VIROID AND VIROID INDUCED DISEASES

The Grouping of Citrus Viroids: Additional Physical and Biological Determinants and Relationships with Diseases of Citrus

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ABSTRACT. A consensus catalogue for the grouping of citrus viroids (CV) utilizing the physical parameters of size and nucleotide sequence homology as tested with group-specific cDNA probes has been furthered developed. Analysis of cachexia disease sources by sequential polyacrylamide gel electrophoresis (sPAGE) has revealed a third member of the CV-II Group, designated as CV-IIc. A viroid characterized by a similar size and sequence homology is common to all "xyloporosis" disease sources analyzed. Chrysanthemum, which has been identified as a selective host for CEV and the Group II viroids, produces a leaf spotting reaction when inoculated with CV-IIa, the viroid inducing the most mild reaction in citron.

Mixed infections of viroids from different groups has indicated a range of responses on citron including: 1) both enhanced and delayed severe dwarfing symptoms of CEV, 2) synergism resulting in severe dwarfing and epinasty mimicking CEV symptoms, and 3) a variation in leaf symptoms ranging from no effect to severe epinasty. Field testing of pure citrus viroids and mixed viroid inoculations of commercial scion on exocortis sensitive rootstock combinations has been initiated.

Analysis of the viroid profiles of citrons inoculated with field sources of some diseases of suspected viroid etiology such as, "xyloporosis" of Palestine sweet lime, "gummy bark" of navel orange, and "Kassala" of grapefruit, indicated the presence of a complex pattern of several viroids.

Index words. Citrus viroids, Etrog citron, cDNA viroid probes, molecular hybridization, electrophoresis, sequential PAGE, exocortis, cachexia.

With the revelation of the large collection of citrus viroids (6), an attempt has been made to rationalize these viroids into specific groupings on the basis of physical and biological properties. The existence of viroid-like pathogenic agents in citrus was originally detected by a symptom reaction on Etrog citron, the bioassay host for exocortis (4, 19). These were, therefore, assumed to be isolates of the citrus exocortis viroid (CEV). The citrus viroids have now been demonstrated to be distinctly different from the 371 nucleotide CEV and to comprise a diverse collection of independently-transmissible RNA molecules in a size range of 275-340 nucleotides. In addition to the type isolate of classical CEV, four discrete groups of the citrus viroids were proposed on the basis of molecular size, conformation, and sequence homology as well as host range and symptom reactions on Etrog citron (7, 8).

In the studies reported here, a complete series of cDNA probes representative of each citrus viroid group has been tested against a standard range of citrus viroids. The homology reactions verify the specificity of the distinct groups. Chrysanthemum is introduced as an additional selective host which may be useful in detection of the CV-II group noted for the most mild symptom reaction and lowest titer in citron.

With the collection of over 12 citrus viroids and the identification of exocortis and cachexia as the only two citrus diseases induced by viroids, the significance of the citrus viroids as agents of specific diseases or possibly even more subtle physiological conditions affecting citrus production remains unclear.

DEFINITION OF CITRUS VIROID GROUPS BY PHYSICAL PROPERTIES

Tissue selection and sequential PAGE. Essential to the description of the citrus viroids was the utilization of Etrog citron as the common host for detection and analysis of viroids from various citrus sources. The standard procedure adopted in these studies involved the primary inoculation into citron of a bud or tissue piece of source materials derived from commercial plantings or disease collections. After a period of 3 to 6 months, the new flush of tissue was collected, the nucleic acids extracted (8) and analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) (17).

If it was desirable to either transport or store samples, freshly collected tissue was frozen in liquid nitrogen, pulverized to small fragments to assure random sampling, and lyophilized. With this treatment, excellent recovery of biologically-active viroid preparations could be assured from tissues maintained under dry conditions without any special temperature requirements.

A representative of each citrus viroid group is presented in Fig. 1 as a discrete silver stained band in a denaturing gel, the final stage in the detection of viroids by sPAGE. With the exception of CEV and CV-IV which comprise single viroid groupings, the CV-I, -II, and -III Groups each contain 2-4 viroids. Furthermore, it is not unexpected that additions to these families will be made as viroid surveys employing the standard sPAGE procedure are extended to the different citrus growing regions.

