Partial Characterization of a Distinct Potyvirus Isolated from Watermelon in Florida

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ABSTRACT

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Conspicuous, unusual nuclear inclusions in stained epidermal strips of leaves implicated a virus (designated isolate 2932) as the cause of foliar mosaic in a watermelon plant (*Citrullus lanatus*) received for analysis from South Florida in 1990. In greenhouse tests, mechanically inoculated plants of *Cucurbita pepo* (Small Sugar pumpkin and Early Prolific Straightneck squash) and watermelon (Crimson Sweet) developed mosaic or mottle symptoms. Isolate 2932 caused foliar symptoms in 16 cultivars of *Cucurbita pepo*, including Freedom II and Prelude II, and in six cultivars of watermelon. None of five cultivars of melon (*Cucumis melo*) or 11 cultivars of cucumber (*Cucumis sativus*) developed consistent, distinctive symptoms, but all of these cultivars were systemically infected based on back-inoculations to squash. No systemic infection of mechanically inoculated plants of 25 species representing 13 noncucurbitaceous plant families was detected. Crystalline nuclear inclusions, cytoplasmic amorphous inclusions, and cytoplasmic cylindrical inclusions were detected by light and electron microscopy in leaf tissues of infected squash and watermelon. Electron microscopy of squash leaf extracts revealed filamentous particles, and 86% of 159 particles measured ranged from 800 to 890 nm in length. The virus was transmitted in a nonpersistent manner by *Myzus persicae* from squash to squash in two of three trials. Immunodiffusion tests with polyclonal antisera prepared to partially purified 2932 or its capsid protein showed that the isolate was antigenically different from papaya ringspot virus type W, watermelon mosaic virus 2, and zucchini yellow mosaic virus. In limited testing of field samples of squash and watermelon since 1990, no additional isolates of the 2932 type have been found. The characteristics of isolate 2932 obtained thus far indicate that it is a distinct potyvirus. It is tentatively named watermelon leaf mottle virus to distinguish it from other potyviruses commonly isolated from cucurbits in Florida.

Cucurbit crops in the United States are adversely affected by numerous viruses (14,16,20,27). Three potyviruses, papaya ringspot virus type W (PRSV-W, formerly watermelon mosaic virus-1) (18), watermelon mosaic virus-2 (WMV-2), and zucchini yellow mosaic virus (ZYMV), commonly infect and can cause losses in squash, watermelon, cantaloupe, pumpkin, and cucumber (14,16,27). The viruses differ in their host ranges and the reactions they induce in certain hosts, and they can be distinguished readily by serological means. Both PRSV-W and WMV-2 have caused problems in cucurbits in Florida for many years (1). In 1981, ZYMV also was discovered in the state (1,17). A survey of cucurbit crops, mainly squash and watermelon, indicated that all three vi-

ruses were prevalent in Florida in 1987 and 1988 (20).

In the spring of 1990, several watermelon samples with viruslike symptoms were submitted to the Florida Extension Plant Disease Clinic (FEPDC) in Gainesville for analysis. Unusual and very prominent cellular inclusion bodies were detected in stained epidermal strips of one of the samples. Serological tests gave no indication that the sample was infected with any of six other mosaic viruses of cucurbits known to occur in Florida (20). A virus isolate (2932) in this sample was transferred by mechanical inoculation to pumpkin and squash for further study. This report deals with the partial biological, serological, and cytological characterization of isolate 2932, and presents evidence that the virus is distinct from the three other potyviruses that commonly occur in cucurbit crops in Florida. A preliminary report was published previously (19).

MATERIALS AND METHODS

Source of the virus. A watermelon plant with foliar mosaic symptoms was submitted to the FEPDC at the University of Florida. The sample, designated isolate 2932, was a field collection from Hendry

County, Florida, submitted by the Agricultural Extension Service and Glades Crop Care, Inc. The watermelon leaves were used as inoculum for mechanical transmission to pumpkin (*Cucurbita pepo* 'Small Sugar'), which developed a mild mosaic. The virus was then maintained by serial mechanical inoculation to squash (*Cucurbita pepo* 'Early Prolific Straightneck'), which also served as an assay host.

Inoculation and assay of test plants.

Usually, at least five young, actively growing test plants of each type were used for the host range trials. Cucurbit test plants were inoculated by rubbing expanded cotyledons. Inocula consisted of extracts from squash infected with isolate 2932, triturated in 20 mM potassium phosphate, pH 7.5, to which Carborundum was added. Test plants were observed for symptoms, and about 3 weeks after inoculation (range 19 to 40 days), leaves above the point of inoculation were collected and assayed by mechanical inoculation on 6 to 12 squash plants. The squash plants were observed for symptoms over a period of 2 weeks and then assayed by sodium dodecyl sulfate (SDS)-immunodiffusion for isolate 2932.

