Cytology and Histology

Barley Stripe Mosaic Virus but Not Brome Mosaic Virus Binds to Wheat Streak Mosaic Virus Cylindrical Inclusions In Vivo

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ABSTRACT

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Immunogold detection was used to follow interactions of three wheat viruses in doubly infected tissue. Most cylindrical inclusions (CI) of wheat streak mosaic virus (WSMV) in young, doubly infected cells were labeled with antibody to barley stripe mosaic virus (BSMV) or antibody to WSMV but not with antibody to brome mosaic virus (BMV), indicating an association of BSMV or WSMV with WSMV CI. Antibody to BSMV-coat protein absorbed with an excess of intact BSMV virions no longer labeled WSMV CI but still labeled BSMV virus aggregates, indicating that intact virions rather than coat protein adsorbed to CI. The amount of BSMV and WSMV virions and cylindrical inclusions was about the same in doubly infected as in singly infected plants whether analyzed by electron microscopy or by gel electrophoresis of extracts from infected tissue. Interactions between the two viruses were not detectably influenced by greenhouse temperatures or light intensity. Cylindrical inclusions of WSMV were not labeled with monoclonal antibodies to six contractile proteins that are widely conserved in the plant and animal kingdoms.

Various aspects of double infections of plants with related or unrelated viruses have been described. Synergism, cross protection, enhanced synthesis or movement, genomic masking, and others have been reviewed and discussed (21). Advances in immunocytochemical tracing of early events in intracellular plant virus capsid protein synthesis (18,19) have made it possible to detect viral coat protein antigen at the ultrastructural level. The immunogold procedure is sensitive, specific with a low nonspecific

background, and high resolution is attained with ultrathin sections.

It was reported earlier that in double infections of soilborne wheat mosaic virus (SBWMV) and wheat spindle streak mosaic virus (WSSMV) or wheat streak mosaic virus (WSMV), cylindrical inclusions (CI) of both WSSMV and WSMV were labeled with antibody to SBWMV (13,14). Virus coat proteins of both WSSMV and WSMV also attached to their respective CI (15). It seems then that CI of WSSMV and WSMV will accept, or have associated, homologous capsid protein and virions as well as those of an unrelated non-CI producing rod-shaped virus (SBWMV). It is further noteworthy that virions of oat golden stripe virus (OGSV) have been reported to be ranked along the arms of CI of oat mosaic virus (OMV) (24). There have also been reports of specific association of animal viruses, or their proteins, with cell components (5,6) or of plant viruses with cell organelles or structures. Barley stripe mosaic virus (BSMV) is one plant virus shown to be associated with plastid membranes, microtubules, or the endoplasmic reticulum (4,19).

Information on the relationship of virus synthesis and assembly with the host's cytoskeleton or virus-coded structures, such as CI, is still limited for animal viruses (23) and even more so for plant viruses. Much remains to be catalogued and understood. It was, therefore, of interest to determine if WSMV-CI would accept, or have associated with it, any other virus than SBWMV coinfecting the same cell and whether the observed phenomenon was also true for the rod-shaped barley stripe mosaic virus (BSMV) or the icosahedral brome mosaic virus (BMV).

MATERIALS AND METHODS

Hosts and virus. Wheat plants (*Triticum aestivum* L. 'Centurk') were manually inoculated singly or in combination with WSMV (Type strain ATCC PV57), BSMV strain ND 18, BSMV white leaf strain (22), or BMV (Type strain ATCC PV47). Plants were maintained in a greenhouse at 20 or 28–33 C without supplemental light during the spring, summer, and fall of 1985 and 1986.

Electron microscopy. Ten days to 3 mo after inoculation, leaves of various ages were sampled and vacuum infiltrated with 0.1 M K_2HPO_4, adjusted to pH 7.2 with citric acid, cold treated, and glutaraldehyde fixed as described (12). No OsO_4 postfixation was used since it reduces the serological reactivity of antigens and interferes with UV polymerization of the resin used to embed tissues. Tissues were dehydrated in a graded ethanol series at room temperature and infiltrated under N_2 with London Resin Gold or London Resin White resins (Agar Aids, Stansted, England) as previously reported (13–15).

