Lack of Cross-Protection Between Citrus Exocortis Viroid and Citrus Viroids Associated with Mild Symptoms in Etrog Citron*

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ABSTRACT. Etrog citron plants systemically infected with citrus viroid (CV) isolates, which cause mild symptoms in citron, developed the same typical severe symptoms of citrus exocortis viroid (CEV) infection as noninfected plants when challenge-inoculated by graft or stem-slash inoculation with CEV. There was no difference in incubation period for expression of symptoms of the challenge isolate between plants with a primary infection of a mild viroid and noninfected plants. When extracts from citrons infected with the viroids inducing mild symptoms were subjected to sequential gel electrophoresis (sPAGE) under native and denaturing conditions and silver stained, no CEV band was found, but bands corresponding to citrus viroids CV-IIa and CV-IIIb were present. No hybridization was observed between cDNA probes to CEV and either mild citron viroid. The lack of cross-protection, or interference by mild viroids with infection by the CEV challenge, supports the lack of relationship between CEV and the other mild citrus viroids studied.

Index words. sequential gel electrophoresis, stem-slash inoculation

Etrog citron was used extensively for testing citrus for exocortis infection following its introduction as a diagnostic indicator for citrus exocortis (4). Some variation in symptom expression in citron indicators was noted (17), and some inoculum sources caused only very mild symptoms (8,18). At the time, it was assumed that the causal agent of exocortis was a virus and that the variation in symptom expression indicated presence of isolates or strains of different severities. The viroid nature of exocortis later became evident, but the assumption remained that the mild symptoms caused by some isolates reflected strain differences among a single viroid pathogen.

Several cross-protection experiments were initiated in Florida to test different isolates which produced mild symptoms for ability to protect citrons against several isolates that produced classical severe symptoms. No evidence was obtained for cross-protection in these tests. These results were puzzling since cross-protection had been reported in citrus with citrus tristeza virus (13), and cross-protection had been reported between mild and severe strains of potato spindle tuber viroid (PSTV) (2,14) and between citrus exocortis viroid (CEV), PSTV, and chrysanthemum stunt (CSV) viroids (14) in tomato or chrysanthemum. Infectivity assays and purification attempts by PAGE indicated that the mild isolates were present in lower concentrations than CEV in comparable tissue (1,8). It was also observed that mild symptoms could be obtained by infecting less reactive clones of citron with CEV (8). These observations suggested that failure of cross-protection could be associated with either poor distribution or poor replication of the protecting isolate, and this was investigated in subsequent experiments.

As these protection studies were progressing, evidence accumulated that the symptoms in Etrog citron, which were once all attributed to CEV, were caused by several viroids with distinct differences in molecular weight and other properties (3,6). Examination of the viroid isolates used in the various cross-protection studies by sequential PAGE (sPAGE) procedures revealed that there were several viroid species present, and that the mild isolates test for protection did not contain typical CEV.

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Recent reports have been published on interference or protection between two CEV isolates (7) and between members of the CV-II group (15,22).

This paper reports that cross-protection did not occur between CV-II or CV-III and CEV. The lack of cross-protection observed is a further indication of a lack of relationship between Group II and Group III citrus viroids which cause mild symptoms in Etrog citron and CEV. A preliminary report of these results has been presented (9).

MATERIALS AND METHODS

Host plants. All cross-protection studies were done with clonal propagations of three selections of Etrog citron: Arizona 861, 861-S1, and OES-4. Their responses to CEV infection have been described (8,18). All receptor plants were propagated as rooted cuttings and grown in individual containers in a sterilized potting medium. Plants were maintained in a partially shaded glasshouse cooled with an evaporative cooling system. Temperature conditions during the periods of experimentation followed a normal diurnal fluctuation with night temperatures of 21-24 C and day temperatures of 27 to 34 C. Plants were fertilized as required to promote vigorous growth and sprayed periodically to control pests. Precautions were observed in handling and pruning plants to avoid accidental viroid infection by contamination (10,17). Cutting tools were dipped in diluted sodium hypochlorite or a mixture of formalin and sodium hydroxide (10).

