OTHER CITRUS VIRUS DISEASES

Studies on the Psorosis Disease of Citrus and Preliminary Characterization of a Flexuous Virus Associated with the Disease

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ABSTRACT. Symptomatic shoot-tips from Etrog citron infected with a California isolate of Psorosis A (Ps203m) produced local lesions in *Chenopodium quinoa*, *Nicotiana megalosiphon*, and *Gomphrena globosa* 7, 10, and 14 days post inoculation respectively, when diluted 1:3 (w:v) with ice-cold 0.05 M sodium borate pH 8.3 plus 0.5% 2-ME and maintained under glasshouse conditions at UC Riverside. Under the same conditions systemic vein clearing (SVC) was observed in *Capsicum annuum* 12-16 weeks postinoculation, and a mild systemic mosaic in *N. benthamiana* 14-20 days postinoculation. Dodder (*Cuscuta subinclusa*) was used to transmit psorosis from citron infected with Ps203m to healthy *C. annuum* resulting in SVC 8-10 weeks postestablishment. Reciprocal experiments involving dodder transmission from *C. annuum* infected with psorosis to healthy Etrog citron resulted in necrotic shock at 10-12 weeks postestablishment followed by chlorotic flecking in young leaves. A disease-specific dsRNA pattern was established in Etrog citron infected with Ps203m, and observed in systemically infected *C. annuum* and *N. benthamiana*. Genomic dsRNA had a Mr of 5.3 x 10^6 and contained two additional dsRNAs (4.5, 4.1 x 10^6 Mr). A flexuous rod-shaped particle measuring 660-665 x 12nm was observed in pre-nectrotic local lesion tissue from *C. quinoa*, and purified from citron infected with Ps203m. A 29-Kd nucleoprotein was observed in 12% SDS-PAGE in virus containing gradient fractions verified by electron microscopy. A polyclonal antiserum was generated in chickens to the flexuous particle which weakly detected Ps203m and additional psorosis strains in an antigen coated indirect ELISA.

Psorosis is the oldest virus disease documented on citrus. In 1896 when the disease was described and named in Florida by Swingle and Webber (32), its viral nature was not known. A similar disease was identified in California in 1908 by Smith and Butler which they named scaly bark (31). This disease was determined to be the same disease identified years earlier in Florida based on similar symptoms and longterm effects on citrus. It was not until 1933, that the first evidence for the viral nature of psorosis was recorded (11). This evidence initiated descriptive research on other diseases that appeared related to psorosis based on similar leaf symptoms. These diseases included psorosis A, psorosis B, concave gum, blind pocket, crinkly leaf, and infectious variegation (14, 15, 16, 19, 32). Leaf symptoms in many of the above diseases are very transitory or non-existent in the field making early detection of virus-free budwood sources nearly impossible. An improvement in detection was elucidated by Wallace in 1945 (37). Wallace discovered that inoculation of sweet orange indicator seedlings with buds from psorosis-infected citrus reduced the time for detection of psorosis from 12 yr to 6-10 weeks based on young leaf symptoms. This was a major breakthrough in the detection of psorosis and psorosis-like diseases. Detection of psorosis A, B, and citrus ringspot virus were hastened by the development of a necrotic shock reaction in sweet orange as early as 5 weeks. For other psorosis-like diseases (concave gum, blind pocket, infectious variegation, and crinkly leaf) young leaf symptoms are the only symptoms which are manifested in indicator hosts 6-10 weeks post-graft-inoculation. With the implementation and refinement of biological assays several scientists began to

investigate the relationships among psorosis diseases, specifically by employing cross protection. One of the first investigations (38) showed that sweet orange previously inoculated with a non-lesion bark form of psorosis (psorosis A) showed no lesions, mature leaf symptoms, or twig die-back when inoculated with a lesion bark form of psorosis B, thus establishing a relationship between psorosis A and psorosis B. In similar experiments, citrus inoculated with psorosis A showed no protection against concave gum (28), infectious variegation virus (6), and crinkly leaf (15). The failure of psorosis A to cross protect against these viruses was taken as evidence that they are distinct from psorosis A. The above diseases were originally grouped by investigators in what is called the psorosis complex based on the young leaf symptoms induced on citrus seedling index hosts. There are, however, many symptoms which can be used to differentiate these diseases. Citrus variegation and crinkly leaf viruses are no longer included in the psorosis complex because the nature of the causal agents are known. In 1961 it was reported (19) that infectious variegation could be easily transmitted to citrus hosts and herbaceous hosts. Shortly thereafter, a 30 nm isometric particle was purified from plants infected with infectious variegation virus (5, 10). These viruses were shown to have physical characteristics, and a serological relationship to viruses in the ilarvirus group (16, 35).

Recently investigations were attempted to identify the etiological agent associated with citrus ringspot virus (8). Citrus ringspot is highly transmissible to a large number of herbaceous host plants, and can be moved back into citrus from one herbaceous host, *Gomphrena globosa* (17, 34). A two-component filamentous virus has been associated with the disease (7, 8). The particles are of two sizes and can be separated by sucrose density gradient centrifugation. Partially purified filamentous virus particles of each size class induce few if any local lesions in *Chenopodium quinoa*. However, the combination of each size class of partially purified particles from specific gradient fractions induce numerous local lesions in *C. quinoa*. Re-inoculation to citrus hosts with combined or individual gradient fractions has not been reported to reproduce the citrus ringspot virus syndrome, and therefore, the etiologic nature of these particles has not been demonstrated.

A flexuous rod-shaped particle that has an appearance and dimensions unlike that described for citrus ringspot virus has also been observed to be associated with an isolate of psorosis A (3).

What is known about psorosis and psorosis-like diseases is based on biological data accumulated from observations in the field and greenhouse. With the exception of those diseases from which the causal agents have been characterized and placed in appropriate virus groups, the nature of the etiological agents and their relationship to one another remains to be elucidated.

MATERIALS AND METHODS

Virus isolates. The primary virus isolate used throughout this study was an isolate of psorosis A designated ps203m and maintained under glasshouse conditions at the University of California, Riverside. The original source was introduced into the citrus variety collection at UC Riverside in 1930 in a Kao Panne pummelo selection from Thailand. The isolate was reported to be a mechanically transmissible form of psorosis in 1980 because it displayed the following biological properties in citrus indicator seedlings: no reaction in Mexican lime, strong leaf mottle in *Citrus excelsa* and King mandarin, leaf curl and mottle in sour orange and rough lemon, a severe shock reaction of shoot-tips, leaf curl, flecking, and nectrotic lesions on the leaves of Etrog citron. The ps203m strain was further characterized as a psorosis A strain by failure of psorosis B lesions to develop in sweet orange seedlings pre-inoculated with ps203m,

when challenge inoculated with psorosis lesion bark inoculum (29).

Virus isolates for the following research were bud inoculated into Etrog citron seedlings and first flush tissue (post shock reaction) was harvested for all experiments. Various psorosis isolates used for serological experiments were maintained in Madame Vinous sweet orange. Symptomatic first flush tissue was selected for use in all experiments.

