

Purification, Characterization, and Immunological Analysis of Nuclear Inclusions Induced by Bean Yellow Mosaic and Clover Yellow Vein Potyviruses

C.-A. Chang, E. Hiebert, and D. E. Purcifull

Plant Pathology Department, University of Florida, Gainesville, Florida 32611. Present address of first author: Department of Plant Pathology, Taiwan Agriculture Research Institute, Taichung, Taiwan, Republic of China.

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ABSTRACT

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The nuclear inclusions (NI) induced by the PV-2 isolate of bean yellow mosaic virus (BYMV-PV-2) and the Pratt isolate of clover yellow vein virus (CYVV-P) were partially purified by treating infected tissue extracts with Triton X-100 followed by low-speed centrifugations. Purified NI from both viruses had cubic symmetry, but from the surface view the NI induced by BYMV-PV-2 were square-shaped, while those induced by CYVV-P were diamond-shaped. The NI of both viruses contained two distinct types of protein monomers with estimated M_r of 54,000 (54K) and 49K for BYMV-PV-2 and 60K and 49K for CYVV-P. The large NI monomers of both viruses were antigenically and chemically distinct from their respective small NI monomers. The NI proteins also were antigenically distinct from cylindrical inclusion and capsid proteins. A 98K protein and a 100K protein

were consistently associated with NI preparations of BYMV-PV-2 and CYVV-P, respectively. These high M_r proteins were shown by immunological analyses and by peptide mapping to be polyproteins that contained sequences of both the large and the small NI monomers. Antisera prepared against SDS-dissociated NI proteins reacted specifically with NI in immunofluorescence studies of infected tissues. The large NI protein monomers of tobacco etch virus (TEV), BYMV-PV-2, and CYVV-P were serologically closely related to each other, while the small NI protein monomers of these three viruses were more viral specific as determined by SDS-immunodiffusion and by immunoprecipitation tests. In the cases of nine out of 14 potyviruses, extracts from infected tissues gave positive reactions for large NI protein-related antigens by indirect ELISA.

Infections with potyviruses induce the synthesis of nonstructural proteins in infected cells (18). Some of these proteins aggregate in different parts of the infected cells (12) to form distinct structures known as inclusion bodies, such as amorphous (irregular) inclusions (AI), cylindrical inclusions (CI), and nuclear inclusions (NI). A nonstructural protein known as helper component (HC) was first identified in soluble form (25) but may also accumulate to form inclusions (E. Hiebert, D. Thornbury and T. Pirone, *unpublished data*). All of these proteins except NI proteins have been purified and characterized from at least two different potyviruses. The CI, for example, are consistently associated with infections of potyviruses (4,11) and all contain a single species of M_r 67,000–73,000 (67K–73K) protein monomer (14,15). Although NI are associated with infections by several members of the potyvirus group (4,12), only the NI induced by tobacco etch virus (TEV) have been purified and characterized (10,16). The NI induced by TEV contain two protein monomers with M_r of 54K and 49K. Antiserum to TEV 54K NI reacts with certain in vitro translation products of all potyviruses, which have been translated in the rabbit reticulocyte lysate system (E. Hiebert, *unpublished data*). This antiserum has also been found to immunoprecipitate some in vitro translation products of tobacco vein mottling virus RNA (13), pepper mottle virus RNA (7), and peanut mottle virus RNA (28). These findings indicate that the antigenic properties of the large NI protein monomers are highly conserved among different potyviruses. This NI protein may have an important and common biological function. To help resolve the biological significance of NI protein, more information about NI induced by potyviruses is needed.

We have isolated and characterized the NI induced by bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV), both of which induce NI in infected tissues (4,19). In this paper, characteristics of the NI and NI proteins induced by BYMV and CYVV, and the serological detection of NI protein antigen in infected tissue are described. Serological comparisons between NI proteins induced by BYMV, CYVV, and TEV are also presented.

MATERIALS AND METHODS

Viruses. An isolate (PV-2) of BYMV (24) and an isolate (CYVV-P) of CYVV (21) kindly provided by F. W. Zettler were used throughout the study. Both viruses were cultured in pea (*Pisum sativum* L. 'Alaska') and maintained at a temperature of approximately 26 C in a growth room. The 204-1, Scott, and orchid isolates of BYMV were obtained from M. McLaughlin, O. W. Barnett, and N.-J. Ko, respectively. The other viruses used in the serological tests were from the collection of the third author.

Partial purification of NI. Generally, the procedures for purification of tobacco etch virus (TEV) NI developed by Knuhtsen et al (16) were followed. However, because of differences in the shape and size between the NI, the procedures for purifying NI from tissues infected with BYMV-PV-2 or CYVV-P were carefully evaluated. The preparations from each step and from different treatments were saved and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to determine the most suitable treatments.

Alaska pea leaves systemically infected by BYMV-PV-2 were harvested 3–4 wk after inoculation. The leaves, chilled 1–2 hr in the cold room before blending, were homogenized in a Waring blender for 2 min with 3 ml of sodium phosphate buffer (100 mM, pH 7.2, containing 0.2% sodium sulfite) per gram of tissue. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth. The filtrate was treated with 5% Triton X-100 and stirred in the cold room for 1.5–2 hr, followed by centrifugation at 2,000 g for 10 min in a GSA rotor. The pellet was resuspended with resuspending buffer (RB) (20 mM sodium phosphate buffer, pH 8.2, containing 0.5% sodium sulfite) and centrifuged again at 2,000 g for 10 min. The pellet was resuspended again with RB and Triton X-100 was then added to a concentration of 2%, and the mixture was stirred in the cold room for 1 hr. The preparation was centrifuged at 2,000 g for 10 min, and the resulting pellet was resuspended with RB containing 20% sucrose. This preparation was homogenized in a Sorvall Omnimixer at position 7 for 1 min and then centrifuged through a sucrose step gradient (50:60:80% sucrose in RB) at 7,000 g for 12 min. The fractions

containing 60 and 80% sucrose were collected, diluted with RB and centrifuged at 3,000 *g* for 15 min. The pellet was resuspended with a small volume of 20 mM Tris-HCl, pH 8.2 (2 ml/100 g of tissue). Hereafter, this preparation will be referred to as BYMV-PV-2 semipurified NI.

To monitor the distribution of NI proteins during purification, preparations saved from each step were dissociated by adding equal volumes of Laemmli dissociation buffer (100 mM Tris-Cl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol [ME], and 5% sucrose) (17) and heating in a boiling water bath for 3 min. The samples were loaded in a 7.5–15% gradient gel and electrophoresed at constant 60 V for 16 hr (15). The acrylamide gel and running buffer were prepared as described by Laemmli (17). Proteins were stained with Coomassie blue R-250.

Purification procedures for CYVV-P induced NI were similar to those for BYMV-PV-2 NI, except that the sucrose step gradient centrifugation was omitted and that the centrifugal force needed to sediment CYVV-P NI was greater. The filtrate was treated with 5% Triton X-100 and stirred 1.5–2 hr in the cold room, followed by centrifugation at 164 *g* for 10 min. The supernatant was centrifuged at 16,300 *g* for 10 min, and the pellet was resuspended with RB. The sample was centrifuged again at 16,300 *g* for 10 min and then treated with 2% Triton X-100 for 1 hr, followed by centrifugation at 16,300 *g* for 10 min. The pellet was resuspended in a small volume of 20 mM Tris-Cl, pH 8.2 (4 ml/100 g of tissue). This preparation will be referred to hereafter as CYVV-P semipurified NI.

Further purification of NI proteins by gel electrophoresis. The proteins of semipurified NI were further purified by preparative gel electrophoresis as described by Hiebert et al (15) with modifications. Semipurified NI from CYVV-P and BYMV-PV-2 were dissociated by adding an equal volume of Laemmli dissociation buffer and heating in a boiling water bath for 3 min before loading on a 7.5–15% gradient preparative gel. The gel and running buffer were prepared as described by Laemmli (17). Samples were electrophoresed at a constant 55 V for 16 hr. The inclusion protein bands were visualized by soaking the gel in cold 200 mM KCl for a few minutes and excised with a sharp razor blade. The excised bands were washed three times with deionized water for 5 min each and then frozen at –20 C for at least 2 hr. To ensure the purity of protein, we usually loaded excised protein bands on a second preparative gel and electrophoresed again under identical conditions. The gel slices were thawed and pressed through a cushion of 1–2 ml of Laemmli dissociation buffer on the top of a stacking gel. The gel slices were carefully loaded to avoid trapping of air bubbles. After electrophoresis the protein band was visualized and excised as described above. If required, the protein band could be electrophoresed again without any appreciable loss of protein during manipulation. The protein was eluted from the gel as described by Hiebert et al (15). Protein yields were estimated by absorbance at 280 nm, and the proteins were stored at –20C. Purity of protein was determined by analysis in SDS-PAGE and visualization by silver staining.