Viroid isolates. Four viroid sources were used extensively in various tests. Isolate E9 is a standard isolate of CEV, which has been used in many studies, and is also entered in the collection of the American Type Culture Collection as PV194. In our assays, and in other independent tests (12), it has been shown to contain only CEV. It produces typical CEV symptoms in Etrog citron. Source E22 was obtained by graft-inoculation from a Temple orange tree (Florida Dept. of Agriculture and Consumer Services, Division of Plant Industry, code Te 20-3-5) to Etrog citron and subsequent passage by slash-cut transmission to other Etrog citrons. It also causes typical, severe CEV symptoms in citron and contains CEV, CV-IIa, and CV-IIIb (23). Source E10 was obtained originally by graft-inoculation to Etrog citron from a Persian lime tree on Rangpur lime rootstock and subsequent passage by slash-cut inoculation to Etrog citron. It produces only mild symptoms in Etrog citron. Extracts from E10-infected plants do not carry CEV, but contain CV-III. Source E11 was obtained by graft-inoculation to citron from a propagation of the Temple orange Te 20-3-5 (see E22), which had undergone heat therapy treatment. It was also transferred by mechanical inoculation to citron. E11 causes mild symptoms in Etrog citron similar to those produced by E10. Extracts from E11-infected plants contain both CV-II and CV-III. The CV-II is apparently CV-IIa and not CV-IIb (21) since the original source tree had been propagated on Orlando tangelo for more than 10 yr without cachexia symptoms (16). Isolates E16b and E25 used in one limited test produce typical CEV symptoms in Etrog citron, but presence of other viroids was not determined.

Inoculation procedures. Graft-inoculation was done by conventional T-bud procedures with a chip of stem tissue as the inoculum source. Mechanical inoculation was done by a stem-slash procedure (10). The cutting blade was contaminated either by dipping it into an extract from infected plants, or by cutting into the stem of a viroid-infected plant, and then immediately making a cut in the stem of the receptor. Ten cuts were made per plant with the blade freshly contaminated prior to each cut. Receptor plants were cut back following inoculation to force new growth which was confined to a single shoot.

Challenge inoculations to previously inoculated plants were not made until definite symptoms of the primary

isolate had been observed, normally 4 to 8 months postinoculation.

Symptom evaluation. The leaf epinasty symptoms typical of CEV infection in citron (Fig. 1b) were rated visually on a 0 to 4 scale where 1 indicated a deflection in the leaf blade from zero to 90 degrees; 2, a deflection of 90 to 180 degrees; 3, a deflection of 180 to 270 degrees; and 4 indicated that the leaf had curled into at least a full circle (360 degrees). A composite score per plant was compiled by totaling the reading for all leaves on a flush and dividing by the number of leaves. Stunting was determined by measuring shoot growth at different intervals after inoculation. Incubation period was determined as the time between challenge inoculation and appearance of symptoms of the challenge isolate. Because of the periodicity in growth flushes in citrus, incubation period data is less precise than in rapidly and continuously growing herbaceous plants. Under our conditions, symptoms of severe isolates of CEV normally appeared at the onset of the second flush of growth following inoculation that was approximately 40 to 50 days. Symptoms can appear within 20 days postinoculation if expressed in the first flush.

PAGE and cDNA hybridization procedures. Bark and leaf midrib tissue from young, symptomatic flushes of Etrog citron were powdered in liquid nitrogen and extracted immediately or lyophilized for extraction at a later date. Extraction, concentration, and electrophoresis were as described previously (5,20) and included phenol extraction, partitioning with 2M lithium chloride, partial purification on CF11 cellulose, and sPAGE.

A cDNA probe to a California isolate of CEV was synthesized from the viroid template by random priming in the presence of ^{32}P. Hybridizations were done as previously described (5).

RESULTS

Cross-protection. In the first cross-protection test attempted, Arizona 861 citron plants systemically infected with E10 and E11 isolates and non-protected controls were challenged with E9. There were nine replications per treatment. In addition, graft and mechanical challenge were tested for each combination. There was essen-

Fig. 1. Arizona 861 Etrog citron plants: A) uninoculated, B) systemically infected with mild citrus viroid isolate E10, C) systemically infected with citrus exocortis viroid isolate E9.

tially no difference in results between the methods of inoculation. The results for the mechanical inoculation, which was the less severe form of challenge, are summarized in Table 1 and also illustrated in Fig. 2. Based on incubation period, leaf epinasty reaction, and stunting, there was no evidence of any modification in symptom severity or delay in symptom expression resulting from the primary infection by E10 and E11. There was, in fact, some slight indication of an additive effect of symptoms of the primary and the challenge isolates.