Host range. Symptomatic first flush shoot-tips from citron seedlings inoculated with ps203m were ground in ice cold inoculation buffer (0.05M sodium borate pH 8.3 plus 0.5% 2-mercaptoethanol) in a prechilled mortar and pestle. Citrus shoot-tips were ground in inoculation buffer at a dilution no greater than 1:3 and applied to the following plants dusted with carborundum (600 mesh): *Capsicum annum* L. cv. California Wonder, *Capsicum annum* L. cv. Yolo Wonder, *Capsicum annum* L. cv. Jalapeno, *Capsicum frutescens* cv. Mexican chili, *Catharanthus roseus* (L.) G. Don cv. Little Pinkie, *Chenopodium amaranticolor* Coste & Reyn., *Chenopodium quinoa* Willd., *Cucumis melo* L. cv. Topmark, *Cucumis sativus* L. cv. Straight Eight, *Cucurbita pepo* L. cvs. Richgreen and Small Sugar Pumpkin, *Datura stramonium* L., *Glycine max* (L.) Merr., *Gomphrena globosa* L., *Gynura aurantiaca* (Bl.) DC, *Lycopersicon esculentum* L. cv. Rutgers, *Nicandra physaloides* L., *Nicotiana benthamiana* L., *Nicotiana clevelandii* A. Grey, *Nicotiana glauca* Grah., *Nicotiana megalosiphon* Heurch & Muell., *Nicotiana rustica* L., *Nicotiana sylvestris* Speg. & Comes, *Nicotiana tabacuum* L. cvs. Turkish and Xanthi-nc, *Phaseolus vulgaris* L. cv. Black Turtle 2, *Pysalis floridana* L., *Solanum nigrum* L., *Vigna unguiculata* (L.) Walp. cv. California blackeye, and *Zinnia elegans* Jacq.. Inoculated leaves were washed with water shortly after inoculation to prevent injury, kept in the headhouse overnight, and subsequently maintained under glasshouse conditions at UC Riverside for up to 24 weeks. All host range plants were evaluated by visual observation for obvious symptoms, ability to induce necrotic local lesions when back inoculated to *Chenopodium quinoa*, and presence of psorosis-specific dsRNAs.

Graft inoculation. Etrog citron and Madame Vinous sweet orange seedlings were graft inoculated by T-grafts using infected buds, and bark or leaf pieces. Inoculated seedlings were maintained under glasshouse conditions at the Rubidioux facility of the University of California for a minimum of 5 weeks to observe the necrotic shock reaction characteristic of psorosis. Plants that failed to show the shock reaction in that period of time were cut back for a second incubation. Plants failing to develop the shock reaction after this second incubation were considered negative for the presence of psorosis. Those plants showing the necrotic shock reaction after the five week period were observed for the characteristic flecking in young leaves 4-6 weeks later (these leaves were considered first flush tissue).

Symptomatic Mexican Chili peppers from the host range study were used as a source of inoculum for Mexican Chili, Jalapeno, Yolo Wonder, and California Wonder pepper cultivars by the following methods: whole and half-leaf side grafts, insertion of leaf pieces into pepper stems, and placement of the infected shoot-tips onto a pepper "rootstock". Recipient peppers were prepared for grafting by decapitation and defoliation to a single leaf pair. Pepper stems were sliced to 3/4 of the diameter with a sterile razor blade at a 45° angle, and donor pepper leaves were excised from infected plants and petioles trimmed at an angle to allow maximum contact between petiole of donor leaf and vascular tissue of recipient pepper stems. All grafts were tightly secured with latex grafting bandage and maintained either in sealed plastic bags under greenhouse conditions, or in growth chambers. Experimental conditions for growth in chambers was determined by main-

taining a light/dark regime at 12/12hr, temperature constant at 28 C, and varying the level of relative humidity in different experiments at 10% intervals starting at 65% and ending at 95%. Experiments were maintained at the various growth chamber conditions for a minimum of 26 days postgraft.

Dodder transmission. The dodder culture used for this study was a culture of *Cuscuta subinclusa* maintained in the greenhouse at UC Riverside for over 20 years and is known to be free of transmissible viruses. The transmission of citrus ringspot virus (CRSV) to herbaceous hosts via dodder has been reported (9). Dodder was established on Etrog citron infected with ps203m and used to transmit psorosis from infected citron to healthy citron. This strain was then referred to as ps203md. An experiment was designed to test the transmission of psorosis A from systemically infected Mexican Chili hosts to healthy citron seedlings and in an opposite manner from infected citron to healthy Mexican Chili using dodder. Dodder was established on citron infected with ps203m for a period of several weeks. Resulting free dodder shoots were connected to 4-6 week old healthy Mexican Chili peppers that had been decapitated and defoliated to a single leaf pair. These experiments were maintained under glasshouse conditions at UC Riverside until symptom development occured (usually 8-10 weeks). Conversely, dodder was established on infected Mexican Chili peppers for a period of several weeks and resulting free strands were connected to healthy Etrog citron seedlings which had been cut back to a single stem. The experiments were maintained under glasshouse conditions at UC Riverside until symptoms developed 8-12 weeks post-dodder-establishment.

Leaf-dip analysis. Leaf-dips were performed using all systemic hosts of ps203m. A 1-g sample of infected and healthy leaf material was ground with an equal volume of ice cold 0.05M sodium borate buffer pH 8.3 plus 0.5% 2-mercaptoethanol in a pre-chilled mortar and pestle. The resulting homogenate was centrifuged at 4 for 5 min at 3000 x g and the supernatant was removed. The supernatant was clarified with a 1/4 each volume of ice cold chloroform and carbon tetrachloride, and centrifuged as above. The aqueous phase was made 4% and 1.8% with polyethylene glycol (PEG) and sodium chloride, respectively, and shaken on ice until dissolved (10 min). The suspension was spun in a microfuge at 4 C for 30 min at 11,000 rpm. The resulting pellet was resuspended in 50 µl of extraction buffer and applied to carbon-backed, formvar coated electron microscope grids (100 mesh). Grids were washed briefly with deionized water, stained with 2% uranyl acetate, and examined at a minimum magnification of 35,000x with an Hitachi H600 electron microscope operating at 75KV accelerating voltage.

