An Unusual Viruslike Particle Associated with Golden Yellow Mosaic of Beans

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ABSTRACT

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The whitefly-transmitted pathogen that causes bean golden yellow mosaic disease in Puerto Rico was efficiently sap-transmitted to *Phaseolus vulgaris* 'Top Crop' or 'Diablo' by rubbing. Infectivity was associated with a viruslike nucleoprotein particle approximately 18 nm in diameter. Virus particles often occurred as aggregates in glutaraldehyde-fixed negatively stained preparations. Large amounts of phytoferritin also were present in extracts from infected plants. Phytoferritin in much smaller amounts, but not viruslike particles, was present in extracts of healthy plants. The nucleoprotein particles were separated from phytoferritin by rate-zonal sucrose density gradient centrifugation or polyethylene glycol precipitation. Plants inoculated with the purified nucleoprotein particles showed symptoms typical of bean golden yellow mosaic disease and were used successfully in transmission trials with the whitefly vector, *Bemisia tabaci.*

Golden yellow mosaic (GYM) comprises a group of bean diseases found in many areas of tropical America. The causal agents, which are transmitted by the whitefly *Bemisia tabaci* Genn., are the most important whitefly-borne pathogens of *Phaseolus vulgaris* L. in Central America and Brasil (8, 9, 12). The incidence of a similar disease can be extremely high in Puerto Rico (up to 100%) in the drier sections of the southern coast where beans are grown near infected stands of *Macroptilium lathyroides (Phaseolus lathyroides).*

According to Varma (20) *B. tabaci* is the most important whitefly vector of plant viruses, and in India it is the only known aleyrodid vector of plant pathogens. In Puerto Rico, whitefly-borne viruslike pathogens have been studied in detail (4), and all of them are spread by *B. tabaci.* Marchoux et al. (15) listed most of the diseases in which the causal agents are spread by whiteflies and gave tabulated information on their geographical distribution, modes of transmission, and host ranges.

The whitefly-transmitted pathogens causing bean golden yellow mosaic and other diseases were presumed to be viruses, but little progress was made in etiological studies until recent reports by Gálvez and Castaño (11) and Matyis et al. (16) who reported that spherical geminate particles were associated with GYM in El Salvador, Brasil, and Colombia. We have discovered that GYM disease in Puerto Rico is associated with a viruslike nucleoprotein similar to that reported to be associated with GYM in Central and South America. In this report we describe purification methods for and some properties of this new virus.

MATERIALS AND METHODS

The Puerto Rican isolate of the GYM agent used was obtained originally from *Phaseolus lunatus* L. 'Haba de Tocon.' It was maintained in that cultivar and frequently was used for testing new lines of beans and lima beans. The isolate was found to be identical with respect to symptoms and host range to GYM isolates affecting beans and *M. lathyroides* in the field. Additional details about GYM and its causal agent in Puerto Rico have been published previously (1, 2, 3, 4, 5).

The primary leaves of 10-day-old bean seedlings (*P. vulgaris* 'Top Crop') were inoculated by rubbing with inoculum prepared from GYM-infected Top Crop beans approximately 2 weeks after inoculation. Freshly inoculated plants were placed in an ISCO growth chamber operating at 32 C with 14-hour day length.

Tissue for virus purification was harvested from infected plants about 14 days after inoculation and from buffer-rubbed or noninoculated plants of the same age. Tissues were frozen on dry ice, crushed to a coarse powder, and homogenized with a Waring Blendor in 0.1 M sodium phosphate buffer, pH 7.8, containing 10 mM sodium ethylenediaminetetraacetate (EDTA) and 1 mM cysteine (3 ml buffer per gram of tissue). Homogenized

tissues were squeezed through four layers of cheesecloth and clarified by centrifugation (12,000 rpm, 20 minutes, in a Sorvall SS-34 rotor). Clarified homogenates were subjected to three cycles of ultracentrifugation (Beckman 30 rotor, 4 hours, 27,000 rpm) and low-speed centrifugation (Sorvall SS-34 rotor as above).