A similar experiment was done where E11 was used again as the primary isolate, but the challenge isolate was E22 instead of E9, and 861-S1 and OES-4 citrons were used in place of Arizona 861. The E11 and E22 protection-challenge combination was chosen because at the time the test was initiated, it was felt that E11 had arisen as a heat therapy-induced mild variant of E22 and could possibly be more closely related to E22 than to E9, which had a different origin. The OES-4 citron showed a stronger reaction to mild isolates than Arizona 861 (8) and was considered a potentially more favored host for those isolates. Challenge was by graft-inoculation 3 months after

TABLE 1
CITRUS EXOCORTIS VIROID SYMPTOMS, GROWTH RESPONSE, AND INCUBATION PERIOD IN HEALTHY ETROG CITRON AND ETROG CITRON INFECTED WITH TWO MILD CITRON VIROIDS

Inoculations		Shoot growth (cm)[x]	Leaf epinasty[w]	Incubation period (days)[v]
Primary[z]	Challenge[y]			
None	None	70	0.0	–
E10	None	69	0.3	–
E11	None	66	0.7	–
None	E9	24	2.5	51
E10	E9	22	2.6	50
E11	E9	19	2.8	49

[z]Plants graft-inoculated with mild isolates 8 months prior to challenge.
[y]Challenge by stem-slash inoculation.
[x]Growth measured 128 days after challenge. Average for 10 replicates.
[w]0 = no epinasty, 4 = leaf curled full 360°. Average for all leaves on flush after challenge inoculation.
[v]Days after challenge inoculation.

TABLE 2
CEV SYMPTOMS AND GROWTH RESPONSE IN TWO ETROG CITRON SELECTIONS INOCULATED WITH A MILD VIROID ALONE, CEV ALONE, AND A MILD VIROID FOLLOWED BY CEV CHALLENGE

Host	Inoculation		Growth (cm)	Symptoms[y]
	Primary	Challenge[z]		
861-S1	None	None	176	O
861-S1	E11	None	141	M
861-S1	E11	E22	25	S
861-S1	None	E22	46	S
OES-4	None	None	194	O
OES-4	E11	None	164	M
OES-4	E11	E22	93	S
OES-4	None	E22	84	S

[z]Plants challenged by graft-inoculation after symptoms of the primary mild isolate had appeared. Five replicates per treatment.
[y]O = no symptoms, M = mild leaf epinasty, S = severe epinasty.

Fig. 2. Arizona 861 citron plants 124 days after challenge inoculation with citrus exocortis viroid isolate E9. A) uninoculated control with no primary or challenge inoculation, B) challenge inoculation by E9 without primary inoculation by E11, C) inoculated with mild citrus viroid isolate E11 without challenge by E9, and D) primary inoculation with E11 and subsequent challenge-inoculation by E9. E11 infected plants were showing systemic symptoms at the time of challenge by E9. All plants were cut back at the time of challenge inoculation, and growth shown was formed after the challenge inoculation.

symptoms of E11 appeared in the receptor plants receiving a primary inoculation prior to challenge. There were five replications per treatment.

The results are summarized in Table 2. Symptoms were not scored numerically in this test, but all challenge plants clearly showed the severe symptoms of E22 infection along with the severe stunting indicated.

An experiment was also conducted to investigate the effect of challenge inoculum concentration. Healthy and E11-infected citron plants were chal-

lenge-inoculated with two dilutions of three different CEV isolates as shown in Table 3. Again, there was no evidence of protection, even when challenge inoculum titer was below the threshold to give 100% infection in healthy control plants.

Hybridization probes. Hybridization tests with different Florida viroid isolates to a labeled cDNA to a California isolate of CEV showed strong positive reactions to all sources, which caused typical severe CEV symptoms in citron, but no reaction to mild sources, including E10 and E11.

PAGE analysis. Initially, single-dimensional-PAGE (1) of extracts from citrus infected with different Florida isolates revealed presence of an infectious viroid band from extracts of mild and severe sources. Concentrations were consistently higher from extracts infected with severe isolates than from those infected with mild sources, but no other physical discrimination was made at that time.

Further evaluation of extracts of different Florida viroid isolates used in the cross-protection tests described was attempted with the development of sPAGE procedures, coupled with silver staining and refinements in differential viroid species recovery from CF11 by elution with different concentrations of ethanol (19,20). These sPAGE tests were done several times. Different sources of tissue were used for each assay. Extracts from tissue infected with all Florida isolates that cause severe, typical CEV symptoms in citron contained a prominent viroid band that co-migrated with CEV standards. In some cases, other faster migrating viroidlike species were also present. The CEV band was absent in gels run from extracts of tissue infected with mild isolates including E10 and E11. These mild sources contained one or more of the faster migrating species which co-migrated with CV-II or CV-III standards from California. Patterns for E9, E10, E11, and E22 are shown in Fig. 3.