An additional Group-II viroid, designated as CV-IIc, was found when analyzing the cachexia disease source collection maintained at UC-Riverside. CV-IIc is a pure single viroid isolate contained in the cachexia source Ca 905 which originated from Prior Lisbon lemon. Although CV-IIc induces a more moderate (6.5/10) reaction in Parson Special mandarin

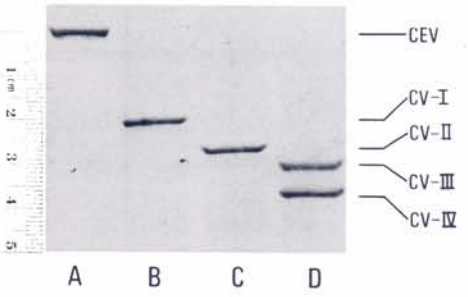

Fig. 1. Polyacrylamide (5%) gel containing 8M urea and silver stained after processing by the sequential PAGE procedure of CEV (A), CV-I (B), CV-II (C), and CV-III + CV-IV mixture (D).

than the severe reaction (8/10) produced by the type citrus cachexia viroid (CCaV) or CV-IIb (26, 27), both CV-IIb and CV-IIc must be considered as cachexia disease agents even though they represent physically separable molecular entities.

Group specific sequence homology by molecular hybridization. Using viroid templates, cDNA probes were synthesized by the random priming procedure in the presence of ^{32}P. To test the group specificity of the probes, a standard range of citrus viroids were electrotransferred from denaturing gels to nytran membranes, hybridized to the radioactive cDNA probes, and autoradiographed. In this way, the relative intensity of the homology reaction of the specific citrus viroids, as defined by the characteristic electrophoretic mobility, could be monitored.

The marked specificity of the citrus viroid groups was essentially sustained throughout the test (Table 1). The sole intergroup reaction observed was a very weak signal CV-IV produced when probed with the CEV-cDNA. This suggests that a closer relationship may exist between the genome of CEV, the largest (371 nucleotides), and CV-IV, the smallest (275 nucleotides) of the citrus viroids than among the almost continuous array of viroids contained in Groups I, II, and III. From this, it can be speculated that a more significant

TABLE 1
COMPARATIVE PHYSICAL PROPERTIES OF CITRUS VIROID GROUPS

GROUP	CITRUS VIROID	BASES	HYBRIDIZATION REACTION TO cDNA PROBE				
			CEV	CV-Ib	CV-II	CV-IIIb	CV-IV
CEV	CEV	371	++++	—	—	—	—
I	CV-Ia	340	—	++++	—	—	—
	CV-Ib	330	—	++++	—	—	—
II	CV-IIa	302	—	—	++++	—	—
	CV-IIb (CCaV)	299	—	—	++++	—	—
	CV-IIc[z]	297	—	—	++++	—	—
III	CV-IIIa	292	—	—	—	++++	—
	CV-IIIb	290	—	—	—	++++	—
	CV-IIIc	285	—	—	—	++++	—
	CV-IIId	280	—	—	—	++++	—
IV	CV-IV	275	+	—	—	—	++++

[z]A new viroid (isolate Ca 905, UC-Riverside collection).

portion of the genomes of CEV and CV-IV is composed of a common nucleotide sequence than can be accounted for by the central conserved region common to all viroids (11).

DIFFERNTIATION OF CITRUS VIROIDS BY BIOLOGICAL CHARACTERISTICS

Screening hosts. Alternate herbaceous hosts have been invaluable in the definition of the groupings of the citrus viroids which are known to replicate in citron. This has been especially useful in studies of the CV-II group which induces the most mild response in citron and yet the most severe reaction in cucumber. Furthermore, the availability of high titers of CV-II viroids in cucumber has stimulated considerable activity in the sequencing of these viroids (16, 21). Since cucumber does not sustain replication of viroids of the CV-I or -III Groups, the detection and recovery of CV-II viroids, which display similar electrophoretic migration rates with CV-III viroids, is greatly facilitated.

The symptom expression and high level of accumulation of CV-II viroids in cucumber taken with the molecular size (297-305 nucleotides) supports an (implicit relationship with the hop stunt viroid (HSV) (14, 15). However, since the reaction of HSV on citrus hosts has not yet been reported, the Group II exocortis agent, CV-IIa, as well as the citrus cachexia viroid (CCaV) or CV-IIb, should be considered as members of the hop stunt viroid family but not as biologically equivalent to the hop stunt viroid.

