Replication/Accumulation and Symptom Expression of Citrus Viroids on Some Species of Citrus and Related Genera

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ABSTRACT. Plants of 32 species of *Citrus* and related genera grafted on rough lemon rootstock were inoculated with an artificial mixture of citrus viroids containing *Citrus exocortis viroid*, Citrus viroid I, Citrus viroid II, *Citrus viroid III* and *Citrus viroid IV*. Infection and viroid titers were determined by sPAGE and molecular hybridization analysis. Plants in which viroid infection could not be detected were further indexed by inoculation on Etrog citron 861 S1. Under the conditions of this assay most of the species were symptomless carriers. Only *Citrus excelsa, C. ichangensis, C. karna, C. latifolia*, *C. meyeri* and *C. pyriformis* developed symptoms as a result of viroid infection. Comparative analysis of nucleic acid extracts from bark versus leaf blade tissues indicated that in 10 species, viroid that were readily detectable from bark were undetectable from leaf blade tissues by sPAGE.

Citrus can be infected by several viroids (7) including *Citrus exocortis viroid* (CEVd) and specific variants of Citrus viroid II (CVd-II, *Hop stunt viroid*) that cause the exocortis and cachexia diseases respectively (17, 24, 25). Most viroid-host combinations appear to be symptomless, and exocortis and cachexia symptoms are only observed when their viroid agents infect sensitive species. Trifoliate orange, its hybrids (the citranges) and Rangpur lime, used as rootstocks, and the Etrog citron indicator have been described as exocortis sensitive species. Alemow, Rangpur lime and Palestine sweet lime used as rootstocks, and several mandarins, including clementines, satsumas and their hybrids are sensitive to cachexia.

Reports dealing with the expression of exocortis and cachexia symptoms on sweet limes (23), citrumelo (3) and Cleopatra mandarin (13) were based on the observation of symptoms on plants infected with field isolates characterized only by the response of exocortis and cachexia indicators. At present, the effect of specific viroids species has only been tested on Etrog citron (7) and trifoliate orange (21), whereas the response of most species of *Citrus* and related genera to viroid infection remains unknown.

The objective of this study was to evaluate the response of 32 accessions of the Instituto Valenciano de Investigaciones Agrarias (IVIA) germplasm bank to viroid infection. Such a wide-ranging study has not been previously reported.

MATERIALS AND METHODS

Plant materials and viroid inoculation. Plants of 22 species of *Citrus* and 10 species of related genera (Table 1) of the IVIA germplasm collection (www.ivia.es) grafted on rough lemon rootstock were bark-graft inoculated with an artificial mixture of citrus viroids maintained in Fino lemon. The inoculated plants were kept under greenhouse conditions at 28-32°C for 5 yr before initiating this study.

The mixture of citrus viroids used as inoculum had been previously obtained by graft inoculation of Fino lemon plants with the following viroid sources: CEVd (E-117) (9), Citrus viroid I (CVd-I, *Citrus bent leaf viroid*) (variant Ia) (8), Citrus viroid II (CVd-II, *Hop stunt viroid*) (IIa-117 and X-707) (14), *Citrus viroid III* (variant IIIb) (8), and *Citrus viroid IV* (CVd-IV) (16).

