A New Type of Elongated Virus Isolated from Apple Trees Containing the Stem Pitting Agent

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ABSTRACT

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A flexuous, filamentous virus was always transmitted by mechanical inoculation to *Nicotiana occidentalis* from apple trees infected with the agent, causing apple stem pitting disease. A virus with particles 12–15 nm wide and 800 nm long was purified from infected *N. occidentalis* leaves. The virus particles readily formed end-to-end aggregates. Prominent peaks in particle length distribution of 800, 1,600, 2,400, and 3,200 nm were observed in leaf dip preparations of diseased *N. occidentalis* subsp. *obliqua*. The particles contained a single species of RNA of M_r 3.1 × 10^6 and a major coat protein of M_r 48,000. The virus particles were found in the cytoplasm of mesophyll cells. These data suggest that the virus does not fall into any recognized group of plant viruses.

Apple stem pitting disease was first recognized in 1954 (13). In 1956, Guengerich and Millikan (3) transmitted the causal agent of stem pitting disease with buds and bark patches. Since then, the causal agent has been considered to be apple stem pitting virus (ASPV) although, until now, no viruslike particles have been associated with the disease.

ASPV causes a severe topworking disease in Japan where ASPV-sensitive *Malus sieboldii* (Regel) Rehd. is used as one of three common rootstocks (17). Although a virus-certification program is being developed in Japan, there is no quick and easy way to index ASPV, which infects many apple trees but remains latent. Currently, the only way to test for ASPV is by grafting to sensitive hosts such as Virginia Crab, Spy 227, or *Malus sieboldii* 'MO-65' (19). However, van Dijk et al (16) and van der Meer (15) succeeded in transmitting a virus from apple trees with stem pitting to *Nicotiana occidentalis* Wheeler '37B.' This paper describes some of the properties of a virus that has been transmitted to *N. occidentalis* from apple trees in Japan and its association with apple stem pitting disease. We also demonstrate that ASPV is a new type of elongated virus.

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Abstracts describing part of this research have been published (7,18).

MATERIALS AND METHODS

Virus source. The isolates of ASPV used in this experiment, B-12, B-30, B-39, B-49, and B-72, were gifts from G. I. Mink (2) and were maintained in apple seedlings. Most of the work was done with the B-39 isolate. Reactions of *Malus* indicator plants to these isolates have been described previously (17,19). The isolates are free from other known apple viruses.

Mechanical inoculation and plants. Apple petals or young leaves were ground in 0.01 M phosphate buffer (pH 7.0) containing 2% nicotine and were inoculated to *Nicotiana occidentalis* subsp. *obliqua* Wheeler. Once the virus was transmitted to *N. occidentalis*, the inoculum was prepared in 0.01 M phosphate buffer, pH 7.0. For host range studies, six seedlings of each of 15 species were inoculated. Local or systemically infected tissue from test plants were back-inoculated to *N. o.* subsp. *obliqua* after 15–20 days. Carborundum was used as an abrasive in all mechanical inoculations. For purification, the virus was propagated in *N. occidentalis* '37B,' which was grown under short-day conditions. *N. o.* subsp. *obliqua* was used as a local lesion host.