In other experiments, differential centrifugation was preceded by treatment with polyethylene glycol. Clarified extracts were adjusted to 0.2 M with NaCl and then 4 g of polyethylene glycol (PEG = Carbowax 6000, Union Carbide Corp.) was added per 100 ml of extract. The mixture was stirred at 4 C for 2 hours, and the precipitate was collected by centrifugation (10,000 rpm, 20 minutes). The precipitated virus was resuspended in 0.1 M sodium phosphate, pH 7.8, and clarified by low-speed centrifugation. Both the virus solution and the supernatant liquid remaining after PEG precipitation were subjected to differential centrifugation.

Analysis of purified preparations was by rate-zonal density gradient ultracentrifugation through 10 to 40% (w/v) linear sucrose gradients (Beckman SW 27.1 rotor, 4 hours, 25,000 rpm). Samples collected from the gradients were concentrated by ultracentrifugation and resuspended in 0.1 M potassium phosphate, pH 7.8, for infectivity tests and electron microscopy.

Material for electron microscopy was negatively stained with 2% phosphotungstic acid, 1.5% ammonium molybdate, or 1% uranyl acetate on carbon-stabilized Formvar-coated grids and observed in a JOEL JEM 100-C electron microscope operated at 60 KV. Some samples were fixed for 20-30 minutes in 1.5% glutaraldehyde before negative staining.

Plants inoculated with purified virus were used in transmission trials with *B. tabaci*. Twenty-five virus-free adult whiteflies first were caged for 24 hours on infected plants inoculated 1 month earlier, and then on healthy Top Crop seedlings on which they were allowed to feed for several days. Plants exposed to whiteflies that had fed on infected plants, and other plants either exposed to whiteflies not fed on infected plants or not exposed to whiteflies at all, were observed for symptoms typical of golden yellow mosaic disease.

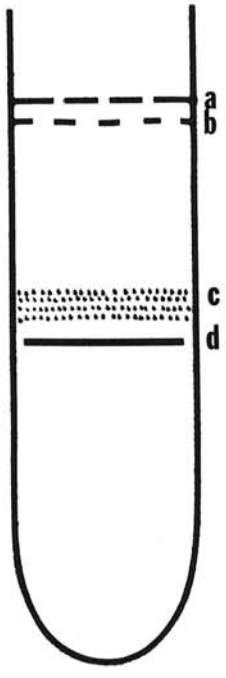

Fig. 1. Diagram showing the relative positions of phytoferritin and nucleoprotein bands following rate-zonal sucrose density gradient centrifugation of extracts from GYM-infected Top Crop beans for 4 hours at 25,000 rpm (Beckman SW 27.1 rotor). Legend: a, top of sample; b, top of 10% to 40% linear sucrose gradient; c, a diffuse yellow-orange band containing phytoferritin; and d, a narrow light-scattering band containing viruslike particles.

RESULTS

Mechanical transmission and symptom development.—The GYM agent was sap-transmitted with high efficiency (up to 100%) by standard techniques when inoculum was prepared from systemically infected plants inoculated 2 weeks earlier and the inoculated plants were kept at 32 C. Use of inoculum from older plants gave less efficient transmission, although newly emerged leaves from older infected plants also were used successfully. Top Crop beans showed symptoms within 7 days and often as early as 5 days after inoculation. Symptom appearance following greenhouse inoculation of Diablo or Red Kidney beans in Puerto Rico took 14 days or more. No careful comparisons between bean cultivars have been made in the growth chamber, but in a few experiments conducted in the greenhouse, Top Crop beans showed symptoms several days sooner than did Diablo beans when the plants were grown under the same conditions and inoculated at the same time.