In all instances viroid infection was determined by sPAGE and

TABLE 1
DETECTION OF CITRUS VIROIDS ON INOCULATED SPECIES OF CITRUS AND CITRUS RELATIVES

Citrus and citrus relatives	Accession number	Viroid detection and titer[z]					Symptoms
		CEVd	CVd-I	CVd-II	CVd-III	CVd-IV	
Citrus bergamia Risso and Poit. (cv. Calabria)	IVIA–254	4	2	3	3	4	—
Citrus depressa Hay	IVIA–238	4	3	2	4	3	—
Citrus excelsa Wester	IVIA-167	1	3	2	4	4	+
Citrus grandis L. Osb.	IVIA-321	1	1	2	2	1	—
Citrus halimii B.C. Stone	IVIA-278	1	2	1	4	4	—
Citrus hystrix DC.	IVIA-178	2	0	1	2	2	—
Citrus ichangensis Swing.	IVIA-235	2	2	0	3	3	+
Citrus karna Raf.	IVIA-242	4	4	3	4	3	+
Citrus latifolia Tan. (cv Bearrs)	IVIA-124	1	0	1	4	4	+
Citrus limon L. Burm f. (cv. Verna)	IVIA-50	4	2	2	3	2	—
Citrus macroptera Montr.	IVIA-279	1	2	0	3	2	—
Citrus madurensis Lour.	IVIA- 135	4	1	2	4	4	—
Citrus meyeri Y. Tan. (cv. Meyer)	IVIA-145	0	2	2	4	4	+
Citrus myrtifolia Raf. (Hoja G)	IVIA-136	1	3	3	4	3	—
Citrus myrtifolia Raf. (Hoja P)	IVIA-137	1	2	3	4	4	—
Citrus pyriformis Hassk. (cv. Ponderosa)	IVIA-268	3	1	3	3	3	+
Citrus shunkokan Hort. ex Tan.	IVIA-241	0	1	0	4	0	—
Citrus sunki Hort. ex Tan.	IVIA- 239	1	2	2	2	2	—
Citrus tachibana (Mak.) Tan.	IVIA-237	1	1	2	2	1	—
Citrus temple Hort. ex Y.Tan.	IVIA-81	3	3	2	4	4	—
Citrus unshiu (Mak.) Marc. (cv. Clausellina)	IVIA-19	0	1	1	1	0	—
Citrus webberi Wester	IVIA-234	1	2	2	3	3	—
Atalantia citroides Pierre ex Guill	IVIA-180	0	0	0	0	0	—
Fortunella crassifolia Swing.	IVIA-280	1	0	1	2	1	—
Fortunella margarita Lour. Swing.	IVIA-138	0	2	3	4	4	—

[z]Viroids were detected by sPAGE and molecular hybridization analysis of bark tissue samples. Viroid titers were rated accordingly with the intensity of the sPAGE bands and the hybridization signal: (0) not detected, (1) sPAGE negative and very weak hybridization signal, (2) sPAGE positive and weak hybridization signal, (3) sPAGE positive and intense hybridization signal, (4) sPAGE positive and very intense hybridization signal.

TABLE 1 (CONTINUED)
DETECTION OF CITRUS VIROIDS ON INOCULATED SPECIES OF CITRUS AND CITRUS RELATIVES

Citrus and citrus relatives	Accession number	Viroid detection and titer[z]					Symptoms
		CEVd	CVd-I	CVd-II	CVd-III	CVd-IV	
Fortunella obovata Tan.	IVIA-312	0	2	2	4	4	—
Microcitrus australis (Planch.) Swing.	IVIA-313	0	0	0	0	0	—
Microcitrus australasica (F. Muell.) Swing.	IVIA-150	1	0	1	1	0	—
Microcitrus warburgiana (F.M. Bail.) Tan.	IVIA-315	0	2	2	4	3	—
M. australis × *M. australasica*	IVIA-378	3	2	2	2	2	—
Pleiospermium sp. Engl. Swing.	IVIA-389	0	3	2	4	2	—
Severinia buxifolia Poir. Tenore	IVIA-147	1	1	1	1	1	—

[z]Viroids were detected by sPAGE and molecular hybridization analysis of bark tissue samples. Viroid titers were rated accordingly with the intensity of the sPAGE bands and the hybridization signal: (0) not detected, (1) sPAGE negative and very weak hybridization signal, (2) sPAGE positive and weak hybridization signal, (3) sPAGE positive and intense hybridization signal, (4) sPAGE positive and very intense hybridization signal.

molecular hybridization analysis of bark samples from the inoculated plants. In some cases viroid infection was confirmed by inoculation on Etrog citron (three bark-grafts/plant) followed by nucleic acid analysis 3 mo after inoculation.

