PAGE analysis and by bioassay in Parson's Special mandarin.

Close inspection of the PAGE profiles of these cachexia isolates suggests that the CCaV component may not display an identical relative mobility. This suggests that strains of CCaV may exist with minor differences in the total nucleotide number similar to that recently reported for CEV (20). Alternatively, the PAGE analyses may detect distinct viroids that produce a similar reaction in the same bioassay host. The latter situation has been recently established among the other citrus viroids and the response of Etrog citron interpreted as the exocortis disease (5). Determination of which of these possibilities is the case for cachexia must await more comparative studies of the physical and biological properties of the citrus viroids as a group.

Cucumber as an alternate host for the citrus cachexia viroid. The low concentration of CCaV detected in citron extracts coupled with difficulty experienced in separation of the viroid from host components migrating in a similar region after PAGE (fig. 1), stimulated the search for an alternate herbaceous host for CCaV. *Gynura aurantiaca*, the preferred herbaceous host for CEV, was resistant to CCaV infection.

When cucumber was inoculated with several different extracts from citron infected with Ca 902, a range in response from a very mild stunting reaction to a disease syndrome involving severe stunting, irregular leaf rugosity (fig. 5), internode shortening (fig. 6), floral bud proliferation and general dark green coloration resulted. These symptoms became more intense on regrowth tissue after plants were trimmed 3 weeks post inoculation. Reinoculation into cucumber of nucleic acid extracts of this tissue resulted in a moderation of this severe reaction. Nevertheless, a
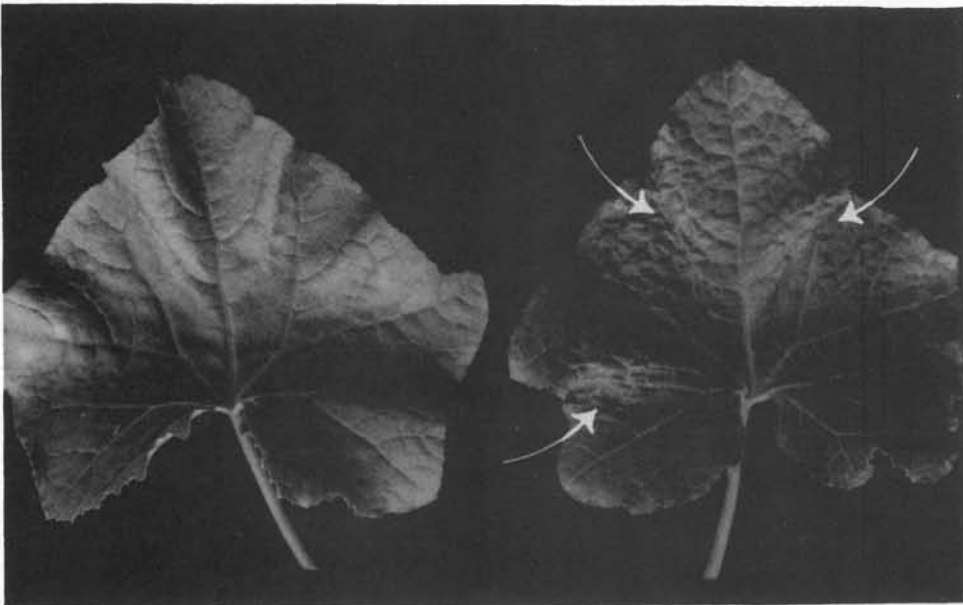

Fig. 5. Cucumber displaying mild leaf rugosity (right, see arrows) 12 weeks post inoculation with CCaV. Comparable healthy control (left).
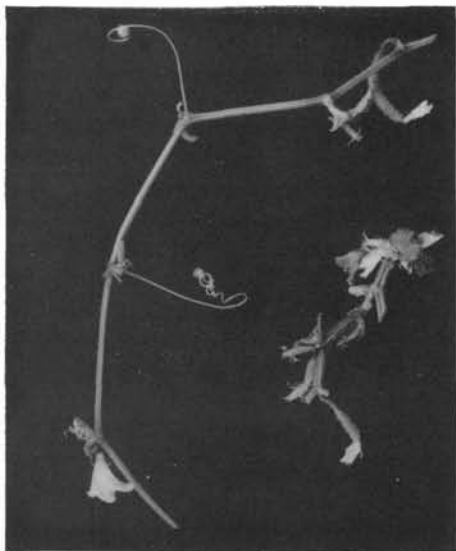
Fig. 6. Cucumber displaying internode shortening and floral bud proliferation (right) 12 weeks post inoculation with CCaV. Comparable healthy control (left).
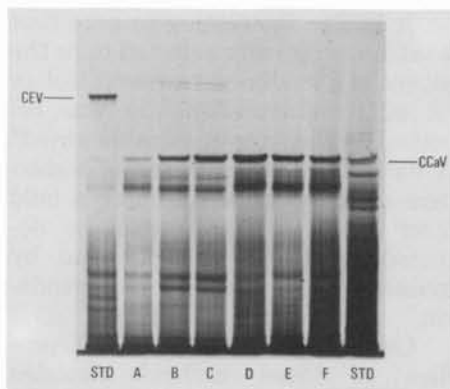

