Interactions Among the Group II Citrus Viroids: A Potential for Protection From the Cachexia Disease?

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ABSTRACT. Viroids classified in Group II of the Citrus Viroid Catalogue contain agents of both the exocortis and cachexia diseases as determined by reactions on the indexing hosts Etrog citron and Parson's Special mandarin. In spite of these distinct biological expressions, CV-IIa and -IIb display a high degree of nucleotide sequence homology.

A third member of the CV-II Group, designated as CV-IIc, has been defined on the basis of relative electrophoretic migration. Because of a 2-4 nucleotide differential within a size range of 296-302 nucleotides, the three Group II viroids can be resolved by sequential polyacrylamide gel electrophoresis (sPAGE). All of the CV-II viroids react positively in homologous and heterologous hybridization tests with cDNA probes.

To study interactions among the Group II viroids, buds from established citron sources containing single viroids, along with a healthy bud were grafted to a common seedling citron and forced as four equivalent growing points. The viroid content of successive growth was monitored at 4-6 week intervals. All tissues remained symptomless throughout the experiment.

In tissues derived from the healthy bud, the three viroids rapidly became established in equal titers as a mixed infection. By contrast, tissues from the CV-IIc source bud contained either CV-IIa or CV-IIb as a predominant second viroid component but not both CV-IIa plus CV-IIb. This demonstration of a possible interference or mutual antagonism between CV-IIa and CV-IIb was most pronounced in tissues derived from source buds of the two viroids. This suggests that the mild "exocortis" agent, CV-IIa, impedes the replication and/or accumulation of the severe cachexia agent, CV-IIb, in citron and might represent a potential agent for the suppression of cachexia in commercial plantings.

Index words. Citrus viroids, Etrog citron, sequential PAGE, exocortis, cachexia, interference, cross-protection.

The Group II citrus viroids of the Citrus Viroid Catalogue (2,3,12) have become of particular interest because the high titers in alternate hosts which facilitate the description of physical properties and the implicit relationship between the causal agents of two distinct citrus diseases, exocortis and cachexia.

CV-IIa produces the most mild reaction and the lowest yield of all citrus viroids on the "exocortis" bioassay host, Etrog citron (9), and yet it induces a very severe reaction on cucumber and can be purified in relatively high concentrations. These properties are common to all Group II viroids and similar to the latent viroid of citrus reported by Sano *et al.*, (10,11) and the "citrus B viroid" (1), both demonstrated to be related to hop stunt viroid (HSV) by size and sequence homology.

Two slightly smaller Group II viroids within the 297-302 nucleotide range, CV-IIb or CCaV and CV-IIc, have also been shown to replicate well in cucurbits and display a high nucleotide sequence homology by molecular hybridization analysis (13). Another potential Group II viroid of 299 nucleotides has been recovered from grapefruit (6) and may be identical to one of these forms.

It appears that viroids in the size range of about 300 nucleotides with subtle composition differences are readily perpetuated as variant forms. The Group II citrus viroids can be considered generically as a family of viroids related to the hop stunt viroid by molecular structure and sequence (4,5). Nevertheless, dramatic biological distinctions can be described among the viroids of this group. The causal agents of two diseases of citrus, exocortis and cachexia, as well as the hop stunt viroid which produces the characteristic severe stunting of hops and cucurbits are contained in this group. It is yet unknown whether HSV, the classical type viroid for this group, induces any reaction in citrus indicators.

Investigation of the interactions among some of the Group II citrus viroids in a common host, reported here, indicates that distinctly different relationships, including interference and complete compatibility, exist between specific viroids. It may be possible to exploit these effects to define a strategy for the suppression or control of cachexia in the field.

IDENTIFICATION AND PROPERTIES OF SELECTED GROUP II CITRUS VIROIDS

The origin of the sources of the three Group II citrus viroids employed in these studies are presented in Table 1. CV-IIa, -IIb, and -IIc have been maintained as stable pure isolates in sweet orange over a period of many years. These were transmitted to citron and maintained for 1-2 yr prior to serving as bud donors. CV-IIa has been designated as an exocortis source, E818, on the basis of the faint tip browning and petiole necrosis induced on Etrog citron. CV-IIb and CV-IIc induce a strong browning reaction in Parson's Special mandarin indicative of causal agents of cachexia.