Viroid detection. Bark or leaf (with petiole and mid-rib removed) samples (5 g) were homogenized in 5 ml of extraction buffer (Tris-HCl 0.4 M, pH 8.9; SDS 1% (w/v); EDTA 5 mM, pH 7.0; mercaptoethanol 4% (v/v) and 15 ml of water saturated phenol (25). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspension in TKM buffer (Tris-HCl 10 mM; KCl 10 mM; $MgCl_2$ 0.1 mM; pH 7.4) (24). These preparations were subjected to sPAGE and slot-blot hybridization analysis using specific viroid probes. For some specific viroid-host combinations, the recovery of viroids from bark versus leaf lamina tissues were compared.

For sPAGE (5%, 39:1) analysis, 20 µl aliquots of the nucleic acid preparations equivalent to 300 mg of fresh tissue were subjected to electrophoresis under non-denaturing conditions (2 h, 60 mA) and 8M urea denaturing conditions (4 h, 18 mA) (19). Circular forms of viroids were visualized by silver staining (10).

For slot-blot hybridization, aliquots (10 µl equivalent to 150 mg of fresh tissue) were pre-treated with 6×SSC and 8% formaldehyde for 15 min at 60°C and blotted onto positively charged Nylon membranes (Boehringer Mannheim®) using an Hybri-slot filtration manifold (BRL®), immobilized by UV cross-linking and hybridized with DIG-labeled DNA probes. DIG-labelled viroid specific probes were synthesized by PCR from plasmid templates containing full-length viroid cDNAs as described by Palacio et al. (15). Prehybridization and hybridization were carried out in the presence of 50% formamide and 6×SSPE and the DIG-labelled hybrids were detected with an alkaline phosphatase-anti-DIG Fab fragment conjugate and visualized with the chemiluminescence substrate CSPD (Boehringer Mannheim).

Viroid titers were visually rated from 0 to 4 according to the intensity of the silver stained sPAGE bands and the molecular hybridization signals.

RESULTS AND DISCUSSION

Infectivity. The five viroid species (CEVd, CVd-I, CVd-II, CVd-III and CVd-IV) were detected directly from bark tissues in 17 of the 32 genotypes studied. None of the inoculated viroids could be detected from tissues of *Atalantia citroides* and *Microcitrus australis*. In the remaining 13 accessions, at least one of the viroids was undetectable: CEVd (*C. meyeri, C. shunkokan, C. unshiu, Fortunella margarita, F. obovata*, *M. waburgiana* and *Pleiospermium* sp.), CVd-I (*C. hystrix, C. latifolia, F. crassifolia* and *M. australasica*), CVd-II (*C. ichangensis, C. macroptera* and *C. shunkokan*), and CVd-IV (*C. shunkokan, C. unshiu* and *M. australasica*) (shown shaded in Table 1). These plants were graft-inoculated on Etrog citron and the inoculated citrons were subjected to sPAGE and molecular hybridization analysis to determine their infection status. In all instances the graft survived and the results suggest that 11 of these 15 species may be truly resistant to one or more of the inoculated viroids (Table 2). These results should be further confirmed to avoid an erroneous interpretation due to uneven viroid distribution as already reported for satsuma and Navelina sweet orange (6).

The detection results rated 1 (Table 1) should be considered with caution since the hybridization signal was only slightly above background. These plants are in the process of being retested by graft-inoculation on Etrog citron.

Symptom expression. Under our greenhouse conditions, *C. excelsa*,

TABLE 2
DETECTION OF CITRUS VIROIDS USING ETROG CITRON AS AN AMPLIFICATION HOST

Citrus and citrus relatives	Viroids detected in inoculated citron[z]				
	CEVd	CVd-I	CVd-II	CVd-III	CVd-IV
Citrus hystrix	+	+	+	+	+
C. ichangensis	+	+	+	+	+
C. latifolia	+	+	+	+	+
C. macroptera	+	+	+	+	+
C. meyeri	—	+	+	+	+
C. shunkokan	—	+	+	+	+
C. unshiu	—	+	+	+	+
Atalantia citroides	—	—	—	—	—
Fortunella crassifolia	+	—	+	+	+
F. margarita	—	+	+	+	+
F. obovata	—	+	+	+	+
Microcitrus australis	—	—	—	—	—
M. australasica	—	+	+	+	+
M. warburgiana	—	+	+	+	+
Pleiospermium sp.	—	+	+	+	+

[z]The viroids were detected by molecular hybridization on the inoculated citrons.