Purification. Fresh leaves (75 g) were homogenized in 300 ml of 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1% thioglycollic acid and squeezed through four layers of gauze. After centrifugation at 5,000 *g* for 10 min, the supernatant was clarified by adding, drop by drop, 6 ml of 1 M Na_2HPO_4 followed by 1.5 ml of 2 M $CaCl_2$ per 100 ml of extract. The mixture was stirred gently for 30 min and centrifuged at 5,000 *g* for 10 min. The supernatant was centrifuged over a 5-ml cushion of 20% sucrose in phosphate buffer at 120,000 *g* for 1.5 hr in a Hitachi RP42 rotor. The pellet was suspended in 0.01 M phosphate buffer (pH 8.0) containing 0.5% Triton X-100 and centrifuged for 10 min at 5,000 *g*. The supernatant was then centrifuged at 100,000 *g* for 1.5 hr in a Hitachi 55T rotor over a 1-ml cushion of 30% sucrose in phosphate buffer. The pellet was resuspended in 0.01 M borate-HCl buffer containing 0.5 M urea (pH 8.0), and the suspension was stored overnight at 4 C before centrifugation through a linear 10–40% sucrose gradient prepared in 0.01 M borate-HCl buffer containing 0.5 M urea (pH 8.0) at 80,000 *g* for 3 hr in a Hitachi SRP28SA rotor. The virus-containing zones were collected and dialyzed against phosphate buffer. Virus particles were alternatively purified by equilibrium density gradient centrifugation. The virus suspension was layered onto a 50–70% (v/v) linear Omnipaque 350 gradient prepared in phosphate buffer. Centrifugation was at 8 C for 20 hr at 80,000 *g* in a Hitachi SRP28SA rotor or at 130,000 *g* in a Hitachi RPS50 rotor. Omnipaque 350 (Daiichi Seiyaku Co. Ltd., Tokyo) is a nonionic radiographic contrast medium containing 754.9 mg of iohexol and 0.1 mg of calcium disodium edetate per 1 ml. The virus zone was collected, diluted with phosphate buffer, and pelleted.

Protein analysis. The purified virus preparation was suspended in 0.0625 M tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol and boiled for 2 min. Electrophoresis was performed on a 12% gel with the use of the Laemmli discontinuous buffer system (8). (Standards for molecular weight determinations were purchased from Bio Rad, Richmond, CA.)

Nucleic acid analysis. Virus particles were suspended in STE buffer (0.1 M NaCl, 50 mM tris, 1 mM EDTA, pH 7.2) containing 0.25% SDS and treated with proteinase-K at a concentration of 500 μg/ml for 2 hr at 37 C. After adding SDS to a final concentration of 1%, the nucleic acid was extracted by shaking

with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. After low-speed centrifugation, the aqueous layer was recovered and then re-extracted with water-saturated phenol. The nucleic acid was precipitated from the aqueous layer by adding 2.5 vol of ethanol and stored at −20 C. Leoning's (9) buffer system was used to electrophorese extracted nucleic acid on 2% polyacrylamide-0.5% agarose composite gels. Gels were stained with ethidium bromide. The nature of the nucleic acid was determined by post-staining digestion of the gel with ribonuclease (Type 1A, Sigma, St. Louis, MO) in the presence of water or 0.3 M NaCl, or DNAse I in the presence of 5 mM $MgCl_2$ as described by Jordan et al (6). For molecular weight determination, the extracted nucleic acid was denatured with glyoxal and analyzed by electrophoresis in 0.75% agarose gels, following the method of Murant et al (11). The gels were stained with acridine orange as described by McMaster and Carmichael (10). Standards for molecular weight determinations (RNA ladder) were purchased from Bethesda Research Laboratories, Gaithersburg, MD.

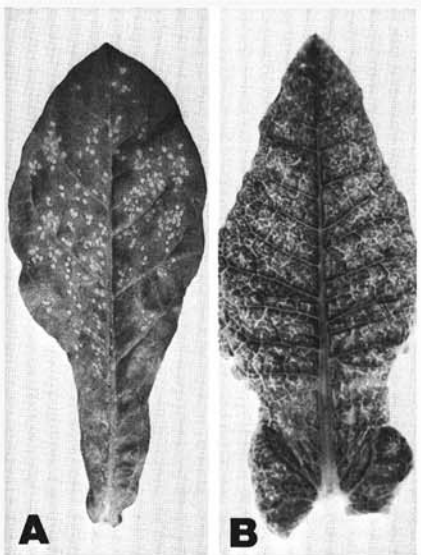

Fig. 1. Leaf symptoms on *Nicotiana occidentalis* '37B' induced by a virus (isolate B-39) associated with apple stem pitting disease. **(A)** Necrotic local lesions on *N. occidentalis* subsp. *obliqua*, **(B)** systemic vein yellowing on *N. occidentalis* '37B.'

