Partial Characterization and Serological Analysis of Pseudo-Curly Top Virus

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ABSTRACT

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A polyclonal antiserum was prepared to partially purified virions of pseudo-curly top virus (PCTV), a provisional member of the geminivirus group. The PCTV virions were detected by double antibody sandwich enzyme-linked immunosorbent assay in *Lactuca sativa, Solanum melongena, S. nigrum*, and *Lycopersicon esculentum*, as well as in the treehopper vector *Micrutalis malleifera*. Indirect ELISA tests showed a relationship between PCTV and beet curly top virus. Nucleic acid extracted from partially purified PCTV virions was digested by DNase I but not by RNase A.

A tomato disease resembling beet curly top was reported from the eastern (14) and western (6) coasts of Florida in the early 1950s. Identification of the causal agent as beet curly top virus (BCTV) was dismissed when the vector was determined to be a treehopper, *Micrutalis malleifera* Fowler (13), whereas BCTV is transmitted only by a specific leafhopper, *Circulifer tenellus* (Baker) (1). The disease was renamed pseudo-curly top and the viral agent was presumed to be pseudo-curly top virus (PCTV) (13). A nuclear inclusion showing geminiviruslike particles and cytopathic effects similar to those induced by geminiviruses was observed by light and electron microscopy (3,5,7,15) in PCTV-infected tobacco tissue, making PCTV a provisional member of the geminivirus group (9). Although pseudo-curly top was suspected to be endemic in much of southern Florida (13), no reliable means of assay was developed to differentiate PCTV from other geminiviruses or to study the economic impact and geographic distribution of this disease. Tomato plants presumed to be infected with PCTV have been noted regularly each year in lower coastal areas in Florida (J. H. Tsai, *unpublished*). An incidence of pseudo-curly top greater than 50% has been reported on tomato (18). This paper presents data on partial purification of geminiviruslike particles from PCTV-infected plants, the development of a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for detection of PCTV in insect and plant tissues, and the serological relationship between PCTV and BCTV as determined by indirect ELISA.

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MATERIALS AND METHODS

Virus and vector maintenance. Colonies of *M. malleifera* were reared on eggplant (*Solanum melongena* L. 'Florida Market 10') and maintained in an insectary provided with 12 hr of 6,000-lx light per day at 25 ± 1 C. Reared insects were periodically checked to ensure that they were pathogen-free. All acquisition and inoculation feedings were performed in an air-conditioned room at approximately 25 C. Seeds of nightshade (*S. nigrum* L.), eggplant, tomato (*Lycopersicon esculentum* Mill. 'Walter PF'), lettuce (*Lactuca sativa* L.), sugar beet (*Beta vulgaris* L.), and groundcherry (*Physalis floridana* Rydb.) were germinated under mist, and uniform seedlings were transplanted into 16-cm pots containing a 1:1:1 sand:loam:peat mixture. Seedlings in the three- to four-leaf stage were used as test plants.

Four isolates of PCTV originally collected from naturally infected nightshade plants in Palm Beach and Broward counties, Florida, were compared by *M. malleifera* transmission trials in 1985. One isolate from Palm Beach County that was found to be transmitted more efficiently than the others was used in all subsequent tests. PCTV was maintained in nightshade plants using viruliferous *M. malleifera* 1–2 days after acquisition access feeding on PCTV-infected nightshade source plants. After an inoculation access period of 2–3 days on nightshade plants, treehoppers were killed with malathion spray and the plants were kept in a glasshouse for observation of symptom development.

Virion purification. Leaves and stems of PCTV-infected nightshade plants were harvested 2–4 wk after inoculation and either processed for purification immediately or stored at −20 C. The extraction and resuspension buffers were essentially the same as those described by Brown and Nelson (2); sodium sulfite was replaced by 0.05% 2-mercaptoethanol as the antioxidant. Reagents and equipment in contact with the virus suspension were precooled. Healthy tissues were similarly processed.