DsRNA analysis. DsRNAs were extracted according to published methods (1, 20, 25). Briefly, tissue ground to a fine powder in liquid nitrogen was extracted with phenol by rapid aggitation, separated from cellular debris and organic solvents by low speed centrifugation, and the aqueous supernatant made 16.5% with 95% ethanol. Further purification was achieved by selectively binding dsRNA to CF-11 cellulose columns at an ethanol concentration of 16.5%, extensively washing with a buffer containing ethanol at a concentration of 16.5% to remove ssRNA and DNAs, and eluting retained dsRNA in a buffer containing no ethanol. DsRNAs were further concentrated by two sequential ethanol precipitations containing a 1/20 dilution of 3M sodium acetate. Some samples required enzymatic digestion of contaminating DNA. Appropriate samples were treated with DNase I (Sigma) at 10 µg/ml in the presence of 5mM magnesium chloride for 1 hr at 37 C (30) and extracted with two volumes of STE (0.1M sodium chloride, 0.05M Tris, 0.0001M EDTA) saturated phenol and one volume of chloroform:isopentanol (24:1) on ice for 20 min. The

aqueous phase collected after centrifugation was precipitated with ethanol in the presence of sodium acetate. DsRNAs were analyzed using 6% polyacrylamide mini-vertical slab gels (8.3 x 10.2 x .15 cm) run at 110 V for 2.5 hr. Polyacrylamide gels were stained with 50 ng/ml ethidium bromide at the conclusion of electrophoresis. DsRNA was visualized with the aid of a UV transilluminator (260 nm) and photographed using red and yellow Wratten filters with a Polaroid MP4 Landcamera using Polaroid type 667 film.

DsRNAs utilized in this study were from the following sources; eggplant mild mottle carlavirus, potato virus S carlavirus, and dandelion latent carlavirus obtained from J. Allan Dodds at UC Riverside; tobacco mosaic tobamovirus strain U5 plus its associated satellite virus obtained from R. A. Valverde at Louisiana State University; red clover vein mosaic carlavirus obtained from D. J. Gumpf at Riverside.

Virus purification. Shoot-tips for purification were ground to a powder in liquid nitrogen. Ground plant material was thawed on ice at 4 C by stirring with extraction buffer (0.5M sodium borate pH 8.3, 0.1% sodium sulfite, and 0.5% 2-mercaptoethanol) at a ratio of 1:5 (w/v) for 30 min. The extract was filtered through two layers of miracloth (Calbiochem), and clarified with the addition of 1/2 volume of a 1:1 mixture of chloroform and carbon-tetra-chloride. The emulsion was stirred on ice at 4 C for 10-15 min and then centrifuged for 10 min at 6000 rpm. The supernatant was made 6% and 1.8% with PEG and sodium chloride, respectively, and stirred slowly for 2 hr at 4 C on ice. The solution was centrifuged for 30 min at 8,300 rpm, and pellets resuspended in 1/3 original extracted volume in extraction buffer and slowly stirred at 4 C on ice for 2 hr. One sixth of the volume used for resuspension was initially withheld and made 0.5% with respect to Triton X-100 and added to the resuspended pellets after 2 hr and stirring was continued under the same conditions for an additional 30 min. After low speed centrifugation at 5,000 rpm for 10 minutes the supernatant was retained and adjusted to 4% and 1.8% with PEG and sodium chloride, respectively, and stirred slowly on ice at 4 C for 1 hr. The suspension was centrifuged at 8,300 rpm for 30 min. The pellets recovered were resuspended in 0.5M sodium borate pH 8.2 by gentle grinding in a glass tissue grinder on ice. After low speed centrifugation, the virus in suspension was further purified by cesium chloride/density gradient centrifugation. Virus preparations were underlayed with an equal volume of cesium chloride in 0.5M sodium borate buffer pH 8.2 (starting density of 1.15 g/ml), and centrifuged in a Beckman Ti-50 rotor at 56,560 RCF at 6 C for 15-18 hr. Gradient fractions of 1-2 ml were hand collected (the fraction at the bottom of the gradient tube was used to resuspend any pellet which formed during centrifugation). All fractions were diluted to 10 ml in 0.005M sodium borate pH 8.2 and adjusted to 4% PEG and 1.8 sodium chloride. Precipitated virus was collected by centrifugation at 8,300 rpm for 20 min and resuspended in 0.5 ml of 0.005M sodium borate pH 8.2. Presence of the virus was assayed by electrophoresis of viral proteins in SDS-PAGE, inoculation to *Chenopodium quinoa*, and by direct examination in the electron microscope of PEG concentrated gradient fractions after preparing 2% uranyl acetate stained grids.

Citron tissue used for purification was first flush symptomatic shoot-tips approximately 8-10 weeks after graft inoculation. Symptomatic second and third flush shoot-tips were also used if the tissue continued to shock severely after previous tissue harvests. In all cases, very symptomatic shoot-tips were used.

Protein analysis. Proteins present in concentrated aliquots from individual gradient fractions from virus purification were denatured by heating to 100 C for 4 min in the presence of 0.07M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol. Proteins were analyzed on 12% SDS-polyacrylamide gel with a 4% stacking

gel using the discontinuous buffer system of Laemmli (23). Gels were electrophoresed for 3-4 hr with constant cooling to 6 C at 30 mA. Gels were stained using silver nitrate by first fixing overnight with 50% methanol and 0.1% formaldehyde. Fixed gels were reacted with silver nitrate in the presence of sodium hydroxide and ammonium hydroxide, and developed in the presence of citric acid and formaldehyde according to published method of Wray (41).

Molecular weight markers were purchased from Sigma and included the following protein markers: albumin from bovine plasma-66Kd, albumin from egg (ovalbumin)-45Kd, pepsin from porcine stomach mucosa-34Kd, trypsinogen (PMSF treated) from bovine pancreas-24Kd, beta-lactoglobulin from bovine milk-18.4Kd, lysozyme from egg white-14.3Kd.

Polyclonal antisera production. Polyclonal antiserum was generated in chickens according to the following procedure. White leghorn chickens were injected with purified virus at a concentration of 0.25 mg/ml intramuscularly in alternating thigh muscles at weekly intervals for a period of 3 weeks. The initial injection was emulsified 1:1 with Freund's complete adjuvant, each subsequent injection was emulsified with Freund's incomplete adjuvant. Antiserum was collected from egg yolks according to published methods (26, 27). Briefly, egg yolks were collected from eggs starting at 7 days post-initial-injection. The yolks were equilibrated in 1/2 strength PBS at a volume of 1:3 (yolk:buffer), and PEG added to a concentration of 3.5%. The mixture was stirred at room temperature until the PEG was dissolved and then centrifuged at 6,000 rpm for 10 min. The supernatant fluid was removed and made 8% with respect to PEG, stirred at room temperature until dissolved and left standing at room temperature for 10 min. The mixture was centrifuged at 8,300 rpm for 30 min and the resulting pellet resuspended with 12 ml of 1/2 PBS. The resuspended pellets were made 12% with respect to PEG and stirred at room temperature until dissolved and left standing at room temperature for 10 min. The mixture was centrifuged at 8,300 for 30 min and pellets resuspended with 2 ml of 1/2 PBS. To remove excess polyethylene glycol, the preparation was cooled to O C and mixed 1:1 (v:v) with 50% ethanol (prechilled to -20 C) and centrifuged in a prechilled rotor at 0 C at 8,300 rpm for 30 min. The pellets which contained IgY were resuspended 2 ml of 1/2 PBS.