Purification.—Pellets obtained from extracts of infected plants after the third cycle of differential centrifugation consisted of nearly transparent areas

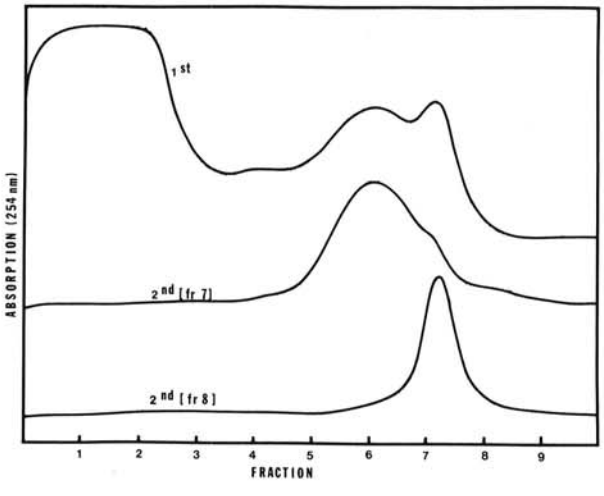

Fig. 2. Sedimentation profile of partially purified extract from infected Top Crop beans after first rate-zonal density gradient ultracentrifugation (upper curve) and separation of phytoferritin (fraction 7) and viruslike nucleoportein (fraction 8) by a second rate-zonal ultracentrifugation (lower curves).

mixed with unevenly distributed red or orange pigment. Rate-zonal centrifugation in sucrose density gradients showed two bands (Fig. 1) corresponding in position to the two peaks observed when the contents of the gradients were scanned at 254 nm (Fig. 2). The upper band (Fig. 1) was somewhat diffuse and orange-yellow in color; the lower band was colorless and discrete. The bands were separated further by repeating the rate-zonal density gradient centrifugation with each band (Fig. 2, lower curves). The lower discrete band having the light-scattering and ultraviolet-light absorbing (Fig. 3) properties of nucleoprotein was obtained essentially free of material that sedimented at the position of the upper orange-yellow diffuse band. Separation of the two bands also was achieved with PEG at 4% (w/v) in the presence of 0.2 M NaCl which completely precipitated the material corresponding to the lower band (nucleoprotein), but not that corresponding to the upper band (Fig. 4).

Electron microscopy and sedimentation properties.—Early attempts to detect particles in sucrose density gradient fractions from infected plants yielded only phytoferritin particles (Fig. 5-B). These were concentrated in gradient fractions corresponding to the orange diffuse band (Fig. 1) or the middle curve of Fig. 2. When the peak fractions represented in the bottom curve of Fig. 2 or Fig. 4 were fixed with glutaraldehyde before staining, the particles in Fig. 5-A were observed. The diameters of the particles in Fig. 5-A and 5-B are 18.0 nm and 10.4 nm, respectively. In corresponding sucrose density gradient fractions from extracts of healthy plants, phytoferritin particles were seen occasionally, but the larger 18-nm particles were not. The sedimentation coefficient of these particles was estimated by comparing their sedimentation velocity on linear sucrose density gradients with that of top, middle, and bottom components of cowpea mosaic virus (19). Values of 95S and 76S were obtained for the nucleoprotein and the phytoferritin, respectively (Fig. 6).

Infectivity tests.—Fractions collected from sucrose gradients of purified extracts of healthy and GYM-infected Top Crop beans were each used to inoculate 10 bean plants. No plants inoculated with fractions from the gradient made with the healthy tissue became infected. From the extract of infected tissue only those fractions that contained nucleoprotein were infectious. Results of other experiments with nucleoprotein and phytoferritin separated by density gradient centrifugation (Fig. 2) or PEG fractionation (Fig. 4) also showed that the infectivity was associated only with the nucleoprotein band.

Transmission trials.—The purification and electron microscopy experiments described above were conducted with an isolate that originated from a plant that was mechanically inoculated in Puerto Rico, and the isolate was maintained for 10 months by graft transmission and mechanical inoculation in insect-free cages and isolated growth chambers at Urbana. After the purification experiments described here, we did whitefly transmission trials in Puerto Rico with plants mechanically inoculated with purified nucleoprotein to confirm that it was still whitefly transmissible. The results showed that whiteflies were able to transmit the pathogen to healthy Top Crop beans resulting in the same disease symptoms as those exhibited by the source plants. Noninoculated control plants remained free of symptoms.