Fig. 7. Polyacrylamide gel electrophoresis as presented in fig. 1 of extracts from cucumber healthy tip tissue (A), and CCaV infected plant parts including asymptomatic leaf tissue (B), rugose leaf tissue (C), tip tissue (D), flowers (E), and immature fruit (F). Extracts from CEV and CCaV infected citron included as standards (STD).

densely staining band in the CCaV region could be recovered by PAGE from all infected cucumber regardless of symptom intensity.

PAGE analysis of extracts from different plant parts from infected cucumber indicated the highest concentration of CCaV occurred in actively growing tip tissue (fig. 7D), and was significantly greater than that recovered from citron (fig. 7, CCaV-STD). Even cucumber flowers (fig. 7E) and immature fruit (fig. 7F) yielded CCaV in amounts comparable to foliar tissues, again in excess to that recovered from citron.

Detection and homology of CCaV from citron and cucumber as determined by cDNA hybridization. Preparative purification of CCaV from cucumber permitted the synthesis of a random-primed cDNA probe to CCaV. Various CCaV containing samples from citron and cucumber as well as comparable extracts from healthy tissue were subjected to sequential gel electrophoresis and electrotransferred directly from the denaturing gel (fig. 8A-G) to Nytran membrane. These preparations were then hybridized with ^{32}P labelled

CCaV-cDNA. Figure 8 presents the positive reactions of the CCaV-cDNA probe to the CCaV purified from cucumber (F'), to an extract from Ca 902 infected citron (A'), to CCaV$_C$ purified from Ca 902 infected citron (B'), and to extracts from healthy seedling citron inoculated with CCaV$_C$ (D'). Negative reactions resulted with hybridization against healthy citron seedling extract (C'), healthy cucumber extract (E'), and the CEV electrophoresis standard.

These results demonstrate homology between the viroid (CCaV) transmitted to and purified from citron and cucumber and the viroid associated with the original cachexia disease source.

DISCUSSION

The CCaV as related to other citrus viroids. On the basis of electrophoretic analysis, a new small RNA with viroid-like structural properties has been identified in nucleic acid extracts from cachexia disease sources. Transmission of this RNA to citron followed by secondary passage to the bioassay host, Parson's Special mandarin, was successful in producing a strongly positive disease reac-
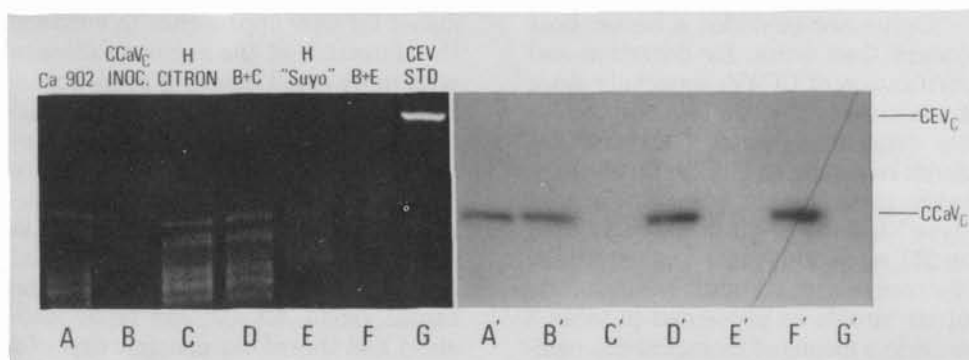
Fig. 8. Segment form denaturing polyacrylamide gel prepared by sequential electrophoresis, stained with ethidium bromide (A-G) containing an extract from citron containing Ca 902 (A), purified CCaV$_C$ (B), extract from healthy citron (C), extract from citron inoculated with CCaV$_C$ (D), extract from healthy cucumber (E), extract from cucumber inoculated with CCaV$_C$ (F), standard CEV (G). Autoradiograph (A'-G') of Nytran filter onto which preparations (A-G) had been electrotransferred and hybridized with ^{32}P-cDNA made to CCaV purified from cucumber.

tion. It is significant that the bioassay reaction is a direct expression of the cachexia disease symptoms. This is distinct from assays of certain other citrus pathogens where other hosts are chosen for bioassay convenience. For example, although Etrog citron is used as an indicator of the exocortis disease, a positive disease reaction in the host to certain viroids may not always correlate with the production of the classical bark shelling reaction of exocortis on trifoliate orange.