A narrow molecular size range of 297-302 nucleotides encompasses CV-IIa, -IIb, and -IIc (Table 2). Nevertheless, the three viroids can be resolved as discrete bands following sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Fig. 4A). All CV-II viroids respond similarly to CF-11 cellulose chromatography and in molecular hybridization tests with homologous and heterologous cDNA probes.

Figure 1 presents the common molecular hybridization reaction of the three Group II viroids after sPAGE and electrotransfer to Nytran membrane. The total absence of any reaction with the size standards (STD) CEV and avocado sunblotch viroid (ASBV), representing the largest and smallest viroids is apparent. The positive reactions of the cachexia isolate, Ca 907, (Fig. 1, lane D) indicates the presence of two Group II components. The intense ethidium bromide staining band in the Group II region results from the presence of CV-IIIb which migrates near the Group II region. These results indicate the value of hybridization analysis following sPAGE especially in the detection of low concentration viroids such as the Group II members that can be masked in the stained gel.

CV-IIa induces a somewhat stronger reaction in cucumber and a leaf spotting symptom in chrysanthemum (13). This may provide an additional bioassay host of potential importance in indexing for CV-IIa which induces the most mild reaction in citron of all exocortis isolates.

INTERACTIONS AMONG GROUP II CITRUS VIROIDS

Citron was chosen as the model host system for these studies since CV-IIa, -IIb, and -IIc are know to replicate and accumulate in about equivalent titers when maintained as single viroid cultures. Also, distinctions among the symptoms induced by the three viroids when maintained over an extended period are minimal to unapparent.

TABLE 1
IDENTIFICATION OF SELECTED MEMBERS OF THE GROUP II CITRUS VIROIDS

Group II Viroid	Isolate	Source	Etrog Reaction	Parson Reaction	Disease Agent
IIa	E 818	Atwood Navel orange	+ +	–	Exocortis
IIb	Ca 902	Old-line Navel orange	–[z]	+ + + +	Cachexia
IIc	Ca 905	Prior Lisbon lemon	–[z]	+ + +	Cachexia

[z]"Symptomless carrier" exhibiting positive viroid replication.

TABLE 2
COMPARATIVE PROPERTIES OF SOME GROUP II CITRUS VIROIDS

Group II Viroid	Bases	Chromatography Cf-11 Cellulose	Hybridization cDNA Probe			Infectivity	
		25% Ethanol	IIa	IIb	IIc	Cucumber	Chrysanthemum
IIa	302	+ + +	+	+	+	+ + + +	+ + + +
IIb	299	+ + +	+	+	+	+ +	+ +
IIc	297	+ + +	+	+	+	+ +	+ +

Buds were taken from citrons maintained as pure single viroid source trees of CV-IIa, -IIb, or -IIc for at least 1-2 yr and budded to a viroid-free seedling citron along with a healthy bud (Fig. 2). Tissue was forced from the buds to produce a tree with four main shoots with about equal growth (Fig. 3). Apex tissue was collected successively from each shoot at 6-week intervals over a period of 8-10 months. Nucleic acids were recovered by phenol extraction, partitioned by LiCl, and the final preparations enriched in CV-II viroids by CF-11 cellulose chromatography (14,15,16). Analysis of viroids was accomplished by sPAGE (14) as modified by Rivera-Bustamante *et al.* (7).

The typical resolution achieved among the Group II citrus viroids after sPAGE and silver staining is presented in Fig. 4. These data clearly indicate the distinctly different viroid profile that is recovered from tissue collected from the buds which originally carried a single Group II viroid but propagated on the same viroid-free citron. The patterns

