Accumulation of Viroid RNA in Tumor Cells After Double Infection by Agrobacterium tumefaciens and Citrus Exocortis Viroid

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ABSTRACT

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The presence of citrus exocortis viroid (CEV) in tumors induced by *A. tumefaciens* in CEV-infected tomato has been detected by infectivity of pathogenic RNA and visualization of viroid RNA after polyacrylamide gel electrophoresis. These data correlate directly with the increased level of RNase-resistant ^{125}I-CEV following molecular hybridization of nucleic acid preparations from CEV-infected tissue. Primary tumors contained higher levels of the viroid than did apex tissue or fruit. In neoplastic cell cultures CEV can be detected through four subculture passages.

The low-molecular-weight (10^5 daltons) RNAs classed as "viroids" present a unique opportunity to study the process of pathogenesis by a minimal infectious molecule (7). The dramatic expression of biological activity can be compared to a transformed condition with respect to the permanent alteration in normal cell development (12). The major obstacles to the study of viroid synthesis and pathogenesis center on the extremely low concentration of the pathogenic RNA and the asynchrony of intact plant systems (13).

The accumulation of the citrus exocortis viroid in neoplastic plant cell growth following double inoculation with CEV and *Agrobacterium tumefaciens*, introduces a novel system for the investigation of interaction of the viroid with the host cell as coordinated with cell division.

MATERIALS AND METHODS

Culture and bioassay of citrus exocortis viroid.—CEV was rub-inoculated to the first true leaves of tomato *(Lycopersicon esculentum* Mill 'Rutgers') seedlings and by stem puncture with a tRNA-like preparation (10) from infected tissue. Infectivity was determined by bioassay on *Gynura aurantiaca* (13).

Culture and inoculation of Agrobacterium tumefaciens.—Cultures of *Agrobacterium tumefaciens* (ATCC 15955) were maintained at 4 C on 2.3% nutrient agar (Difco) slants containing 0.3% beef extract and 0.5% peptone. Inoculum was prepared by incubating a seeded flask containing 50 ml of 0.8% nutrient broth, 0.5% sucrose, 0.1% yeast extract, and 0.002 M $MgSO_4$ for 18-24 hr at 28-30 C (with rotary shaking). Cells were collected, washed with water, and adjusted to contain about 1-3 × 10^6 cells/ml. Tomato seedlings were inoculated by a single needle puncture of the hypocotyl at a point above the site of CEV inoculation about 3-7 days after inoculation with the viroid.

Tumor culture.—Tumors induced by *A. tumefaciens* were excised from 1- to 2-mo-old infections on tomato hypocotyls. After surface sterilization with 10% hypochlorite and 1 drop of Tween-80 in 100 ml, the tumor tissue was cut into 2- to 3-mm cubes and dipped into sodium hypochlorite solution and 70% ethanol for 1-3 min each before being placed on minimal medium (6) supplemented with neomycin sulfate (50 mg/liter), penicillin G (80 mg/liter), streptomycin sulfate (50 mg/liter), and 50,000 units/liter polymyxin B sulfate. Neoplastic outgrowths were subcultured on nonsupplemented minimal medium. All operations were carried out in a laminar flow hood (Microvoid IIC) and cultures were maintained in the dark at 28-30 C.

Nucleic acid extraction and analysis.—Tomato plant apex tissue, fruit, and primary tumors as well as cultured neoplastic tumors were phenol-extracted under conditions previously reported (10). Detection of viroid RNA was performed either by 5% cylindrical or slab polyacrylamide gel electrophoresis (5, 9). Molecular hybridization with ^{125}I-CEV (kindly provided by Elizabeth Dickson) was performed utilizing conditions of 4× SSC (SSC: 0.15 M NaCl/0.015 M sodium citrate at pH7) and 50% formamide at 42 C as recently reported (3).

RESULTS

Effect of double infection on whole plant system.—Under standard conditions of being inoculated with the citrus exocortis viroid, tomato does not demonstrate the sensitivity reported for *G. aurantiaca* (13). Inoculum which produces a 90-100% infection in *G. aurantiaca* inoculated by stem slashing, induces viroid symptoms on only 30-50% of combined stem-punctured

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and foliar-rubbed tomatoes. This pattern is not altered by subsequent inoculation with *A. tumefaciens*.