Aphid transmission trials. Individuals of *Myzus persicae* were allowed 10 to 15 s acquisition access probes on an infected squash leaf. The aphids were transferred to squash plants, allowed to remain overnight, and were removed or sprayed the next day with insecticidal soap. The squash leaf used as a virus source for aphids was macerated in buffer and used as inoculum for mechanical transmission to verify infectivity.

Purification of virus, preparation of antisera, and serological tests.

Isolate 2932 was purified from infected Small Sugar pumpkin leaves harvested 14 to 19 days after inoculation. One hundred g of tissue was homogenized in 270 ml of buffer consisting of 0.3 M potassium phosphate, pH 8.5, 0.2% sodium diethyldithiocarbamate, and 0.1% mercaptoethanol for 10 s in a Waring blender. One hundred eighty-five ml of Freon 113 (trichlorotrifluoroethane) was added as a denaturant, and the mixture was homogenized for 70 s. The mixture was centrifuged at 4,000 × g for 10 min. Triton X-100 was added to the supernatant to a final concentration of 1%. The mixture was stirred for 20 min at 4°C, then centrifuged in a Beckman Ti 70 rotor at 100,000 × g for 90 min at 4°C. The pel-

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let was resuspended in 8 ml of buffer (0.02 M HEPES, pH 8.2) with stirring for 1 h at 4°C. The resuspended material was centrifuged at $1,500 \times g$ for 2 min. The supernatant was centrifuged at $140,000 \times g$ in a Beckman SW 41 rotor for 16 h at 4°C after layering 2 ml of supernatant per tube, each containing 5 ml of Cs_2SO_4 solution (10 g of salt plus 27 ml of 20 mM HEPES, pH 8.2). The virions were concentrated in a visible band at a position about 23 to 25 mm from the bottom of the tube, and were collected by droplet fractionation. The collected fractions containing virions were combined and diluted with 1 vol buffer and centrifuged at $12,000 \times g$ for 10 min. The virions were precipitated from the supernatant by adding 20% polyethylene glycol (MW 6,000 to 8,000) to a final concentration of 4%, incubating for 20 min at 4°C, and then centrifuging at $12,000 \times g$ for 10 min. The pellet was resuspended in 1 to 2 ml of HEPES buffer. The virions were stored at -80°C . The ultraviolet spectrum indicated a high 260/280 value of 1.4, possibly due to contaminating host DNA carried with the virions during purification, as has been noted for other potyvirus purifications (7). Analytical polyacrylamide gel electrophoresis of purified virus was conducted using a modification (3) of Laemmli's procedures (10).

Two polyclonal antisera were prepared in rabbits for use in studying virus relationships and for assays. Both rabbits were immunized intramuscularly with antigen emulsified with Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for two subsequent injections. For one antiserum (1170), untreated, purified virus was used as immunogen. For the other antiserum (1163), protein was recovered from sucrose density gradients following dissociation of virus (8) and used as immunogen. Immunodiffusion tests were conducted in medium containing 0.8% Noble agar, 0.5% SDS, and 1.0% sodium azide (17,20). Test antigens were prepared by triturating leaf tissues in SDS (1.0 ml of distilled water and 1.0 ml of 3% SDS per g of tissue). In most cases, the extracts were heated in a boiling water bath for 4 min and then used at room temperature, or stored frozen for later use.

Assays of field samples. Squash and watermelon samples with possible virus-like symptoms collected in commercial and experimental plantings were typically assayed by SDS-immunodiffusion tests against antisera to isolate 2932 and against antisera to six other viruses that affect cucurbits in Florida (20). Selected samples also were assayed by mechanical inoculation to squash.

Light and electron microscopy. Stained epidermal strips were examined by light microscopy to facilitate detection of inclusions. The strips were removed from the underside of symptomatic leaves and stained with the combination of Cal-

comine Orange and Luxol Brilliant Green dyes (4).

Symptomatic leaf tissues, in which inclusions had been detected by light microscopy, were prepared for transmission electron microscopy according to standard preparative techniques (fixation in glutaraldehyde, postfixation in osmium tetroxide, and embedding in Epon-araldite epoxy resin). Thin sections were stained with uranyl acetate and lead citrate.

Filamentous particles in extracts from infected leaves chopped in phosphate buffer were stained with 2% uranyl acetate. Particles in electron micrographs were measured by comparison with micrographs of a diffraction grating.