Polyclonal preparations of IgY were fractionated according to published methods (4). Briefly, immunoglobulins were precipitated by addition of saturated ammonium sulfate at a concentration of 1:10 (immunoglobulins:ammonium sulfate), collected by centrifugation, and the resulting pellets resuspended in 1/2 PBS and dialyzed against two changes of 1/2 PBS for 4 hr and overnight at 4 C. Immunoglobulins were fractionated by chromatography on DEAE-cellulose equilibrated in 1/2 PBS. Columns were monitored at a wavelength of 280 nm, and uv absorbing areas containing IgY was collected. The immunogen source for polyclonal production in chickens was purified non-aggregated virus boosted at the final injection with non-aggregated virus.

When necessary, polyclonal antiserum was cross absorbed by the following procedure. Healthy citron tissue was ground to a frozen powder in liquid nitrogen, extracted in 0.5M sodium borate buffer pH 8.3 and clarified by the addition of 1/2 volume of a 1:1 mixture of chloroform and carbontetrachloride. Healthy proteins in the aqueous phase was concentrated by the addition of 6% PEG precipitation (plus 1.8% sodium chloride) centrifuged at 8,300 rpm for 15 min. The 6% supernatant was then made 4% with respect to PEG (plus 1.8% sodium chloride) and centrifuged as above. The 4% pellet containing healthy plant proteins was resuspended in ml of 0.005M sodium borate pH 8.2 and added at a 1:1 ratio with antiserum, heated for one hour at 37 C, cooled to 4 C for 4 hr

(18), and centrifuged at 5,000 x g for 15 min. The resulting supernatant was frozen at -20 C for 1 hr to overnight, and centrifuged at 5,000 x g for 15 min. The supernatant was the antiserum preparation that was used for serological studies.

ELISA. Psorosis infected and healthy citrus shoot-tips were ground 1:10 in sample buffer (0.1M phosphate pH 6.4, 0.33M sodium chloride), applied immediately to immulon II microtiter plates (Dynatech) and incubated at room temperture for 1-2 hr. Plates were blocked with either PBS + 3%BSA and 1% Carnation non-fat dry milk, or PBS + 3%BSA for 1 hr at room temperature. Various antiserum preparations (non-absorbed or cross-absorbed) were diluted in blocking buffer, applied to the microtiter plates and incubated at 4 C overnight. Rabbit anti-chicken IgG conjugated with alkaline phosphatase (Sigma) was diluted in 1/10 blocking buffer to a concentration of 1:1500 and incubated at 30 C for 5 hr. Reactions were observed following the addition of substrate (p-nitrophenyl phosphate) diluted in diethanolamine substrate buffer at a concentration of 0.6 mg/ml, and incubated at room temperature for 1-4 hr. Results were analyzed spectrophotometrically at a wavelength of 405nm using a Molecular Devices Emax microtitre plate reader. Positive results were those samples with absorbances at 405nm twice the healthy control.

RESULTS

Host range. Ps203M induced local lesions on *C. quinoa* 7-10 days post-inoculation. The number of lesions and incubation period varied with the quality of inoculum from citrus, level of dilution in buffer, pH of the buffer, and presence or absence of reducing agents.

The largest numbers of lesions developed when first flush tissue was used as the source. Earlier shock tissue and subsequent flush tissues produced fewer lesions and required longer incubation periods. Two additional local lesion hosts were found during this study, however, both proved to be unreliable at times. *Nicotiana megalosiphon* developed a few local lesions (5 per leaf) 10-14 days post-inoculation. Spontaneous small necrotic lesions also developed in buffer-inoculated and healthy uninoculated control plants prior to or shortly after inoculation (depending on the age of the *N. megalosiphon* plants used). These spontaneous lesions were not transmissible to additional *N. megalosiphon* plants. It was later determined that the spontaneous lesions were formed in response to insect damage from soil fungal gnats present in the greenhouse, leading to confusion when interpreting experimental results and was therefore not used routinely. Inoculation of extracts from true lesions from *N. megalosiphon* to *C. quinoa* developed 4-6 local lesions per leaf 10 days post-inoculation. *Gomphrena globosa* produced a spreading purple lesion in response to inoculation by ps203m tissue 14-21 days post-inoculation. Back inoculation of these lesions to *C. quinoa* produced 6-8 lesions per leaf 10 days post-inoculation. Transmission to *G. globosa* was very infrequent. *Nicotiana benthamiana* was inoculated with first flush tissue only, and developed a mild systemic mosaic 14-20 days post inoculation. Shoot-tip tissue from *N. benthamiana* back inoculated to *C. quinoa* produced 4-6 local lesions per leaf 10-12 days post-inoculation. During many of the experiments, the infection was latent and required back inoculation to *C. quinoa* and dsRNA analysis to verify the presence of the virus. Mechanical transmission of ps203m first flush citron shoot-tips to 3-6 week old *Capsicum frutescens* cv. Mexican Chili required an incubation period of 12-16 weeks when maintained under glasshouse conditions. At 12 weeks vein clearing was observed in only 1-2 veins on older leaves. Development of systemic vein clearing was observed during the following 2-4 week period usually corresponding with a period of flower break (Fig. 1). No vein clearing symptoms were observed in buffer in-

Fig 1. Systemic vein clearing in *Capsicum annum* Mexican Chili 16 weeks post-mechanical-inoculation from Etrog citron shoot-tips infected with ps203m.

oculated or healthy control plants in the three year duration of these experiments. The susceptibility of peppers to infection by mechanical inoculation with ps203m appears related to photoperiod as peppers were only susceptible in early spring.

Plants inoculated in the period February-April developed symptoms during May-July in Riverside. This was verified by visual appearance of symptoms, ability to induce local lesions on *C. quinoa* following back inoculation, and the presence of psorosis disease-specific dsRNAs. This phenomenon was reproduced in each of three consecutive years in Riverside. All peppers in these experiments were analyzed using the same three parameters. DsRNAs in ethidium stained 6% polyacrylamide gels were not detectable in infected peppers until 1-2 days prior to symptom expression.

Graft inoculation. Systemically infected Mexican Chili peppers were used as donors for graft experiments to additional Mexican Chili pepper plants. Systemically infected Mexican Chili "scions" were whip-grafted to healthy Mexican Chili pepper "rootstocks". In addition whole leaves were side-grafted to 4-6-week-old decapitated and defoliated healthy Mexican Chili peppers. These plants were covered with plastic bags and maintained under glasshouse conditions. In these experiments, all grafts began to decline in 6 hr and were dead in 1-2 days. These experiments were analyzed after an incubation period of 24 weeks after graft inoculation by visual examination for symptoms and for the presence of psorosis-specific dsRNAs and found to be negative in both tests. The experiments were then modified and conducted in growth chambers maintained at 12/12hr day/night light regime, 28 C, and various relative humidity (RH) levels from 65% to 95%. At all levels of RH whip-grafts of infected "scions" began to decline in 12 hr post-inoculation and were dead in 2-3 days. Analysis of these experiments for the presence of dsRNA after 12 weeks were negative for the presence of psorosis disease-specific dsRNAs. Whole leaf area side-grafts began to decline at RH levels 65%-80% three days after graft inoculation, and were dead in five days. DsRNA analysis after 12 weeks were negative for the presence of specific dsRNAs. Whole leaf area side-grafts at higher RH levels, 85%-95%, began to decline 5 to 6 days post-inoculation and were dead at 7 days. DsRNA analysis was likewise negative for the psorosis disease-specific pattern. Half-leaf area grafts, prepared according to the Materials and Methods, maintained at RH levels above 75%, survived for the duration of the experiments with no decline. Systemic vein clearing symptoms were observed routinely 23-26 days post-graft-inoculation. Psorosis-specific dsRNAs were detected in plants from these experiments. These experiments were reproduced in 40 Mexican Chili pepper plants every 2 months over a 3-yr period with comparable results. Successful graft transmission was dependent on the use of pepper leaves with symptoms of vein clearing.