Three classes of response can be recorded for tomatoes after inoculation with CEV and subsequent inoculation with *A. tumefaciens*: Class-1 plants with phenotypically normal apical growth (CEV^-) and displaying large tumors, Class-2 plants which develop viroid infection symptoms (CEV^+) relatively late (15-30 days postinoculation), displaying medium-sized tumors, and Class-3 plants which develop viroid infection symptoms (CEV^{++}) relatively early (10-15 days postinoculation) and display small tumors. Figure 1 presents the typical effect of viroid infection on tumor development. The most dramatic effect on tumor development, which is observed in the Class-3 plants, coincides with severe stunting and an almost complete cessation of normal plant growth. In this condition, development of about 80% of the tumors also are markedly reduced. Nevertheless, even in plants which have escaped CEV infection (CEV^-) as judged by symptom development, tumor development also is reduced (Fig. 1-b) when compared to plants not inoculated with CEV (Fig. 1-a).

Distribution of citrus exocortis virus (CEV) in tomato tissues.—The impaired vigor of severely affected CEV^{++} plants certainly must be a contributing factor in tumor size reduction. Nevertheless, a question remains as to the effect of the viroid on tumorigenesis in less seriously affected plants. Fundamental to approaching these questions is an analysis of nucleic acid extracts of infected tissues for the presence of CEV. The parameters of relative infectivity and RNase-resistant ^{125}I-CEV, previously utilized (8) to identify CEV-infected tissues, were applied to apex and tumor tissues (Table 1). Tissues from viroid-inoculated plants bioassayed positive for CEV. This established the presence of the viroid even in tissues of the CEV^-, or normal, phenotype (Class-1). In all cases the relative infectivity of the tumor extracts was greater than that of the corresponding apex tissue. Confirmation of the presence of CEV RNA in extracts of these tissues is presented in polyacrylamide gel patterns (Fig. 2).

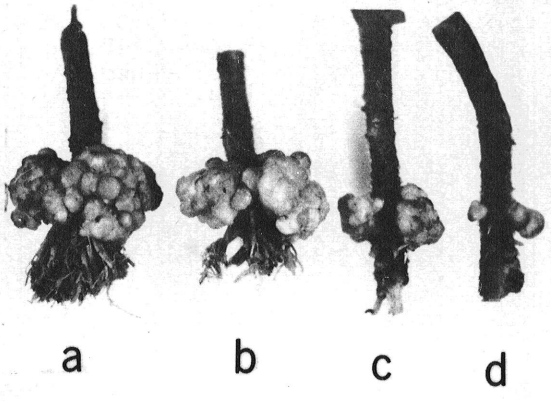

Fig. 1-(a to d). Tumors produced on tomato about 2 mo after inoculation with **a)** *Agrobacterium tumefaciens* only; (b, c, d) after inoculation with *A. tumefaciens* and citrus exocortis viroid (CEV) demonstrating **b)** no CEV symptoms, CEV^-; **c)** late CEV symptoms, CEV^+; or **d)** early CEV symptoms, CEV^{++}, respectively.

Molecular hybridization of ^{125}I-CEV with 2 M LiCl supernatant from healthy *G. aurantiaca* always yields lower levels of RNase-resistant ^{125}I-CEV than do similarly treated extracts from infected *G. aurantiaca*. Nucleic acid extracts from infected apex and tumor tissue from tomato respond similarly when compared to extracts from healthy tissue, even though potato spindle tuber viroid-infected tomatoes have been reported not to demonstrate this effect (4). Since the background RNase-resistant ^{125}I level observed with extracts from tumor tissue exceeds that of apex tissues, it is important to make hybridization correlations within tissue types.

If a comparison is made of the CEV levels in the apex, fruit, or tumors (Table 2), the viroid level is highest in the tumor tissue. This experiment was performed with CEV^+ plants since severely infected (CEV^{++}) plants did not set fruit. The relative concentration of viroid is displayed in Fig. 3 which represents data for samples with equal A_{260} units were analyzed on 5% cylindrical polyacrylamide gels.

