Soilborne Wheat Mosaic Virus Antigen Binds to Cylindrical Inclusions of Potyviruses in Doubly Infected Cells of Wheat

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ABSTRACT

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Both intact virions and capsid proteins of soilborne wheat mosaic virus bound to cylindrical inclusions of the potyviruses Hordeum mosaic virus or Agropyron mosaic virus in doubly infected wheat cells but not to other cell organelles. Double labeling of ultrathin sections first with primary antiserum to one of the viruses and then with goat anti-rabbit attached to colloidal gold could precisely localize each antigen. The results confirm earlier findings that proteinaceous plates that form the cylindrical inclusions or pinwheels of potyviruses can bind heterologous as well as homologous virions or capsid proteins of rod-shaped viruses.

Additional keywords: immunogold, localization of viral proteins, viral protein attachment.

Soilborne wheat mosaic virus (SBWMV) is a rod-shaped furovirus with a bipartite genome (3). It is transmitted to wheat and other small grains by the obligate, root cortex-inhabiting, fungal parasite *Polymyxa graminis* Led. (3). In the spring, virions of two lengths, 281 and 138 mm, can be isolated from infected wheat. Virus with particles of these lengths has been named wild type (WT) (19). In previous publications it was shown that capsid protein of SBWMV-WT virions can associate with cylindrical inclusions (CIs) of the potyviruses wheat spindle streak mosaic virus (WSSMV) and wheat streak mosaic virus (WSMV) in doubly

infected wheat (11,12). Cylindrical inclusions of WSMV and WSSMV also can bind virions or coat proteins of their homologous viruses (13).

It is unclear at this time if capsid protein, polymerized capsid-protein-mimicking virions, or virions only attach to CI of WSMV or WSSMV. It also is not clear if attachment is peculiar to SBWMV-WT or an intrinsic property of the CIs of these two potyviruses. Virions of barley stripe mosaic virus (BSMV), but neither its capsid protein nor brome mosaic virus (BMV), attach to CIs of WSMV (15). It was, therefore, of interest to determine if SBWMV-WT protein also would associate with CIs of other potyviruses, such as Hordeum mosaic virus (HMV) (21) or Agropyron mosaic virus (AMV) (20) in wheat already infected with SBWMV-WT. Both HMV and AMV are similar to WSMV in virion length (700 nm) and intracellular appearances. They are only distantly serologically related to each other and differ in host range (21). This report shows that SBWMV-WT virions as well as capsid proteins also adhere to CIs of HMV and AMV in wheat but not to host cell organelles. This and earlier findings (11–13,15) could lead to an understanding of the role of CIs in potyvirus infection and multiplication processes.

MATERIALS AND METHODS

Plant and viruses. Wheat plants (*Triticum aestivum* L. 'Scout 66') were transplanted from a test plot naturally infected with