Chrysanthemum is now shown to restrict the replication of not only CV-I and CV-III viroids but also CV-IV (Table 2). A leaf spotting symptom has been observed with the inoculation of CV-IIa (isolate E 818) under hot seasonal growing conditions in California (Fig. 2).

MIXED VIROID INFECTIONS AND GROUP SPECIFIC SYMPTOMS ON CITRON

Group specific symptoms in citron have been previously presented (7, 8). The principal distinguishing feature of each group can be described as: 1) a severe dwarfing with CEV, 2) a "bent leaf" pattern caused by point necrosis of midveins with CV-I, 3) faint necrosis of leaf tips and petiole wrinkle with CV-II, and 4) a "drooping leaf" pattern caused by petiole necrosis with CV-III and -IV.

These distinctive symptom expressions in citron could be modified in mixed viroid infections (Table 3). The severe symptoms induced by CEV were either delayed by the addi-

TABLE 2
TRANSMISSION AND SYMPTOM INTENSITY OF SOME CITRUS VIROID INDICATORS

GROUP	CITRUS VIROID	CITRON	CUCUMBER	CHRYSANTHEMUM	GYNURA
CEV	CEV	++++	+	++++	++++
I	CV-Ia	++	—	—	—
	CV-Ib	++	—	—	—
II	CV-IIa	+	++	+++	—
	CV-IIb (CCaV)	+	++	++	—
	CV-IIc[z]	+	++	++	—
III	CV-IIIa	+++	—	—	—
	CV-IIIb	+++	—	—	—
	CV-IIIc	+++	—	—	—
	CV-IIId	+++	—	—	—
IV	CV-IV	+++	+	—	—

[z]A new viroid (isolate Ca 905, UC-Riverside collection).

tion of either CV-Ia and -IIa, or enhanced by the addition of CV-Ia plus -IIa. Pronounced dwarfing resulted when CV-Ia was mixed with CV-IIa (Fig. 3A) or -IIId (Fig. 3B). A most severe reaction which resembled the response of citron to CEV resulted when the viroid inoculum contained CV-Ia, -IIa, and -IIId (Fig. 3C).

An intermittent or "variable" leaf reaction, in which either a severe stunting and epinasty or essentially a symptomless condition could prevail depending on climatic conditions (Fig. 3D), was observed when CV-Ib, -IIa, -IIb, and IIIb were present. This viroid profile was detected in citron tissues infected with an isolate formerly

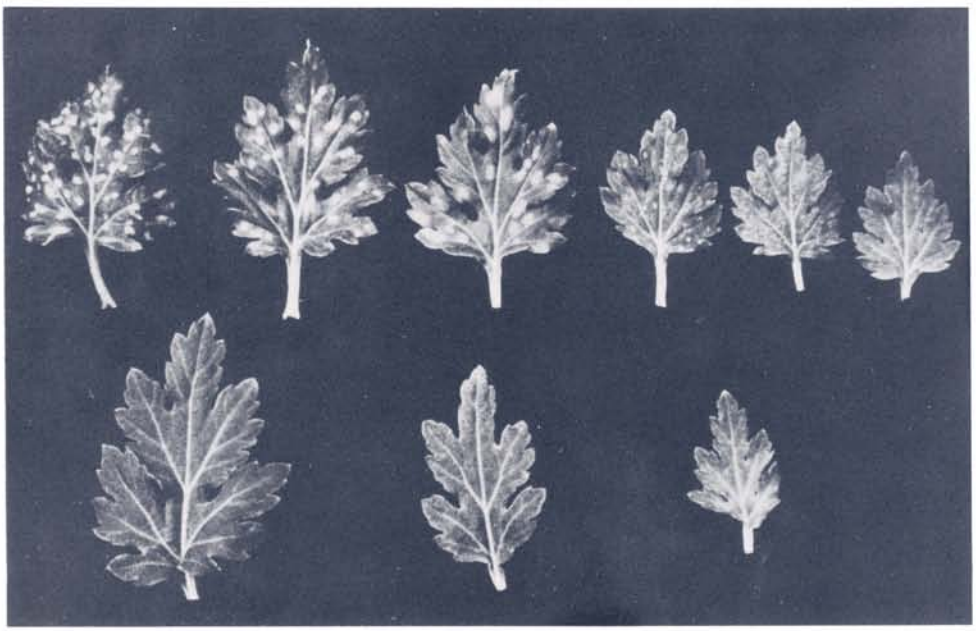

Fig. 2. Systemic symptoms of flecking and yellow spotting on chrysanthemum (*Chrysanthemum* X *morifolium* Ramat. cv. Bonnie Jean) inoculated with Citrus Viroid (CV)-IIa (upper row). Healthy leaves of different developmental provided by stages are shown for comparison (lower row). Photograph provided by G. M. Marco.