Detection of citrus exocortis virus (CEV) in cultured transformed cells.—The significant accumulation of the viroid RNA in tumor tissue of CEV-infected tomato agrees with observations that indicated the association of viroid with mitotically active tissues (Semancik, *unpublished*). Since extracts of *A. tumefaciens* have been reported to contain tumorigenic RNA (15) which may be viroid-like (1), an attempt was made to culture tumorigenic cells in vitro in the absence of the tumor-inducing bacterium.

Several selections which had been passaged through three or four subcultures and maintained on minimal, antibiotic-free media were analyzed. The cultures displayed wide variation in growth patterns ranging from slow-growing, dark-pigmented to rapidly-growing, white friable types. Correlation of the relative CEV-infectivity and RNase-resistant ^{125}I-CEV was not as consistent as

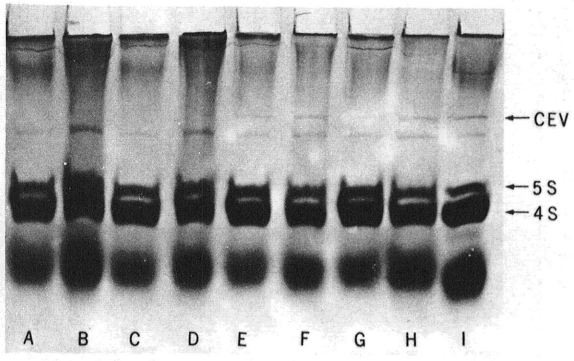

Fig. 2-(A to H). Polyacrylamide slab gel (5%) electrophoresis pattern of 2 M LiCl supernatant preparations from tomatoes about 2 mo postinoculation with *Agrobacterium tumefaciens*, (apex, A, and tumors, B); following inoculation with citrus exocortis viroid (CEV) (C-H); demonstrating no symptoms, (apex, C, and tumors, D); late CEV symptoms, (apex, E, and tumors, F); and early CEV symptoms, (apex, G, and tumors, H). Preparations were equalized for 45 μg DNA and treated with 2.5 μg of DNase prior to electrophoresis. Standard CEV preparation (I) indicating migration of CEV RNA, 5S and 4S components of LiCl supernatant preparations. The gels were stained with toluidine blue.

the role of "viroid-like" molecules in the process of tumorigenesis by *A. tumefaciens* (1, 15) can only be conjectured.

The positive correlation of the ^{125}I-CEV probe with infectivity and detection of the viroid RNA by gel electrophoresis confirm the validity of the hybridization probe (8, 11). Furthermore, a more recent analysis of the technical aspects (3) of the molecular hybridization demonstrates the presence of viroid-complementary RNA species in CEV-infected *G. aurantiaca* as well as tomato. The inability of Hadidi et al. (4) to reproduce these data using the potato spindle tuber viroid might center on the isolation procedure and ultimate quality of the nucleic acid preparations or the conditions of hybridization. In many cases, especially with nucleic acid preparations from old infections, the differential effect between healthy and CEV-infected tomato is more subtle than with *G. aurantiaca*.

The apparent accumulation of viroid RNA in tumorigenic cells is compatible with emerging concepts involved in CEV infection (2, 9). Viroid infection appears to be initiated in discrete receptor or target cells of the meristematic region (12). This implies transitory susceptibility associated with cellular development. Furthermore, viroid synthesis is favored by cell division or occurs in intimate association with mitotic activity (Semancik, *unpublished*). This observation is supported by the demonstrated association of viroid-complementary molecules with nuclear preparations (11) and DNA-rich preparations (8).

These observations lend credence to the analogy of a viroid infection with a form of cellular transformation. The symptom of a viroid infection can be best described as an impairment of a regulatory function which becomes permanently fixed as a result of viroid RNA interacting with the host genome as a controller RNA (14). The fact that CEV replicates in the tumors induced by *A. tumefaciens* from mature stem tissue, which does not readily support viroid synthesis, indicates (i) a homology between the tumor cells and some cells of the meristematic apex, and (ii) the presence of CEV target or receptor cells in the tumor mass.

A significant result of the observation presented here is the application of the tumorigenic cell cultures for studies of viroid synthesis and pathogenesis. As previously indicated (2) the low concentration of the viroid in vivo, the slow rate of synthesis, and the technical problems and asynchrony associated with whole plants or excised tissue fragments have inhibited progress. The tumorigenic cell system offers a valuable tool for investigation of the association of a viroid with cell division as well as the postulated importance of "viroid-like" molecules in the process of regulating cell development.