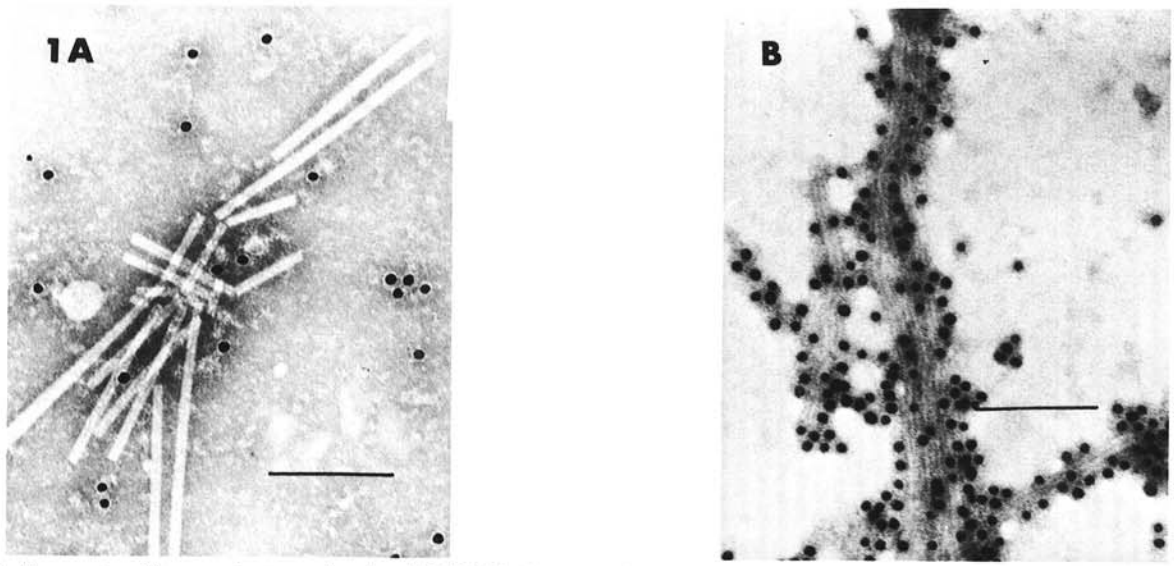

Fig. 1. A, Antiserum to soilborne wheat mosaic virus (SBWMV) absorbed with an excess of aggregated SBWMV no longer labels intact virions. **B,** Aggregate of SBWMV labeled positively by nonabsorbed antiserum to SBWMV and then gold-labeled goat anti-rabbit IgG. Bar represents 200 nm.