TABLE 3
EFFECT OF MIXED VIROID INFECTION ON GROUP SPECIFIC SYMPTOMS IN CITRON

VIROID MIXTURE	SYMPTOMS
CEV, -Ia	Delayed symptoms of CEV
CEV, -IIa	Delayed symptoms of CEV
CEV, -Ia, -IIa	Enhanced symptoms of CEV
CV-Ia, -IIa	Enhanced dwarfing and epinasty
CV-Ia, -IIId	Enhanced dwarfing and epinasty
CV-Ia, -IV	Enhanced dwarfing and epinasty
CV-Ia, -IIa, -IIId	Pronounced dwarfing and epinasty
CV-Ib, -IIa, -IIb, -IIIb (Citron variable viroid)	Variable leaf symptoms—severe to symptomless
CV-IIIa, -IV	Variable leaf symptoms—severe to symptomless

analyzed as a single viroid and therefore termed, the "citron variable viroid" (CVaV) (23). It has now been found that the symptom response occurs following inoculation of a viroid mixture. Since no single viroid has been demonstrated to induce these characteristic symptoms, the term "citron variable viroid" or CVaV as introduced by Schlemmer (23) is not an accurate term and should be no longer used.

It should be emphasized here that the reactions described above relate exclusively to symptoms produced in citron as a bioassay host. Data on the effect of the citrus viroids on commercial scion/rootstock combinations is not yet available. Initial field testing of pure viroid isolates and viroid mixtures has been established in Spain on sweet orange and clementine on Carrizo citrange rootstock and in Corsica on mandarin on trifoliate orange rootstock.

This information will undoubtedly provide a valuable insight into the significance of viroids to citrus production. Nevertheless, the limited scope of these tests in terms of the citrus germplasm sources employed plus the potential ramifications introduced by crop management and climatic conditions in different citrus growing regions may require regional or local testing programs to definitively determine the full range of effects possible with the citrus viroids.

CITRUS VIROIDS AND AS RELATED TO DISEASES OF CITRUS

The present collection of five groupings of citrus viroids of related molecular forms contains about 12 distinct species. It is most probable that each viroid may be composed of a range of variants, as has been already described for CEV (28). Furthermore, it would be presumptuous to assume that the full complement of citrus viroids has already been identified. In fact, it might be predicted that a virtual continuum of citrus viroids in the range of 275 to 375 nucleotides may eventually be described. From this perspective, it seems incredible that to date only two citrus diseases, exocortis and cachexia, have been attributed to viroids.

Thus, the consideration may be entertained whether the terms "exocortis" and "cachexia" actually define specific diseases or simply reflect more generic condition which might be better defined into more distinct "diseases" or altered developmental conditions. It should be recalled that a great many of the citrus viroids were originally identified as "strains" of "exocortis" solely by a leaf symptom response on the accepted exocortis bioassay host, citron. These isolates were never tested as true exocortis agents by the demonstration of the classically defined

Fig. 3. Symptoms on Etrog citron induced by mixed viroid infections among representatives of different citrus viroid (CV) groups. (A) healthy (left) and CV-Ia + CV-IIa (right), (B) healthy (left) and CV-Ia + CV-IIId (right), (C) healthy (left) and CV-Ia + CV-IIa + CV-IIId (right), and (D) typical "variable" symptom pattern induced by either mixture of CV-Ib + CV-IIa + CV-IIb + CV-IIIb or CV-IIIa + CV-IV.

bark scaling reaction on *P. trifoliata* rootstock. It would not be surprising, therefore, to anticipate that a wide range of differential symptom reactions could result if each citrus viroid were tested on a battery of citrus indicators.