Purification of cylindrical inclusions. Cylindrical inclusions of singly (WSMV only) or doubly infected plants were isolated by the method of Brakke et al (3). For cylindrical inclusions examined in leaf dips, hydroquinone sulfonic acid was used instead of 2-mercaptoethanol.

Antisera. Antiserum to BMV was received from L. C. Lane. Antisera to WSMV and BSMV were produced in rabbits injected with intact virions and to BSMV-coat protein by immunization with BSMV-coat protein produced by the $CaCl_2$ method (8) (courtesy of Brakke and Ball, 1968, and of E. M. Ball). BSMV and BMV were purified by differential centrifugation and WSMV by differential and density-gradient centrifugation. All anti-viral antisera were preabsorbed with an equal volume of sap expressed from healthy wheat or barley. Antibody to BSMV-coat protein was additionally absorbed with excess BSMV virions that had been fixed in glutaraldehyde and neutralized. Antibody to BSMV was additionally absorbed with excess BSMV-coat protein. Monoclonal rat or mouse antibodies to the contractile proteins tubulin, vimentin, and desmin (Accurate Chemical & Scientific Corp., Westbury, NY), glial fibrillary acidic protein (GFAP) (Labsystems Inc., Chicago, IL), actin, and myosin (Miles Scientific, Naperville, IL) were purchased.

Leaf dips. Leaf dips and decoration of viruses and cylindrical inclusions was with the method of Lin (17).

Immunohistochemistry. Antisera for immunolabeling were diluted 1/200 with 0.1 M K_2HPO_4, adjusted to pH 7.2 with citric acid, containing 1% normal goat serum and 0.05% NaN_3. Ultrathin sections were labeled as previously described with the two-step method (18). To detect mouse or rat monoclonal antibody, gold-labeled goat anti-mouse or anti-rat was prepared as for goat anti-rabbit.

Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM10A at 60 kV.

Quantitative analyses for BSMV, WSMV, and WSMV 66K protein. The youngest fully expanded leaves of plants infected with either strain of BSMV, WSMV, or doubly infected were harvested from 10–90 days postinoculation. Several independent leaf dip tests of doubly infected plants at different times after inoculation showed that doubly inoculated plants were indeed infected. Both viruses remained in high enough concentration throughout the 10–90-day time frame to be detected. Two to three grams of infected leaves was ground in 5 ml of cold 0.01 M K_2HPO_4 in a cold mortar and pestle for 2 min. Expressed sap was incubated 1 hr at 40 C (WSMV) or 25 C (BSMV), Triton X-100 was added to 2% by volume, and virus content was analyzed by density gradient centrifugation (2). Cylindrical inclusion body protein of WSMV (66K protein) was assayed by SDS-polyacrylamide gel electrophoresis with a discontinuous buffer system according to Laemmli (11) after partial purification (3). Each experiment was repeated three times or more. Plants doubly infected with WSMV and BMV or with BMV alone were not assayed for virus concentration or 66K WSMV inclusion body protein.

RESULTS

Membranes normally present as electron dense lines in micrographs of tissue are present here as electron translucent areas since the tissues were not postfixed in OsO_4, except for tissue shown in micrograph 6, which was briefly (10 min) postfixed in 0.1% OsO_4. Not all virions or cylindrical inclusions are labeled in thin sections. Only those at the very surface of the section are accessible to specific antibody.

BSMV and WSMV. Antibody to WSMV as the first label. Cylindrical inclusions were present in large numbers and in aggregates in the cytoplasm of cells doubly infected with WSMV and BSMV (Fig. 1). Virions of WSMV were fewer in number than BSMV and were only seen in small aggregates in tissue sampled from expanding regions of the emerging leaf. No BSMV aggregates or virions were labeled with antibody to WSMV. Double infection with either BSMV strain gave the same result. Antibody to WSMV immunolabeled WSM virion aggregates and some cylindrical inclusions only (Fig. 2) and was highly specific with little or no background.