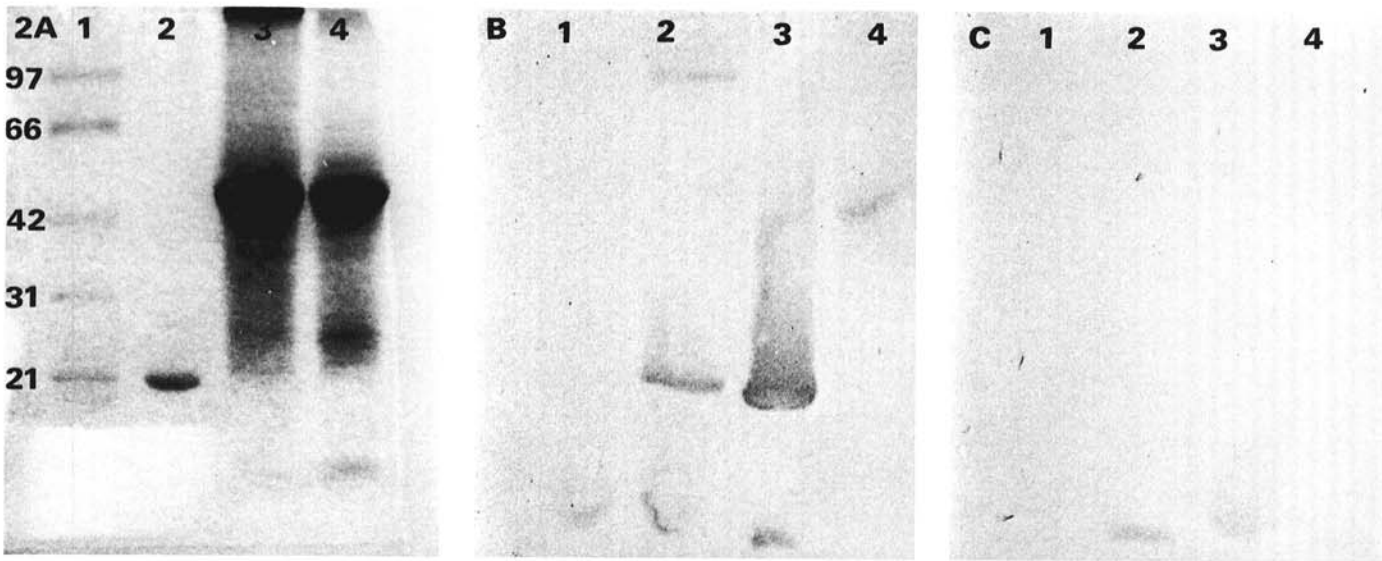

Fig. 2. Detection of proteins after separation on mini sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels. **A,** Stained with Coomassie Brilliant Blue. Lane 1: protein standards; lane 2: aggregated purified soilborne wheat mosaic virus (SBWMV) virions; lane 3: sap expressed from SBWMV-diseased wheat; and lane 4: sap from healthy wheat. **B,** Western blot of a gel identical to the one in A, labeled with immunogold after labeling with a 1:4,000 dilution of antiserum absorbed exhaustively with intact aggregated SBWMV virions. Lane 2 shows that the extracted antiserum reacts with capsid protein of SBWMV (band opposite 21K standard). The lighter labeling band opposite the 97K standard is a multiple aggregate of the 21K capsid of SBWMV. Lane 3 shows label of SBWMV capsid protein in sap from a diseased plant. Lane 4 shows a lightly labeling band at the position of the large subunit of ribolosebiphosphate carboxylase (Rubisco). This band was also visible, but rarely so, in lane 3. The antiserum to SBWMV used here is known to have some antibodies to Rubisco (10). **C,** Western blot from a gel as in A and B but labeled with normal rabbit serum and then goat anti-rabbit colloidal gold. No label is present. The bands visible at the very bottom of the Western blots are from Coomassie Brilliant Blue in the sample loaded onto the gels. The dye also transferred and bound to the blot.

SBWMV to the greenhouse and maintained at 15 C. Plants were tested by the leaf-dip method of Ball and Brakke (2), as modified by Lin (16), for the occurrence of viruses other than SBWMV, which in Nebraska in early spring can be WSMV and WSSMV. These were not found to coinfect the SBWMV-infected plants. Individual plants found to be free of WSMV and WSSMV were superinfected with HMV (American Type Culture Collection, PV 81) or AMV (American Type Culture Collection, PV 75) (1), maintained at 20–25 C for up to 5 mo, and tested for the appearance of long flexuous HMV or AMV particles by the leaf-dip method in diluted homologous antiserum. Systemic superinfection with AMV or HMV took 7–10 days.

Electron microscopy. Plants, doubly infected with SBWMV-WT and one of the two potyviruses, or singly infected with each virus by itself as a control, were fixed, flat embedded in L. R. White plastic, and sectioned as previously described (13).

Antisera. Antisera to HMV and AMV were prepared by injection of rabbits with virus purified as described for WSMV by Brakke and Ball (4). Polyclonal rabbit antiserum to SBWMV-WT was received from E. M. Ball (Department of Plant Pathology, University of Nebraska, Lincoln). Antisera were absorbed with crude sap expressed from uninfected wheat leaves (1:1, v/v) and used at 1/200–1/400 dilution in 1% normal goat antiserum, 0.1 M phosphate buffer, pH 7.2, containing 0.05% sodium azide to label ultrathin sections.

Preparation of IgG with specificity for SBWMV capsid protein only. Removal of antibodies to intact SBWMV virions from rabbit antiserum to SBWMV was accomplished as follows: To 2 ml of a 1/200 dilution of antiserum in phosphate buffer was added approximately 5 mg of aggregated, water-insoluble SBWMV virions. Virus was purified as described by Shirako and Brakke (19). The diluted antiserum and added virus was mixed vigorously, kept at room temperature for 2 hr, centrifuged at 10,000 rpm in a Sorvall HB4 rotor for 10 min, and stored at 4 C until used. The SBWMV-containing pellet was not removed and remained in the diluted antiserum. The supernatant of the low-speed centrifugation was tested with dips of aggregated SBWMV virions for the presence of antibodies to intact virions by the colloidal gold immunostaining method of Lin (16). A dilution of 1/4,000 of the extracted antiserum was used to label Western blots of antigens separated in 12% polyacrylamide slab gels as described by Brakke et al (5) and Hsu (9). Gels were cast and run in Mini Protean II Slab Cells (Bio-Rad Laboratories, Richmond, CA).

Immunoelectron microscopy. Ultrathin sections were labeled with the two-step method of Lin and Langenberg (17) with rabbit antisera as the primary label. Serial sections were labeled with rabbit anti-HMV, anti-AMV, or anti-SBWMV sera, or with rabbit anti-SBWMV specific for SBWMV capsid protein only. Primary label was detected with goat anti-rabbit IgG labeled with gold of about 18 nm in diameter (17). Ultrathin sections were contrasted with uranyl acetate and lead citrate and viewed in a Zeiss EM10A electron microscope (Carl Zeiss, Inc., Thornwood, NY).