RESULTS

Host range. Isolate 2932 systemically infected 10 of the 11 species of Cucurbitaceae that were inoculated mechanically. No systemic infection of mechanically inoculated plants of 25 other species representing 13 noncucurbitaceous plant families was detected.

The Early Prolific Straightneck cultivar of squash showed veinal chlorosis of systemically infected true leaves within 4 to 8 days after inoculation with isolate 2932, followed by appearance of mosaic symptoms. When inoculated on expanded cotyledons just as the first true leaf was beginning to appear, transfer from squash to squash (Early Prolific Straightneck) usually resulted in 100% infection. All other cultivars of *Cucurbita pepo* tested also were readily infected and showed foliar symptoms. Yellow squash types tended to show mosaic and chlorotic mottle symptoms, whereas the zucchini types showed veinal chlorosis and chlorotic flecks or spots. The following cultivars of *Cucurbita pepo* also were infected: Black Beauty, Burpee Hybrid Zucchini, Bush Table Queen, Destiny III, Dixie, Dividend, Fordhook Zucchini, Freedom II, Lemon Drop, Liberator III, Jaguar, Multipik, Prelude II, Revenue, Small Sugar pumpkin, and Tigris. In the case of *Cucurbita maxima*, Pink Banana developed chlorotic spots in one of two tests, and veinal chlorosis, chlorotic mottling, and spotting were seen in leaves of Big Max. A cultivar of *Cucurbita moschata*, Waltham Butternut, showed chlorotic mottling, and Butterbush showed mottling and veinal chlorosis. No distinctive symptoms were observed in *Cucurbita okeechobeensis* (PI 561112), but the bioassays indicated this species also was systemically infected. *Cucurbita ecuadorensis* (Grif 9446) showed no symptoms, and the virus was not recovered by back-inoculation tests.

Cultivars of watermelon (*Citrullus lanatus*) tested developed systemic symptoms of veinal chlorosis and mosaic or mottling consisting of green patches or slightly raised green spots on a lighter green or chlorotic background. The cultivars were Charleston Gray, Crimson

Sweet, Fordhook Hybrid, Jubilee, Sugar Bush, and Sunsweet.

None of the cultivars of melon (*Cucumis melo*), or cucumber (*Cucumis sativus*) showed definitive, consistent symptoms, but isolate 2932 was recovered from true leaves above the inoculated cotyledons of all inoculated cultivars. *Cucumis melo* cultivars included Edisto 47, Hale's Best Jumbo, Imperial 45, Sierra Gold, and Summer Dream Hybrid. The *Cucumis sativus* cultivars were Burpee Hybrid II, Conquistador, Eureka, Indy, Marketmore 76, Matador, National Pickling, Pickalot, Picklebush, Poinsett 76, Revenue, Straight 8, Sweet 'n Crunchy Hybrid, Sweet Slice, and TMG-1.

Reactions of *Cucumis metuliferus* depended on the accession. Accession 2459 (kindly provided by R. Provvidenti) (24) was systemically infected but showed only mild vein-clearing and chlorosis of systemically infected leaves. Some plants of *C. metuliferus* (PI 292190 and PI 482459) showed chlorotic and necrotic symptoms. Some plants inoculated in the cotyledonary stage died, and some recovered. Plants that recovered showed scattered veinal necrosis on leaves above the inoculation point. Isolate 2932 was detected in systemically infected leaves of Accession 2459 by mechanical inoculation to squash, and also from symptomatic leaves of PI 292190 and PI 482459 above the point of inoculation.

Lagenaria siceraria (Grif 11938) showed only faint veinal chlorosis of leaves above the inoculation point. *Luffa aegyptiaca* (PI 555606) showed a few yellow spots in systemically infected leaves of two plants, but other plants showed no symptoms. Both species were systemically infected with isolate 2932 based on bioassays. The virus was recovered readily from systemically infected *Lagenaria siceraria* but with more difficulty and inconsistency from *Luffa aegyptiaca*.

The following noncucurbitaceous plants did not show symptoms, and the virus was not recovered: *Abelmoschus esculentus* 'Long Pod Green', *Brassica rapa* 'Seven Top', *Capsicum annuum* 'Early Cal Wonder', *Carica papaya*, *Catharanthus roseus*, *Chenopodium quinoa*, *Gomphrena globosa*, *Helianthus annuus* 'Mammoth', *Lactuca sativa* 'Black-Seeded Simpson', *Lupinus angustifolius*, *Lupinus albus*, *Lycopersicon esculentum* 'Homestead', *Nicotiana benthamiana*, *N. × edwardsonii*, *N. tabacum* 'Burley 21' and 'NN', *Passiflora edulis*, *Phaseolus vulgaris* 'Burpee Stringless', 'Topcrop', and 'Midnight', *Pisum sativum* 'Alaska' and 'Little Marvel', *Raphanus sativus* 'Red Devil B', *Sesamum indicum*, *Tagetes erecta*, *Tropaeolum majus*, *Vigna unguiculata* 'California Blackeye' and 'Purple Hull', *Zea mays* 'Golden Cross Bantam', and *Zinnia elegans* 'Giant Double'.