Purification of cylindrical inclusion protein and virus capsid proteins. Cylindrical inclusions and CYVV-P and BYMV-PV-2 were purified from infected tissues as described by Nagel et al (19) except that virus preparations were suspended in Hepes buffer (20 mM, pH 8.2, containing 10 mM EDTA). The CI proteins and capsid proteins of CYVV-P and BYMV-PV-2 were further purified by preparatory gel electrophoresis as described above.

Antisera preparation, SDS-immunodiffusion, and indirect ELISA tests. Antisera to NI, CI, and capsid proteins were prepared in New Zealand white rabbits as described by Hiebert et al (15). The reactivities of antisera were evaluated routinely by SDS-immunodiffusion tests. The SDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (22). When purified antigens were used, they were adjusted to optimum concentration by addition of 0.1% SDS.

In attempts to detect NI protein antigen in plant tissue, indirect enzyme-linked immunosorbent assays (ELISA) were conducted. The procedures of indirect ELISA were similar to those described by Yeh and Gonsalves (29), except that the plates were treated with

3% BSA (A-7030, Sigma, St. Louis, MO) in PBS after antigen coating to minimize the nonspecific reactions. Reactions were recorded with a plate reader (Model EL 307, Bio-Tek, Inc.) at 405 nm 60 min after addition of the substrate.

Protein blotting procedure. Protein blotting was conducted according to the method of Towbin et al (26). Proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes (0.45- μ m pore size, Bio-Rad, Richmond, CA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), at 0 C for 5 hr at constant 100 V using a TE transphore-electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, CA). After transfer, the membrane was incubated 2 hr or overnight with a blocking solution containing 3% BSA in Tris-buffered saline (TBS) (20 mM Tris, pH 8.2, 0.9% NaCl). The membrane was then incubated for 2–3 hr with various antisera, which were diluted in TBS containing 0.1% BSA and 1% normal goat serum. After three 15-min washes with washing buffer (TBS containing 0.1% BSA), goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-8025) was used at 1/1,500 dilution in washing buffer and incubated with the membrane for 3 hr. After further washing as described above, followed by 5 min of incubation in 1 M diethanolamine buffer (pH 9.6), the immune complex was visualized by incubating the membrane with a development solution that was prepared as described by Parent et al (20) with some modifications. It consisted of 18 ml of diethanolamine buffer (1 M, pH 9.6), 2 ml of 1 mg/ml of *p*-nitroblue tetrazolium (Gibco 870-1180) in diethanolamine buffer, 0.2 ml of 5 mg/ml *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate (ICN 100368) in diethanolamine buffer and 40 μ l of 2 M magnesium chloride. The membrane was incubated at room temperature until distinguishable color development occurred. Reactions were stopped by rinsing the membrane in water.

Peptide mapping by partial digestion with V8 protease. The NI proteins were compared by partial proteolytic digestion with *Staphylococcus aureus* V8 protease as described by Cleveland et al (5).

Light microscopy, immunofluorescence, and electron microscopy. Presence of NI in infected cells was observed by light microscopy of epidermal strips from Alaska pea leaves stained with the luxol brilliant green BL-calcomine orange 2RS technique (4), hereafter referred to as O-G stain. Immunofluorescence tests were conducted as described by Hiebert et al (15). Inclusion preparations were mounted on carbon-coated Formvar membranes and stained with uranyl acetate for electron microscopy.

RESULTS

Light microscopy of nuclear inclusions. Nuclear inclusions were observed consistently in leaves infected with BYMV-PV-2 and CYVV-P. No inclusions were seen in healthy control tissue. The NI induced by BYMV-PV-2 were morphologically different from those induced by CYVV-P. The former induced cuboidal nuclear inclusions (Fig. 1A). The sizes of BYMV-PV-2 NI were highly variable (Fig. 1B). Unlike BYMV-PV-2, CYVV-P induced clusters of granular NI that were individually much smaller than BYMV-PV-2 NI (Fig. 1C and D). Both types of NI were easily stained with O-G stain but not by azure A stain, indicating they were proteinaceous. The BYMV-PV-2 also induced structures in the cytoplasm, hereafter referred as cytoplasmic crystals (CC), which were morphologically indistinguishable from the NI (Fig. 1B). No similar cytoplasmic crystals were seen in healthy tissue or in the tissue infected by CYVV-P.

Purification of NI and NI proteins. The semipurified NI from BYMV-PV-2, when observed under light microscopy, were cuboidal, square from the top view, highly variable in size, and indistinguishable from those seen in situ (Fig. 2A). Attempts to observe the substructure of these NI by electron microscopy were unsuccessful. Only very darkly stained NI could be seen. Unlike BYMV-PV-2, no distinct structure of the semipurified CYVV-P NI could be resolved by light microscopy. Occasionally, very small granular structures were seen. When examined by electron

microscopy, structures that were diamond-shaped in surface view were easily observed in the semipurified CYVV-P NI preparations (Fig. 2B). No substructure could be resolved on the CYVV-P NI by electron microscopy (Fig. 2C).

During purification, most of the BYMV-PV-2 NI sedimented during centrifugation at 2,000 g. By contrast, most of the CYVV-P NI required at least 6,870 g to be sedimented (data not shown). After sucrose step gradient centrifugation, the majority of the BYMV-PV-2 NI were in the 80 and 60% fractions, while the majority of the CYVV-P NI were in the 50% fraction. These observations were consistent with the findings of the microscopic studies.

The semipurified BYMV-PV-2 NI samples contained two types of protein monomers with M_r of 54K and 49K when analyzed in SDS-PAGE (Fig. 3). A minor 98K protein was consistently associated with the semipurified BYMV-PV-2 NI (Fig. 3). These three proteins were not observed when healthy tissue was processed by the same purification procedure (Fig. 3). Similarly, the CYVV-P NI also contained two species of protein monomers, but the M_r were 60K and 49K (Fig. 3). Because these two NI monomer bands were well separated from host contaminants in our gel system, the sucrose step gradient centrifugation was omitted from the purification procedure. Although this modification resulted in the occurrence of some protein bands with M_r smaller than 49K (Fig. 3), they were possibly host proteins and they apparently did not affect further purification of the NI protein monomers by preparative PAGE. Similar to BYMV-PV-2 NI,

semipurified CYVV-P NI also consistently contained a larger protein with M_r of 100K (Fig. 3). A protein that moved faster and two minor proteins that moved slower than the 100K protein were sometimes detected in the preparations (Fig. 3); however, they were not consistently observed and their staining intensities varied in different preparations (data not shown). A 65K protein that was later found to be CI protein of CYVV-P was also associated with semi-purified CYVV-P NI. The protein monomers of CYVV-P and BYMV-PV-2 NI were further purified by preparatory gel electrophoresis. Because of the closeness between 54K and 49K protein bands in SDS-PAGE and the possible contamination with 53K Fraction 1 protein, we routinely purified 54K protein by electrophoresing the protein excised from the gel four consecutive times in preparatory SDS-PAGE. The BYMV-PV-2 49K NI protein, 98K NI protein, and the three CYVV-P NI proteins were always purified twice in preparatory SDS-PAGE. The purity of each NI protein was assessed by analytical SDS-PAGE, which indicated a single band in each preparation (Fig. 4). The yields of the NI proteins were estimated spectrophotometrically (A_{280nm} of 1.0 estimated as 1 mg/ml) to be 2–3 mg per 100 g of infected tissue for 60K, 54K, and the two 49K NI proteins. However, the yields of the 100K and 98K proteins were 2–3 mg and 0.5–0.8 mg per 100 g of infected tissue, respectively.