RESULTS

In interpreting the figures shown, it must be remembered that IgG cannot penetrate ultrathin sections. The sections, silver to gold interference color, are approximately 100 nm thick which is many times the diameter of virions which is 14 nm for AMV or HMV and 20 nm for SBWMV. Only surface antigen is available for reaction with antibody. However, the uranyl acetate and lead citrate stains penetrate the entire section as do electrons, thus enabling the viewer to see the contents of the section.

Soilborne wheat mosaic virus occurs in moderate amounts in infected wheat tissue. Unfortunately, it has a pronounced tendency to aggregate during purification. Aggregates of SBWMV virions have resisted attempts at solubilization in water. This problem is reflected by the six published purification schedules for this virus (6). The insolubility of SBWMV aggregates in water was used to absorb antibodies specific for intact virions out of the polyclonal rabbit anti-SBWMV antiserum. The low-speed supernatant of centrifuged absorbed antiserum no longer labeled aggregates of SBWMV (Fig. 1A) in contrast to that of the pre-absorbed antiserum (Fig. 1B). Water-insoluble aggregates of SBWMV are no longer destabilized in homologous antiserum as are virions in leaf dips of SBWMV-infected plants (14). Heavy label is associated with aggregates of SBWMV.

That the absorbed antiserum still contained IgG with specificity for SBWMV capsid protein is shown in an immunoblot (Fig. 2B, lanes 2 and 3) of an identical mini-slab gel (Fig. 2A). A normal rabbit serum labeled control blot did not label (Fig. 2C).

AMV and SBWMV. It was further necessary to establish that the sera and viruses did not cross-react. Figure 3 depicts an area of a cell from tissue infected with SBWMV only. Shown is part of an SBWMV inclusion body and an SBWMV aggregate. The section was labeled with rabbit antiserum to AMV. Only a few background gold particles are visible; no reaction is evident. Figure 4 is the opposite control: A thin section of AMV-only infected tissue labeled with rabbit anti-SBWMV. No label is visible over the CI or the AMV virus aggregate. These controls were performed with every independent fixation and embedding experiment and also prevented stored or mislabeled vials from being mixed up inadvertently with diluted antiserum.

Figure 5 shows serial sections of young doubly infected tissue. Figure 5A was treated with rabbit anti-AMV antibody. The aggregate in the lower right-hand corner labels positively. Light

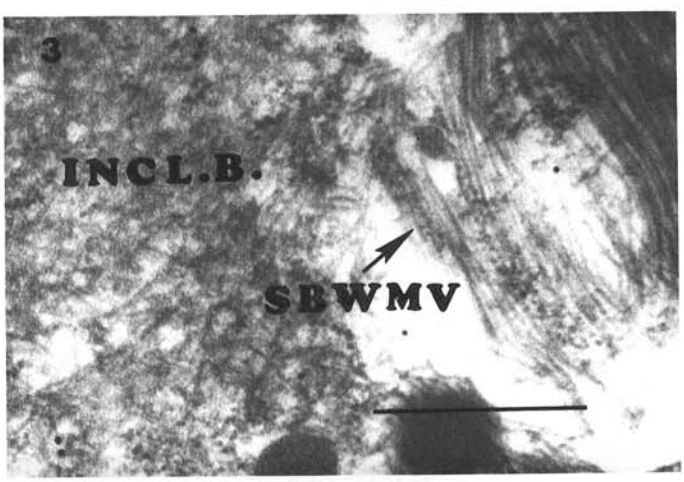

Fig. 3. Soilborne wheat mosaic virus (SBWMV)-infected wheat treated with rabbit anti-Agropyron mosaic virus serum and then gold-labeled goat anti-rabbit IgG. No label is evident above background. INCL. B = inclusion body. Bar represents 500 nm.