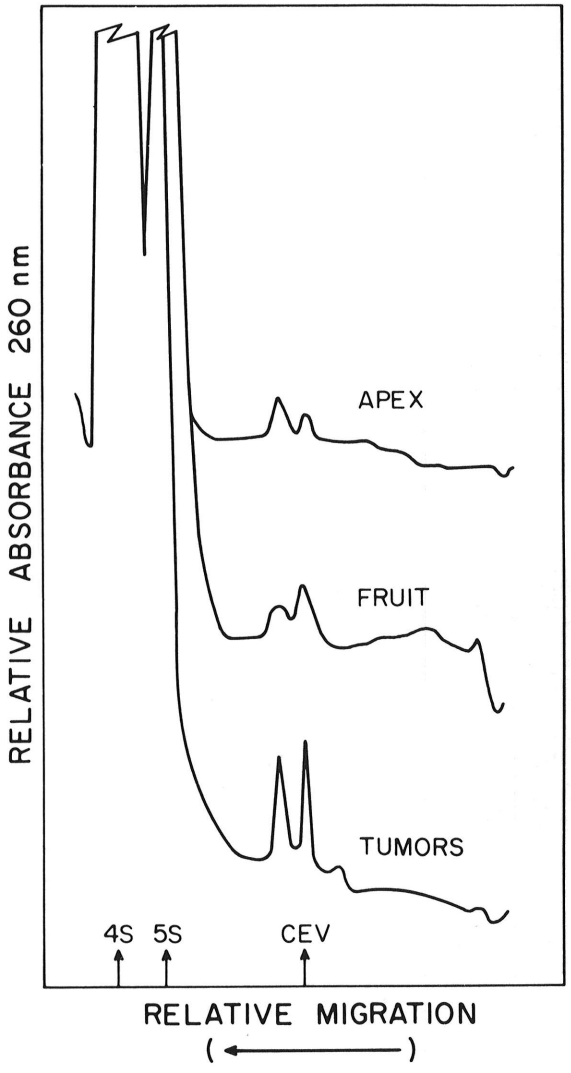

Fig. 3. Absorbance (A_{260}) scanning patterns of cylindrical 5% polyacrylamide gels of 2 M LiCl supernatant preparations from *Agrobacterium tumefaciens* and citrus exocortis viroid (CEV)-infected tomatoes. Samples were equalized for A_{260} and treated with 2.5 μg of DNase prior to electrophoresis.

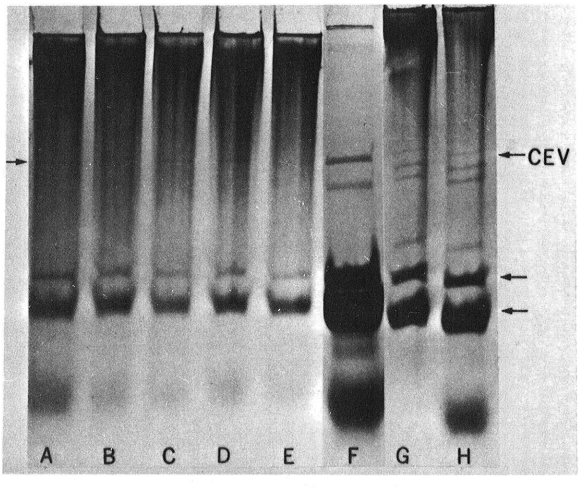

Fig. 4-(A to H). Polyacrylamide slab gel (5%) electrophoresis patterns of 2 M LiCl supernatant preparations from *Agrobacterium tumefaciens* and citrus exocortis viroid (CEV)-infected tomatoes. In vitro cultured neoplastic cells (A-E), fruit (F), and primary tumors (G and H, treated with DNase). The migration positions of CEV, 5S, and 4S RNA are indicated by arrows. The gels were stained with toluidine blue.

those made with apex and primary tumor extracts. Nevertheless, detection of viroid RNA in cultured cells also could be made by direct observation after polyacrylamide gel electrophoresis [Fig. 4-(A to E)]. More interestingly, gel patterns of tumor extracts often contained a multiplicity of RNA species migrating in the same region as CEV-RNA (Fig. 4-G, H). Whether these "viroid-like" RNA species are related to the high level of RNase-resistant ^{125}I-CEV in tumor tissue is presently unknown. Even when high concentrations of viroid are detected, as in the extracts from infected fruit (Fig. 4-F), similar bands are not observed.