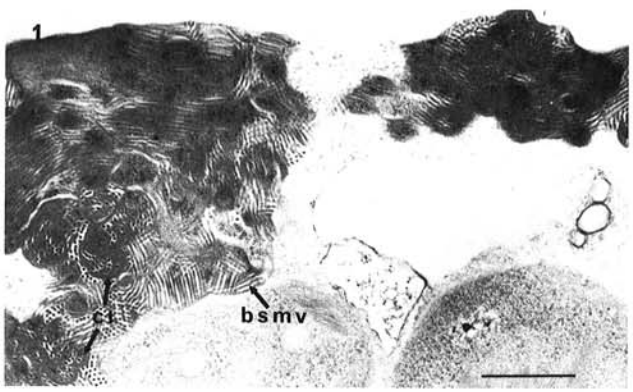
Antibody to BSMV as the first label. Cylindrical inclusions of WSMV showed associated BSMV. The cylindrical inclusion in Figure 3 is labeled with antibody to BSMV as is the nearby plastid. Vacuolation of pro-plastids at their periphery is indicative of active BSMV-protein synthesis (18). Cylindrical inclusions that labeled with antibody to BSMV could be attached perpendicularly to cell walls (Fig. 3) but were also oriented in other directions. On the other hand, some cylindrical inclusions aligned at right angles to cell walls did not label with antibody to BSMV and apparently had little BSMV attached or were below the cut surface and thus not available for labeling. Pinwheels were present with adsorbed BSMV virions (Figs. 1 and 4). Such virions, when observed, were oriented parallel to the long axis of the pinwheel only. It should be noted that BSMV virions formed aggregates only with self (Fig. 5) or cylindrical inclusions (Figs. 1 and 4). In Figure 5, only BSMV virions and, to some extent, cylindrical inclusions are labeled. Virions were not seen associated with normally occurring cell organelles or structures. Fixation conditions did not favor preservation of microtubules. WSMV did not form large mixed virus inclusions with BSMV. Pinwheels, bundles, and CI, however, had considerable affinity for BSMV (Figs. 1 and 4), more so than for the homologous WSMV. Each virus formed its own distinct and discrete aggregate. Label again was highly specific with little or no background. Results of reactions with either BSMV strain were

indistinguishable. No differences were noted when WSMV was inoculated into an established BSMV infection or vice versa, or with simultaneous inoculation with both viruses.

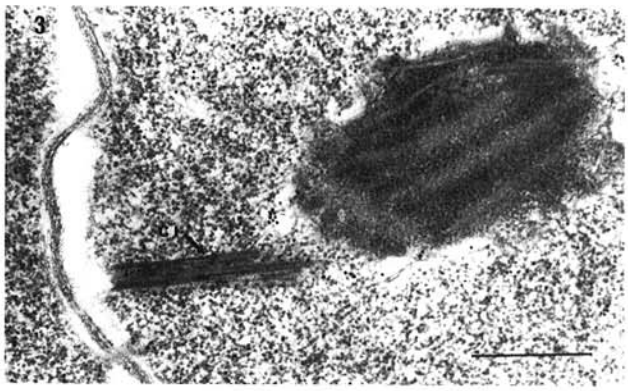
Antibody to BSMV-coat protein before and after absorption with excess intact virions. Antiserum to BSMV-coat protein also reacts with intact virions in leaf dip preparations and in ultrathin sections (19). By adding an excess amount of purified glutaraldehyde-fixed and ammonium chloride-neutralized intact virions (± 10 mg) to 2 ml of 1/200 diluted anti-BSMV coat protein antiserum, all IgG with specifity for intact virions could be removed. The remaining IgG did not react with BSMV virions in leaf-dips as shown by the gold-enhanced antibody decoration method of Lin (17) (inset Fig. 6). The remaining IgG still labeled BSMV in thin sections (Fig. 6) but no longer labeled CI. Conversely, antiserum to BSMV absorbed with excess fixed and neutralized BSMV-coat protein still labeled BSMV aggregates and also CI in doubly infected cells (Fig. 7).