Reconstitution experiments. It was originally believed that the 98K protein of BYMV-PV-2 and the 100K protein of CYVV, like the 95K protein of TEV NI, were dimers that resulted from incomplete dissociation during SDS-PAGE of the two NI protein

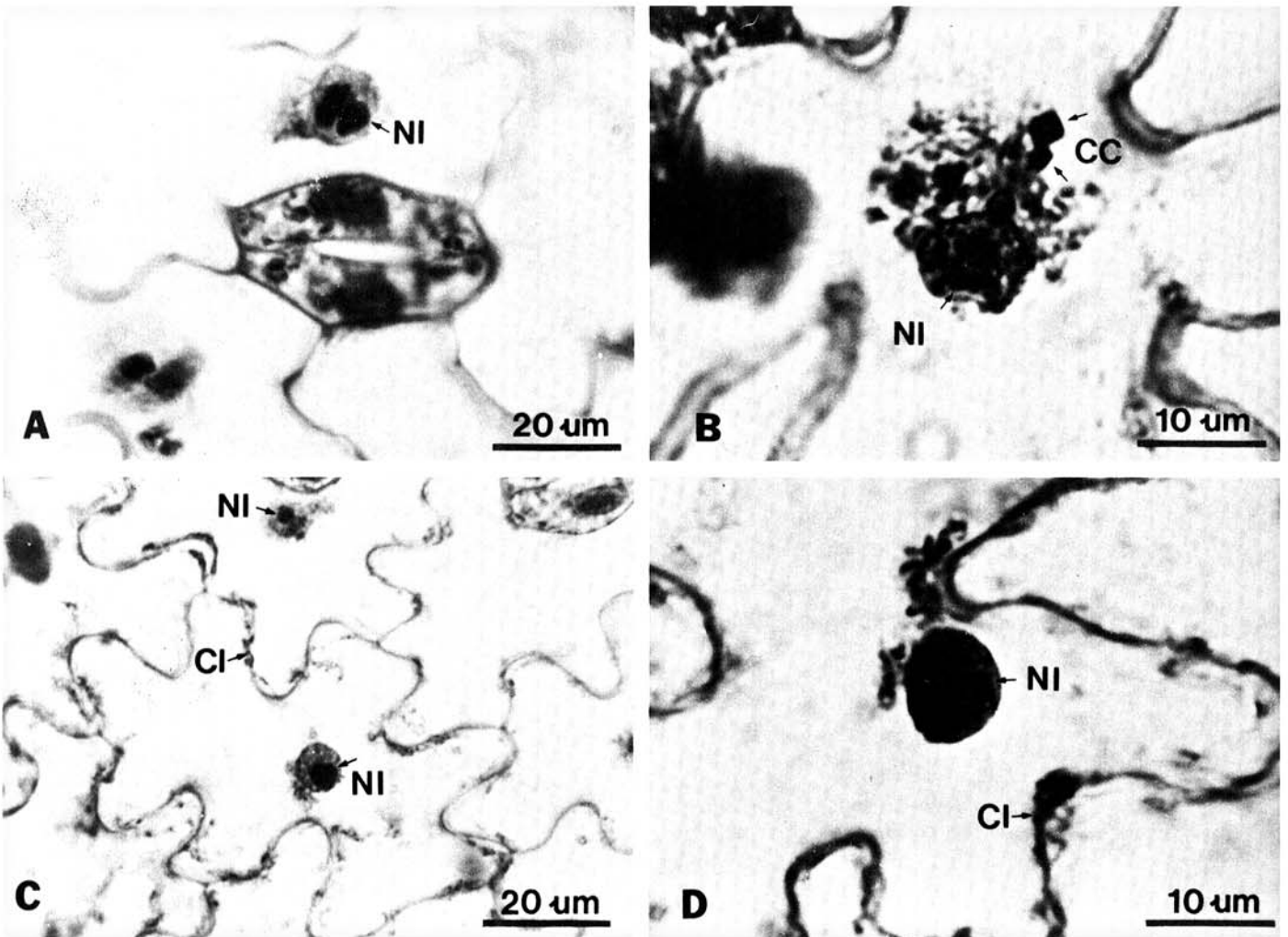


Fig. 1. Light micrographs of infected epidermal tissue of Alaska pea stained with Luxol brilliant green/calcomine orange showing A, bean yellow mosaic virus (BYMV)-PV-2 induced nuclear inclusion; B, BYMV-PV-2 induced nuclear inclusions and cytoplasmic crystals; C, clover yellow vein virus (CYVV)-P induced nuclear inclusion and membrane-associated cylindrical inclusions; D, CYVV-P induced clusters of granular nuclear inclusions and cylindrical inclusions under higher magnification. NI = nuclear inclusions, CI = cylindrical inclusions, CC = cytoplasmic crystals.

monomers (15). However, when the 98K and 100K proteins were isolated, retreated with Laemmli dissociation solution, and reanalyzed in SDS-PAGE, they still maintained the same M_r (Fig. 4). This indicated that those proteins were resistant to dissociation and were probably not homodimers of any of the NI proteins. To test this further, we mixed purified 54K NI and 49K NI proteins of BYMV-PV-2 without adding any dissociation or reducing agents and analyzed the mixture in SDS-PAGE after 4 hr of incubation (Fig. 5). The results showed that no high M_r protein formed under these conditions, which presumably were favorable for formation of dimers of either 54K or 49K protein. Furthermore, when the BYMV-PV-2 NI were purified from infected tissues with 1.0%, 0.5%, or no reducing agent (sodium sulfite) in the extraction buffers, no differences were found in protein profiles between different treatments based on analysis by SDS-PAGE (Fig. 5).

Immunological analyses of NI proteins. Antisera to NI proteins of BYMV-PV-2 and CYVV-P were obtained over a period of several months, beginning 2 wk after the final injections. Antigenic properties of NI proteins were first analyzed by SDS-immunodiffusion tests, and the results are summarized in Tables 1 and 2. The 54K and 49K NI proteins of BYMV-PV-2 were serologically distinct from each other and also distinct from CI protein and capsid protein induced by BYMV-PV-2 (Table 1). Both antisera reacted with the 98K NI protein of BYMV-PV-2;

however, antiserum to 98K NI protein reacted reciprocally only to 49K protein. Similar to BYMV-PV-2, the 60K and 49K NI proteins of CYVV-P were also serologically distinct from each other, and from CI and capsid proteins of CYVV-P (Table 2). Antisera to both CYVV-P 60K and 49K NI proteins reacted with 100K NI proteins, and reciprocally the antiserum to 100K NI protein reacted with both 60K and 49K NI proteins. These results indicated that the 98K and 100K proteins contained sequences from both large and small NI proteins. All antisera to NI proteins gave prominent bands when tested against extracts from infected plants in SDS-immunodiffusion tests. No reactions were found between these antisera and healthy control plant extracts.

Distinct serological properties between large NI monomers and small NI monomers were also confirmed by the fact that antisera to large NI monomers immunoprecipitated a completely different set of *in vitro* translation products of BYMV-PV-2 RNA from that immunoprecipitated by antisera to small NI proteins (data not shown).

Antigenic properties of NI proteins were further analyzed by Western blot analysis. Antisera to 100K protein of CYVV-P

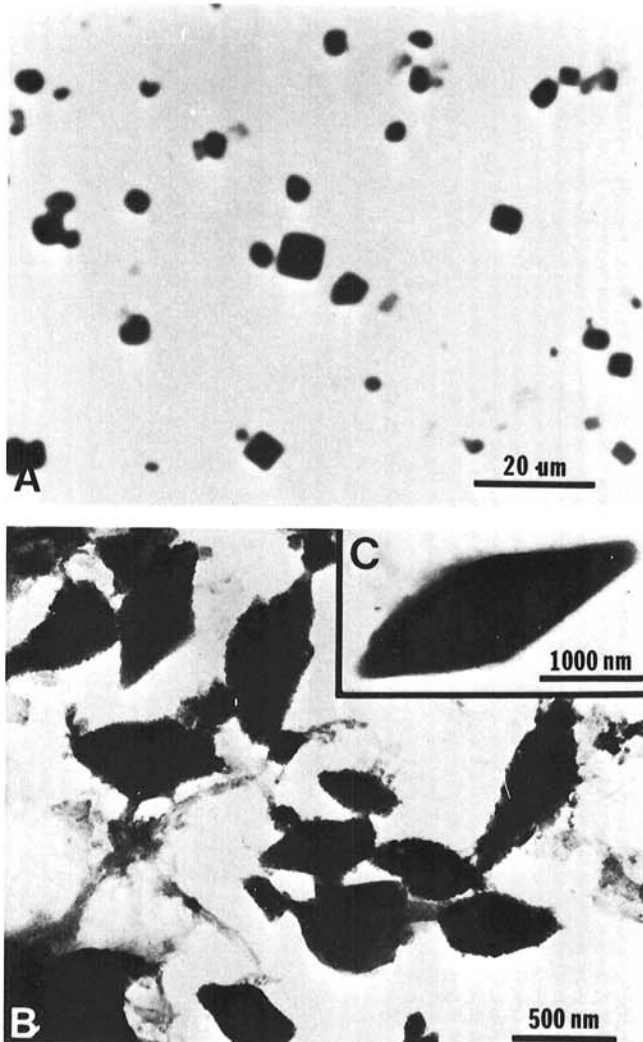


Fig. 2. Micrographs of semipurified nuclear inclusions of bean yellow mosaic virus (BYMV)-PV-2 and clover yellow vein virus (CYVV)-P. A, Semipurified BYMV-PV-2 nuclear inclusions stained with Luxol brilliant green/calcolmine orange under light microscopy; B, semipurified CYVV-P diamond-shaped nuclear inclusions stained with uranyl acetate under electron microscopy; C, electron micrograph of individual CYVV-P nuclear inclusion.