Peppers grafted with half leaves from healthy plants remained negative for visual symptoms and presence of specific dsRNA. As observed in mechanical transmission experiments, symptom expression followed a period of flower break. Half-leaf area grafts were utilized to transmit psorosis to three other pepper cultivars (California Wonder, Yolo Wonder, and Jalapeno) with comparable results. These peppers were not used routinely due to the presence of indigenous dsRNAs associated with some of these cultivars. In two experiments, the RH was maintained at 90%, light regime at 12/12hr day/night, and temperature reduced to 23 C which resulted in a delay of symptom expression of dsRNA detection by 7-10 days.

Dodder transmisison. Dodder established on Etrog citron infected with ps203m was subsequently attached to healthy Mexican Chili peppers and maintained under glasshouse conditions until symptom development. Vein clearing symptoms developed 10 weeks after dodder establishment (Fig. 2). Systemic infection was confirmed by dsRNA analysis, half leaf area side-grafts to additonal Mexican Chili peppers, and back transmission to healthy citron. To accomplish the final transmission, dodder was reestablished on infected Mexican Chili peppers from the original dodder transmission experiments and subsequently attached to healthy citron seedling trained to a single stem cut to 35cm in height. Twelve weeks post-dodder-establishment the psorosis shock reaction was observed followed in 4-6 weeks by flecking in the young leaves. DsRNA analysis of citron flush shoot-tips detected psorosis disease-specific dsRNAs.

Fig. 2. Dodder (*Cuscuta subinclusa*) transmission of ps203m from Etrog citron infected with ps203m to healthy *Capsicum annum* Mexican Chili inducing systemic vein clearing 8-10 weeks after dodder establishment. (Arrow indicates a cleared vein)

Leaf dip analysis. Leaf dip analysis was performed as described in the Materials and Methods section. The "virion" prep was prepared for all hosts and observed after negative staining with 2% uranyl acetate with the use of an Hitachi H600 transmission electron microscope.

Flexuous rod-shaped virus particles were observe in samples from *N. benthamiana, C. frutescens* cv. Mexican Chili, and *C. quinoa.* Only a few particles (1-2 per grid) were observed with all samples with the exception of those from *C. quinoa* local lesions. When yellow/green local lesions prior to necrosis were used, 4-6 flexuous rod-shaped virus particles were observed per field of view measuring 650-665nm x 12-13nm.

DsRNA Analysis. Disease-specific dsRNAs were detected in citron infected with ps203m 8 weeks after T graft inoculation with infected buds. When 28-36 g of infected citron, instead of the standard 7 g, processed through a single column a 12-16 g equivalent was loaded per lane on 6% polyacrylamide mini-vertical slab gels a dsRNA pattern containing two bands (5.3 and 4.5 x 10^6 Mr) was observed. A large amount of DNA was carried over from CF-11 columns into the sample using these procedures (Fig. 3, lane D). When samples were subjected to DNase treatment as described in the Materials and Methods, a third sub-genomic dsRNA band was observed with a Mr of 4.1 x 10^6 (Fig. 3, B). Subsequent samples were routinely treated with DNase before being analyzed in polyacrylamide gels.

The psorosis disease-specific dsRNA pattern was similar in size and

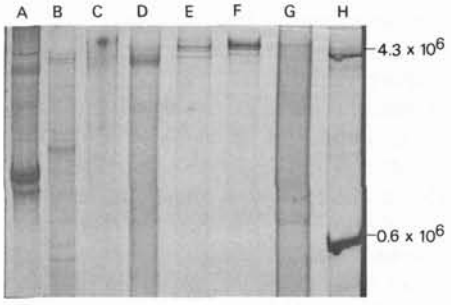

Fig. 3. Polyacrylamide vertical slab gel (6%) comparison of isolated dsRNA from ps203m infected systemic hosts, and systemic hosts of other viruses. Lane, A) Red clover vein mosaic carlavirus, B) Mexican Chili infected with ps203m, C) Etrog citron infected with ps203m (DNase treated), D) Etrog citron infected with ps203m, E) Potato virus S carlavirus, F) Dandelion latent carlavirus, G) Eggplant mild mottle carlavirus, H) Tobacco mosaic tobamovirus strain U_5 plus Satellite tobacco mosaic virus. (This gel picture is a printed negative image from a positive photo of the ethidium bromide stained gel to enhance weakly fluorescent dsRNA gel bands).

appearance to dsRNA patterns established for the Carlavirus group. Several carlavirus cultures were reactivated from the collection of Dr. J. Allan Dodds and analyzed in tandem with citron shoot-tips and Mexican Chili vein cleared leaves infected with ps203m on 6% polyacylamide mini-vertical slab gels (Fig. 3). The Mr of dsRNAs from hosts infected with ps203m was comparable to that of dsRNAs of red clover vein mosaic virus, dandelion latent virus, and potato virus S (Fig. 3, lanes A, E, F). Disease-specific dsRNAs were not detected in citron shoot-tips during the period of shock reaction, in non-symptomatic shoot-tips, or in shoot-tips not preceded by a period of necrotic shock (unpublished data). The most abundant accumulation of specific-dsRNAs were detected in symptomatic citron shoot-tips following a necrotic shock period.

Virus purification. First flush shoot-tips from ps203m infected citron equiliberated with extraction buffer plus sodium sulfate, clarified with chloroform:carbontetrachloride, and sequentially (6 and 4%) PEG concentrated resulted in almost complete aggregation of virus particles which concentrated in cesium chloride gradient pellets. This procedure was modified to include 0.1% Triton X-100 in all resuspension steps which decreased the level of aggregation from 80% to 30% based on the concentration of virus in various gradient fractions. The purified virus preparations were visibly free of phytoferritin contamination (a major contaminant of earlier purification attempts), after electron microscopic analysis, however, a high degree of fragmentation was observed possibly due to prolonged exposure to cesium chloride (Fig. 4). A total of 450 particles on 20 separate electron microscope grids negatively stained with uranyl acetate were measured and the results indicated a consistent particle width of 12-13nm. Full length particles varied in length from 650-665nm, however, several particles were also measured at 600, 450, 250, and 200nm in length which may indicate several structural weak points along the virus particle.