Electron microscopy. Virus preparations were negatively stained with 2% sodium phosphotungstate (pH 7.0) or 2% uranyl acetate. Tobacco mosaic virus and catalase crystals were used as size references. For observation of thin sections, the samples were fixed in 5% glutaraldehyde for 2 hr, followed by post-fixation for 2 hr with OsO_4 solution. After dehydration in graded dilutions of ethanol, they were treated with QY-1 and embedded in Spurr's resin. The ultra-thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were made with a Hitachi H-300 electron microscope.

RESULTS

Host range. All five isolates of ASPV produced similar necrotic local lesions on *N. o.* subsp. *obliqua* and systemic vein yellowing on *N. occidentalis* '37B' (Fig. 1). The isolate B-39 produced the most abundant necrotic local lesions on *N. occidentalis*. The host range of ASPV isolates, B-12 and B-39, is shown in Table 1. The most sensitive test plant was *N. o.* subsp. *obliqua*, which expressed necrotic local lesions 4–7 days after inoculation followed by leaf necrosis. There was a slight difference in host range between B-39 and B-12 (Table 1).

Stability in sap. Infectivity of isolate B-39 in *N. o.* subsp. *obliqua* sap was lost at dilutions between 1×10^{-2} and 1×10^{-3} and after storage for 9–24 hr at room temperature. The thermal inactivation point was between 55 and 60 C.

Purification. Treatment of tissue extracts with butanol (8%) caused total loss of infectivity. Clarification of the homogenate with calcium phosphate (14), magnesium bentonite (1), or acidification (5) removed much of the host materials with little loss of infectivity. The method chosen for further study was acidification followed by calcium phosphate clarification. Precipitation of the virus by polyethylene glycol (6%) resulted in pellets that were difficult to suspend. The resuspension of pellets was easier when the virus was sedimented through a sucrose cushion.

Isolate B-39 prepared by the previous methods produced two major and several minor bands (designated as zones 1,2,3, ... from top to bottom) in sucrose density gradients (Fig. 2A). These bands were absent in gradients of healthy material subjected to the same purification procedure. The fastest sedimenting components (zones 2,3 ...) probably represent end-to-end aggregation of the slowest sedimenting particles (zone 1). Only faint bands were observed when virus preparations were suspended in 0. 1 M tris (pH 8.0–9.0), with or without urea.

The virus particles were unstable in CsCl, Cs_2SO_4, Renografin (meglumine diatrizoate), and Angio-Conray (sodium salt). The virus particles produced a single band in Omnipaque density gradients (Fig. 2B). Simple iohexol or Iopamiron 370 (Nihon Schering Co. Ltd., Ohsaka) instead of Omnipaque gave the same results.

Electron microscopy. Long, flexuous, rod-shaped virus particles were found consistently in negatively stained dip

Table 1. Symptoms of virus isolates associated with apple stem pitting disease following mechanical inoculation to various herbacious species

Species[a]	Virus isolates[b] B-39	B-12
Nicotiana occidentalis '37B'	NL, VY	NL, VY
N. occidentalis subsp. *obliqua*	NL, LN	NL, LN
N. sylvestris	0	...[c]
N. rustica	0	0
N. glutinosa	0	...
N. tabacum	0	...
N. clevelandii	S	0
Celosia cristata	NL	NL
Gomphrena globosa	NL, M	0
Beta vulgaris subsp. *cicla*	S	S
Tatragonia expansa	(CL)	CL
Physalis floridana	S	0
Cucumis sativus	S	S
Phaseolus vulgaris	0	0
Chenopodium quinoa	(CL)	...
C. murale	CL	CL
Sesamum indicum	CL	...

