Purification and Further Characterization of Panicum Mosaic Virus

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ABSTRACT

Panicum mosaic virus (PMV) infected 15 additional grass species, but 60 dicotyledonous species were immune. Desiccated tissues contained infectious virus after 7 years of storage at 4 C, but purified PMV was unstable. PMV was purified from Ohio 28 corn by chloroform-butanol clarification and differential centrifugation. $A_{260/280}$ was 1.5 and dilutions to $A_{260} = 0.00004$ were infectious. Two ultraviolet-absorbing components sedimenting at 42 S and 109 S were detected in sucrose density gradients. Only the 109 S component was infectious, and mixing the components did not enhance infectivity. Isometric particles, 28 nm in diameter, were observed in purified, PTA-stained preparations. PMV was not serologically related to brome mosaic, barley stripe mosaic, or foxtail mosaic viruses, but was serologically related to the virus which causes decline of St. Augustine grass in Texas.

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Additional key words: Panicum, Digitaria, Setaria, millet, corn, St. Augustine Decline, grass viruses.

Panicum mosaic virus (PMV) was first observed in 1953 on switchgrass (*Panicum virgatum* L.) and had been reported only in Kansas (10). However, St. Augustine Decline virus (SADV) from Texas was recently shown to be serologically related to PMV (5, 6). PMV is sap-transmissible, and only infects a narrow host range in the Gramineae (4, 7, 9, 10, 11). Canares (4), using crabgrass (*Digitaria sanguinalis* L.) for virus production and assay, found PMV had these properties: dilution end-point 10^{-5}, longevity in vitro 14-16 days, and thermal inactivation point 85 C. PMV was neither seedborne in crabgrass and switchgrass nor transmitted by aphids (9).

We report the purification and additional characteristics of PMV.

MATERIALS AND METHODS.—PMV was increased from infected leaves of switchgrass desiccated and stored at 4 C under $CaCl_2$ in 1965 by Canares. The stock culture was perpetuated in switchgrass, crabgrass, or corn (*Zea mays* L. 'Ohio 28'). Leaves with distinct symptoms were ground, using mortar and pestle, in 0.02 M potassium phosphate buffer, pH 7.0 (PPB), at about 1:50 dilution (w/w). The extract was rubbed on Carborundum-dusted leaves with a cheesecloth pad. All plants were started from seed, grown in the greenhouse, and inoculated while very young. In host-range studies, back assays to crabgrass were done 3 weeks after inoculation to test for symptomless hosts.

Virus purification.—Ohio 28 corn was used for virus purification. Seedlings were inoculated at the two- to three-leaf stage and harvested 14 to 21 days later. Leaves were cut up and blended in cold 0.10 M PPB and 1% 2-mercaptoethanol to give a 1:3 (w:v) dilution. The liquid was squeezed through cheesecloth and used to blend additional batches. An equal volume of a cold chloroform:butanol mixture (1:1, v/v) was added slowly with gentle stirring. The emulsion was kept at 4 C for 30 minutes, then centrifuged 7 minutes at 5,000 *g*. The aqueous phase was filtered through glass wool and centrifuged 2 hours at 147,000 *g* and 4 C. Pellets were resuspended in 0.02 M PPB overnight at 4 C; resuspension was completed the next day by gentle shaking or stirring. Virus suspensions were pooled and centrifuged 10 minutes at 12,000 *g*. After two additional cycles of differential centrifugation (90 minutes at 269,000 *g*, then 10 minutes at 12,000 *g*), final pellets were resuspended in 2-3 ml 0.02 M PPB/100 g tissue. These were combined, centrifuged 10 minutes at 12,000 *g*, and the resulting supernatant (purified virus) was stored at 4 C.

The purification procedure was modified when large volumes were involved. After grinding and cheesecloth filtration, the liquid was centrifuged 10 minutes at 12,000 *g*. Supernatants were combined and brought to 8% (w:v) polyethylene glycol 6,000 (PEG) and 0.2 M NaCl. The mixture was stirred for 2 hours at room temperature and the virus collected by centrifugation for 10 minutes at 8,000 *g*. Pellets were dispersed in one-eighth the original volume of 0.10 M PPB and stored overnight at 4 C. The resulting suspension was slowly mixed with an equal volume of a cold chloroform:butanol mixture (1:1, v/v) and kept at 4 C for 30 minutes. The remaining purification steps were as described above.