The condition of gummy pitting of Palestine sweet lime known as "xyloporosis" has been variously described as: 1) a distinct disease of possible viroid etiology, 2) a disease identical to "cachexia", and 3) a physiological condition induced by hot drying winds (18). With the suspicion that a viroid etiology may be involved, "xyloporosis" disease sources were obtained from Israel (kindly provided by M. Bar-Joseph), inoculated to citron and analyzed following our standard procedures for viroid detection.

Since isolates of pure "xyloporosis" do not exist, field sources were employed. The viroid profiles of the citrons inoculated with three "xyloporosis" sources tested indicated a complex of 4-6 viroids (Table 4). Sufficient diversity exists in the viroid patterns from the three "xyloporosis" sources to demonstrate coincidence in only two viroids, CV-IIc and CV-IV. With the implied relationship of "xyloporosis" to cachexia, the focus of attention for a putative viroid causal agent for "xyloporosis" would logically be directed to the CV-II group. From this we can deduce that if a distinct causal agent as the "citrus xyloporosis viroid" does exist, CV-IIc would presently represent the prime single viroid candidate.

Alternatively, from the characteristic effects mixed viroid infections produce on citron presented here, some specific combination of citrus viroids must also be considered as a potential "causal viroid complex" responsible for the induction of a specific disease syndrome. The ultimate resolution of this question must await biological indexing on specific bioassay hosts.

A similar reasoning can also be applied to two other diseases of citrus which have also been suspected of a viroid etiology, the "gummy bark" condition of sweet orange occurring in North Africa and the Middle East (3, 13) and the "Kassala" disease of grapefruit reported in the Sudan (2). Currently, only field sources of these diseases are available. "Gummy bark" samples from the Middle East were kindly supplied by J. Bove and C. N. Roistacher. The "Kassala" field sources were collected by J. Bove in the Sudan and propagated in Corsica in the collection of R. Vogel.

As in the case of the "xyloporosis" sources, when inoculated to citron, a

TABLE 4
VIROID COMPONENTS FROM CITRONS INOCULATED WITH SOURCES OF "XYLOPOROSIS" OF PALESTINE SWEET LIME, "GUMMY BARK" OF SWEET ORANGE AND "KASSALA" OF GRAPEFRUIT

GROUP	CITRUS VIROID	XYLOPOROSIS			GUMMY BARK		KASSALA	
		A	B	C	A	B	A	B
CEV	CEV	−	+	+	+	−	−	+
I	CV-Ia	−	−	−	−	−	−	+
	CV-Ib	+	−	+	+	−	−	−
II	CV-IIa	+	−	−	+	−	−	−
	CV-IIb	−	+	+	−	+	−	−
	CV-IIc	+	+	+	+	−	−	−
III	CV-IIIa	−	−	−	−	+	+	+
	CV-IIIb	−	+	+	+	−	−	−
	CV-IIIc	−	−	−	−	−	−	−
	CV-IIId	−	−	−	+	−	−	−
IV	CV-IV	+	+	+	−	+	+	+

complex of viroids was also detected (Table 4). It has been shown that the recovery of mixtures of viroids from field sources is not uncommon and complex mixtures can accumulate in commercial citrus even in the absence of any disease expression. No consistent correlation could be made between the viroid profiles from the field isolates and disease expression for both "gummy bark" and "Kassala." Therefore, the implication that the viroids recovered from these disease sources are in any way related to the disease condition must be viewed with caution.

THE RELATIONSHIP OF THE CITRUS VIROID CATALOGUE TO RECENT REPORTS OF VIROID DETECTION IN CITRUS SPECIES

Since the original presentation of the model for the grouping of citrus viroids, reports have been made for either a concurrence with and/or proposed additions to the Citrus Viroid Catalogue (9, 10, 20, 21) as well as the introduction of a different nomenclature (5, 12). For the most part these studies employed detection systems compatible with those used to differentiate the citrus viroid groups. Therefore, an attempt can be made to rationalize these data into a common focus.

The survey of Australian citrus viroid sources conducted by Gillings *et al.*, (9) resulted in a coincidence in viroids with properties similar to CEV, CV-Ib, -IIa, -IIb, -IIIa, and -IIIb. In addition, two new viroids with relative molecular sizes of 317 and 298 nucleotides were proposed, and designated as CV-Ic and CV-IIc, respectively. When viewed from a size factor alone, CV-Ic with 317 nucleotides occupies a sufficiently unique position between the smallest Group I component CV-Ib (330) and the largest Group II component CV-IIa (302) to qualify as a new entry to the catalogue. Testing against the group specific cDNAs as well as symptom expression in citron and cucumber are required to confirm the suggested inclusion into Group I.