[a] Six plants of each species were inoculated.
[b] NL = necrotic local lesion, LN = leaf necrosis, VY = vein yellowing, M = mottle, CL = chlorotic local lesions, (CL) = occasional chlorotic local lesions, S = symptomless systemic infection, 0 = no infection.
[c] Not tested.

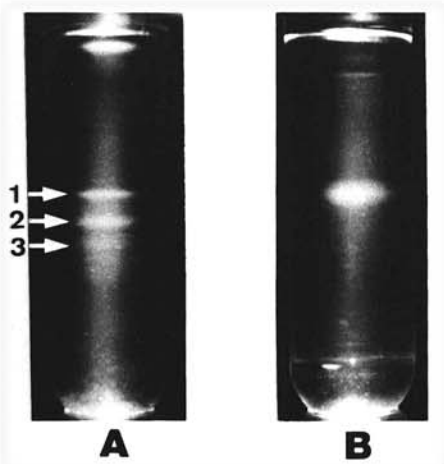

Fig. 2. Light-scattering bands of a virus associated with apple stem pitting disease. **(A)** Sucrose density gradient centrifugation, **(B)** Omnipaque 350 equilibrium density gradient centrifugation. Zones 1, 2, and 3 indicate zones referred to in the text.

preparations from diseased leaves of *N. occidentalis*. It is difficult to determine the precise width of the virus particle. The particle in leaf homogenates became very thick, possibly because of the absorption of host proteins. Four prominent peaks appeared at 800 nm, 1,600 nm, 2,400 nm, and 3,200 nm in the length distribution of virus particles (Fig. 3). These values represent a multiple of the basic unit, 800 nm. The virus particle seemed to be relatively stable in phosphotungstate and uranyl acetate. Short, fragmented particles were also observed but were not counted.

The virus preparations purified by sucrose density gradient centrifugation contained large numbers of flexuous, filamentous particles of various lengths. The modal length of particles in zone 1 was 800 nm and in zone 2 was 1,600 nm. The width of particles was 12–15 nm. The particles purified by Omnipaque equilibrium centrifugation aggregated to such a degree that recognition of single entities was difficult (Fig. 4). The virus particles did not show the obvious cross-banding common in closteroviruses, but some of the particles exhibited a central canal.

Infectivity of purified virus. Fractions of the zones containing the virus were inoculated to *N. o.* subsp. *obliqua* singly or after mixing in order to find whether both components are necessary for infection. Before inoculation, each sample was adjusted to the same nucleoprotein concentration with 0.01 M phosphate buffer, pH 7.0. There was no significant difference between the infectivities of zones 1 and 2 and their mixture. The preparations purified by Omnipaque equilibrium centrifugation also retained high infectivity.

Proteins and nucleic acid. Polyacrylamide gel electrophoresis of SDS-disrupted virus preparations revealed one major and several minor protein bands. The M_r of the major protein was estimated to be 48,000 (Fig. 5). It is uncertain whether minor bands were derived from virus particles, degradation products of the particle, or contamination with host proteins.

Nucleic acid extracted from purified virus was digested by RNAse in 0.3 M NaCl and water but not by DNAse, indicating the virus contains single-stranded RNA. The RNA consisted of a single species of M_r 3.1×10^6.

Cytopathology. Elongated, flexuous rods were found in mesophyll, epidermal, and vascular parenchyma cells of infected leaves but not in comparable cells of uninoculated leaves. They were observed sometimes singly and scattered in