DISCUSSION

Double infection of tomato by the citrus exocortis viroid and *A. tumefaciens* does not appear to alter the response of the plant to viroid infection. On the other hand, tumor size is reduced in CEV-inoculated plants in the . presence or absence of discernible symptom expression. Whether this effect on tumor growth simply reflects reduced metabolic activity or a more direct action of the viroid is unknown. This observation may be pertinent to many additional metabolic parameters because of the severely debilitated condition of tomato following viroid infection. We observed that following onset of symptoms at early time postinoculation (CEV^{++}), little additional growth occurs. This is contrary to the effect of CEV on *G. aurantiaca* which although it displays pronounced symptoms sustains growth over extended periods. Nevertheless, CEV-infectivity, analysis of viroid RNA species after gel electrophoresis and RNase-resistant ^{125}I-CEV confirms the presence of the viroid in tumor tissue. Whether these data can be used to support

TABLE 1. Analysis of 2 M LiCl supernatant preparations from tomato apex tumor tissue infected by citrus exocortis viroid (CEV) and *Agrobacterium tumefaciens*[a]

Inoculum	Nucleic acid source[b]	Relative CEV infectivity[c]	RNase-resistant ^{125}I-CEV	
			Cpm[d]	Cpm equalized A_{260}[e]
A) Buffer + *A.t.*[f]	CEV-free apex	0	98	205
	CEV-free tumors	0	188	300
B) CEV + *A. t.*	CEV^- (Class-1) apex	24	98	231
	CEV^- (Class-1) tumors	38	345	350
C) CEV + *A. t.*	CEV^+ (Class-2) apex	52	130	574
	CEV^+ (Class-2) tumors	72	700	993
D) CEV + *A. t.*	CEV^{++} (Class-3) apex	49	245	496
	CEV^{++} (Class-3) tumors	57	851	994

[a]Tissue harvested 6-8 wk postinoculation. The data represent a typical experiment from three replicates.
[b]CEV^- = inoculated plants remaining phenotypically healthy. CEV^+ = late CEV symptom development, 15-30 days postinoculation. CEV^{++} = early CEV symptom development, 10-15 days postinoculation.
[c]Relative infectivity = sum of infected plant days/equal volume of extract.
[d]Cpm/equalized volume of extract.
[e]Nucleic acid concentrations were estimated from an extinction coefficient of 25 A_{260} units/ml·mg^{-1} as 1 mg/ml. With these values, hybridization procedures were done with initial concentration of nucleic acids (moles of nucleotides per liter) × time (sec) equal to 50. Each aliquot of reaction mixture contained 10^4 cpm of ^{125}I-CEV.
[f]The abbreviation *A.t. Agrobacterium tumefaciens* at an inoculum concentration of 1-3 × 10^6 cfu/ml.

TABLE 2. Comparison of 2 M LiCl supernatant preparations from tomato tissues following infection by citrus exocortis viroid (CEV) and *Agrobacterium tumefaciens*[a]

Nucleic acid source[b]	Total A_{260}/g fresh weight	DNA μg/g fresh weight	Relative CEV infectivity	RNase resistant ^{125}I-CEV cpm[c]
CEV-free apex	4.0	95	...	205
CEV-infected apex[d]	2.9	90	23	574
CEV-free fruit	0.4	5.0	...	222
CEV-infected fruit[d]	0.4	6.4	35	620
CEV-free tumors	1.3	24	...	300
CEV-infected tumors[d]	2.2	100	74	993

[a]The data represent a typical experiment from three replicates.
[b]Tissue harvested 6-8 wk postinoculation.
[c]Nucleic acid concentrations were estimated from an extinction coefficient of 25 A_{260} units/ml·mg^{-1} as 1 mg/ml. With these values, hybridization procedures were done with initial concentration of nucleic acids (moles of nucleotides per liter) × time (sec) equal to 50. Each aliquot of reaction mixture contained 10^4 cpm of ^{125}I-CEV.
[d]Late CEV symptom development, 14-21 days postinoculation.

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