DISCUSSION

The results of purification experiments showing a viruslike nucleoprotein particle of unusually small size associated with GYM-infected beans, together with positive infectivity tests on fractions containing nucleoprotein and subsequent whitefly transmission trials, strongly support the conclusion that GYM from Puerto

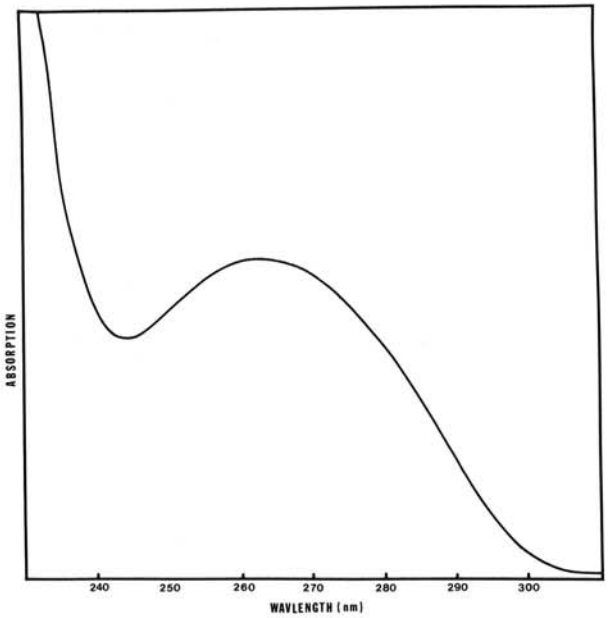

Fig. 3. Ultraviolet light absorption spectrum of purified golden yellow mosaic virus following purification by rate-zonal sucrose density gradient ultracentrifugation. The ratio of absorption at 260 nm and 280 nm is 1.4.

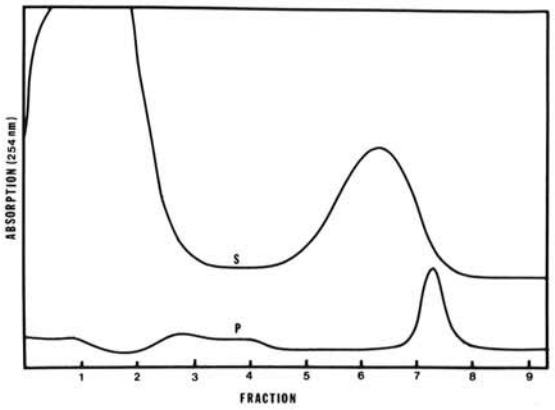

Fig. 4. Sedimentation profiles of partially purified extracts from GYM-infected Top Crop beans following fractionation with PEG. Full-scale deflection corresponds to 2.5 absorbance units at 254 nm. Abbreviations: S, profile of portion of extract remaining in supernatant liquid after PEG precipitation; and P, profile of portion of extract precipitated by PEG treatment.

Rico is caused by the viruslike particle. Logistical and quarantine considerations have so far prevented us from attempting artificial feeding or injection of whiteflies with purified nucleoprotein. Evidence from such experiments together with our mechanical transmission results would complete the requirements of Koch's postulates as nearly as can be accomplished with viruslike pathogens.