Sedimentation coefficients of PMV components.—Linear-log sucrose density gradients (2) were used to estimate the sedimentation coefficients of PMV components by comparison with those reported for other viruses. Viruses used as standards were brome mosaic (BMV), tobacco mosaic (TMV), and cowpea mosaic virus (CPMV). BMV was purified from Reno barley and TMV from Havana 38 tobacco as described by Brakke and Van Pelt (2). CPMV was purified from Early Ramshorn cowpea as described by Semancik and Bancroft (8). Sedimentation coefficients used for these viruses were: BMV, 79 (2); TMV, 190 (2); and CPMV, 60, 98, and 119 (3).

Density gradients were prepared essentially as described by Brakke and Van Pelt (2). However, it was necessary to reduce the volume of the two most concentrated sucrose solutions to compensate for the shorter axis of rotation of the International B-60 ultracentrifuge. Gradients were stored 12 to 16 hours at 4 C, and immediately prior to use, 1.5 ml was carefully removed from the top of the gradient and 1 ml of sample (A_{260} = 0.5 to 1.2) layered on the gradient. After centrifugation for 2-3 hours at 110,000 *g* and 15 C, gradients were fractionated using an ISCO Model D

density-gradient fractionator. Fractions were dialyzed overnight in 0.02 M PPB, concentrations were adjusted (spectrophotometer), and the fractions were inoculated to crabgrass, millet [*Setaria italica* (L.) Beauv., German strain "R"], or Ohio 28 corn.

Electron microscopy.—Purified PMV was stained by incubating a mixture (1:1, v/v) of neutralized 1% phosphotungstic acid (PTA) and PMV for 20 to 25 minutes at 4 C prior to depositing a drop of the preparation on a carbon-backed, formvar-coated grid. Purified TMV was the calibration standard. Immediately after preparation, grids were examined under an RCA-EMU-4 or Hitachi Hu-11B electron microscope.

Serology.—One milliliter of purified PMV (A_{260} = 5 to 7) was emulsified with 1 ml of Difco incomplete Freund's adjuvant and injected intramuscularly into a rabbit. A total of eight injections were spaced at 4-day intervals, and the rabbit was bled for antiserum 7 days after the last injection.

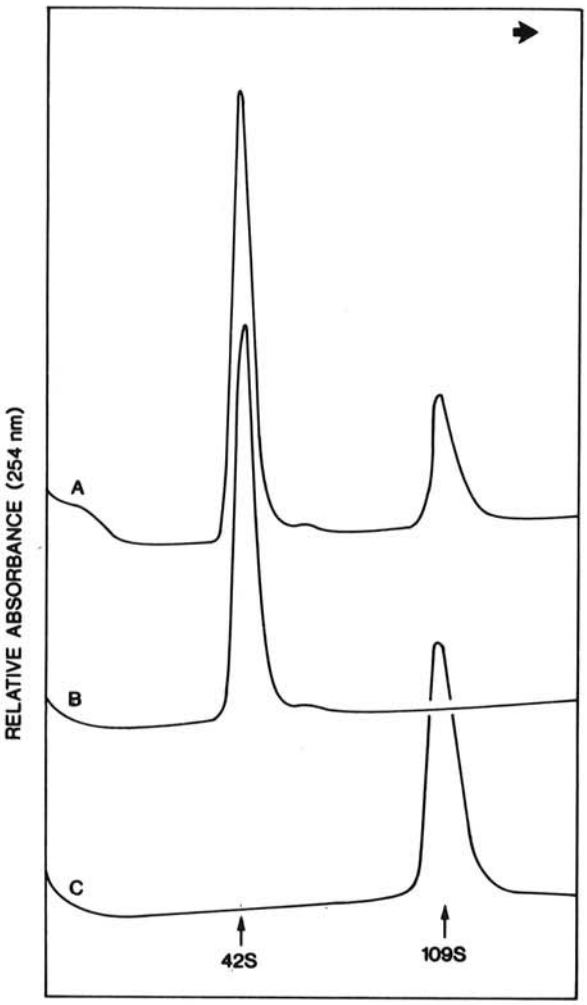

Fig. 1-(A to C). Sedimentation patterns of **A)** unfractionated Panicum mosaic virus, and the separated **B)** 42 S and **C)** 109 S components. Samples (A_{260} = 0.4 to 1.0) were centrifuged in sucrose linear-log density gradients for 3.0 hour at 110,000 *g* and 15 C. The direction of sedimentation is indicated.