Although isolate 2932 was not recovered from noninoculated leaves of *N. benthami-*

ana, it was recovered from the inoculated leaves of 10 of 17 plants by assay to squash.

Aphid transmissibility. In two of three trials, isolate 2932 was transmitted from squash to squash in a nonpersistent manner by *Myzus persicae*. Using two aphids per test plant, none of 10 plants were infected in one test, but in a subsequent test with five aphids per plant, four of 10 plants were infected. Using this aphid-transmitted culture as an acquisition source, the virus was then transmitted to seven of 10 plants using two aphids per plant.

Analysis of purified virus. Aggregated filamentous particles were observed in electron micrographs of a purified preparation of isolate 2932. The preparation was infectious to squash. Analytical polyacrylamide gel electrophoresis of purified virus revealed three protein components: a major component of 32 kDa, and minor components of 30 kDa and 26 kDa.

Serological relationships. Both of the antisera prepared in this study reacted in SDS-immunodiffusion tests with sap from Early Prolific Straightneck squash and watermelon leaves infected with isolate 2932, but the test was not sensitive enough for virus detection in some cucurbits. Antiserum to untreated virus (1170) was used initially for virus detection and for some of the studies on serological relationships. Antiserum to the protein (1163) was then used for most detection and relationship studies, because it gave stronger immunoprecipitin bands with isolate 2932.

Isolate 2932 cross-reacted weakly with some of the potyvirus antisera (e.g., zucchini yellow mosaic virus, Moroccan wa-

termelon mosaic virus, papaya ringspot virus types W and T). These reactions, however, were ones of partial identity (cross-reacting bands were always spurred over by the band with the homologous virus). In reciprocal tests, isolate 2932 was serologically distinguishable from each of the following members or possible members of the Potyviridae: bean common mosaic virus (a peanut stripe virus strain), bean yellow mosaic virus isolate PV-2 (3), clover yellow vein virus (3), Moroccan watermelon mosaic virus (13,21), PRSV types W and T (21), peanut mottle virus, soybean mosaic virus, turnip mosaic virus, WMV-2, zucchini yellow fleck virus (23), and ZYMV (6,17). The serological distinction of isolate 2932 from PRSV-W, WMV-2, and ZYMV is illustrated in Figure 1. Isolate 2932 did not react serologically with cucumber mosaic virus (CMV), squash mosaic virus (SqMV), and a virus from *Trichosanthes* spp. (20) that has the characteristics of a potexvirus.

Cytology and particle morphology. Light micrographs of stained epidermal strips from symptomatic watermelon leaves from the greenhouse revealed the presence of prominent, elongated, nuclear inclusions (Fig. 2). They stained green with the Calcomine Orange-Luxol Brilliant Green combination. In addition, two other types of inclusions were seen. One was a mass of cytoplasmic, cylindrical inclusions, and the other was similar to the irregular or amorphous cytoplasmic inclusions induced by PRSV-W. All three types of inclusion bodies were also seen in squash leaves infected with isolate 2932.

Electron microscopy also revealed three types of inclusions in watermelon (Fig. 3) and squash tissues infected with isolate 2932. Crystalline inclusions were observed in the nuclei, and cylindrical inclusions (pinwheels and scrolls) were observed in the cytoplasm (Fig. 3). Another type of inclusion, which in some sections appeared as two layers or bands alternating with a layer of circles, was seen mainly in the cytoplasm (Fig. 3), but also occasionally in the nuclei (15). Filamentous particles also were observed in the cytoplasm.

Flexuous, filamentous particles were also observed in electron micrographs of leaf dips. Eighty-six per cent of 159 particles measured were 800 to 890 nm in length.

Analysis of field squash and watermelon samples. In April and May of 1991, 44 samples of squash and 44 samples of watermelon were collected in Hendry and Collier counties in South Florida, including the area where isolate 2932 had been collected in 1990. Most of the samples had mosaics or other viruslike symptoms. Samples were assayed by SDS immunodiffusion. The samples were tested against antiserum to isolate 2932 and against antisera to six other viruses of cucurbits, i.e., PRSV-W, WMV-2, ZYMV, CMV, SqMV, and the possible potexvirus (20). None of the samples reacted with antiserum to isolate 2932. Forty-two of the 44 squash samples and 43 of 44 watermelon samples reacted with PRSV-W antiserum, and 1 squash sample reacted with ZYMV antiserum. No other reactions were seen.