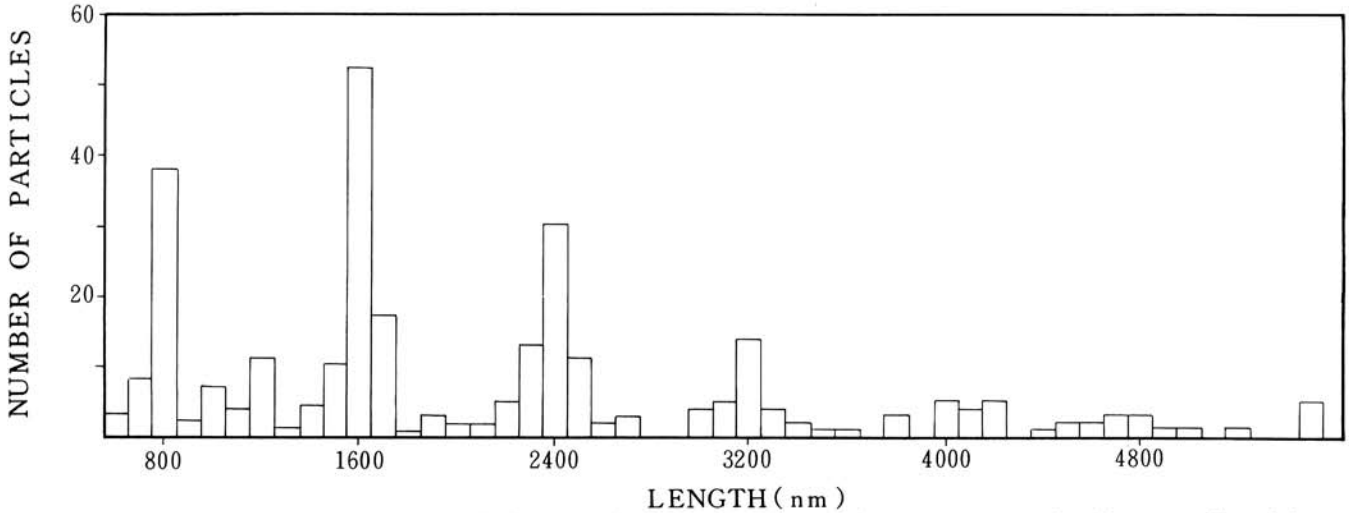

Fig. 3. Length distribution of virus particles found in dip preparations. Samples were taken from *Nicotiana occidentalis* subsp. *obliqua* infected with apple stem pitting virus isolate B-39 and negatively stained with uranyl acetate.

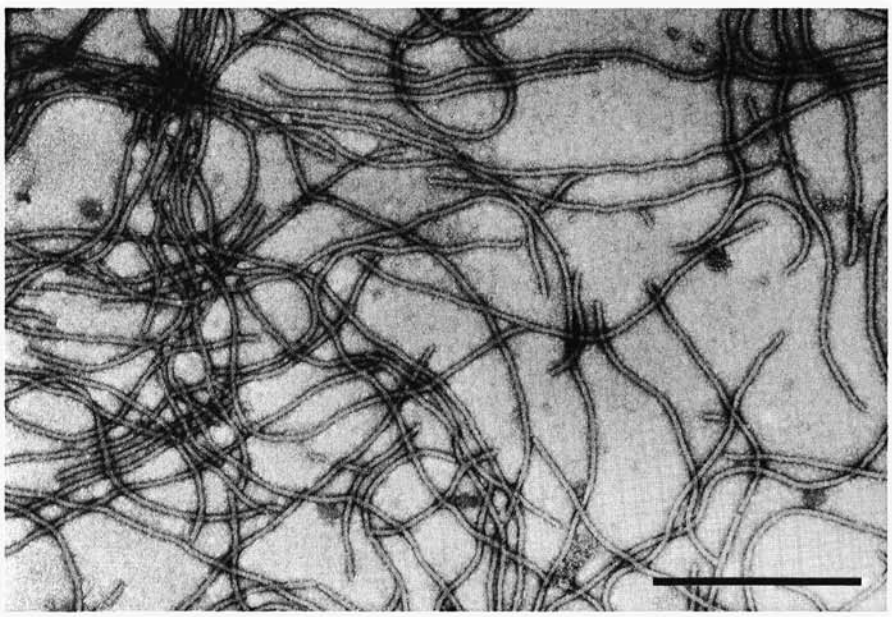

Fig. 4. Electron micrograph of a virus associated with apple stem pitting disease purified by Omnipaque 350 equilibrium density gradient centrifugation. Bar = 500 nm.