The cachexia viroid (CCaV) displays properties distinct from the other two major viroids of citrus, CEV and the CV-Ib component of the "CVaV" isolate (table 3). The three viroids can be easily discriminated, by molecular size and nucleotide sequence homology. In fact, the marked lack of homology within a group of nucleic acid molecules characterized by highly conservative regions of the genome is, indeed, surprising. With a total nucleotide number of about 300, CCaV is included within the suggested citrus viroid classification as CV-IIb (5, 12), and therefore potentially related to CV-IIa, the only other group II viroid.

Although all three viroids replicate in citron, accumulation of viroid as judged by analysis of extracts and symptom expression of each is quite distinct. CEV, which is severe in citron, and CV-Ib, which produces a more moderate response in citron both accumulate to levels 5-10 fold greater than CCaV. This undoubtedly has contributed to the difficulty experienced in the detection and characterization of the causal agent of the cachexia disease.

TABLE 3
COMPARATIVE PROPERTIES OF THE MAJOR CITRUS VIROIDS

Viroid[z]	Bases[y]	cDNA			Infectivity		
		CEV	CV-Ib	CCaV	Citron	Cucumber	Gynura
CEV	371	++++	+/−	−	++++	(+)[x]	++++
CV-Ib	330	−	++++	−	++	−	−
CCaV	300	−	−	++++	(+)[x]	++	−

[z]CEV = citrus exocortis viroid; CV-Ib = citrus viroid Ib, the major component of the "citron variable viroid" isolate reported by Schhemmer et al., 1985 (15). CCaV = citrus cachexia viroid.
[y]Determined by relative mobility in dPAGE with CEV and avocado sunblotch viroid (ASV).
[x](+) = symptomless carrier of the viroid.

Cucumber provides a better host system than citron for detection and purification of CCaV, especially since it constitutes a symptomless host for low concentrations of CEV and remains resistant to CV-Ib. Gynura appears to be highly selective for the "type" isolate of CEV characterized by 371 nucleotides (5). The responses of citron, cucumber and Gynura to the citrus viroids as presented in table 3 provide a means of biological discrimination among these pathogens.

CCaV "strains" and the specificity of the Parson's Special reaction. The detection of a new electrophoretic species in itself does not establish a single nucleic acid moiety. Distinct nucleotide sequences of an identical total number demonstrate the same electrophoretic mobilities. Thus, CCaV from Ca 907 which produces a weak (1-2) reaction in bioassay on Parson's Special mandarin migrates much as CCaV from Ca 902, a strongly reactive (8) isolate. However, by preliminary examination, CCaV from Ca 907, another weak (1-2) isolate, appears to migrate more slowly than CCaV from Ca 902, indicating a slightly larger nucleotide number.

These observations indicate the existence of "strains" of CCaV of identical molecular size as well as viroids of different size, which all produce a reaction on Parson's Special mandarin. This measure of added variation to the cachexia disease picture might be anticipated, but because of the specificity of the bioassay reaction, it should not approach the complexity recently defined (5) for the wide range of distinct citrus viroids which have been indexed on citron as isolates of exocortis disease.

Potential for the rapid indexing of the cachexia disease. With these studies, the potential has been introduced for new approaches to enhance the detection of the cachexia disease with more rapid indexing procedures. These include direct sequential PAGE analysis of nucleic acid extracts from citron and the alternate herbaceous host, cucumber, inoculated with suspected cachexia sources. We have also demonstrated that a cDNA probe specific for the causal viroid, CCaV, can be synthesized and therefore, construction of a cloned cDNA probe would appear most feasible.

Direct analysis of field trees may pose some problems because of the low concentration of the viroid and the general symptomless nature of many citrus varieties to cachexia. Preliminary analyses of extracts from foliar tissues of Parson's Special mandarin and rough lemon for CCaV by PAGE was unsuccessful. Nevertheless, we remain confident that with the experimental tools introduced here, the present bioassay procedure can be improved.

Relationship between the cachexia disease and xyloporosis. It is now possible to begin to resolve the question of the relationship between cachexia and xyloporosis (11). With the description of a definitive molecular species as the causal agent of the cachexia disease, a comparative study of the infectious agents of the two diseases can be initiated.

ACKNOWLEDGEMENTS

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