Tissue (70–100 g) was chopped into small pieces and disrupted in an electric blender with 3–4 vol of extraction buffer. This brei was stirred at 4 C for 30 min, then expressed through four layers of cheesecloth to remove fibrous matter. The extract was emulsified with 1/10 vol of chloroform, followed by separation of the two phases by centrifugation in a Beckman JA-14 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 15 min at 15,000 *g* (av.). The aqueous phase was removed, poured through polyester fiber, and centrifuged in a Beckman SW 28 rotor for 3 hr at 100,000 *g* (av.) at 5 C. Pellets were combined into a single tube and resuspended in 5–6 ml of resuspension buffer (0.1 M Tris-HCl [pH 7.2], 0.05% Triton X-100) overnight at 4 C. The suspension was centrifuged in a Beckman JA-20 rotor for 10 min at 12,000 *g* (av.), and the supernatant liquid was layered onto each of two 10–40% linear sucrose density gradients in 0.1 M Tris buffer, pH 7.2, previously prepared in Beckman SW 28 rotor tubes. Gradients were centrifuged for 3 hr at 100,000 *g* (av.) at 10 C, and the fraction containing the single, opalescent band located near the middle of the tube was removed by means of a large-bore needle and syringe. Sucrose was removed by diluting the sample at least 1:5 with 0.1 M Tris buffer, pH 7.2, and centrifuging in the SW 28 rotor under the same conditions as for the sucrose density gradients. The pellet was resuspended in 0.1–0.5 ml of the 0.1 M Tris buffer. Purity of the PCTV sample was assessed by means of transmission electron microscopy (TEM) and ultraviolet spectroscopy. Samples examined by TEM were stained with 0.5% aqueous uranyl acetate. A sample from healthy nightshade was similarly prepared.

To assay for the infectivity of partially purified PCTV virions, treehoppers were injected with a suspension of viruslike particles from the first high-speed centrifugation step. The pellet was resuspended in a sterile phosphate buffered saline (PBS) solution containing sucrose (0.1 M sodium phosphate [pH 7.4], 0.15 M sodium chloride, and 5% sucrose). *M. malleifera* adults were immobilized, held under stretched Parafilm, and injected in the abdomen with a capillary glass needle inserted through the Parafilm. Two injected treehoppers per nightshade seedling were allowed an inoculation access period of 7 days, and virion infectivity was assessed on the basis of symptom development, Ouchterlony double diffusion serology tests, and TEM assays. Thirty *M. malleifera* adults were injected with a sterile PBS sucrose solution to serve as controls.

Nucleic acid. The PCTV virions were partially purified from 70.0 g of infected nightshade tissue using three cycles of differential centrifugation. The final pellet was suspended in 500 µl of a Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1% sodium dodecyl sulfate and heated at 58 C for 45 min. Total nucleic acid was extracted with a phenol, phenol/chloroform series and precipitated with ethanol as previously described (10). The nucleic acid was pelleted, resuspended in 80 µl of Tris-EDTA buffer, and stored at −20 C. Samples (10 µl) were mixed with Tris-EDTA buffer containing Mg^{++} and either 10 mg/ml of DNase-free RNase A (Sigma Chemical Co., St. Louis, MO) or bovine pancreatic DNase I (0.2 units/ml). These solutions and controls (19 µl total volume per tube) were placed in a 37 C water bath for 3.5 hr. Samples were analyzed for type of nucleic acid by electrophoresis in a horizontal 1% agarose gel (10 × 6.5 cm; type II agarose, Sigma) using a Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 0.6 vol/cm^2 for 2 hr at room temperature. A *Hin*dIII digest of lambda bacteriophage DNA (Sigma) was used for molecular size standards (23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125 base pairs). Gels were stained with ethidium bromide (0.5 mg/ml) for 30 min, destained for 10 min, and visualized by ultraviolet irradiation (302 nm).

Serology. Partially purified PCTV virions (absorbance ratio 260/280 nm = 1.87; uncorrected for light scatter) suspended in PBS were emulsified with Freund's incomplete adjuvant and injected intramuscularly into both hips of a New Zealand white rabbit. This initial immunization emulsion contained 132 ng of PCTV, which was calculated using the extinction coefficient of bean golden mosaic virus (BGMV) (8). Freund's complete adjuvant was used in three subsequent intramuscular immunizations to deliver 560 ng of PCTV every second week. Blood was collected and processed 1 wk after the final injection. The homologous titer of this serum in intragel, cross-absorbed Ouchterlony assays was 1:16; no precipitin bands were visible between wells containing PCTV antiserum and sap from healthy plants.