C. ichangensis, *C. karna*, *C. meyeri*, *C. latifolia* and *C. pyriformis* developed symptoms in response to the infection (Table 1). Since the symptoms have developed during the last growing season, other species that are still symptomless may develop symptoms later on. The symptoms observed (Table 3) are similar to those associated with viroid infection in other sensitive species. Although the bark cracking symptoms observed in *C. excelsa, C. ichangensis, C. latifolia* and *C. pyriformis* were resembled mild exocortis but it has been demostrated to be a specific response of trifoliate orange to CVd-II infection (4, 21). The necrotic lesions and gum exudates resemble those of cachexia in sensitive hosts (12, 18). Yellow blotching observed in *C. karna* and *C. meyeri* is similar to the symptoms previously observed on trifoliate orange stems (26).

The sensitivity of *C. karna* and *C. latifolia* to viroid infection confirms early reports from Brazil (22, 23) indicating that these species were exocortis sensitive, however in contrast to the results reported here, *C. excelsa, C. hystrix* and *C. ichangensis* were found to be tolerant. Since the isolate used there was considered to be an exocortis source on the basis of biological indexing results and field observations, differences in terms of the viroids present in this isolate versus the complex source used in the present study, may account for the observed discrepancies. Others have suggested that a

TABLE 3
SYMPTOMS OBSERVED IN SIX CITRUS SPECIES AS A RESULT OF VIROID INFECTION

Species	Symptoms
Citrus excelsa	Bark cracking and gum deposits
C. ichangensis	Bark cracking and necrotic lesions
C. karna	Stem yellow blotching, necrotic lesions and gum deposits
C. meyeri	Yellow blotching
C. latifolia	Bark cracking and gum deposits
C. pyriformis	Leaf vein corking, bark cracking and gum exudates

"wood pocket" disorder of *C. latifolia* that is characterized by bark cracking, wood staining, dieback of branches and tree decline is a genetic disorder characteristic of large fruited limes growing under very warm conditions (21). However, the viroid indexing results of affected lime trees growing in Mexico (2) as well as the results of the present study, are in favor of a viroid etiology of the symptoms.

Previous studies have reported that *C. pyriformis* developed weak cachexia symptoms following viroid infection, whereas *C. sunki* and *C. tachibana* did not (5, 12), observations that are compatible with the results reported here. The reports on the sensitivity of *C. depressa* are contradictory since this species was found to be sensitive by Calavan and Christiansen (5) and asymtomatic by Nauer and Roistacher (11). Under our assay conditions *C. depressa* did not develop symptoms.

Since the study reported here was conducted with an artificial mixture of viroids, the results should be considered as an only indication of viroid susceptibility. Single inoculations of each species that developed symptoms will be required to establish the relationship between the specific viroid/s and the appearance of specific symptoms.

Viroid accumulation in infected plants. The results of this study show that the level of replication/accumulation of each viroid was species dependent. The accumulation of CEVd on inoculated plants ranged from very high in *C. bergamia, C. depressa, C. karna, C. limon* and *C. madurensis* (rated 4 in Table 1), to undetectable, even after transfer to citron as an intermediate host (Tables 1 and 2), as in *C. meyeri*, *C. shunkokan*, *C. unshiu*, *A. citroides, F. margarita, F. obovata*, *M. australis. M. austalasica*, *M. waburgiana* and *Pleiospermium* sp. The accumulation of CVd-I also ranged from high (rated 3 in Table 1) to only detectable after transfer to citron (Tables 1 and 2), as in *C. hystrix, C. latifolia* and *M. australasica.* With only a few exceptions (Table 1), CVd-III and CVd-IV reached easily detectable levels in the inoculated plants, whereas the titers of CVd-I and CVd-II were generally lower. *C. karna* was the only species where CVd-I titer was rated 4.