Other viruslike particles similar in some respects to

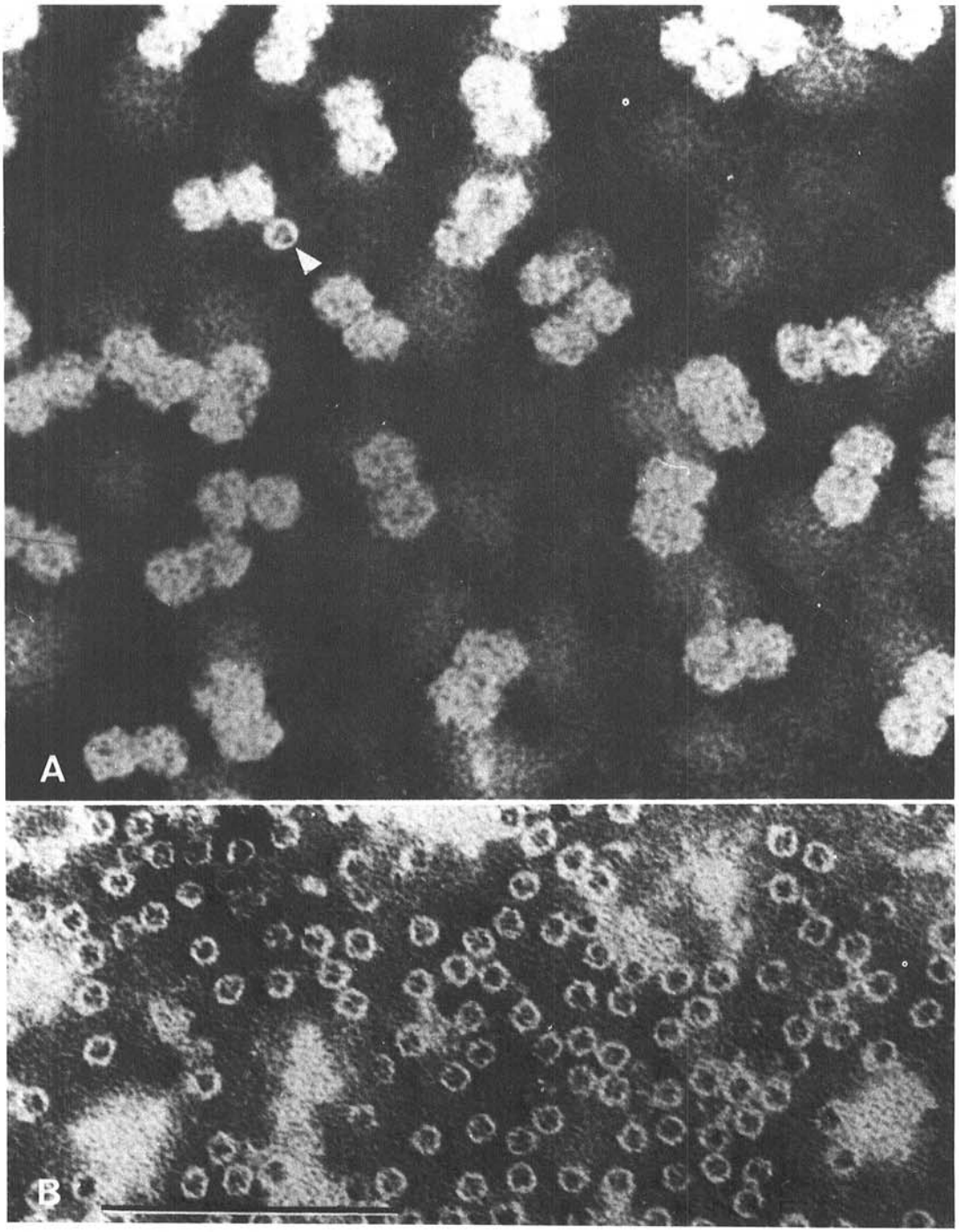

Fig. 5-(A, B). Electron micrographs of purified nucleoprotein and phytoferritin negatively stained with 2% phosphotungstic acid, pH 7.0. **A)** Nucleoprotein fixed 30 minutes with 2% glutaraldehyde. Arrow marks a phytoferritin particle. **B)** Phytoferritin without fixation. Bar equals 100 nm.

those described here have been reported in the past. Bock et al. (6) described preparations of maize streak virus from East Africa containing predominantly geminate particles with configuration and dimensions similar to GYMV. The report by Gálvez and Castaño (11) indicated that the virus associated with golden yellow mosaic of beans in El Salvador and Colombia was geminate and had a diameter of 15-20 nm. Matyis et al. (16) reported spherical geminate particles 12-13 nm in diameter associated with three diseases, including one of beans, whose causal agents were transmitted by *B. tabaci* in Brasil, but they failed to establish the infectivity of the purified particles obtained from diseased beans. Curly top virus purified from Turkish tobacco was reported to be 18-20 nm in diameter (17). Finally, Tuveson et al. (18) found viruslike particles in *Neurospora crassa* having some properties similar to those of GYMV. These viruses and viruslike particles may represent a new class of virus pathogens.