DAS-ELISA was used in polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) and conducted as previously described (4). IgG was obtained by 6,9-diamino-2-ethoxyacridine lactate (Rivanol, Sigma) precipitation (16). Alkaline phosphatase (type VII-T, Sigma) was conjugated to IgG as previously described (17). The coating antibody was used at 10 µg/ml and the coating buffer and conjugated antibody were used at a 1:500 dilution in PBS buffer containing 0.05% Tween 20, 1% polyvinylpyrrolidone (PVP 10), and 0.2% bovine serum albumin (fraction V). The substrate was *p*-nitrophenyl phosphate (1.0 mg/ml of diethanolamine, pH 9.8). Plates were incubated for 60 min at room temperature, then 30 µl of 3 N NaOH per well was added to stop the reaction. Absorbance of well contents was recorded at 410 nm with a Minireader II (Dynatech). Antigen samples were prepared by grinding a plant tissue sample in five volumes (w/v) of PBS-Tween buffer with a mortar and pestle and removing fibrous material with a cheesecloth pad. Treehoppers (one or two insects per set) were each ground in 0.50 ml of PBS-Tween buffer using a glass tissue homogenizer. The plates with reagents or samples were incubated at 37 C—coating antibody for 3 hr, antigens for 3 hr, conjugate for 2 hr, and substrate for 1 hr.

Antigens for the indirect ELISA were prepared by grinding tissues of nightshade (1:1,000, w/v) and freeze-dried

sugar beet (1:80, w/v) in coating buffer containing 1% PVP and 1% powdered nonfat milk. Samples were incubated overnight in microtiter plates at 4 C. Primary antibody was prepared (1:1,000 v/v) in PBS-T containing PVP and powdered nonfat milk and incubated in wells for 2 hr at 37 C. Labeled (alkaline phosphatase) goat antirabbit antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN) were similarly diluted and incubated. Absorbance of well contents was obtained at 405 nm using a Titertek Multiskan reader (Flow Laboratories, Inc., McLean, VA). The average buffer control absorbance value was subtracted from the other values before completion of the statistics.

RESULTS

Symptomatology. Initial symptoms on nightshade (Fig. 1A) included enations and veinclearing followed by some upward, but mostly downward, leaf curling. Infected plants were generally stunted and bushy. Symptoms on plants at the three- to four-leaf stage appeared about 5 days after inoculation. Symptoms on infected tomato plants (Fig. 1B) included chlorosis of the leaf edge and upward leaf curling, as well as apical shoot proliferation. These plants became brittle as they matured. Young tomato plants inoculated with PCTV rarely set fruit, and such fruit was greatly reduced in size. The principal symptom on infected lettuce (Fig. 1C) and eggplant was leaf puckering, although lettuce later showed veinbanding and chlorosis. Such symptoms were not observed on inoculated groundcherry, and veinclearing was noted only occasionally. Leaves of inoculated sugar beet (Fig. 1D) were curled and leathery, and new leaves sometimes were spindle-shaped (Fig. 1D).

Virion purification. The purification technique provided PCTV relatively free from large contaminants, as judged by TEM examination. Numbers of intact, unaggregated, or otherwise undegraded particles were insufficient for accurate measurement of particle dimensions.

The PCTV recovered from the first high-speed centrifugation step and injected into treehoppers was infective. Symptoms of psuedo-curly top developed on 20 of 39 healthy nightshade plants on which PCTV-injected insects were placed. No symptoms developed on 15 nightshade plants on which PBS buffer-injected insects were allowed to feed. Sap extracted from these symptomatic nightshade plants was examined by TEM and found to contain viruslike particles, whereas sap of healthy plants did not. The sap from PCTV-affected plants also tested positive for PCTV in Ouchterlony assays using PCTV antiserum, whereas healthy controls were negative.

Nucleic acid. The PCTV nucleic acid was digested by DNase I but not by RNase A (Fig. 2). No corresponding nucleic acid was observed in the preparation from healthy tissue. The undigested PCTV nucleic acid migrated the same distance as the 2.027-kbp bacteriophage DNA digest fragment in the nondenaturing gel.