Non-aggregated virus particles from purifications utilizing Triton X-100, induced 2-4 local lesions per leaf on *Chenopodium quinoa*, however, stem-slash inoculation or mechanical inoculation by rubbing young leaves of Etrog citron seedlings failed to reproduce the psorosis disease syndrome. Difficulty with mechanical inoculation of citron seedlings with purified virus

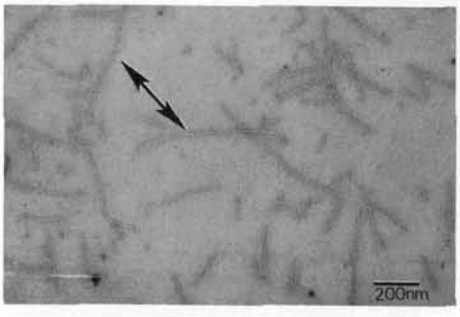

Fig. 4. Flexuous virus particles from Etrog citron infected with ps203m purified following cesium chloride equilibrium gradient centrifugation. (Stained with 2% uranyl acetate, 94,500x)

or local lesion tissue from *C. quinoa* gave rise to the need for indirect "routes" for back inoculation to citron or sweet orange. Purified virus and local lesion tissue from *C. quinoa* rub inoculated to the leaves of 4-week-old Mexican Chili seedlings failed to develop vein clearing symptoms in 16 weeks. Psorosis-disease-specific dsRNAs also failed to develop these pepper plants.

Protein analysis. PEG concentrated cesium chloride gradient fractions containing putative viral proteins were denatured and subjected to 12% SDS-PEG analysis. The detection of a single viral coat protein in virus containing fractions was not possible because of the presence of numerous soluble host proteins not detected in electron microscopically determined "clean" virus preparations. It was observed that few if any contaminating soluble proteins were present in pellet fractions from cesium chloride gradients, and purification attempts that excluded Triton X-100 resulted in the partitioning of 80% of purified virus into gradient pellets. Utilizing these two results, a virus specific polypeptide was detected in virus containing pellet fractions from purifications which did not include Triton X-100. A 29Kd protein was the major protein detected in the gradient pellet from infected preparations (Fig. 5, lane 5) which by electron microscopic analysis contained many virus particles. A small amount of this protein was detected in fraction 2 from infected preparations (Fig. 5, lane 11) in addition to numerous soluble proteins. Electron microscopic analysis of fraction 2 revealed a small population of virus particles.

Polyclonal antisera and ELISA. Chickens were selected to generate polyclonal antiserum because very high titre antisera could be produced in a short period of time (10-20 days) using small amounts of protein (259 μg/ml). The first polyclonal antiserum produced, CPCAS1-15, generated against dispersed virus particles (Fig. 4) was used in an attempt to develop

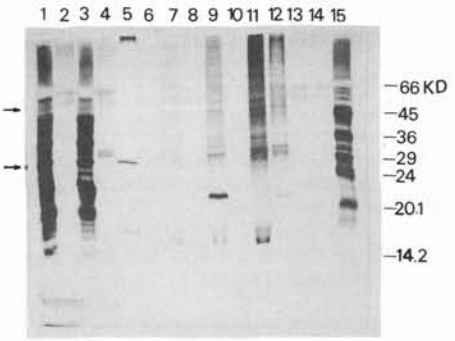

Fig. 5. Separation by SDS polyacrylamide vertical slab gel (12%) electrophoresis of proteins from gradient fractions from a citrus psorosis virus purification without the incorporation of Triton X-100 in the procedure. Lane 1) Molecular weight standards (SDS 7 Sigma), 2) Eggplant mild mottle carlavirus, 3) Molecular weight standards (SDS-7), 4) Red clover vein mosaic carlavirus, 5) infected fraction five, 6) healthy fraction five, 7) infected fraction four, 8) healthy fraction four, 9) infected fraction three, 10) healthy fraction three, 11) infected fraction two, 12) healthy fraction two, 13) infected fraction one, 14) healthy fraction one, 15) Molecular weight standards (SDS-7). Electrophoresed for 3-4 hr at 30mA, stained with silver nitrate.

an antigen coated, indirect ELISA for the detection of psorosis in citron and sweet orange infected with ps203m. The chicken antiserum gave a high background reaction to healthy sweet orange and citron shoot-tips regardless of antigen or antiserum dilution.

Several blocking agents were tested to decrease the background absorbance (nonfat milk, BSA, Tween-20) with limited success. The combination of 1% nonfat milk and 3% BSA in PBS gave the best results, however the improvement was minimal. When CPCASI-15 antiserum was cross absorbed by the procedure described in the Materials and Methods, an improvement in the detection of psorosis in citron shoot-tips infected with ps203m was observed (Table 1). Although the discriminating ability of this antiserum is weak, this is the first antiserum produced which detects psorosis in first flush, symptomatic shoot-tips of Etrog citron infected with ps203m in an ELISA system. The ELISA conditions for detection are

TABLE 1
INDIRECT ELISA DETECTION OF PSOROSIS AND PSOROSIS-LIKE DISEASES USING CROSS-ABSORBED CPCA1-15 POLYCLONAL ANTISERA[z]

Sample[y]	A_{405nm}[x]
Concave Gum-301 (in Madame Vinous)	0.499
11542 (psorosis A field strain in Madame Vinous)	0.525
Madame Vinous control	0.204
Lesion bark psorosis-251-3 (in Madame Vinous bark)	0.586
Madame Vinous bark control	0.221
Ps203m (psorosis A in 2nd and 3rd flush Etrog citron)	0.556
Indian psorosis (Beltsville strain 168 in Etrog citron)	0.455
Indian psorosis (Beltsville strain 169 in Etrog citron)	0.409
Etrog citron control	0.402
Florida citrus ringspot (Beltsville strain 85 in Duncan)	0.469
Duncan grapefruit control	0.276

[z]CPCASI-15 is antisera developed in chickens to dispersed virus particles.
[y]Citrus shoot-tips or sweet orange green bark diluted 1:10 in sample buffer.
[x]absorbance at 405nm.