DISCUSSION

The results obtained in the different cross-protection trials failed to demonstrate that the viroids in the mild isolates E10 and E11 conferred any protective effects to Etrog citron plants when these were subsequently challenge-inoculated by CEV. A delay in expression of symptoms of the challenge isolate is commonly used as evidence for protection or interference (7,11,14). The lack of any delay in symptom expression in previously inoculated versus noninoculated plants suggests that infection by the challenge isolate was unimpaired, and that subsequent systemic movement of the challenge isolate was also unrestricted. There was no evidence that varying the host, the primary isolate, or the challenge isolate affected the results. The level of challenge inoculum was

TABLE 3
INFECTION RATES BY THREE CITRUS EXOCORTIS VIROID ISOLATES CHALLENGE-INOCULATED AT TWO DILUTIONS INTO ETROG CITRON PLANTS WITH AND WITHOUT PRIMARY INOCULATION WITH A MILD CITRUS VIROID

		Challenge inoculcum concentration[y]					
		10^{-2}			10^{-3}		
Primary[z]	None	E16B	E22	E25	E16B	E22	E25
None	0/3[x]	3/3	3/3	2/3	2/3	2/3	0/3
E11	0/3	3/3	3/3	3/3	3/3	1/3	1/3

[z]E11 inoculated plants were all showing symptoms at time of challenge.
[y]Inoculum consisted of freshly prepared extract of young bark of Etrog citron systemically infected with the designated isolate.
[x]Number of plants inoculated/number showing typical severe CEV symptoms.

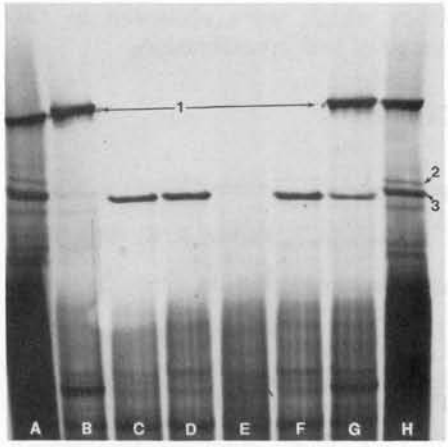

Fig. 3. Polyacrylamide gel electrophoresis of nucleic acid extracts from Etrog citron tissues infected with: B) citrus exocortis viroid isolate E9, C) mild citrus viroid isolate E10, D and F) mild citrus viroid isolate E11, E) noninoculated, and G) CEV isolate E22. Lanes A and H contain standards for CEV (1), Citrus viroid CV-II (2), and citrus viroid CV-III (3). Note absence of CEV band in both E10 (C) and E11 (D,F,) and that CV-III present in E22 (G) is absent in E9 (B).

also not a factor. Plants inoculated by stem-slash with diluted inocula were no less affected than those inoculated by grafting, a much more severe challenge.

We cannot prove that prior to challenge the primary infection of the viroid isolates tested for protection was systemic in all cells of the plants with primary infections. However, in contrast to herbaceous hosts, use of citron hosts allowed an extensive incubation period between primary and challenge inoculations for the primary isolate to become systemic, a factor that favors protection (7,11). Challenge inoculations were made only in sites where symptoms of mild isolate primary infection existed.

The sPAGE and cDNA hybridization data clearly showed that CEV was absent in the protecting isolates tested. The levels of CV-II and CV-III observed in sPAGE gels indicated that the samples were collected and processed under conditions favorable for detection of viroids. Presence of CV-IIIb clearly did not suppress replication or detection of CEV in isolate E22.

Because the sizes of CV-II and CV-III (297-302 and 280-292 nucleotides, respectively) are markedly different from CEV (371 nucleotides) (5,23) and cDNA probes made to CEV do not hybridize appreciably to CV-II or CV-III (19), the logical explanation for the lack of cross-protection observed is that there is no close relationship between CEV and either CV-II or CV-III. The results reported here are a biological confirmation of the molecular evidence already accumulated for this lack of relationship (6,23).

The original assumption that the viroid isolates causing mild symptoms in citron were mild strains of CEV seemed logical at that time, but again it illustrates the hazards of identifying virus or viruslike pathogens solely on symptomatology. This is especially true with viroids where different viroids often induce very similar types of symptoms. The previous references to the lack of cross-protection between CEV isolates are mostly suspect because the assumed relationship between protecting and challenge isolates was based only on symptoms.

Lack of interference cannot be ascribed to a unique property of the citron host because evidence for interference has been demonstrated recently between two biologically distinguishable isolates of CEV (7) and between two members of the Group II viroids (22).

Improved separation and staining techniques have been critical for accurate identification of viroid species and played a key role in this study. For example, early attempts to test relationships between our mild and severe sources with end-labeled viroid from source E22, which had been obtained via a single-phase-PAGE procedure (1), gave misleading results because the multiple viroid species in the probe were not separated. When techniques were developed to separate and purify the different species, the confusion was eliminated.

Our results illustrate the need to characterize by several methods all components used in viroid cross-protection studies; and fortunately, good

collaboration was available to resolve the apparent discrepancies and mysteries which were observed in the course of this investigation.

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