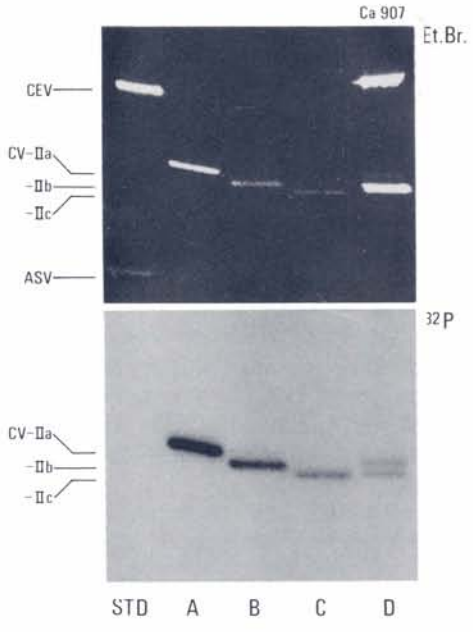


Fig. 1. Polyacrylamide (5%) gel containing 8M urea stained with ethidium bromide (upper) with nucleic acid preparations containing citrus exocortis viroid (CEV) and avocado sunblotch viroid (ASBV) as standards (STD), CV-IIa (A), CV-IIb (B), CV-IIc (C), and the cachexia disease field isolate Ca 907 (D). Autoradiograph (lower) of the matching pattern after electrotransfer onto Nytran membrane and hybridization with ^{32}P-labelled cDNA probe to CV-IIa.

Fig. 2. Etrog citron seedling multiple budded with tissues from healthy citron and buds from citron infected with single Citrus Viroids (CV) of Group II, CV-IIa, CV-IIb, and CV-IIc.

Fig. 3. Etrog citron 3-4 months after multiple budding with healthy and CV-II containing tissue as in Fig. 2.

demonstrate that all three Group II viroids become established in a healthy bud in about comparable titers (Fig. 4, lane A). This response occurs independent of the position of the healthy bud on the seedling citron relative to the viroid containing source buds.

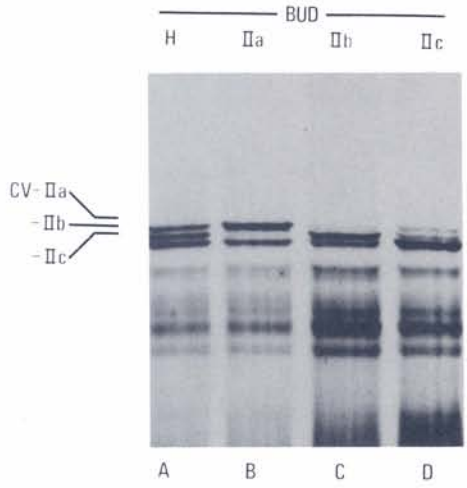


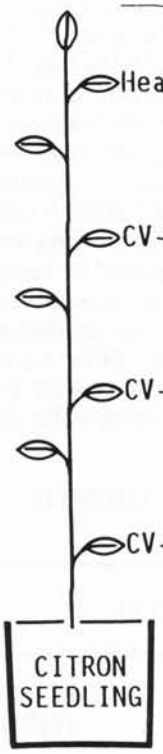
Fig. 4. Polyacrylamide (5%) gel containing 8M urea and stained with silver after processing by sequential PAGE. Nucleic acid extracts from different buds propagated on the same citron seedling and representing tissues which originally were healthy (A), or infected with CV-IIa (B), CV-IIb (C), or CV-IIc (D).

By contrast, the CV-IIa source bud contains CV-IIc in a slightly lower titer, but almost undetectable levels of CV-IIb (Fig. 4, lane B). A similar but heterologous situation occurs in the CV-IIb source bud (Fig. 4, lane C). The source bud for CV-IIC displays a high titer of the resident viroid plus detectable levels of both CV-IIa and CV-IIb (Fig. 4, lane D). The development of these patterns with analysis of successive growth flushes is presented in Fig. 5.

Clearly, some fluctuation occurs in the concentration of the viroid content of the different buds. However, patterns of responses are observed through five successive analyses encompassing a 7-8 month period. The Viroid-free citron bud appears to be invaded and supportive of the replication and accumulation of all three Group II viroids. This confirms that the viroids are independently transmissible and can exist compatibly in the same citron tissues. By contrast, if the tissues already contain a Group II viroid component, different patterns of viroid accumulation are registered. CV-IIc replication suggests that a coexistence with CV-IIa and CV-IIb is possible. However, a bud that originates new growth in the presence of CV-IIa appears to preferentially exclude CV-IIb. The reciprocal reaction is also true of buds containing CV-IIb.