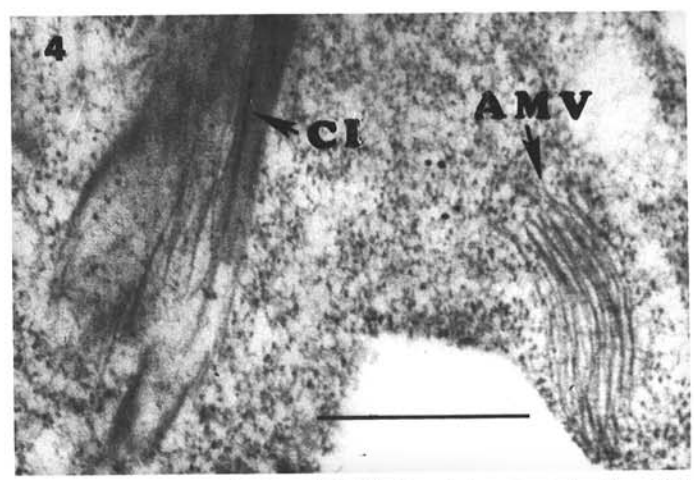

Fig. 4. Agropyron mosaic virus (AMV)-infected wheat treated with rabbit anti-soilborne wheat mosaic virus serum and then gold-labeled goat anti-rabbit IgG. No label over cylindrical inclusion (CI) or AMV aggregate is apparent. Bar represents 500 nm.