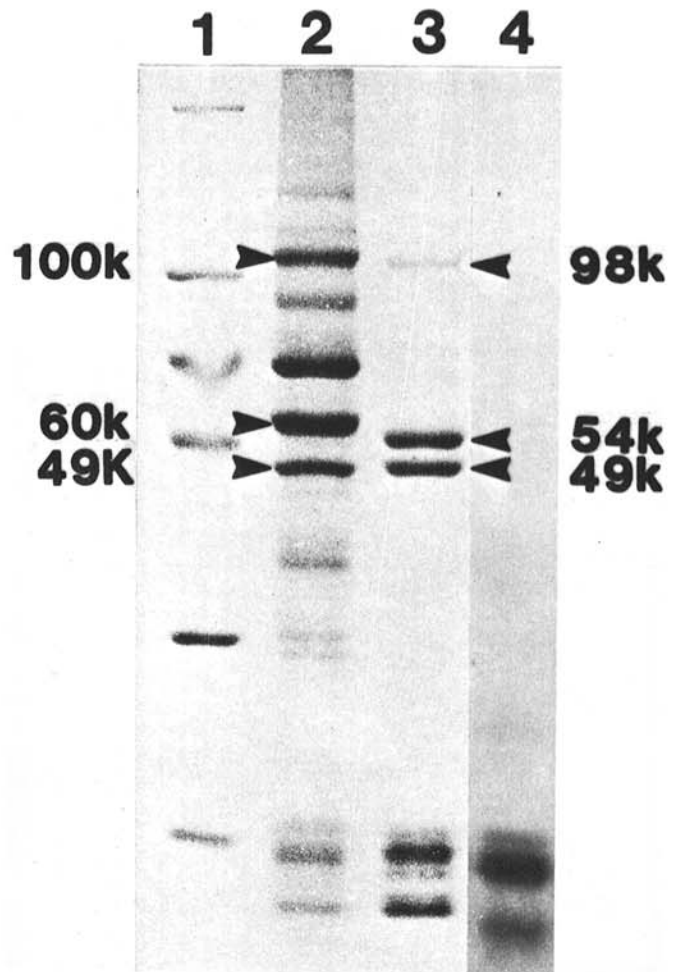


Fig. 3. Analysis of semipurified nuclear inclusions of bean yellow mosaic virus (BYMV)-PV-2 and clover yellow vein virus (CYVV)-P by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Lane 1, protein markers; lane 2, semipurified CYVV-P nuclear inclusions; lane 3, semipurified BYMV-PV-2 nuclear inclusions; lane 4, preparation obtained by the same purification procedure from healthy pea tissue. Nuclear inclusion proteins are indicated by arrows. The molecular weights of CYVV-P nuclear inclusion proteins are indicated on the left hand side of the figure while the molecular weights of BYMV-PV-2 nuclear inclusion proteins are indicated on the right hand side of the figure. Marker proteins are, from bottom to top: tobacco mosaic viral coat protein subunit (17.5K); carbonic anhydrase (29K); glutamic dehydrogenase (53K); bovine serum albumin (67K); phosphorylase b (94K), and myosin (200K).

reacted with 60K NI, 49K NI, and its homologous 100K NI protein (Fig. 6). Antisera to both 60K NI and 49K NI of CYVV-P reacted with 100K protein and their homologous antigens (Fig. 6). A protein with M_r around 80K and several proteins with M_r higher than 100K also reacted with antisera to 60K, 49K, and 100K NI proteins (Fig. 6). These proteins were not consistently associated with the NI preparations, and staining intensities of the bands representing proteins of 80K or greater than 100K varied in different preparations. Therefore, these proteins were probably incompletely dissociated proteins or degraded proteins related to the 100K NI protein. Less of these proteins were found when freshly prepared samples instead of samples stored in the freezer were used in analytical PAGE and Western blotting tests (data not shown).

Similar results were obtained with Western blot analyses of BYMV-PV-2 NI proteins (Fig. 7). Antisera to 54K and 49K NI proteins of BYMV-PV-2 both reacted with the 98K NI protein of BYMV-PV-2 and their homologous antigens. The 98K NI protein antiserum reacted strongly with 49K NI and homologously to 98K protein but only slightly with 54K NI (Fig. 7). The reactivity of the 98K protein antiserum in Western blotting indicated that this antiserum did contain a very small fraction of antibodies specific to 54K NI protein, although the antiserum did not react with 54K NI protein in SDS-immunodiffusion tests. These experiments further confirmed the possible polyprotein nature of the 100K and 98K NI proteins.

Peptide mapping of NI proteins. To determine if the 100K and 98K proteins have a precursor-product relationship to the large NI and small NI proteins of CYVV-P and BYMV-PV-2, we performed partial V8 protease digestions of all NI proteins of CYVV-P and BYMV-PV-2 and compared the resulting peptide profiles by SDS-PAGE. All NI proteins were adjusted to 1 mg/ml before digesting with V8 protease. A ratio of 1:6 (w/w) of V8 protease to NI proteins was used throughout the experiments. In

the case of CYVV-P, six out of seven peptides generated from 60K NI protein comigrated with the peptides of 100K protein, while at least eight out of 10 peptides generated from 49K NI protein comigrated with the peptides of 100K protein. The 100K protein contained two peptides that were unique to those peptides of 60K and 49K NI proteins (Fig. 8). These results are consistent with the proposed polyprotein nature of the 100K protein. Similar results were obtained with the 98K NI protein of BYMV-PV-2 (Fig. 9); however, the 98K protein contained all the detected peptides from the 54K NI protein and at least seven out of 12 peptides generated from the 49K NI protein. Generally the small NI proteins of both CYVV-P and BYMV-PV-2 were more susceptible to V8 protease digestion than the other NI proteins tested. More intense staining of the digested peptides from small NI proteins of both viruses was always observed, even though all proteins were compared under

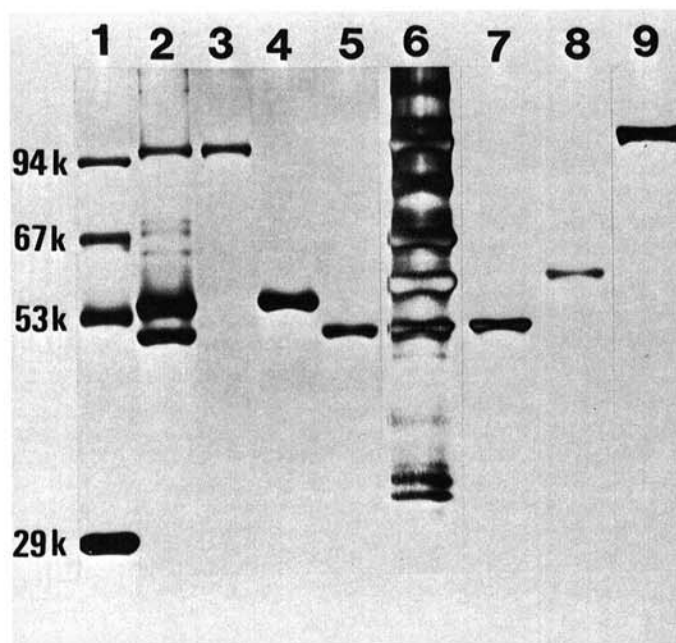


Fig. 4. Analysis of purified nuclear inclusion (NI) proteins of bean yellow mosaic virus (BYMV)-PV-2 and clover yellow vein virus (CYVV)-P in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by silver staining. Lane 1, protein markers; lane 2, semipurified BYMV-PV-2 nuclear inclusions; lane 3, purified BYMV-PV-2 98K NI protein; lane 4, purified BYMV-PV-2 54K NI protein; lane 5, purified BYMV-PV-2 49K NI protein; lane 6, semipurified CYVV-P NI; lane 7, purified CYVV-P 49K NI protein; lane 8, purified CYVV-P 60K NI protein; lane 9, purified CYVV-P 100K NI protein. Molecular weights of marker proteins are indicated on the side of the figures. Marker proteins are, from bottom to top, carbonic anhydrase (29K), glutamic dehydrogenase (53K), bovine serum albumin (67K), and phosphorylase b (94K).