We do not know why the particles of GYMV usually are aggregated, or whether that phenomenon has any biological significance. It may be that the geminate particles represent the infectious entity, as claimed in a recent report by Gálvez and Castaño (11). We have found, however, that extracts made with buffer containing sodium EDTA are infectious, and thus have no reason to believe that the aggregated particles were dissociated by EDTA treatment with loss of infectivity as reported by Galvez and Castano. Moreover, we would expect that fixation with formaldehyde (11) or glutaraldehyde could cause otherwise single particles to become aggregated. We have seen single particles as well as aggregates of three or more particles in addition to geminate aggregates. Repeated attempts to obtain electron microscope images of virus particles without fixation prior to negative staining with phosphotungstic acid, ammonium molybdate, or uranyl acetate were unsuccessful. This result suggests structural differences between GYMV and the viruslike particle from *N. crassa* and maize streak virus, which were detected in unfixed negatively stained preparations (R. W. Tuveson, *personal communication*) (6).

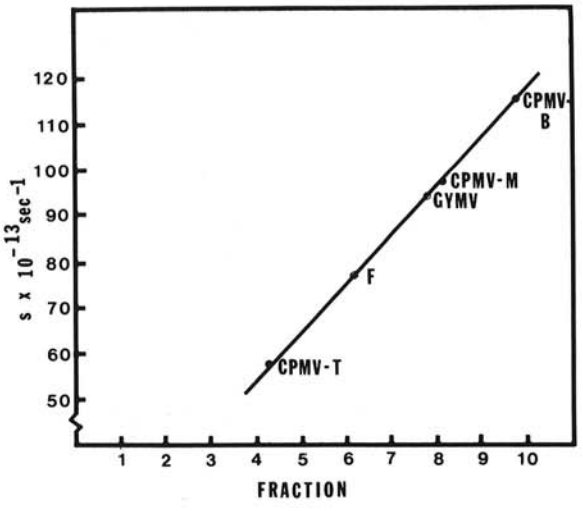

Fig. 6. Estimation of sedimentation coefficient of phytoferritin and golden yellow mosaic virus (GYMV) by comparison with sedimentation of cowpea mosaic virus (CPMV) components in rate-zonal centrifugation through linear sucrose density gradients. Abbreviations: CPMV-T, top component of CPMV (58S); F, phytoferritin; GYMV, golden yellow mosaic virus; CPMV-M, middle component of CPMV (98S); and CPMV-B, bottom component of CPMV (115S).

We estimated the sedimentation coefficient of GYMV particles to be approximately 95S in sucrose density gradients containing 5 mM sodium EDTA. In the same tests, phytoferritin had a mean sedimentation coefficient of approximately 70S to 80S, corresponding to values reported for other ferritin particles from fungi, plants, and animals (7, 10, 13, 14). Even though it is possible that the geminate nucleoprotein particles seen in the electron microscope could have a sedimentation coefficient of 95S, it is also possible the 95S peak corresponds to the monomeric units. A convincing explanation of the predominately geminate aggregates seen in the electron micrographs must await the results of further experiments on nucleic acid content, particle density, analytical ultracentrifugation, and electron microscopy without fixation.

Further analysis of high-resolution electron micrographs will be required to establish the precise morphology of GYMV particles. Preliminary evaluation of our micrographs suggests that the particles are hexagonal in outline when viewed along their presumed axis of 3-fold symmetry, but we note with interest the recent report of Matyis et al. (16) indicating that one of the two particles in their doublet may be hexagonal and the other pentagonal.

Antiserum production with the virus now is possible because we have found methods that result in complete separation of the virus from phytoferritin. We are now attempting to purify sufficient virus for antiserum production, and to characterize the nucleic acid and protein components of this virus.

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