From the summer of 1991 through the fall of 1997, 162 squash and 67 watermelon

Fig. 1. Serological distinction of isolate 2932 from three potyviruses that are commonly found in cucurbits, using immunodiffusion tests in the presence of sodium dodecyl sulfate (SDS). Outer wells contain SDS-treated extracts from *Cucurbita pepo* (squash or pumpkin) leaves infected with: A, papaya ringspot virus type W (PRSV-W); B and E, isolate 2932; C, watermelon mosaic virus-2 (WMV-2); and D, zucchini yellow mosaic virus (ZYMV). F is from noninoculated plants. Antisera are to: 1, PRSV-W capsid protein; 2, WMV-2 virions; 3, ZYMV capsid protein; and 4, isolate 2932 capsid protein.

Fig. 2. Light micrograph of isolate 2932-induced inclusion bodies in watermelon leaf epidermal strips from inoculated plants. Tissues are stained with the Calcomine Orange-Luxol Brilliant Green combination. Note elongated, crystalline nuclear inclusion (NI), massed cylindrical inclusions (CI), and possible amorphous (irregular) inclusion (AI). Bar = 5 μ m.

Fig. 3. Electron micrograph of thin section of watermelon leaf tissue from inoculated plants. Note nuclear inclusion (NI), cylindrical inclusions (CI), and layered, amorphous inclusion (AI). Bar = 1 μ m.

samples, mostly from Central and North Florida, were tested by SDS-immunodiffusion for isolate 2932 and the other six viruses. None of the samples reacted with antisera to isolate 2932, SqMV, or the potexvirus from *Trichosanthes*. The percentages of samples in which viruses were detected in squash were: PRSV (33.9%), WMV-2 (37.6%), ZYMV (30.2%), and CMV (12.9%). The percentages of samples of watermelon in which viruses were detected were: PRSV (11.9%), WMV-2 (59.7%), and ZYMV (34.3%); CMV was not detected in watermelon. Mixed infections of most of the combinations of the various viruses detected were observed in both squash and watermelon.

DISCUSSION

The following evidence indicates that isolate 2932 should be classified as a member of the genus *Potyvirus* (2) in the family *Potyviridae*: flexuous, filamentous particles approximately 800 to 890 nm long were associated with infected plants; cylindrical inclusions were detected in infected plant tissues; the virus was serologically related to several potyviruses; and the virus was aphid-transmitted in a nonpersistent manner. The evidence obtained thus far indicates that isolate 2932 is a distinct member of the *Potyvirus* genus. The sequence of its capsid protein gene differs significantly from the capsid protein genes of PRSV-W, WMV-2, ZYMV, and other potyviruses (5). Although isolate 2932 gave reactions of partial identity in SDS-immunodiffusion tests with some potyviruses, it was serologically different from all potyviruses tested. Such reactions of partial identity between distinct potyviruses have been reported previously (17,21). It should be noted that isolate 2932 has not been compared with several other potyviruses reported to infect cucurbits in other countries. These include bryonia mottle virus (12), caserta mosaic virus (11), melon vein-banding mosaic virus (9), Melothria mottle virus (25), Telfairia mosaic virus (22), Trichosanthes mottle virus (26), and a whitefly-transmitted, poty-like virus tentatively named squash yellow leaf curl virus (28).

Isolate 2932 was detected in Florida in 1990, but it has not been found again in our limited testing. Isolate 2932 has potential importance because it causes mosaic or mottle symptoms in watermelon and some squashes. It causes foliar mosaic symptoms in Prelude II, which has capsid protein-mediated resistance to both WMV-2 and ZYMV. In our tests, isolate 2932 failed to infect *Cucurbita ecuadorensis*. Perhaps this species could serve as a source of resistance to isolate 2932, as it has for certain other viruses that infect squash (16,27). Cucumber and melon are infected but are mostly asymptomatic. No systemic infection of any species outside the Cucurbitaceae was detected. The massive, crystalline, nuclear inclusions and the layered inclu-

sions seen in infected cells help to distinguish isolate 2932 from the other potyviruses that infect cucurbits in Florida. The virus was detected initially on the basis of the nuclear inclusions.

The name watermelon leaf mottle virus is proposed for isolate 2932. Its distribution, economic importance, and precise relationship to other potyviruses await further studies.

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