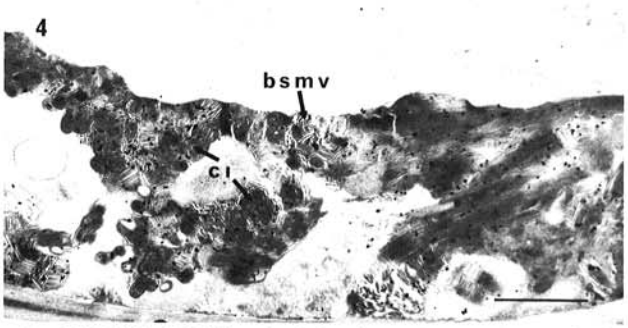
BMV and WSMV. Antibody to WSMV as the first label. Cylindrical inclusions normally formed at right angles to cell walls in young doubly infected tissue cells. Cylindrical inclusions could be labeled positively for associated WSMV or WSMV coat protein (Fig. 8). Specificity of label is shown by label only over the WSMV aggregate and CI. No BMV virions were labeled as evidenced by lack of label over areas other than cylindrical inclusions and the WSMV aggregate. Pinwheels displayed open arms and appeared identical to those in cells infected with WSMV alone and were labeled similarily to bundles.

Antibody to BMV as the first label. Coat protein of BMV or BMV virions was not associated with WSMV aggregates, or cylindrical inclusions (Fig. 9). Lack of label over bundles and pinwheels showed that BMV virions were not entrapped in these structures in spite of the very large numbers of BMV present in

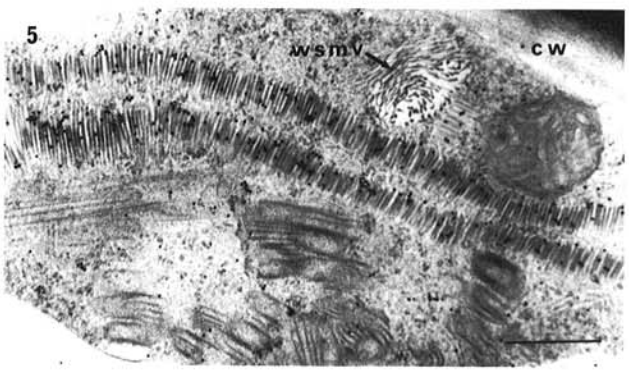


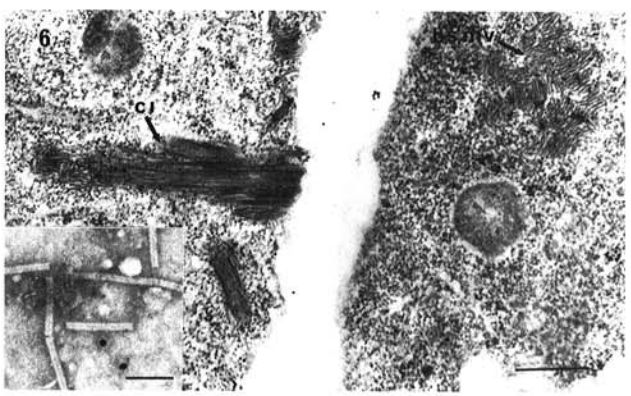
Figs. 1 and 2. 1, Aggregate of wheat streak mosaic virus (WSMV) cylindrical inclusions (CI) and barley stripe mosaic virus (BSMV) in a doubly infected cell (arrows). This particular area of the cell had no clusters of WSM virions. Section treated with rabbit anti-WSMV IgG followed by goat anti-rabbit IgG colloidal gold. Lack of gold label shows that no WSMV protein or virions are associated with CI or BSMV virions. Note that viruslike particles are associated with the pinwheel arms. (Bar is 500 nm.) **2,** Doubly infected cell from the region just behind a root tip meristematic region showing two cylindrical inclusions, perpendicular to a cell wall, that label with antibody to WSMV showing antigen association with the cylindrical inclusions. (Bar is 500 nm.)





Figs. 3 and 4. 3, A doubly infected cell showing a wheat streak mosaic virus (WSMV) cylindrical inclusion labeled with antibody to barley stripe mosaic virus (BSMV). BSMV antigen also labels around the vacuolated periphery of the pro-plastid indicating active BSMV-synthesis (see [18] for details). (Bar is 500 nm.) **4,** Section labeled with antibody to BSMV virions. An area of a doubly infected cell that contains cylindrical inclusions (CI) of WSMV as well as BSMV in a large common aggregate. Both label positive for BSMV. (Bar is 1,000 nm.)