RESULTS.—*Host range and symptoms.*—In addition to the grass species reported as susceptible by Sill and coworkers (9, 10, 11), several new hosts were found. Local lesions were induced on several cultivars, inbreds, selections, and introductions of *Avena sativa* L., *Secale cereale* L., *Hordeum vulgare* L., *Triticum aestivum* L., *T. monococcum* L., *Aegilops bicornis* Jaub. & Spach., *A. variabilis* L., and *Sorghum bicolor* (L.) Moench. Lesions on *S. bicolor* remained faint and small. Lesions on all others ranged from greenish to golden yellow; they enlarged, coalesced, and occasionally became necrotic. Sweet corn cultivars Golden Cross Bantam and Marcross became systemically infected with faint mosaic symptoms. On Ohio 28 corn, a systemic chlorotic striping developed on plants inoculated in the two- to three-leaf stage, but plants inoculated at later stages were generally symptomless. Chlorotic local lesions, followed by systemic mosaic and striping, developed on *Briza maxima* L., *Aegilops juvenalis* L., *A. triuncialis* L., and *Triticum timopheevi* Zhukolv. On *T. durum* Desf., yellow chlorotic lesions were followed by a faint systemic mosaic, but plants eventually recovered. *Coix lacryma-jobi* L. was a symptomless host. On all hosts, symptoms developed best above 24 C.

None of the following 60 species, representing 12 families, were infected in repeated trials: Aizoaceae—*Tetragonia expansa* Murr.; Amaranthaceae—*Amaranthus gangeticus* L., *Celosia plumosa* Hort., *Gomphrena globosa* L.; Apocynaceae—*Vinca rosea* L.; Chenopodiaceae—*Beta vulgaris* L., *Chenopodium amaranticolor* Coste & Reyn., *C. ambrosioides* L., *C. bonus-henricus* L., *C. capitatum* (L.) Aschers., *C. foetidum* Lam., *C. murale* L., *C. quinoa* Willd., *C. rubrum* L., *C. urbicum* L., *Spinacea oleracea* L.; Compositae—*Helianthus annuus* L., *Lactuca sativa* L., *Tithonia speciosa* Hook., *Zinnia elegans* Jacq.; Convolvulaceae—*Ipomoea purpurea* (L.) Roth; Cruciferae—*Brassica oleracea* L. var. *capitata, B. rapa* L., *Raphanus sativus* L.; Cucurbitaceae—*Cucumis melo* L., *C. sativus* L., *Cucurbita maxima* Dcne., *C. pepo* L., *Momordica balsamina* L.; Labiatae—*Torenia fournieri* Lindl.; Leguminosae—*Cassia occidentalis* L., *Cyamopsis tetragonoloba* L., *Glycine max* (L.) Merr., *Medicago sativa* L., *Phaseolus angularis* (Willd.) W. F. Wright, *P. aureus* Roxb., *P. limensis* Macf., *P. vulgaris* L., *Pisum sativum* L., *Vigna cylindrica* (L.) Skeels, *V. unguiculata* (L.) Walp.; Solanaceae—*Capsicum frutescens* L., *Datura metel* L., *D. stramonium* L., *Lycopersicon esculentum* Mill., *Nicandra physalodes* Gaertn., *Nicotiana debneyi* Domin., *N. glauca* Graham, *N. glutinosa* L., *N. megalosiphon* Heurch & Muell., *N. paniculata* L., *N. rustica* L., *N. sylvestris* Speg. & Comes, *N. tabacum* L., *Petunia hybrida* Vilm., *P. violacea* Lindl., *Physalis floridana* Rydb., *Solanum melongena* L.; Umbelliferae—*Apium graveolens* L., *Daucus carota* L.

Seed transmission.—Infected *Setaria italica* (L.) Beauv., *S. lutescens* (Weigel) Hubb., *Panicum maximum* L., and *Briza maxima* L. were allowed to set seed in the greenhouse. More than 1,000 seedlings were grown of each species, and no visual evidence of seed transmission was obtained.

Properties of Panicum mosaic virus.—Infectious virus could be recovered from crabgrass and *Panicum* spp. desiccated in 1965 by Canares, and for at least 1 year from crabgrass tissues lyophilized and liquid-nitrogen frozen by the American Type Culture Collection (ATCC Virus No. PV 140).

Several methods were used to purify PMV. These included differential centrifugation, clarification with organic solvents, PEG precipitation, and combinations of these methods. Differential centrifugation following chloroform-butanol clarification of plant sap or PEG precipitates was most effective. Preparations of purified PMV were opalescent. The $A_{260/280}$ ratio was 1.5 and an average yield of $A_{260} = 0.5$ was obtained per gram of corn tissue. Greater yields were obtained from crabgrass, but these preparations were slightly brownish, even with addition of 2-mercaptoethanol. Preparations were infectious to $A_{260} = 0.00004$ within 1 week after purification, but specific infectivity declined rapidly with storage.

Purified virus preparations contained two components sedimenting at 42 S and 109 S (Fig. 1). These components were readily separated (Fig. 1), and infectivity was associated only with the 109 S component (Table 1). Mixing the two components did not significantly enhance the specific infectivity (Table 1).