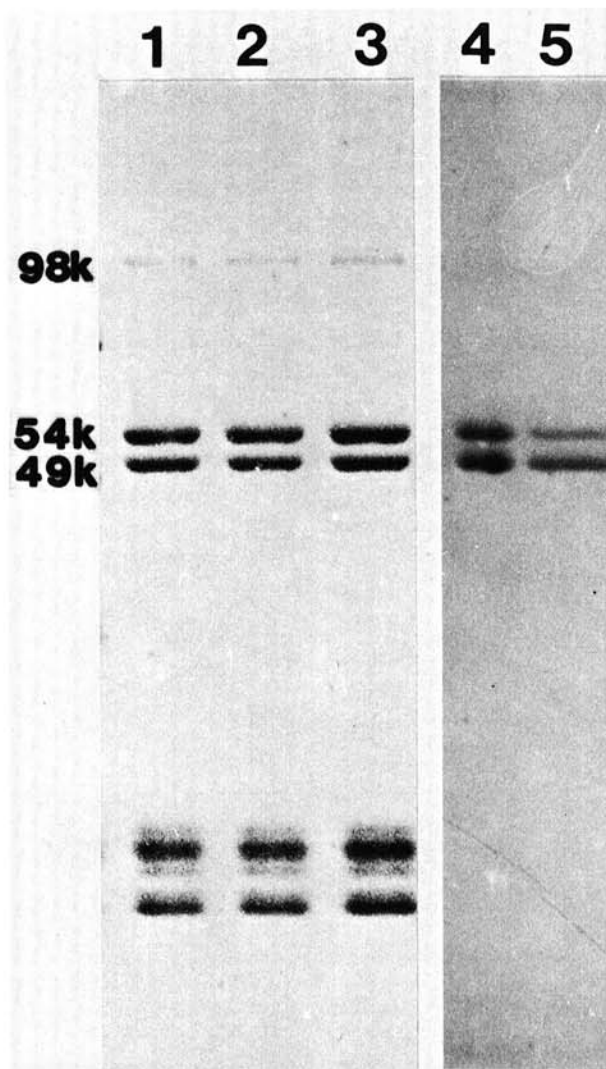


Fig. 5. A reconstitution experiment with bean yellow mosaic virus (BYMV)-PV-2 54K nuclear inclusion (NI) and 49K NI protein monomers and analysis of semipurified BYMV-PV-2 nuclear inclusion preparations obtained by isolating with different concentrations of sodium sulfite. Proteins were separated in 7.5–15% linear gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie blue staining. Lane 1, semipurified NI preparation obtained with sodium phosphate buffer (10mM, pH 7.2) without sodium sulfite; lane 2, semipurified NI preparation obtained with the same buffer containing 0.5% sodium sulfite; lane 3, semipurified NI preparation obtained with same buffer containing 1.0% sodium sulfite; lane 4, 20 µg of 54K NI protein in 20 µl of water was mixed with 20 µg of 49K NI protein in 20 µl of water and analyzed in SDS-PAGE after 4 hr of incubation; lane 5, 10 µg of 54K NI protein in 10 µl of water was mixed with 10 µg of 49K NI protein in 10 µl of water, and analyzed in SDS-PAGE after 4 hr of incubation. Molecular weights of nuclear inclusion proteins are indicated on the side of the figure.

identical conditions (Figs. 8 and 9). On the contrary, strongly staining high M_r peptides, presumably nearly undigested peptides, were always seen in the case of large NI monomers (54K and 60K), 98K, and 100K NI proteins (Figs. 8 and 9). These results indicated that there were differential susceptibilities to protease digestion among the NI proteins.

Immunofluorescence of NI and NI proteins. Antisera to NI proteins of CYVV-P and BYMV-PV-2 were used in immunofluorescence tests with NI in situ. Antisera against 54K, 49K, and 98K proteins of BYMV-PV-2 induced specific fluorescence with BYMV-PV-2 NI in situ, while antisera to 60K, 49K, and 100K NI proteins reacted to induce specific fluorescence with CYVV-NI in situ. Examples are shown in Figure 10. The morphology of NI detected by immunofluorescence tests was indistinguishable from that found by light microscopy (Fig. 10). Antisera to BYMV-PV-2 54K, 49K, and 98K NI proteins also reacted and showed strong immunofluorescence with cytoplasmic crystals induced by BYMV-PV-2 (Fig. 10A). These cytoplasmic crystals did not immunofluoresce with BYMV-PV-2 CI or capsid protein antisera.

Serological comparison of NI proteins induced by TEV, CYVV-P, and BYMV-PV-2. Serological properties of NI proteins induced by TEV, CYVV-P, and BYMV-PV-2 were compared in reciprocal

SDS-immunodiffusion tests. With antisera to large NI proteins (e.g., 54K proteins for TEV and BYMV-PV-2, 60K protein for CYVV-P) to react with crude extracts of these three viruses, all three viruses are related to, but distinct from each other, based on the formation of spurs between homologous and heterologous antigens (Fig. 11). On the other hand, with antisera to small NI proteins (e.g., 49K NI proteins), only BYMV-PV-2 and CYVV-P were related to (Fig. 11) but distinct from, each other based on spur reactions (data not shown). Likewise, antisera to 49K NI proteins of BYMV-PV-2 and CYVV-P reacted homologously and reciprocally, but did not react with TEV NI. This experiment indicated that serological properties of large NI proteins were conserved among these three potyviruses, while those of small NI proteins were more viral-specific. Antigenic conservation of large NI proteins was further confirmed by immunoprecipitating in vitro translation products of pepper mottle virus, a potyvirus distinct from TEV, BYMV-PV-2, and CYVV-P, using antisera to the large NI proteins of the latter three viruses. All three antisera immunoprecipitated an identical set of products (Fig. 12).

Detection of 54K NI related protein in tissue infected by potyviruses. The 54K NI protein was chosen because this protein could represent a conservative, potyvirus specified protein that might be valuable as a target for indexing potyviral infections. The objective was to determine if all potyviruses induced sufficient amounts of serologically related protein in infected tissue, so that this approach could be applied as a potyvirus indexing method. Indirect ELISA was used as the major technique to detect 54K NI related protein in tissue infected by different potyviruses. The system when tested with crude sap of BYMV-PV-2 infected Alaska pea tissue, gave positive reactions (A_{405nm} more than twice as high as those obtained with healthy antigens) with crude sap diluted up to 10×5^8 times (data not shown). Purified BYMV-PV-2 54K NI antigen at concentrations of 32–160ng/ml reacted with BYMV-

TABLE 1. Reciprocal antigenic cross-reactivities of five different bean yellow mosaic virus (BYMV)-PV-2 proteins and their antisera as determined by sodium dodecyl sulfate (SDS)-immunodiffusion tests^a

Antisera	Cross-reactivities ^b to antigen of					
	54K NI	49K NI	98K NI	CI	CP	0.1% SDS
54K NI	+	0	+(I)	0	0	0
49K NI	0	+	+(I)	0	0	0
98K NI	0 ^c	+(I)	+	0	0	0
CI	0	0	0	+	0	0
CP	0	0	0	0	+	0
NS	0	0	0	0	0	0

^aSDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (22). Antigens used were preparative polyacrylamide gel electrophoresis (PAGE)-purified and adjusted to 50 μ g/ml with 0.1% SDS. Antisera were used undiluted and were from blood collected 2–3 mo after the last injections. Tests were done at 25 C and recorded after 48 hr.

^bAntigenic reactivities were: + = precipitation band observed between antiserum and antigen wells; 0 = no precipitation band observed. (I) following + symbol = reaction lines between homologous and heterologous antigens were fused without spurring. NI = nuclear inclusion, CI = cylindrical inclusion, CP = coat protein, NS = normal serum from unimmunized rabbit.

^cThe 98K NI protein antiserum reacted weakly with 54K NI protein in Western blotting tests.

TABLE 2. Reciprocal antigenic cross-reactivities of five different clover yellow vein virus (CYVV)-P proteins and their antisera as determined by sodium dodecyl sulfate (SDS)-immunodiffusion tests^a

Antisera	Cross-reactivities ^b to antigen of					
	60K NI	49K NI	100K NI	CI	CP	0.1% SDS
60K NI	+	0	+(I)	0	0	0
49K NI	0	+	+(I)	0	0	0
100K NI	+(I)	+(I)	+	0	0	0
CI	0	0	0	+	0	0
CP	0	0	0	0	+	0
NS	0	0	0	0	0	0

^aSDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (22). Antigens used were preparative polyacrylamide gel electrophoresis (PAGE)-purified and adjusted to 50 μ g/ml with 0.1% SDS. Antisera applied were undiluted and bleedings collected 2–3 mo after last injections. Tests were done at 25 C and recorded after 48 hr.

^bAntigenic reactivities were + = precipitation band observed between antiserum and antigen wells; 0 = no precipitation band observed. (I) + symbol = reaction lines between homologous and heterologous antigens were fused without spurring. NI = nuclear inclusions, CI = cylindrical inclusions, CP = coat protein, NS = normal serum collected from unimmunized rabbit.