Figs. 5 and 6. 5, Doubly infected cell labeled with antibody to barley stripe mosaic virus (BSMV). BSMV virions in a tier labeled positively and cylindrical inclusions labeled lightly. The wheat streak mosaic virus (WSMV) aggregate (WSMV) next to the cell wall (CW) did not label. (Bar is 500 nm.) **6,** Aggregate of BSMV, positive label, after treatment with anti-BSMV-coat protein that was absorbed with excess intact BSMV. CI not labeled. Inset, lower left, shows no label of intact virions with this absorbed antiserum. (Bar is 500 nm; bar in inset is 100 nm.)

doubly infected cells.

In cells of doubly infected tissue, neither BMV nor WSMV formed specific associations with any cell organelle or structure. Virions of these viruses were only associated with like virions in small or larger aggregates.

Quantitative analysis of singly and doubly infected plants, BSMV and WSMV-only. Results of the assays are shown in Figure 10. Concentrations of WSMV virions in singly or doubly infected tissues were essentially the same within limits of measurement error. Newly expanded young leaves contained approximately the same virus concentrations whether the plants had been inoculated for 10 days or 3 mo, whether the light conditions in the greenhouse were high or low, and whether the temperatures were maintained at 20 or 28–33 C. The amount of extractable WSMV from other leaves may vary (see Discussion [3]). The concentration of BSMV virions in singly infected leaves also equalled that in doubly infected leaves. The viruses coexisted in cells and did not measurably affect one another in spite of the fact that BSMV was adsorbed onto some cylindrical inclusions of WSMV.

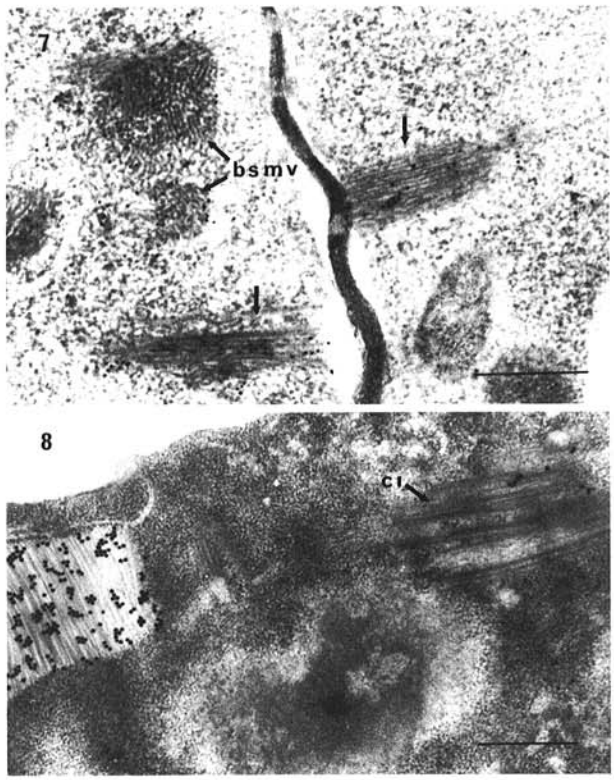
No purification loss of WSMV occurred when sap of BSMV-only and WSMV-only infected plants were mixed. BSMV aggregated under purification conditions used for WSMV and ended up in the low-speed pellet. Some degradation of WSM virions in the bottom pattern of Figure 10 accounts for the broader than usual band of WSMV before the BSMV peak. The shoulder after the main peak of BSMV is a BSMV virion dimer. Each of three upper patterns is extract from, respectively, 1.7, 2, and 1.25+ 1.25 g of leaves, and lower patterns from 2.1 and 2.2. g of leaves. Results of other experiments did not differ much from that shown.

Extractable numbers of CI were also not affected by double infections with BSMV. Polyacrylamide gel analyses of the youngest fully expanded leaf for CI protein in singly and doubly infected plants showed the 66K protein subunit of WSMV CI in comparable concentrations (Fig. 11).