Although this response has been observed only in citron, if other citrus species respond similarly, the antagonistic reaction between CV-IIa and CV-IIb might be exploited for the practical application of inhibiting or suppressing the cachexia disease. CV-IIa is a mild exocortis agent that is very difficult to detect by citron bioassay and has even been found to not seriously affect the production of varieties released through certification programs such as the Atwood and Newhall navels. In fact, the oldest known propagations from the par-

INTERACTIONS AMONG GROUP II CITRUS VIROIDS IN HEALTHY AND VIROID CONTAINING BUDS ON A CITRON SEEDLING



BUD SOURCE	VIROID CONTENT (CV-II)	INTERVAL (6 WEEK) 1st	3rd	5th
Healthy	a	+	+++	+++
	b	+++	+++	+++
	c	++	+++	+++
CV-IIa	a	+++	++++	++++
	b	-	-	+/-
	c	+++	+++	++
CV-IIb	a	-	-	+
	b	++++	++++	++++
	c	+	+++	+++
CV-IIc	a	+	++	++
	b	++	++	+
	c	+++	++++	++++

Fig. 5. Summary of accumulation of Citrus Viroids (CV) of Group II in 6 week intervals after budding as single viroid infected buds and propagation on a common citron seedling. Rating of viroid concentration was made by visual estimation of viroid bands (see Fig. 4) after sequential PAGE and silver staining.

ent Washington navels in Riverside, California have been analyzed by sPAGE for viroids and shown to contain CV-IIa.

CV-IIb, or CCaV, has been demonstrated to be the causal agent of the most severe form of cachexia. The interference between CV-IIa and CV-IIb reported here in citron is being tested with commercial mandarin and tangelo varieties to determine if the preinoculation of CV-IIa functions to deter or suppress the effects of cachexia.

The absence of interference between CV-IIb and CV-IIc, both recognized as cachexia sources, should be viewed in perspective with the suggestion of Semancik and Duran-Vila (13) that CV-IIc represents the best candidate at present for the putative causal agent of "xyloporosis" disease (8).

RELATIONSHIP BETWEEN EXOCORTIS SOURCES AND GROUP II VIROIDS

An additional aspect of these data is the fact that CV-IIa and CV-IIb induce two different diseases of citrus, exocortis and cachexia, respectively.

Causal agents of the exocortis disease have been identified in different groups of the Citrus Viroid Catalogue. What then would be the effect of the presence of a second exocortis source in a similar model system? To this end, a bud source of a severe exocortis source, CEV, not related to the Group II viroids, was grafted to a seedling citron along with buds containing either CV-IIa or CV-IIb and a viroid-free healthy bud.

Figure 6 summarizes the viroid pattern in the tissues forced from the various buds. The severe CEV bud source could not be analyzed since the buds did not generate new tissues, presumably because of the effects of CEV. Nevertheless, the severe CEV bud source did act as an inoculum source because all other buds displayed severe symptoms and high titers of CEV.

Again, as in the previous experiment with the Group II viroids, the healthy bud contained the full complement of all the viroids budded to the seedling. The bud containing the severe cachexia source, CV-IIb, also contained high concentrations of CEV but negligible amounts of CV-IIa. The reciprocal antagonism or interference between CV-IIa and CV-IIb was also noted in the CV-IIa bud. No interference was detected between the mild and severe exocortis sources.

These studies suggest that the physical and biological properties utilized to establish the Citrus Viroid Catalogue indicate closer relationships than may be suggested by bioassay on indexing hosts for a particular disease, such as citron for exocortis. The specificity of the citrus viroid groups provide a powerful tool for not only the development of specific de-

INTERACTIONS AMONG VIROIDS INDUCING MILD AND SEVERE EXOCORTIS AND SEVERE CACHEXIA ON A CITRON SEEDLING

BUD SOURCE	VIROID CONTENT		
	CEV	CV-IIa (E818)	CV-IIb (Ca902)
Healthy	++++	++++	++++
Ca 902 (Cachexia, severe)	++++	+/-	++++
E 818 (Exocortis, mild)	++++	++++	-
E 811 (Exocortis, severe)	++++	[ ]*	[ ]*