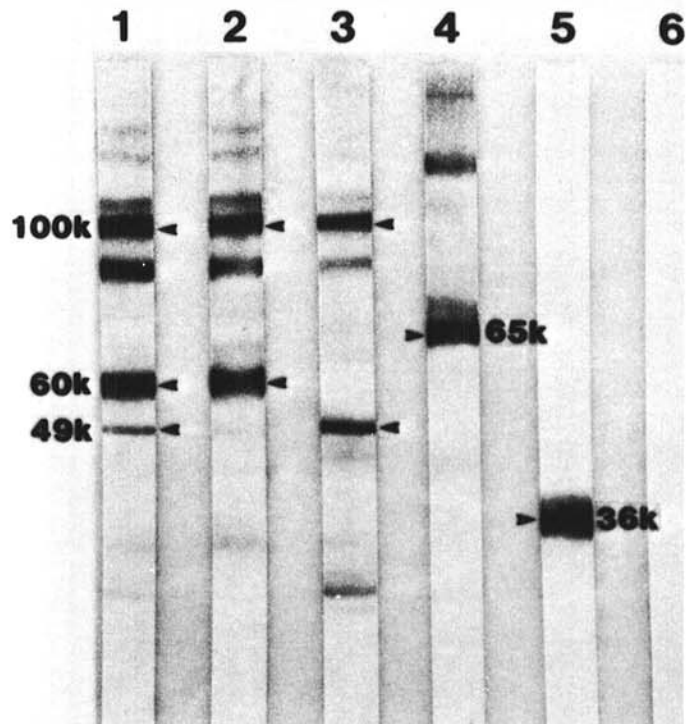


Fig. 6. Western blot analysis of proteins contained in semipurified clover yellow vein virus (CYVV)-P nuclear inclusion (NI) preparations. After protein transfer, nitrocellulose membrane strips were incubated lane 1, CYVV-P 100K⁺ protein antiserum; lane 2, CYVV-P 60K NI protein antiserum; lane 3, CYVV-P 49K NI protein antiserum; lane 4, CYVV-P CI protein antiserum; lane 5, CYVV-P CP antiserum; lane 6, normal serum. Resulting antigen-antibody complexes were detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights of major proteins are indicated on the sides of the lanes.

PV-2 54K NI antiserum.

Extracts from tissues singly infected with 14 potyviruses were tested with antiserum to BYMV-PV-2 NI in indirect ELISA, and the results are shown in Table 3. Infected plant extracts of nine of 14 potyviruses reacted positively with antiserum to 54K NI protein, indicating that some, but not all, potyviruses did induce detectable amounts of antigens related to large NI proteins.

DISCUSSION

Nuclear inclusions induced by BYMV-PV-2 and CYVV-P were successfully obtained in a partially purified form. Because of differences in size and shape of the NI induced by the different viruses, the success of purification depended on careful, step-by-step evaluation of the treatments. Generally, we found NI were very resistant to Triton X-100 treatment, a property that facilitated their purification. The NI could be retreated with Triton X-100 several times and separated from the green pigment of the plant extracts by low-speed centrifugation. A similar approach has been used in purifying amorphous inclusions (6).

As with TEV NI (16), the NI of BYMV-PV-2 and CYVV-P contained two species of protein. This seems to be a common phenomenon for NI induced by potyviruses. The two protein monomers in each NI may be equimolar, as with TEV (16) and BYMV-PV-2, or in apparently different ratios, as with CYVV-P NI. Semipurified NI preparations from both viruses contain proteins other than the two distinct monomers. However, only the 98K and 100K protein are found consistently associated with NI induced by BYMV-PV-2 and CYVV-P, respectively. This is consistent with the analysis of TEV NI (15,16,23). The other

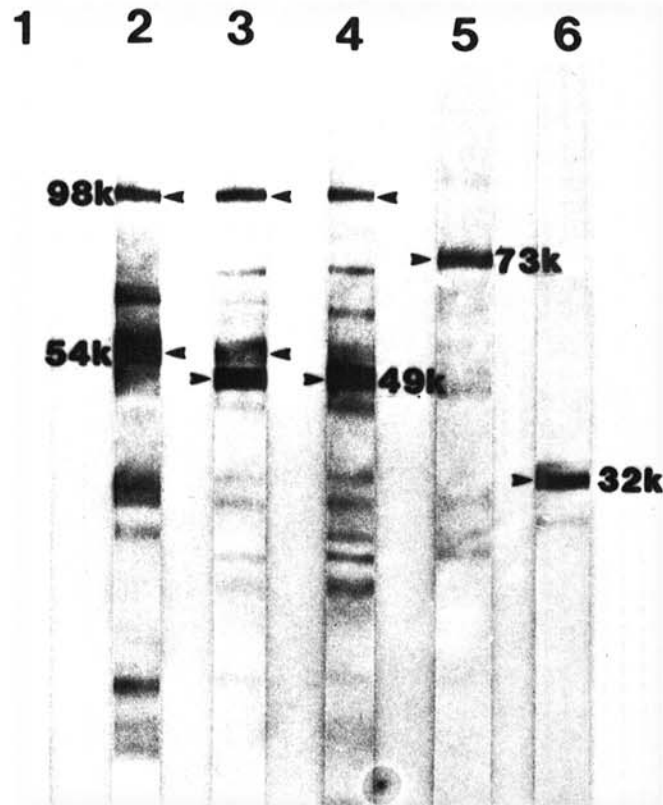


Fig. 7. Western blot analysis of proteins contained in semipurified bean yellow mosaic virus (BYMV)-PV2 nuclear inclusion (NI) preparations. After protein transfer, nitrocellulose membrane strips were incubated with lane 1, normal serum; lane 2, BYMV-PV-2 54K NI protein antiserum; lane 3, BYMV-PV2 98K protein antiserum; lane 4, BYMV-PV-2 49K NI protein antiserum; lane 5, BYMV-PV-2 CI protein antiserum; lane 6, BYMV-PV-2 CP antiserum. Resulting antigen-antibody complexes were detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights are indicated for major proteins.

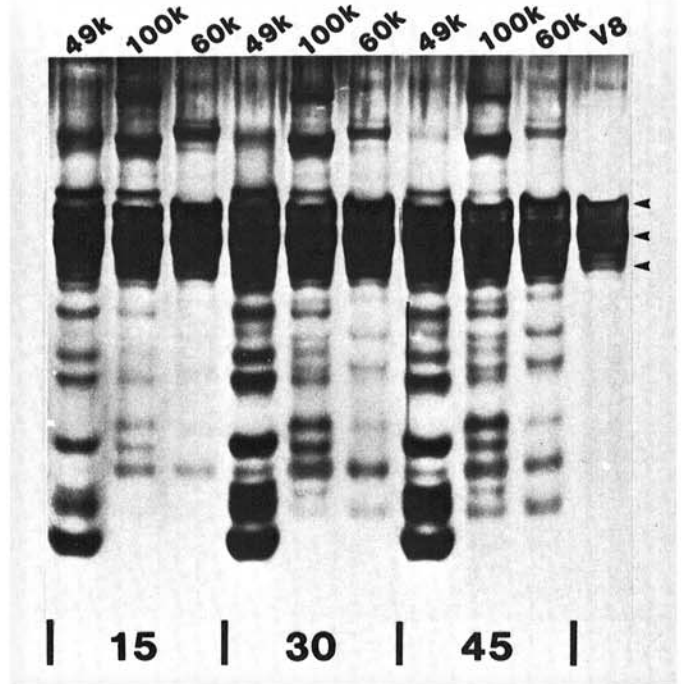


Fig. 8. *Staphylococcus aureus* V8 protease digest pattern comparison of polyacrylamide gel electrophoresis (PAGE)-purified clover yellow vein virus (CYVV)-P 100K, 60K, and 49K nuclear inclusion proteins. The protease digested peptides were separated by sodium dodecyl sulfate (SDS)-PAGE and visualized by silver staining. Arrows indicate components of V8 protease consistently observed in each lane. Identities of proteins are indicated at the top of each lane. Numbers at the bottom indicate digestion time in minutes.