Differences in the number of WSMV aggregates were noted by electron microscopy between young unfurling, emerging leaves and the first fully expanded leaf below it. In the unfolding and expanding leaves of doubly infected plants WSMV CI were aggregated and intertwined with BSMV aggregates (Figs. 1 and 4), while WSMV virion aggregates were small. In expanded leaves CI were still intertwined with BSMV aggregates, but the WSMV virion aggregates were more common and larger than those in emerging leaves (Fig. 5).

Labeling of WSMV CI with antibody to contractile proteins. No positive label was seen over any of the cylindrical inclusions or pinwheels after treatment of thin sections with antibody to tubulin, vimentin, desmin, GFAP, actin, and myosin.

Labeling of isolated CI with antibody to BSMV. Isolated cylindrical inclusions were very unstable and not seen in leaf dips of crude sap. Inclusions had to be partially purified. It was further



Figs. 7 and 8. 7, Doubly infected tissue showing label over wheat streak mosaic virus (WSMV) cylindrical inclusions (arrows) and barley stripe mosaic virus (BSMV) aggregates. Section was treated with antibody to BSMV virions absorbed with excess BSMV coat protein. (Bar is 500 nm.) **8,** Aggregate of WSMV and CI showing label after immunolabeling with antibody to WSMV. BMV distributed evenly in the cytoplasm is not labeled. (Bar is 500 nm.)

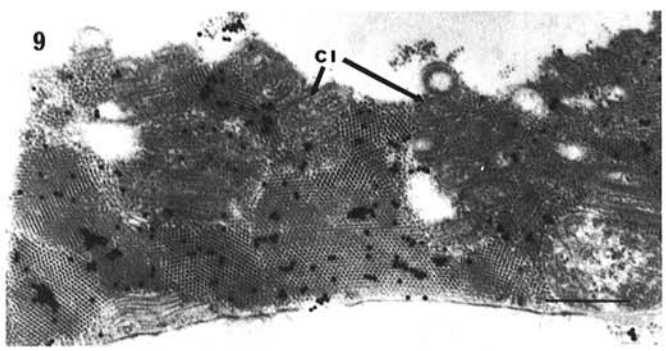


Fig. 9. Cylindrical inclusions (CI) of wheat streak mosaic virus (WSMV) do not label with antibody to brome mosaic virus (BMV). Only BMV has positive label showing no BMV association with CI of WSMV. Central and cytoplasmic vacuoles are also free of label. (Bar is 500 nm.)

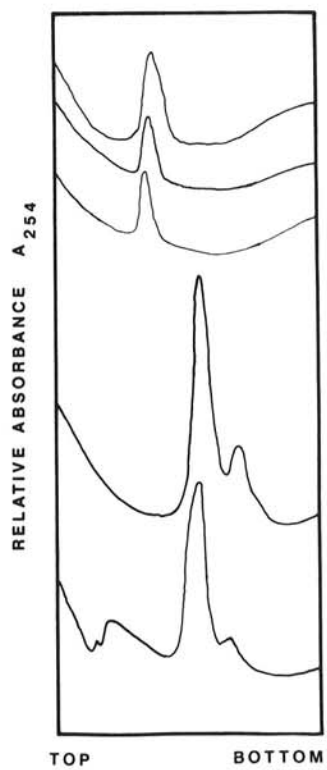


Fig. 10. Sucrose density gradient analysis of virion content of the youngest fully expanded leaf of wheat streak mosaic virus (WSMV)-, barley stripe mosaic virus (BSMV)- and doubly infected wheat leaves 14 days after inoculation. From top to bottom: WSMV-only; mixed sap from WSMV-only and BSMV only, purified as for WSMV; BSMV and WSMV doubly infected leaf, purified as for WSMV; BSMV only; BSMV and WSMV doubly infected leaf purified as for BSMV.

necessary to add hydroquinone sulfonic acid as the reducing agent instead of 2-mercaptoethanol for CI to withstand the handling, labeling, and negative staining procedures. When the inclusions did not label at pH 8, the pH of the isolation medium, other pH's, addition of 0.05 M phosphate, and 0.05 M EGTA or EDTA, were tried in an attempt to prevent possible removal of CI-binding virions. The isolation and labeling procedures were carried out at pH 5.0, 7.0, 8.0, and 9.1 with and without added phosphate, EGTA, or EDTA. Cylindrical inclusions could not be isolated at pH 5.0. The hydroquinone sulfonic acid purified inclusions were stable at higher pH's but at no time showed label after treatment with antibody to BSMV (Fig. 12). However, copurified BSMV virions were unstable under the ISEM procedures and not often seen. Figure 12 was chosen because it shows label of some nearby BSMV.