Serology. DAS-ELISA (Table 1) detected PCTV in inoculated eggplant, lettuce, nightshade, and tomato but not in sugar beet or groundcherry. All inoculated plants tested expressed symptoms not observed in control plants. Samples from inoculated plants were assessed as PCTV-positive if the absorbance values were greater than twice the mean of the healthy controls. Viruslike particles in sap were observed by TEM from all inoculated plants except groundcherry and sugar beet. Attempts by healthy *M. malleifera* to reacquire PCTV from symptomatic lettuce, eggplant, and sugar beet were unsuccessful. DAS-ELISA detected PCTV in single treehoppers that were allowed a 48-hr acquisition access period on infected nightshade plants (Table 2). The indirect ELISA data demonstrated a reciprocal serological relationship between PCTV and BCTV (Table 3).

DISCUSSION

Although PCTV virions in viruliferous treehoppers were easily detected with low background absorbance (Table 2), the

Fig. 1. Symptoms on plants inoculated with pseudo-curly top virus (PCTV): **(A)** Nightshade with leaf curling and enations, **(B)** tomato with leaf curling and apical shoot proliferation, **(C)** lettuce with leaf puckering and veinbanding, and **(D)** sugar beet with leaf curling and leathery texture (sugar beet plants were assessed as PCTV-negative by ELISA and TEM assays).

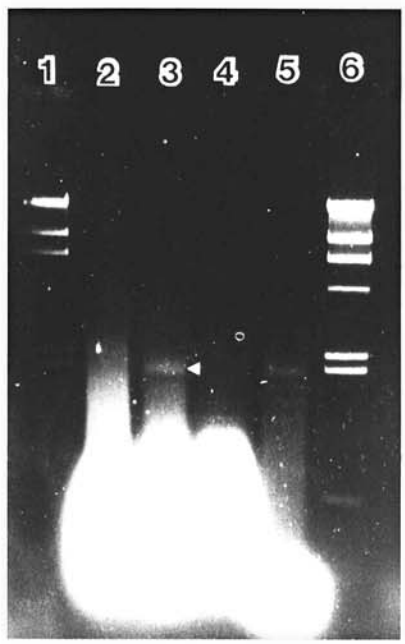

Fig. 2. Nucleic acid extracted from partially purified pseudo-curly top virus (PCTV) virions, incubated with DNase I or RNase A at 37 C for 3.5 hr, and electrophoresed in a 1% agarose gel at 0.6 vol/cm^2 for 2 hr at room temperature. Lanes 1 and 6 contain a *Hin*dIII digest of lambda bacteriophage DNA for a molecular size standard (23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125 base pairs). Lane 2 contains total nucleic acids extracted from healthy nightshade tissue, lane 3 contains nucleic acids from the partially purified PCTV virion preparation, lane 4 contains PCTV virion nucleic acid preparation incubated with DNase I, and lane 5 contains PCTV virion nucleic acid preparation incubated with RNase A. The PCTV virion nucleic acid (arrow, lane 3) was digested by incubation with DNase but not by incubation with RNase. Brightly stained nucleic acid (primarily RNA) at the ends of lanes 2–5 is of plant origin (total nucleic acid preparations made from partially purified virions).

Table 1. Occurrence of pseudo-curly top virus (PCTV), detected by double antibody sandwich enzyme-linked immunosorbent assay, in extracts from six plant hosts inoculated by viruliferous treehoppers[a]

	Absorbance (410 nm)											
	Eggplant		Lettuce		Nightshade		Tomato		Sugar beet		Groundcherry	
Statistic	H[b]	I	H	I	H	I	H	I	H	I	H	I
Mean	0.126	0.691	0.115	0.338	0.321	0.825	0.118	0.429	0.193	0.157	0.305	0.362
SD	0.063	0.261	0.109	0.228	0.232	0.502	0.087	0.168	0.078	0.076	0.073	0.104
No. of samples	9	9	8	8	16	17	9	11	10	14	12	12
No. of PCTV positives	...	9	...	4	...	5	...	10	...	0	...	0

[a] Mean values were determined from the average absorbance of duplicate wells for individual samples; experiments were repeated at least three times. Sample absorbances of inoculated plant extracts greater than twice the mean of the healthy control extract readings were assessed as positive for PCTV.
[b] H = extracts from healthy plants, I = extracts from inoculated (symptomatic) plants.

data in Table 1 show that the serum contained antibodies to healthy plant tissue components. Cross-adsorption of the antiserum twice with a healthy plant tissue preparation was unsuccessful in reducing the background absorbance in DAS-ELISA (L. L. McDaniel, *unpublished*). To reduce this background in subsequent indirect ELISA tests, we prepared 1:1,000 (w/v) antigen dilutions from PCTV-infected nightshade tissues. Ouchterlony agar gel diffusion tests were performed successfully using intragel cross-absorption (16). For higher quality antiserum, refinement of the PCTV purification technique will be necessary to obtain a more suitably purified antigen sample from either infected plant tissue or viruliferous treehoppers.