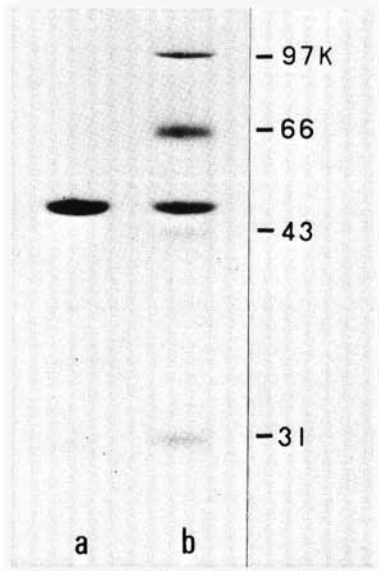

Fig. 5. Electrophoretic analysis of viral coat protein in sodium dodecyl sulfate-polyacrylamide gels (12%). **Lane a**, a virus associated with apple stem pitting disease; **lane b**, the virus plus marker proteins.

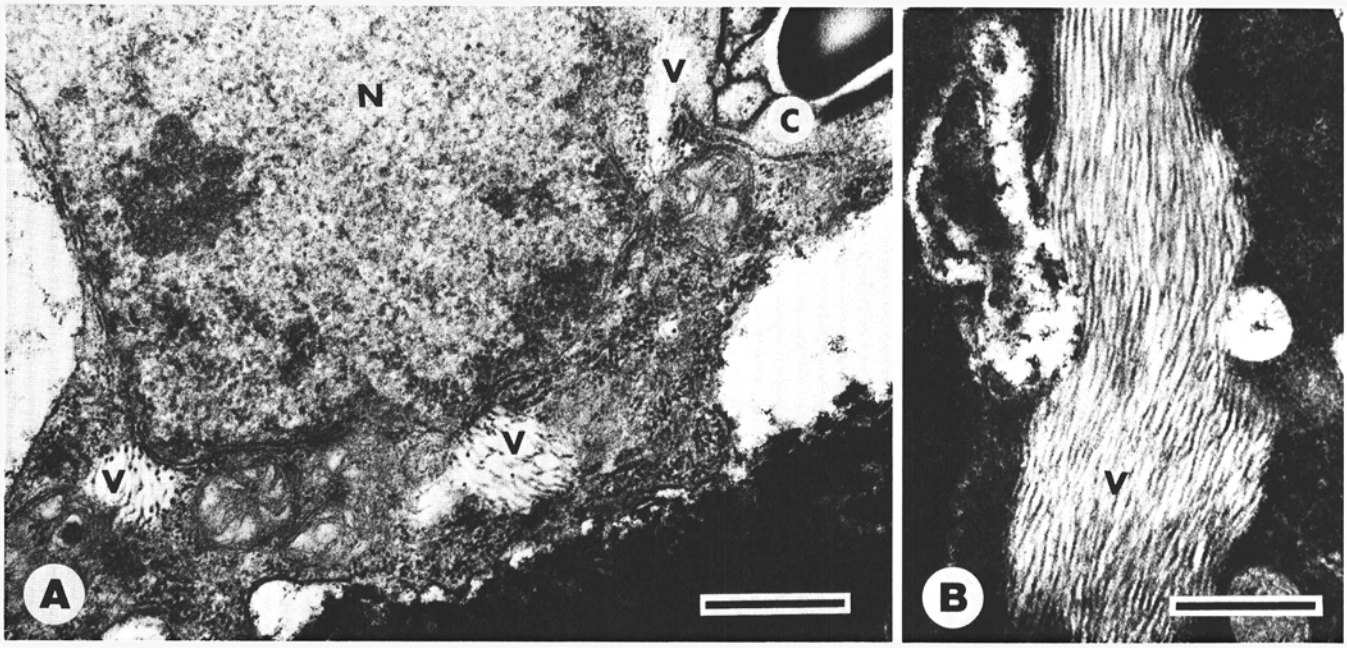

Fig. 6. Ultrastructural studies of *Nicotiana occidentalis* '37B' infected with a virus associated with apple stem pitting disease. **(A)** Virus particles and a disorganized chloroplast in a leaf parenchyma cell, **(B)** virus particle aggregates in a necrotic cell. Bar = 500 nm, V = virus particles, C = chloroplast, N = nucleus.

the cytoplasm but more often in large aggregates (Fig. 6). The lengths of individual rods were difficult to determine. Flexuous rods were not observed in vacuoles, in nuclei, or in other cellular organelles. Some mesophyll cells were necrotic, collapsed, and filled with electron-dense materials (Fig. 6B). Some of the chloroplasts in cells that contained flexuous rods were vacuolated and disorganized, with curved lamellae. The cytoplasmic inclusions specific to potyviruses were not observed.

DISCUSSION

Although purified virus has not yet been back-transmitted to apple indicator plants, the flexuous, filamentous virus described here is likely ASPV. The presumptive evidence for this conclusion is that in the past, the filamentous virus was always isolated from apple trees infected with ASPV alone. No other viruslike particles were isolated from the trees. While other viruses, such as apple chlorotic leaf spot and apple stem grooving viruses, can infect *N. occidentalis*, these viruses were not detected by biological indexing tests of the ASPV-infected apple seedlings used in these experiments (*data not shown*). Back-transmission experiments using purified virus preparations are now in progress.

ASPV seems to be similar to subgroup II closteroviruses and potyviruses in particle length but different from subgroup II closteroviruses in fine structure and molecular weight of coat protein and RNA. The ASPV particles have no obvious cross-banding, and the molecular weights of the coat protein (48,000) and RNA (3.1×10^6) are