At 298 nucleotides, the CV-IIc reported from Australia is virtually identical to the 297 nucleotide viroid, also termed CV-IIc, found in the California cachexia collection (Ca 905) and the "xyloporosis" sources from Israel. However, the symptom expression of "leaf kinking" on citron reported by Gillings *et al.*, (9) is unlike the symptomless behavior of citrons inoculated with the California CV-IIc. From this, the two viroids termed CV-IIc appear to display a distinguishing property necessitating some revision in the common CV designation. Until these can be tested under common conditions of host indexing and sPAGE, it may be advisable to add a location designation, ie. CV-IIc (Australia) and CV-IIc (California) Table 5).

Complete concurrence with CEV, CV-Ib, -IIb, -IIIa, and -IV have been reported from grapefruit dwarfing sources in Israel (10). From this mixed viroid source, a 299-nucleotide viroid has been sequenced after transmission to cucurbits (16).

A viroid which replicates in symptomless citron and symptomatic cucumber has been demonstrated to be related to hop stunt viroid by molecular size and sequence homology (20, 21) and indicated by the authors to be "probably the same" as reported by Duran-Vila *et al.*, (6). These properties are characteristic of Group II citrus viroids.

The hop stunt related viroid termed "citrus B viroid" (CBV) by Diener *et al.*, (5) displayed virtually identical biochemical properties to the viroid described by Sano *et al.* (20, 21). Since the "CBV" isolate was obtained from trees indexed free of cachexia, it is most probable that "CBV" is most closely related to CV-IIa. However, the mild stunting and variable epinasly symptom expression induced by "CBV" on citron (1) is unlike any Group II viroid and more similar to a Group III viroid reaction.

La Rosa *et al.*, (12) presented a scheme for the description of "viroid-like RNAs" with designations of "a1, a2, b1, b2, c, and d." The specific relationship of the "b1" and "b2" viroid-like RNA species to the citrus "B" viroid is unclear. It is difficult to relate this nomenclature to the Citrus Viroid Catalogue since analysis was performed on 7% gels by the 2-dimensional procedure of Schumaker *et al.* (22). Even though the exact molecular size of the various viroid-like RNAs is not provided, the reported size range of 340-275 nucleotides conforms perfectly to CV-Ia and CV-IV which encompass the entire Citrus Viroid Catalogue with the exception of CEV.

TABLE 5
THE CITRUS VIROID CONSENSUS CATALOGUE FOR 1989

GROUP	CITRUS VIROID	NUCLEOTIDES
CEV	CEV	371
I	CV-Ia	340
	CV-Ib	330
	CV-Ic[z]	317
II	CV-IIa	302
	CV-IIb (CCaV)	299
	CV-IIc[z]	298 (Australia)
	CV-IIc[z]	297 (California)
III	CV-IIIa	292
	CV-IIIb	290
	CV-IIIc	285
	CV-IIId	280
IV	CV-IV	275

[z]Newly described viroids provisionally added to the Citrus Viroid Catalogue

THE UPDATED CITRUS VIROID CATALOGUE

Additional data has been obtained which substantiates the parameters employed to formulate the original suggestion for the Citrus Viroid Catalogue (25). Specificity of the citrus viroid groups is well supported by viroid genome homology and differential host reactions. The complete nucleotide sequences of the citrus viroids is required to determine the ultimate structural relationship as well as possible clues to the biological properties. An updated listing of the generally accepted Citrus Viroids is presented in Table 5.

The Citrus Viroid Catalogue remains a valid and convenient organizational scheme for the grouping of this large collection of viroids from plants of a common botanical and commercial relationship. To expedite the comparative analysis of citrus viroids from different regions, it would seem reasonable to name some accepted set of standard reference citrus viroids. An accurate description of a putative new viroid to be added to the catalogue could be made by common sPAGE analysis against this set. In this way, the exchange of unknown viroid isolates could be minimized and the utility of the Citrus Viroid Catalogue could be optimized.

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