Although PCTV virions were detected by DAS-ELISA in symptomatic lettuce and eggplant tissues, attempts to reacquire PCTV using *M. malleifera* were unsuccessful. Since the treehoppers fed well on both plants, especially eggplant, they may have had difficulty in acquiring adequate amounts of infectious virions and transferring them to the assay plants.

No PCTV virions were detected in symptomatic groundcherry and sugar beet plants by TEM and DAS-ELISA. Assays for treehopper acquisition of PCTV virions from sugar beet were also negative. In vector transmission studies (12), groundcherry was not found to be susceptible to PCTV infection, based on symptomatology. Likewise, our experience shows that the veinclearing symptoms induced by inoculation of groundcherry with PCTV are seldom apparent. Since symptoms did not develop on control plants on which nonviruliferous treehoppers fed, PCTV virion concentration in groundcherry and sugar beet may be very low. However, DAS-ELISA sample well absorbances of both healthy and symptomatic groundcherry and sugar beet plants were similar, so further testing must be conducted after development of a more sensitive detection reagent.

A close serological relationship between PCTV and BCTV was suggested by the indirect ELISA data. If confirmed by additional studies, this observation will be significant, because serological relationships between leafhopper-transmitted geminiviruses are very distant, with no relationship established between any whitefly-transmitted and leafhopper-transmitted geminiviruses (11).

Common plants such as ragweed (*Ambrosia* sp.) and common chickweed (*Stellaria media* (L.) Cyr.) are reported to be susceptible to PCTV (12) and may contribute to disease spread. However, because of susceptibility, widespread occurrence, and host plant status for *M. malleifera*, nightshade plants would be an effective reservoir of PCTV, enabling the treehopper to acquire this persistent virus (J. H. Tsai, *unpublished*) and allowing virus to spread throughout much of southern Florida.

Presence of DNA in the geminiviruslike particles and the serological relationship to BCTV further support PCTV placement as a member of the geminivirus group. However, since the virion particles appear to be labile and are difficult to purify, further basic work must be completed in order to better characterize PCTV for definitive placement as a member of this virus group.

Table 2. Detection of pseudo-curly top virus (PCTV) in the treehopper vector *Micrutalis malleifera* by means of double antibody sandwich enzyme-linked immunosorbent assay[a]

	Absorbance (410 nm)			
	One insect		Two insects	
Statistic	H[b]	I	H	I
Mean	0.057	0.442	0.020	0.773
SD	0.140	0.430	0.030	0.782
No. of samples	10	10	10	10
No. of PCTV positives	...	7	...	7

[a] Mean absorbance of all samples, each of which was the mean of duplicate wells; experiments were performed at least twice. Absorbance values for viruliferous insect extracts were assessed as positive for PCTV if exceeding twice the mean of the healthy control absorbance values.
[b] H = healthy or nonviruliferous insects, I = viruliferous insect(s), acquisition access time of 48 hr.

Table 3. Serological relationship between beet curly top virus (BCTV) and pseudo-curly top virus (PCTV) as determined by indirect enzyme-linked immunosorbent assay[a]

	Absorbance (405 nm)							
	BCTV antiserum				PCTV antiserum			
	BCTV		PCTV		BCTV		PCTV	
Statistic	H[b]	I	H	I	H	I	H	I
Mean	0.012	0.434	0.095	0.312	0.000	0.757	0.063	0.732
SD	0.004	0.060	0.008	0.035	0.000	0.022	0.013	0.087
No. of samples	6	6	5	6	6	5	5	5
No. of positives	...	6	...	6	...	5	...	5

[a] Mean values were determined from the average absorbance of duplicate wells for individual samples. Sample absorbances of inoculated plant extracts greater than twice the mean of the healthy control extract readings were assessed as positive.
[b] H = extracts from healthy plants, I = extracts from inoculated (symptomatic) plants (BCTV in sugar beet and PCTV in nightshade).

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