considerably higher than those of subgroup II closteroviruses. The cytological properties of ASPV are apparently different from those of potyviruses as no cytoplasmic inclusions typical of potyviruses were seen in infected *N. occidentalis*. The striking feature of ASPV is a strong tendency to form end-to-end aggregates. At least 75% of particles were aggregated end-to-end in leaf dip preparations. The longest aggregate probably consisted of 10 basic particles. It is common for most elongated viruses to form end-to-end aggregates during purification. However, in dip preparations of most elongated viruses, the percentage of aggregated particles is usually very small. These data suggest that ASPV does not belong to any of the established taxonomic groups of plant viruses.

Hibino and Schneider (4) demonstrated the presence of a filamentous virus in the leaves of pear trees with vein yellows. The length distribution of pear vein yellows virus (PVYV) was almost the same as that of ASPV. Graft transmission experiments indicated that apple Spy 227 epinasty and decline virus, ASPV, PVYV, and pear stony pit virus (PSPV) are merely strains of the same virus (12). van der Meer (15) showed that ASPV, PVYV, and PSPV induced the same symptoms on *N. occidentalis*. Our inoculation tests on woody indicators showed that pear necrotic spot virus was closely correlated with PVYV and ASPV (18). Although none of the other viruses have been characterized to the extent of ASPV, these facts strongly suggest that they are long, flexuous, filamentous particles and may belong to the same taxonomic group.

Loss of virus through aggregation is a major problem in purifying elongated plant viruses. In these experiments, Omnipaque was used in equilibrium density gradient centrifugation. The aggregated particles of ASPV formed a single band in the density gradient, and the resulting virus samples were suitable for production of antisera and characterization studies. This chemical, as well as other nonionic radiographic contrast media, should be helpful for purifying elongated viruses that are unstable in high ionic strength.

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