After centrifugation, an opalescent band was readily observed at the 109 S level when gradients were viewed in a narrow, vertical beam of light. However, no band was visible at the 42 S level except with excessive sample loads ($A_{260} = 8$ to 12). Inoculation with only the 109 S component resulted in typical symptom development and recovery of both the 42 S and 109 S components by purification. Inoculation with only the 42 S component resulted in no symptom development and recovery of neither component.

The ultraviolet (UV) absorption spectra of the 42 and 109 S components were similar and characteristic of nucleoproteins. The absorption maxima were at 262 nm and the minima at 243 nm. The 260/280 ratio of the 42 S component was always equal to or slightly higher than that of the 109 S component. In 10 different preparations the average 260/280 ratios were 1.53 and 1.47 for the 42 S and 109 S components, respectively.

Electron microscopy of purified PMV revealed isometric particles averaging 28 nm in diameter. Many particles were in various stages of degradation, even shortly after purification. PTA staining above 4 C increased degradation. After long storage it was difficult to find any intact particles.

Antiserum prepared to purified PMV possessed a titer of 1:1,280 when tested by the microprecipitin method (1) with PMV at $A_{280} = 0.03$. The Ouchterlony agar double-diffusion method (1) was used to test the serological relationship of PMV to other grass viruses. Antisera to BMV, barley stripe mosaic virus, SADV, and foxtail mosaic virus were tested against purified PMV. Heterologous precipitation lines were observed only with the SADV antiserum; each virus reacted with its homologous antiserum.

DISCUSSION.—Most small grains were previously reported immune to PMV (10). We were able to infect some small grains, but infection was localized in inoculated leaves. Additional susceptible species were found; however, the host range of PMV was limited to grasses, and seed transmission could not be demonstrated.

TABLE 1. Specific infectivity assays of separated and mixed 42 S and 109 S components of Panicum mosaic virus on foxtail millet

A_{260}[a]	42 S	109 S	42 S + 109 S (1:1)
0.01	0/35[b]	43/46	35/37
0.003	0/38	27/38	30/43
0.001	0/34	25/48	21/46
0.0004	0/30	10/39	6/29
0.0001	0/41	3/40	5/48
0.00004	0/40	0/38	2/51

[a]Preparations at $A_{260} = 0.100$ made using spectrophotometer. Dilutions of these are reported above.
[b]Fraction is number infected per number inoculated.

PMV is very stable when frozen, lyophilized, or dried in plant tissue. It is quite stable in sap at room temperature (14 to 16 days) and when heated (85 C). However, purified PMV appeared to be unstable and to undergo spontaneous degradation. This was confirmed by electron microscopy, and by the rapid decline in specific infectivity with storage. Degradation of PMV occurred regardless of the purification procedure or storage medium, and may reflect an intrinsic instability of this virus after purification.

Two centrifugal components were detected in all crude and purified preparations of PMV. It is clear that the 109 S component possesses sufficient genetic information to cause infection and to replicate itself; and, also, that infection by the 109 S component results in the synthesis of the 42 S component. However, the role and origin of the 42 S component are unclear. Inoculation with the 42 S component does not result in detectable infection or replication of that component.

Viral capsids devoid of nucleic acid accompany infection by many plant viruses. However, the UV absorption spectrum of the 42 S component is typical of a nucleoprotein, and its 260/280 ratio is equal to or higher than that of the 109 S component. These properties, lack of infectivity and its production in a 109 S component-directed infection, suggest that the 42 S component may arise from the 109 S component by loss of a significant proportion of coat protein subunits. Such a particle would possess a low sedimentation coefficient and a high 260/280 ratio, be susceptible to host nucleases and thereby noninfectious, and be detectable in the UV in sucrose density gradients. Some alternative possibilities are that the 42 S component originates by encapsidation of host nucleic acid in viral coat protein, or that some other host constituent attaches to the 109 S component rendering it noninfectious and altering its sedimentation characteristics. Experiments to determine the role, origin, and additional properties of the 42 S component are in progress.

Lee (5) and Lee and Toler (6) have demonstrated that antiserum to PMV reacts with SADV from Texas. Our results concur with those of Lee and Toler (6). In addition, we have demonstrated the heterologous serological reaction between PMV and antiserum to SADV. Since PMV was described prior to SADV, Lee (5) has designated SADV as the St. Augustine Decline strain

of PMV. We support this designation. Certain data for these two strains (the number of centrifugal components, sedimentation coefficients, dilution end point, and longevity in vitro) are not in complete agreement. However, this may reflect virus strain differences or differences in experimental procedures. Properties of both strains are presently being compared.

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