Viroid accumulation patterns also differed within a single species. Whereas *C. karna* appears to be an excellent host for all the viroids, *C. unshiu*, *A. citroides*, *M. australis*, *M. australasica* and *S. buxifolia* contained very low titers. Species such as *C. shunkokan* and *C. latifolia* accumulated high titers of, respectively, CVd-III, or CVd-III and CVd-IV, whereas CEVd levels were low or undetectable (Table 1).

Comparative sPAGE analysis of nucleic acid extracted from bark and leaf blade tissue indicated that some viroids that were readily detectable from bark were undetectable in leaf tissue. As shown in Fig. 1, lane 2, bark and leaf blade tissue from *C. temple* gave identical viroid profiles, while viroids could not be detected in leaves from infected *C. karna* (compare lanes 1 and 2 with lanes 3 and 4). Similar results were obtained with *C. excelsa*, *C. grandis, C. hystrix*, *C. karna*, *C. limon*, *C. sunki*, *C. meyeri*, *C. shunkokan, F. margarita* and *M. australis* × *M. australasica*. These observations suggests that in some species viroid spread from the vascular system to other tissues may be impaired.

Indexing strategies in species other than citron. Our results illustrate that, as previously reported, citrus viroids can be detected by sPAGE and molecular hybridization analysis of extracts from bark tissue of species other than citron (1, 11). Although all the plant species studied were grown in a greenhouse at optimal temperatures for viroid replication/accumulation, the titers of some of the inoculated viroids were so low, that detection was not always possible

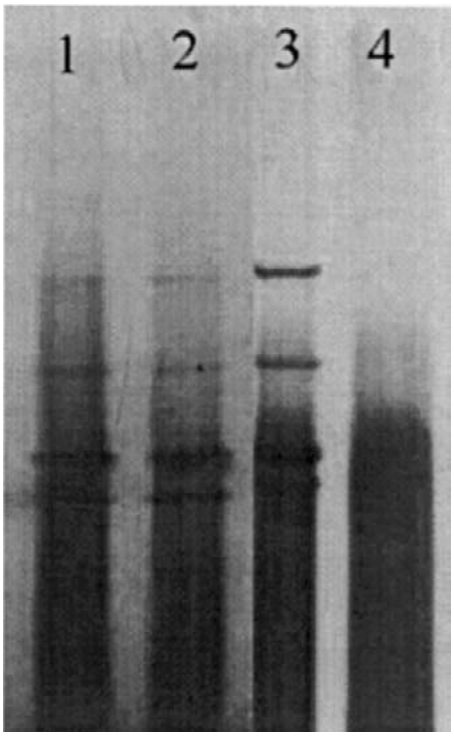

Fig. 1. sPAGE analysis of nucleic acid extracts from *Citrus temple* (1,2) and *C. karna* (3, 4). The nucleic acid extracts were obtained from bark (1, 3) and leaf samples from which the main veins had been removed (2, 4).

(Table 1). Nevertheless, as illustrated in Table 2, they could be satisfactorily detected with the use of citron as an amplification host.

CONCLUSIONS

Citrus viroids have a very wide host range that includes species of *Citrus* and related genera. Viroid titers varied considerably depending the on specific viroid/host combinations, an observation that must be taken into consideration when defining indexing strategies. Six out of the 32 species studied developed symptoms, and the association of the observed symptoms with specific viroids is currently under further study. Ten species appeared to allow only limited viroid spread from vascular tissue to other cells. Two species, *A. citroides* and *M. australis* seem to be viroid resistant. Eight other species may also have some degree of resistance. This should be considered as a preliminary observation that requires further confirmation.

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