Antibody to 66K protein. Isolated CI could be positively identified as such by labeling with IgG to 66K protein (Fig. 13). Figure 13 shows the nondeleterious effect of hydroquinone sulfonic acid on antigenic specificity of cylindrical inclusions.

DISCUSSION

Thin sections of leaves doubly infected with WSMV and BSMV appeared to have more WSMV cylindrical inclusions than leaves infected with WSMV only because BSMV and CI often occurred in large aggregates, which were readily located at low magnifications (Figs. 1 and 4). However, SDS-PAGE analysis showed about the same amount of 66K inclusion body protein in both singly and doubly infected leaves. A possible source of error in reliance on interpretation of EM results (more CI in doubly than in singly infected cells) would be reinforced when comparisons of gel electrophoretic analyses of leaves of different ages are made. The concentration of BSMV is relatively constant in leaves of different ages, whereas WSMV reaches a peak concentration in the youngest fully expanded leaf and is undetectable by density gradient centrifugation in young leaves less than 5 cm long and in old leaves (2,3). It is important, therefore, to compare leaves in the same stage of development.

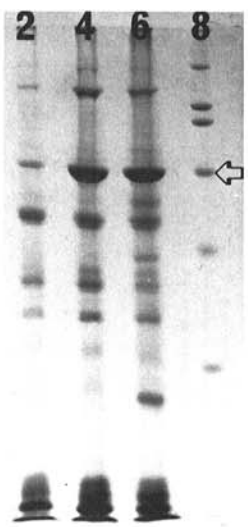
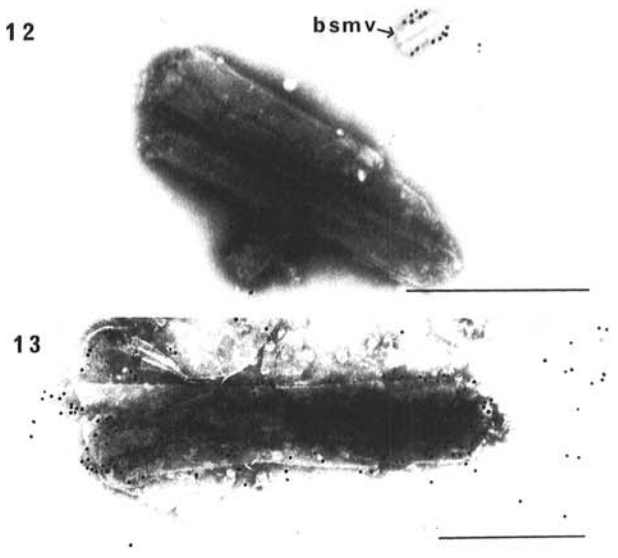


Fig. 11. Polyacrylamide gel analysis of the 66K inclusion body protein of wheat streak mosaic virus (WSMV). Young fully expanded leaves were used. Lanes 2–6, after purification as for WSMV inclusion bodies, essentially a 12,000 rpm pellet of Triton X-100 treated plant extract: lane 2, healthy wheat; lane 4, WSMV-only; lane 6, infected with WSMV and barley stripe mosaic virus (BSMV); lane 8, molecular weight standards: myosin (Mr97,000), beta-galactosidase (Mr116,000), phosphorylase B (Mr97,000), bovine serum albumin (Mr66,000), ovalbumin (Mr45,000), and carbonic anhydrase (Mr29,000). Arrow indicates 66K mol wt band. WSMV capsid protein is not detectable in any lanes, but BSMV capsid protein is readily visible at 23K in lane 6. The 66K WSMV inclusion body protein is equally visible in lanes 4 and 6. The concentrations of the 66K protein in single and double infected plants does not differ significantly.