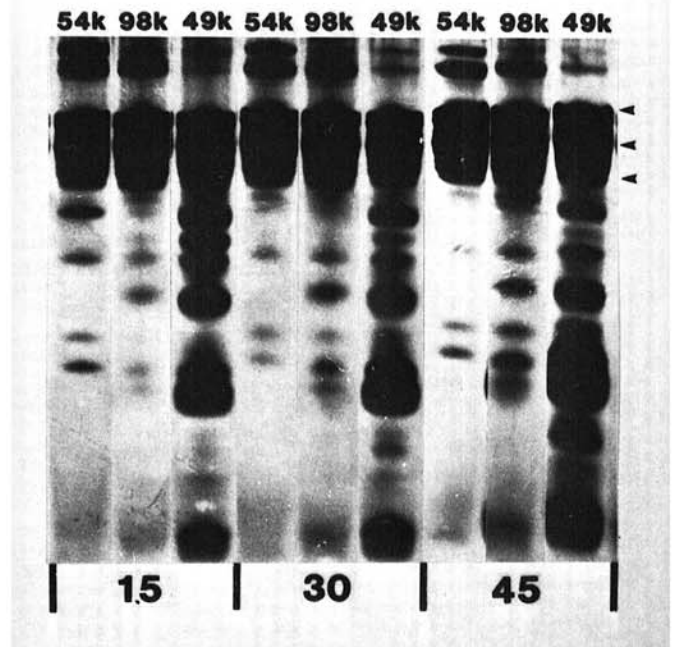


Fig. 9. *Staphylococcus aureus* V8 protease digest pattern comparison of polyacrylamide gel electrophoresis (PAGE)-purified bean yellow mosaic virus (BYMV)-PV-2 98K, 54K, and 49K proteins. The protease digested peptides were separated by sodium dodecyl sulfate (SDS)-PAGE and visualized by silver staining. Arrows indicate components of V8 protease consistently observed in each lane. Identities of proteins are indicated at the top of each lane. Numbers at the bottom indicate digestion time in minutes.

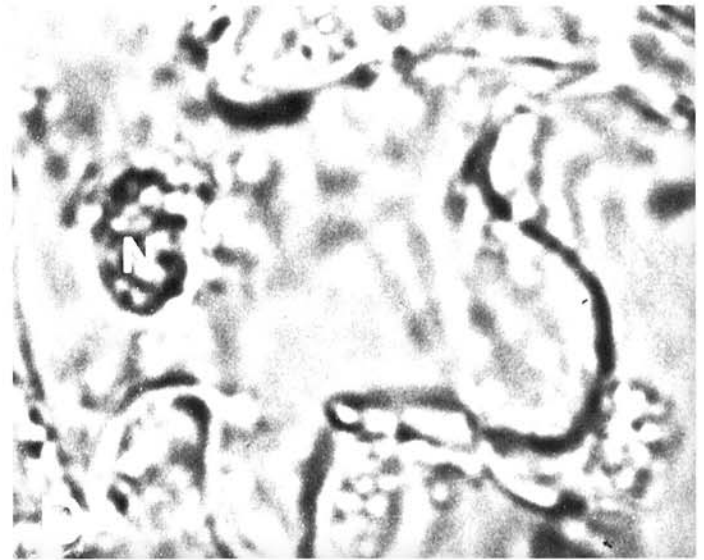
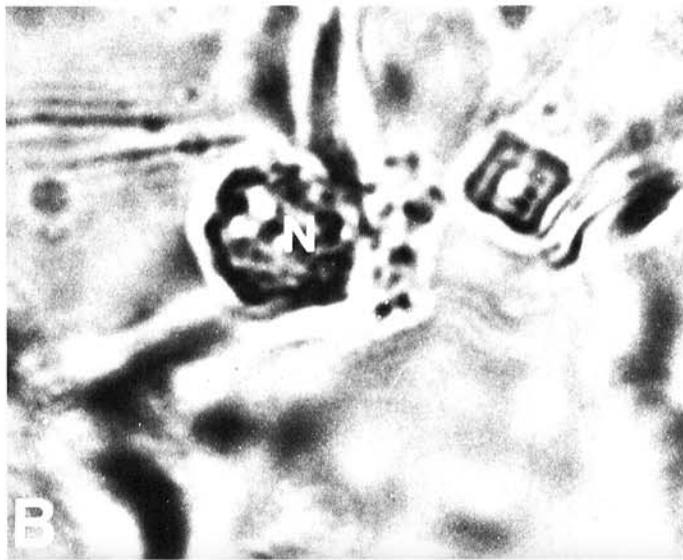
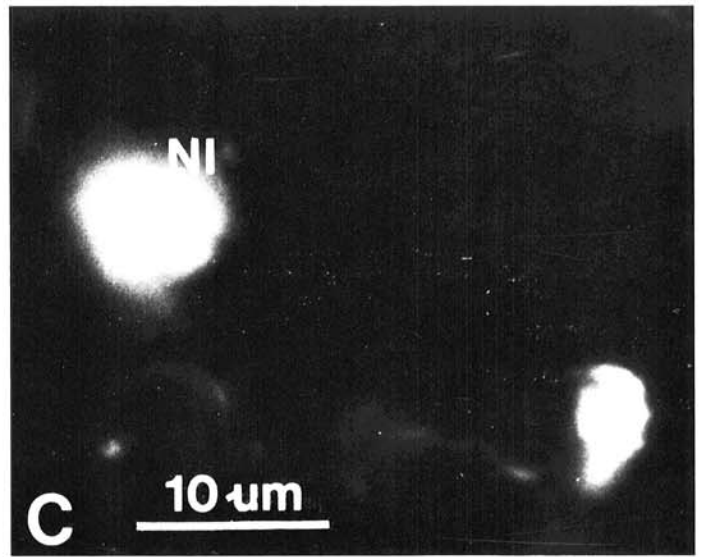
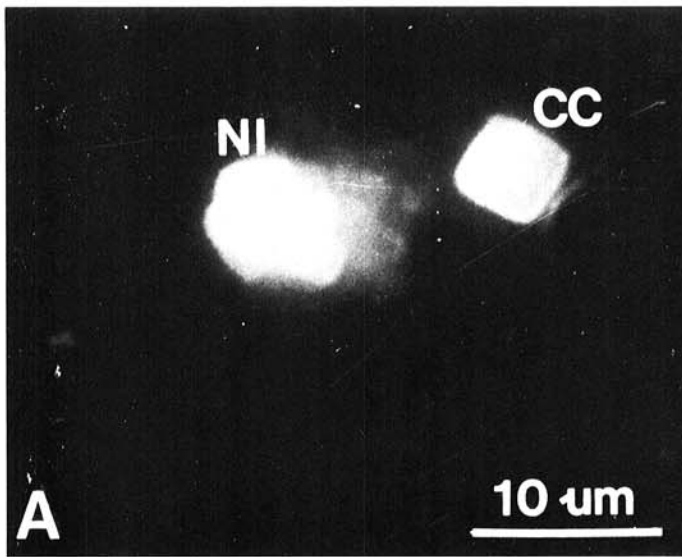


Fig. 10. Detection of nuclear inclusions in situ by immunofluorescence microscopy. **A**, Bean yellow mosaic virus (BYMV)-PV-2 infected pea epidermal strip photographed with epifluorescence optics showing the specific fluorescence from nuclear inclusion (NI) and cytoplasmic crystal treated with BYMV-PV-2 54K NI protein antiserum. **B**, The same field of view photographed with visible light. **C**, Clover yellow vein virus (CYVV)-P infected pea epidermal strip photographed with epifluorescence optics, showing the specific fluorescence from NI treated with CYVV-P 49K NI protein antiserum. **D**, The same field of view photographed with visible light. NI = nuclear inclusion; CC = cytoplasmic crystal; N = nucleus.

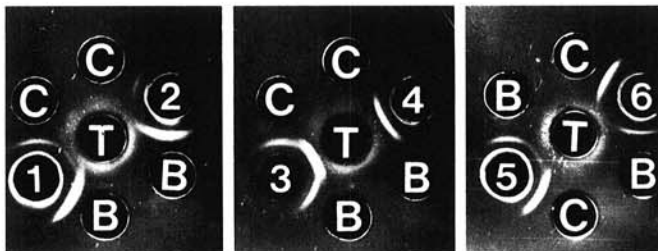


Fig. 11. Serological relationship of large and small nuclear inclusion proteins between tobacco etch virus (TEV), clover yellow vein virus (CYVV)-P and bean yellow mosaic virus (BYMV)-PV-2 in immunodiffusion tests. Wells contained: 1 = BYMV-PV-2 54K nuclear inclusion (NI) protein antiserum, 2 = BYMV-PV-2 49K NI protein antiserum, 3 = TEV 54K NI protein antiserum, 4 = TEV 49K NI protein antiserum, 5 = CYVV-P 60K NI protein antiserum, 6 = CYVV-P 49K NI protein antiserum, C = sodium dodecyl sulfate (SDS)-treated CYVV-P infected Alaska pea extract, B = SDS-treated BYMV-PV-2 infected Alaska pea extract, T = SDS-treated TEV-infected extract of *Datura stramonium*.

proteins, since they do not consistently occur and their staining intensities varied among different preparations, are assumed to be degraded or incomplete dissociation products of NI proteins. This was supported by the cross-reactivities of the proteins with antisera to NI proteins in Western blotting tests. The 98K and 100K NI proteins were originally considered as dimers presumably resulting from incomplete dissociation of either the large NI or small NI proteins during SDS-PAGE (15). Because reconstitution experiments showed that these high M_r proteins appeared independently of the presence of reducing agents, and that they were resistant to further dissociation treatments, these proteins should be considered as separate entities. These proteins contained sequences from both the large NI and small NI proteins, based on immunological analyses and by mapping the partially digested peptides of these proteins. These studies provide evidence for the existence of polyproteins in potyvirus-infected tissue. The significance of these unprocessed polyproteins in the infected tissue is unknown. However, detection of these polyproteins in vivo provides additional evidence that proteolytical processing of polyproteins is part of the translation strategy of potyviruses (2,8,13,27,30).