1:10 dilution of coating antigen (first flush citron shoot-tips), and a 1:500 or 1:1000 dilution of cross absorbed antiserum. Using these conditions CPCASI-15 was tested in an antigen coated indirect ELISA for the comparison of psorosis and psorosis-like diseases in sweet orange, citron, and Duncan grapefruit. The results are summarized in Table 1. Concave gum (CG-301) and a field strain of psorosis (11542) in sweet orange shoot-tips both gave an absorbance twice their healthy controls. Lesions on sweet orange bark infected with a lesion bark strain of psorosis (251-3) gave an absorbance 2.3 times the healthy control bark. Second flush shoot-tips of Etrog citron infected with ps203m did not give positive results when averaged over twelve plates containing three replicates each. Individually, however, several of these samples gave positive results but the data overall was not consistent. The second flush shoot-tip material of infected citron was selected for testing because it best represented the tissue age and quality of citron shoot-tip samples tested which contained two strains of psorosis from India. Failure to observe positive results with second flush shoot-tip material may indicate that older, mild to non-symptomatic citron tissue infected with ps203m was not a good source for detection of ps203m in ELISA with this antiserum. Two strains of psorosis from India were tested in sap from second and third flush citron shoot-tips in citrons increased at the USDA citrus quarantine facility in Beltsville, Maryland. These strains usually give very severe symptoms in citron, however, the tissue available for ELISA showed very few if any symptoms. Not surprisingly, these two strains gave negative results with CPCASI-15 antiserum. Another strain maintained at the Beltsville facility is B-85, the Florida 6E strain of citrus ringspot virus in Duncan grapefruit. CPCASI-15 gave an absorbance just 1.6 times the Duncan shoot-tip control. In all the above ELISAs the substrate was allowed to develop for 2-4 hr at room temperature. Western analysis using CPCASI-15 antiserum was not attempted due to the obvious presence of soluble plant proteins contained in test samples as indicated by the high level of background absorbances observed in ELISA.

DISCUSSION AND CONCLUSIONS

The host range of ps203m (an A-strain of psorosis) was extended to include *Gomphrena globosa* and *Nicotiana megalosiphon* as additional

local lesion hosts. Symptoms on these two hosts, unfortunately, were very difficult to interpret during the course of host range experiments. *G. globosa* developed spreading red, ring-shaped lesions when inoculated with first flush citron shoot-tips infected with ps203m, and when maintained under cool temperatures in the greenhouse. *N. megalosiphon* developed necrotic local lesions in response to the same inoculum. Unfortunately, lesions produced by soil fungal gnats resembled virus induced lesions which made recognition confusing. Both of these hosts would be useful, especially *N. megalosiphon* if control of soil gnats in the greenhouse can be achieved. The successful inoculation of psorosis into two *Capicum* species was by far the most significant result of the host range experiments. *Capsicum annum* and *Capsicum frutescens* have never been reported as experimental hosts of psorosis or psorosis-like diseases. Extracts from citron shoot-tips infected with ps203m mechanically transmitted to *Capsicum frutescens* cv. Mexican Chili produced vein clearing in systemic leaves 12-16 weeks post inoculation. This long incubation period is unusual for development of symptoms in a herbaceous host and is usually characteristic of symptom development in woody hosts from mechanical or graft inoculation. The window of susceptibility of Mexican Chili peppers to infection by ps203m was a 4-5 week period beginning late January to early February in Riverside. This phenomenon was observed in each of 3 consecutive years. Because of the extended incubation period and seasonal constraints on transmission, alternative methods for transmission of ps203m were sought. Vegetative propagation of infected Mexican Chili pepper was successful using symptomatic 1/2 leaf area grafts when maintained under growth chamber conditions. Success of graft transmission was dependent on maintaining grafted plants at relative humidity above 75%. It was also critical that grafted plants be moved to growth chambers no more than 5-10 min after grafting. Under these conditions, grafts survived and retained vein cleared symptoms throughout the duration of the experiment. Humidity levels between 60 and 75% resulted in death of the graft within a few hours to 1-2 days. Graft experiments maintained under greenhouse conditions were unsuccessful due to the inability to maintain relative humidity at a constant level immediately after grafting. The use of Mexican Chili donor leaves greater than 1/2 leaf area was also unsuccessful due to the development of a permanent wilt state which resulted in graft death even when maintained under ideal growth chamber conditions. Previous reports indicated that citrus ringspot virus was dodder transmissible from citrus host to citrus host, and also from citrus to herbaceous hosts (9, 39). Citron seedlings infected with ps203m, just entering the first flush period, reproduced vein clearing symptoms in Mexican Chili systemic leaves 8-10 weeks post-dodder-establishment. More importantly, dodder transmission from vein cleared Mexican chili reproduced the psorosis disease syndrome in healthy citron seedlings by first inducing a necrotic shock reaction 10-12 weeks after dodder establishment, and finally inducing chlorotic leaf-flecking in the young shoot-tips 15-17 weeks after dodder establishment. The results of these experiments demonstrated that the agent transferred and responsible for inducing vein clearing symptoms in pepper could also induce psorosis-like symptoms in citron. Inoculation by rubbing or stem knife-slashing of healthy citron with Mexican Chili sap containing ps203m failed to reproduce the psorosis disease syndrome in citron. The failure of transmission by rubbing or knife-slashing is probably due to the instability of psorosis in sap. Dodder transmission and host range experiments were analyzed by dsRNA analysis. A psorosis disease-specific dsRNA pattern, originally detected in citron infected with psorosis, consisted of a triple-band dsRNA pattern also detected in Mexican Chili during the development of systemic vein clearing

following mechanical or dodder transmission from infected citron. Dodder transmission from Mexican Chili expressing the vein clearing symptom, reproduced the psorosis disease syndrome following connection to healthy citron. DsRNA analysis of the first flush shoot-tips of the above citron detected the presence of the psorosis disease-specific dsRNA triple-band pattern.

Disease-specific dsRNAs were analyzed using ethidium bromide stained 6% tube or vertical polyacrylamide gels. Between 28-36 g of symptomatic citron shoot-tips containing ps203m and *N. tabacum* cv. Turkish containing EMMV were necessary to detect dsRNA in 6% PAGE. DsRNA could be detected in 7.0 g samples of leaf material from plants infected with TMV, RCVMV, DLV, and PVS. Detection of psorosis dsRNA in 6% polyacrylamide mini-vertical slab gels required 10-12 g of citron shoot-tips per gel lane. DsRNA in Mexican Chili vein cleared systemic leaves in *N. benthamiana* systemic leaves could be detected in 6% PAGE containing 2-3 g (fresh weight equivalent) per gel lane. The psorosis disease-specific dsRNA pattern consisted of three high Mr dsRNA bands. The slowest migrating dsRNA, assumed to be the replicative form of ssRNA viral genome, had a Mr of approximately 5.3 x 10^6. Two additional faster migrating dsRNAs had a Mr of 4.5×10^6 and 4.1×10^6, respectively. Occasionally, a faint dsRNA band was observed above the RF in citron and *N. benthamiana* infected with ps203m. The presence of multiple high Mr dsRNAs has been previously reported for plants infected with members of the Carlavirus group (36), The dsRNA pattern associated with psorosis-infected plants was compared to dsRNAs isolated from plants infected with various members of the Carlavirus group (EMMV, PVS, DLV, and RCVMV). Similarities in the Mr of the RF's and number of high Mr dsRNA bands exist between CPSV, PVS, DLV, and RCVMV when analyzed in ethidium bromide stained 6% PAGE.