For the following reasons cylindrical inclusion labeling with antibodies to contractile proteins was attempted: Binding of viral proteins of animal viruses to microtubules (contractile) has been reported (5) as well as binding of BSMV to microtubules (4,19); all contractile proteins have been reported to contain a common antigenic determinant. The monoclonal antibody to this determinant also bound to a 66K protein that could be a component of all intermediate filaments (contractile) (25); the protein of WSMV CI is 66K (3); intermediate filaments and tubulin (contractile) have been highly conserved among the plant and animal kingdoms (7); the hypothesis was advanced that CI serve to facilitate cell-to-cell spread by aligning virus with plasmodesmata (15); the dark staining of cylindrical inclusions in thin sections by uranyl acetate suggested the presence of phosphate groups as in tubulin (20). The uranyl ion has a high affinity for phosphates (10).

However, none of the monoclonal IgG to six contractile proteins labeled CI. Lack of label over CI after treatment with the six monoclonal antibodies does not disprove the hypothesis that CI are contractile. Monoclonal antibodies are identified based on reaction of antibody with antigen in enzyme-linked immunosorbent assay (ELISA) wells. For instance monoclonal antibodies to maize dwarf mosaic virus (MDMV) A and MDMV-B (courtesy of J. Hill, Ames, IA) failed to label the viruses in ultrathin sections of infected corn or in leaf dip decoration tests (*unpublished results*) although they are useful for separating and identifying the viruses by ELISA.

The reason for specific attachment of SBWMV (14), BSMV (Figs. 1, 3, 4, and 7), or WSMV to WSMV cylindrical inclusions (Figs. 2 and 8 and [15]) or of OGSV to OMV CI (24) is still unknown. Attachment of BSMV virions to CI as shown here (Figs. 1, 4, and 7) is not attributed to simple entrapment. BMV virions, which are far more abundant than BSMV virions in infected cells, were not entrapped as shown by lack of label over CI after treatment of BMV- and WSMV-infected tissue sections with antibody to BMV (Fig. 9). Binding of SBWMV or BSMV to WSMV CI is more specific than by a simple, random hydrophobic or ionic interaction. If this were the case, BMV virions would also have been found in association with CI and WSMV and BSMV virions would have formed mixed aggregates, which they did not. Hydrophobic and ionic sites should be abundant and evenly distributed throughout the living cell and not be limited to cylindrical inclusions-only. However, Harrison and co-workers



Figs. 12 and 13. 12, Isolated cylindrical inclusions from young doubly infected wheat labeled with antibody to barley stripe mosaic virus (BSMV). Label on copurified BSMV is positive but inclusions do not label. (Bar is 500 nm.) **13,** Control with antibody to cylindrical inclusions shows label, indicating that the structure is indeed a cylindrical inclusion and not cell debris. (Bar is 500 nm.)

have described the attachment of one virus to another as a result of hydrophobic forces (1,9). Aggregations of virus to self, such as in Figures 1 and 4–9 are common in cells fixed in such a way so as to preserve the very fragile inclusions (12) and ribosomes.

The association of SBWMV protein (13,14) and BSMV with cylindrical inclusions in vivo may be difficult to prove or reproduce in vitro. Cylindrical inclusions were unstable in the leaf dip procedure and also during purification attempts (3). Reducing ribonuclease or phosphatase activity by high pH or by addition of inorganic phosphate, EGTA, or EDTA did not prevent BSMV loss during isolation and ISEM of cylindrical inclusions. To complicate matters, BSMV itself readily dissociates after antibody decoration and negative staining (16). Even though hydroquinone sulfonic acid sufficiently stabilized cylindrical inclusions so that they did withstand handling required in ISEM (Figs. 12 and 13), ways to prevent release of BSMV from cylindrical inclusions were not found.

It has thus been shown that a second non-CI coding rodshaped virus will specifically associate with WSMV-CI but that the icosahedral BMV does not. It remains to be determined if this particular association (rod-shaped versus icosahedral-virus and CI) holds true for other potyviruses and nonrelated rod-shaped or icosahedral viruses and if the nonrelated virus derives an advantage from the CI association such as facilitation of cell-to-cell spread.

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