Antisera prepared against 98K protein of BYMV-PV-2 only reacted with small NI protein (49K NI) but not with large NI protein (54K NI) of BYMV-PV-2 in SDS-immunodiffusion tests. However, it did react slightly with 54K NI protein in Western blot analysis, indicating this antiserum did contain a small quantity of antibodies specific to 54K NI protein. This small quantity of antibodies was only detected by Western blotting because the signal was enhanced by enzyme-substrate reactions during immunoblotting procedures. Possible reasons for this low level of immunoreactivity are: epitopes of the 49K NI were more immunogenic than those of the 54K NI protein, and the individual rabbit did not respond to most epitopes of the 54K NI protein.

Using antisera to TEV NI proteins, Dougherty and Hiebert (10) showed that antisera to 54K and 49K NI proteins each immunoprecipitated a different set of in vitro translation products of TEV RNA. In this paper, we demonstrated that the two NI protein monomers of BYMV-PV-2 and CYVV were antigenically distinct from each other and from CI and CP by SDS-immunodiffusion tests and by Western blotting. Evidence was also presented showing that the two NI protein monomers were chemically different by separating the proteolytic fragments.

As with TEV NI proteins, NI proteins of BYMV-PV-2 are encoded by viral RNA (3). The in vitro translation of CYVV-P RNA (19) and immunoprecipitation analysis of its products (E. Hiebert, unpublished data) provide similar indications that NI proteins of CYVV-P are also of viral origin.

Antisera to NI proteins have been shown in immunofluorescence tests to detect NI in situ despite the fact that SDS-treated NI proteins were used as the immunogens. This not only indicated that the isolated NI proteins were the major constituents of the NI seen

TABLE 3. Serological cross-reactivity of extracts from potyvirus infected plants to antiserum against BYMV-PV-2 54K nuclear inclusion protein in indirect-enzyme-linked immunosorbent assay (ELISA) tests^a

Potyviruses ^b	Host	Absorbance ($A_{405\text{nm}}$) ^c	
		Infected extracts ^d	Healthy extracts
BYMV-PV-2	Pea	1.71 ± 0.04	0.10 ± 0.02
CYVV-P	Pea	1.00 ± 0.05	0.10 ± 0.02
WMV-2	Pea	0.44 ± 0.02	0.10 ± 0.02
CAMV	Cowpea	0.56 ± 0.02	0.08 ± 0.02
PMoV	Pea	1.17 ± 0.03	0.10 ± 0.02
TEV	Tobacco	0.80 ± 0.05	0.05 ± 0.03
BYMV-204-1	Pea	1.60 ± 0.04	0.10 ± 0.02
BYMV-Scott	Pea	1.71 ± 0.03	0.10 ± 0.02
BYMV-Orchid	Pea	1.46 ± 0.03	0.10 ± 0.02
PRSV-P	Squash	0.18 ± 0.02	0.11 ± 0.02
PRSV-W	Squash	0.14 ± 0.02	0.11 ± 0.02
BICMV	Cowpea	0.15 ± 0.02	0.08 ± 0.02
PeMV	Tobacco	0.12 ± 0.02	0.05 ± 0.03
PStV	Peanut	0.17 ± 0.02	0.10 ± 0.02

^aIndirect-ELISA test described by Yeh and Gonsalves (29) with modifications was followed. Immunoglobulin purified from antiserum collected 2 mo after last immunization was used at a concentration of 1 µg/ml. Goat anti-rabbit IgG alkaline phosphatase conjugate diluted 1/1,000 was used.

^bBYMV-PV-2: PV-2 isolate of bean yellow mosaic virus; CYVV-P: Pratt isolate of clover yellow vein virus; WMV-2: watermelon mosaic virus-2; CAMV: cowpea aphid-borne mosaic virus; PMoV: peanut mottle virus; TEV: tobacco etch virus; PRSV-P: Papaya ringspot virus; PRSV-W: papaya ringspot virus type W originally described as WMV-1; BICMV: blackeye cowpea mosaic virus; PeMV: pepper mottle virus; PStV: peanut stripe virus.

^cAbsorbance values and confidence intervals ($P = 0.05$) were obtained at 405 nm. All values were obtained from three replicates in the same test.

^dExtracts from infected and healthy tissue diluted 1/125.

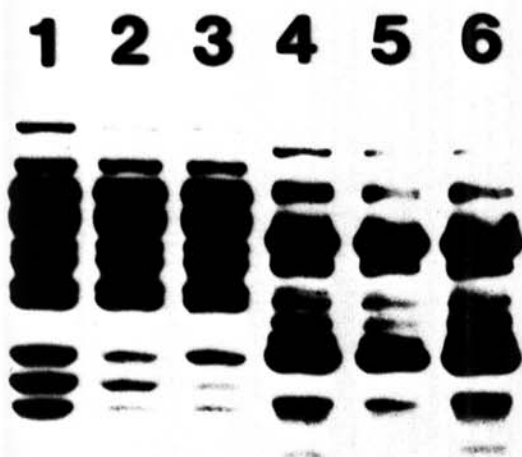


Fig. 12. Protein profiles of in vitro translation products of bean yellow mosaic virus (BYMV)-PV-2 RNA and pepper mottle virus (PeMV) RNA immunoprecipitated with antisera to the (54-60K) large nuclear inclusion (NI) proteins of BYMV-PV-2, clover yellow vein virus (CYVV)-P, and tobacco etch virus (TEV), showing the serological conservativeness of these three large NI proteins. Translations of BYMV-PV-2 RNA (lanes 1-3) and PeMV RNA (lanes 4-6) were done in rabbit reticulocyte lysate system as described in the text. The figure illustrates (S^{35}) methionine-labeled proteins immunoprecipitated by each antiserum that were separated in 7.5-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and detected by fluorography. Lanes 1 and 4, products immunoprecipitated by antiserum to BYMV-PV-2 54K NI protein; lanes 2 and 5, products immunoprecipitated by antiserum to TEV 54K NI protein; lanes 3 and 6, products immunoprecipitated by antiserum to CYVV-P 60K NI protein.

in situ, but also that the SDS-treated proteins still preserved, at least in part, the antigenicities of the natural proteins. Similar results have been consistently obtained with antisera prepared to potyviral CI, AI, and TEV NI (6,15). The NI induced by TEV reveal a distinctive cross-hatched substructure under electron microscopy (16,23). Attempts to observe similar substructures with BYMV-PV-2 and CYVV-P NI were unsuccessful. This may be due to the electron-dense, cubical-shape of their NI, which contrast with the thin platelike structures of TEV NI.

As mentioned, the antigenic properties of the viral-specified protein similar to TEV 54K NI protein may be conserved among different potyviruses as shown by the broad spectrum reactivities of TEV 54K NI antiserum to the in vitro translation products of more than 20 potyviruses (E. Hiebert, unpublished data). We demonstrated here that 54K NI protein of TEV, 54K NI protein of BYMV-PV-2, and the 60K NI protein of CYVV-P are serologically related. The fact that specific antiserum to the large monomer of any of three different NI could immunoprecipitate an identical set of peptides from in vitro translation of a different viral RNA (i.e., pepper mottle virus RNA), further confirmed the serological conservation of the large monomers of potyviral NI. However, attempts to use this conserved potyviral gene product as a target for indexing potyvirus infections were not completely successful because apparently not every potyvirus accumulated enough serologically related antigen in tissue extracts to permit its detection by indirect ELISA using the heterologous BYMV-PV-2 54K NI protein antiserum. If potyviruses, as proposed by most workers, possess a polyprotein type of translational strategy (1,2,9,27,30), then they should theoretically induce equal amounts of every protein that they encode. It is possible that for some potyviruses the NI proteins only exist in soluble form in the infected cells and do not aggregate into inclusion bodies. This soluble protein may easily be degraded and thus escape detection.

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