A flexuous rod-shaped particle was observed in leaf dips of local lesions from *C. quinoa* inoculated with ps203m. Flexuous particles could only be observed in lesion tissue ground prior to the development of necrosis (3-4 days-post-inoculation). Successful purification of this particle was finally achieved after trials with several unsuccessful purification protocols. The method of concentration using subsequent 6 and then 4% PEG precipitation followed by a cesium chloride/equilibrium gradient increased the number of flexuous particles, and addition of Triton X-100 to all resuspension buffers decreased the level of aggregation. The flexuous particles purified were generally in higher concentration and of good quality (when compared to previous purification attempts), however, a high degree of fragmentation resulted possibly due to prolonged exposure of virus particles to cesium chloride. Virus yields were between 0.5 and 0.75 mg/200 g of citron shoot-tips. Inoculation of purified virus to *C. quinoa* induced 2-4 lesions per leaf in 9-11 days. This low level of viral infectivity may reflect the actual percentage of intact virions capable of initiating infection, or the instability of the virus through purification. Purified virus able to produce local lesion in *C. quinoa* failed to reproduce the psorosis syndrome when inoculated to citron. The difficulty of reinfecting citron with purified virus may possibly be overcome by using stem-slashed or puncture inoculated Mexican Chili with purified virus, or *C. quinoa* local lesions as an intermediate system for back inoculation to citron. Dodder transmission of the psorosis agent from puncture inoculated Mexican Chili to healthy citron, could be observed visually for symptoms, and in 6% PAGE for the detection of psorosis disease-specific dsRNAs. This sequence involving an intermediate host may be one way to prove Koch's postulates. Attempts using purified virus failed to induce vein clearing in Mexican Chili when rub inoculated to pepper seedlings. However, only one attempt at

back inoculation occurred during the "window of susceptiblity" in pepper which may account for the failed back inoculation. Better coordination of purification and inoculation during periods of pepper susceptibility is necessary before final conclusions are made.

Use of cesium chloride gradients in purifications that deliberately excluded Triton X-100 resulted in near complete aggregation and partitioning of virus particles to the gradient pellet. Analysis of individual gradient fractions in silver stained 12% SDS-PAGE of equivalent healthy and infected preparations gave evidence for a single polypeptide of 29Kd associated with that part of the gradient tube that contained the pellet. A weakly detectable band in that Mr range was observed in an upper gradient fraction that contained a low concentration of dispersed virus particles. The weak intensity of this band could have been predicted by the low number of virus particles in this fraction verified by electron microscopy. Prior to deliberate concentration of virus particles in the pellet, observation of a putative coat protein was difficult due to the presence of numerous soluble proteins which co-purified in the "viral zone" of the gradients.

The presence of soluble proteins in purified virus preparations made the production of polyclonal antiserum difficult. Purifications which yielded intact CPSV particles visually free of host contaminates, as observed by electron microscopy, were used to develop polyclonal antiserum in chickens because of the ability to generate high titre antiserum from small amounts of immungen (1). Antiserum developed to non-aggregated CPSV particles in upper gradient fractions boosted at the final injection with non-aggregated virus showed no discrimination for psorosis infected tissue. Following cross absorption, weak specificity for psorosis infected material was observed in antigen coated indirect ELISA. This antiserum was designated CPCASI-15 and had a titre of 1:500 in indirect ELISA. CPCASI-15 was used in antigen coated indirect ELISA in an attempt to detect selected psorosis strains maintained at the Rubidoux greenhouse and at the USDA quarantine facility in Beltsville, Maryland. CPCASI-15 weakly detected a concave gum strain (CG-301) and a psorosis field strain (11542). A lesion bark strain of psorosis (251-3) gave an ELISA absorbance 2.3 times the control. CPCASI-15 failed to detect its homologous antigen (ps203m) in citron shoot-tips over 12 plates each with three repetitions when second and third flush tissue was used. This tissue type was selected for testing with the Beltsville isolates because it best represented the age and quality of the tissue available in Beltsville. This result also indicated that quality and age of tissue may be important for the obtaining positive results. CPCASI-15 failed to detect B-169 and B-168 Indian strains of psorosis in citron shoot-tips, and weakly detected (1.6 times control) B-85, the Florida 6E strain of citrus ringspot virus in Duncan grapefruit. Purified virus from cesium chloride gradients and Mexican Chili infected with ps203m gave absorbances 1.9 and 1.6 times background, respectively. Low absorbance readings of purified virus may have resulted from PEG contamination of purified virus preparations following PEG concentration of CsCl gradient fractions. Although the antiserum developed at this point reacts weakly in homologous reactions in indirect ELISA, it is the first antiserum developed to any psorosis agent for use in a diagnostic assay.

The flexuous rod-shaped particle associated with ps203m need further purification to yield particles in higher concentration exhibiting a lower degree of fragmentation. When this is achieved characterization of the virus particle will be possible. Characterization of the viral coat protein was difficult due to the presence of soluble plant proteins. Attempts to further purify the particle resulted in near complete loss of virus particles. Characterization of the viral RNA was attempted with many of the known RNA purifica-

tions. In some purification protocols, large amounts of virus were partitioned to gradient pellets. Unfortunately, the aggregated virus present in gradient pellets could not be denatured to a point where viral RNAs could be released (unpublished data). In fractions containing dispersed virus, the concentration of virus was so low that the RNA purified was at the level of losses during the procedure. Development of a diagnostic reagent would not only be useful for detection of psorosis in budwood sources, but could also be used experimentally to improve purification procedures by following the fate of the virus during purification. The presence of a flexuous rod-shaped particle associated with psorosis A, and a fine filamentous particle associated with citrus ringspot virus may represent the fact that the etiological agents of these diseases are different. Until reliable diagnostic reagents are developed for these two particles, comparative research cannot be conducted. Only after extensive experimentation can the relationship of these diseases to each other be elucidated, in addition to the role these particles have in causing disease. Whether these are the etiological agents of their respective diseases or non-contributing entities remains to be determined. Additional reagents to the CPSV particle are currently being prepared and will be used in direct comparative experiments with the Florida 6E strain of CRSV to determine the relationship, if any, of CPSV and CRSV. The virus particle purified from citron infected with ps203m shares many characteristics to viruses in the Carlavirus group. Carlaviruses as a group are 620-700nm in length and 12-13nm in diameter, have a particle morphology curved to flexuous, and genomes that code for a coat protein with Mr between 27-34Kd. The dsRNA patterns for several members of the Carlavirus group, established by Valverde and Dodds (36), consist of multiple dsRNA bands (usually one genomic dsRNA appximately 5.5 x 10^6 Mr and 1-3 slightly lower Mr dsRNAs) which in some viruses (DLV, and PVS) are clustered together and in other viruses are equidistant from each other (RCVMV). Although some evidence indicates that the properties of the virus particle isolated from citron infected with ps203m are consistent with members of the Carlavirus group, and the dsRNA is very similar in size and nature to RCVMV, the virus is described as Carlavirus-like and additional evidence is necessary to designate this virus as a member of the Carlavirus group.

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