* insufficient tissue to analyze

Fig. 6. Summary of accumulation of the exocortis agent of Citrus Viroid (CV) Group II, CV-IIa, and the cachexia agent, CV-IIb, and the severe exocortis disease type isolate, CEV, after budding as single viroid infected buds and propagation on a common citron seedling. Rating of viroid concentration was made by visual estimation of viroid bands (see Fig. 4) after sequential PAGE and silver staining.

tection procedures for citrus viroids, but also for consideration of potential strategies for the control of citrus diseases of viroid etiology.

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LITERATURE CITED

1. Diener, T. O., D. R. Smith, R. H. Hammond, G. Albanese, R. La Rosa, and M. Davino. 1988. Citrus B viroid identified as a strain of hop stunt viroid. Plant Dis. 72: 691-693.
2. Duran-Vila, N., J. A. Pina, J. F. Ballester, J. Juarez, C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik. 1988. The citrus exocortis disease: A complex of viroid RNAs, p. 152-164. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
3. Duran-Vila, N., C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik. 1988. A definition of citrus viroid groups and their relationship to the exocortis disease. J. Gen. Virology 69: 3069-3080.
4. Ohno, T., J. Akiya, M. Higuchi, Y. Okada, N. Yoshikawa, T. Takahashi, and J. Hashimoto. 1982. Purification and characterization of hop stunt viroid. Virology 118: 54-63.
5. Ohno, T., N. Takamatsu, T. Meshi, and Y. Okada. 1983. Hop stunt viroid: Molecular cloning and nucleotide sequence of the complete cDNA copy. Nucleic Acid Res. 11: 6185-6197.
6. Puchta, H., K. Ramm, R. Hadas, M. Bar-Joseph, R. Luckinger, K. Freimuller, and H. Sanger. 1989. Nucleotide sequence of a hop stunt viroid (HSVd) isolate from grapefruit in Israel. Nucleic Acid Res. 17: 1247.
7. Rivera-Bustamante, R., R. Gin, and J. S. Semancik. 1986. Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system. Anal. Biochem. 156: 91-95.
8. Roistacher, C.N. 1988. Cachexia and xyloporosis diseases. A review., p. 116-124. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
9. Roistacher, C.N., E.C. Calavan, R.L. Blue, L. Navarro, and R. Gonzales. 1977. A new more sensitive citron indicator for detection of mild isolates of citrus exocortis viroid (CEV). Plant Dis. Rep. 53: 333-336.
10. Sano, T., T. Hataya, A. Sasaki, and E. Shikata. 1986. Etrog citron is latently infected with hop stunt viroid-like RNA. Proc. Japan Academy 62: 325-328.
11. Sano, T., T. Hataya, and E. Shikata. 1988. Complete nucleotide sequence of a viroid isolated from Etrog citron, a new member of hop stunt group. Nucleic Acid Res. 16: 347.
12. Semancik, J. S. 1988. The citrus exocortis disease. A review 1976-1986., p. 136-151. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
13. Semancik, J. S. and N. Duran-Vila. 1990. The grouping of citrus viroids: Additional physical and biological determinants and relationships with diseases of citrus, p. 178-188. *In* Proc. 11th Conf. IOCV. IOCV, Riverside.
14. Semancik, J. S. and K. L. Harper. 1984. Optimal conditions for cell-free synthesis of citrus exocortis viroid and the question of specificity of RNA polymerase activity. Proc. Natl. Acad. Sci. USA. 81: 4429-4433.
15. Semancik, J. S., C. N. Roistacher, and N. Duran-Vila. 1988. A new viroid is the causal agent of the citrus cachexia disease, p. 125-135. *In*: Proc. 10th Conf. IOCV. IOCV, Riverside.
16. Semancik, J. S., C. N. Roistacher, R. Rivera-Bustamante, and N. Duran-Vila. 1988. Citrus cachexia viroid, a new viroid of citrus: Relationship to viroids of the exocortis disease complex. J. Gen. Virol. 69: 3059-3068.