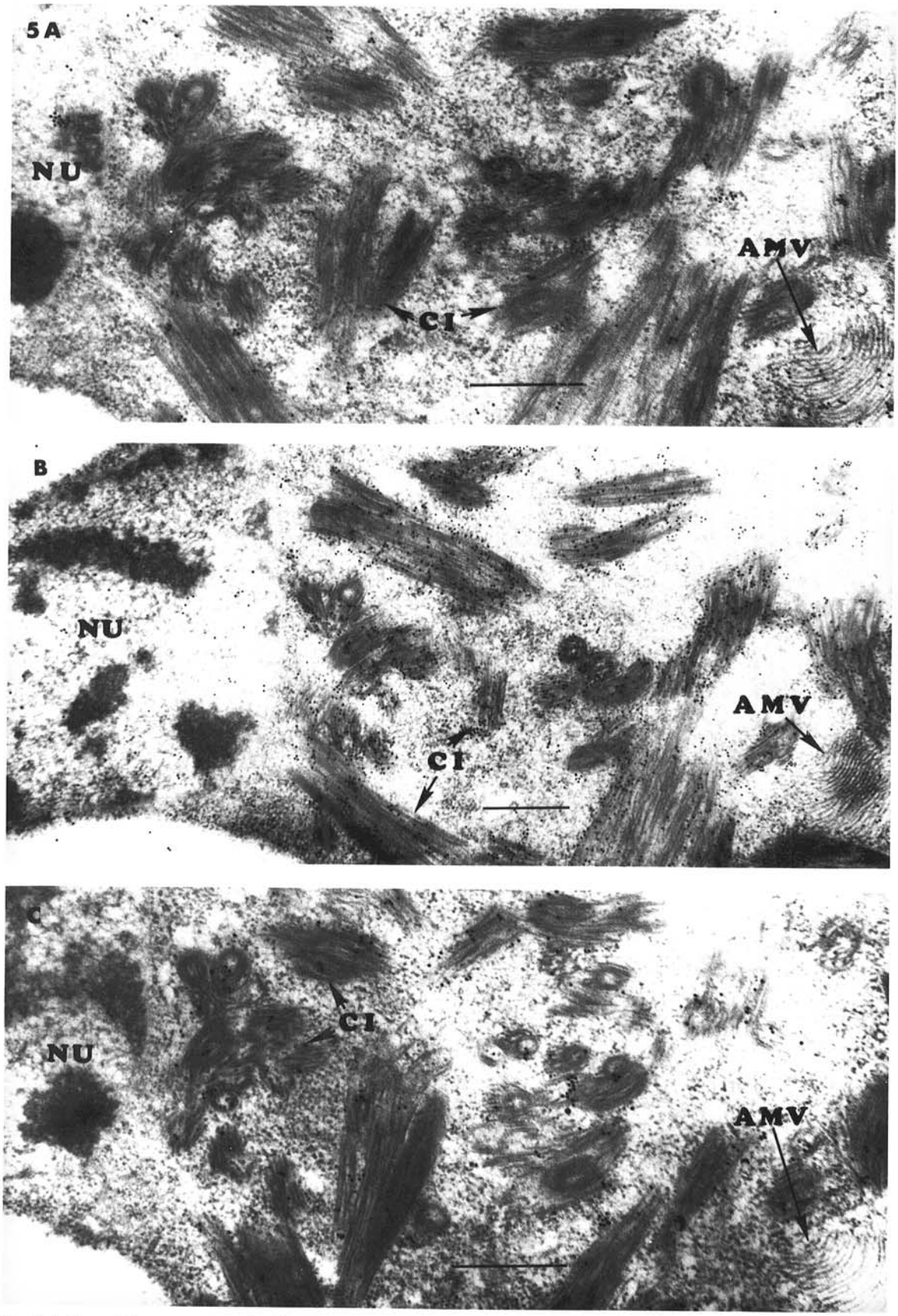

Fig. 5. A, Mixed infection of Agropyron mosaic virus (AMV) and soilborne wheat mosaic virus (SBWMV) labeled with rabbit anti-AMV serum and then goat anti-rabbit IgG bound to colloidal gold. Light label over cylindrical inclusions (CIs) and positive label over AMV aggregate. Bar represents 500 nm. **B,** Serial section of the same tissue block as in A labeled with rabbit anti-SBWMV serum and then immunogold. CIs label heavily whereas the AMV aggregate does not, indicating the presence of SBWMV antigen on AMV CIs. Bar represents 500 nm. **C,** Serial section of the tissue block from which sections in A and B were obtained, treated with rabbit antiserum to SBWMV which had been absorbed with aggregates of intact SBWMV to remove IgG specific for intact virions and then with immunogold as in A and B. CIs still label and thus have some antigen attached, indicating the presence of capsid proteins of SBWMV on CIs. The AMV aggregate again does not label. Nu = nucleus. Bar represents 500 nm.

label over CIs indicates that some AMV virus protein antigen is associated with the CI. This is not unexpected. In young tissue, WSMV and WSSMV were shown to have viral antigen associated with their CIs (13). Figure 5B shows another serial section of the same area, this time labeled with rabbit antiserum to SBWMV. The AMV aggregate in the lower right-hand corner does not label. Cylindrical inclusions are heavily labeled, indicating that not only is AMV antigen present but also SBWMV antigen. Figure 5C shows a third serial section treated with 1:200 diluted antiserum to SBWMV that had been absorbed with a large amount (> 5 mg) of SBWMV water-insoluble aggregates to deplete IgG to epitopes on intact virions, leaving IgG to capsid proteins in solution. Cylindrical inclusions of AMV still labeled although much less than with unabsorbed diluted antiserum to SBWMV. Compare label over the CIs of Figure 5B with label over the same CIs of Figure 5C. Thus, not only intact virions but also capsid proteins adhered to the CIs.

All AMV and SBWMV doubly infected young leaf tissues labeled as just described. Old fully expanded leaves were not processed and examined. In no case were AMV CIs found not to label with antibody to SBWMV. No cells were encountered that were infected with one of the viruses and not the other. These results were obtained from tissues of AMV + SBWMV plants in all six independent fixation and embedding experiments over a period of 5 mo from January to the end of May when plants entered the booth stage.

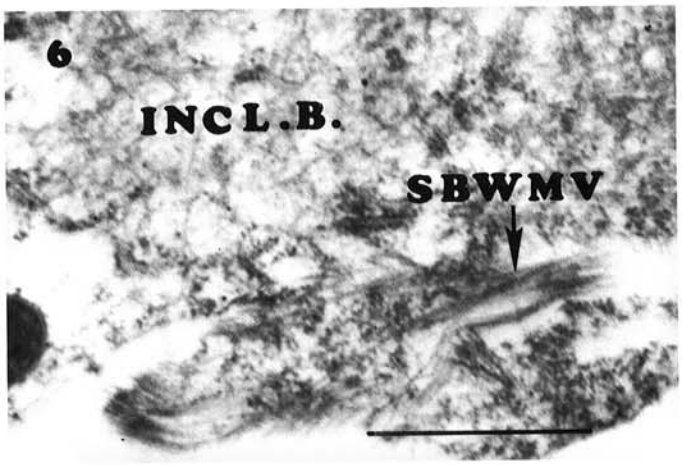

Fig. 6. Ultrathin section of wheat infected with soilborne wheat mosaic virus (SBWMV) only labeled with rabbit anti-Hordeum mosaic virus serum and then immunogold. No label above a few background gold particles is apparent over SBWMV or its inclusion body (INCL. B.). Bar represents 500 nm.

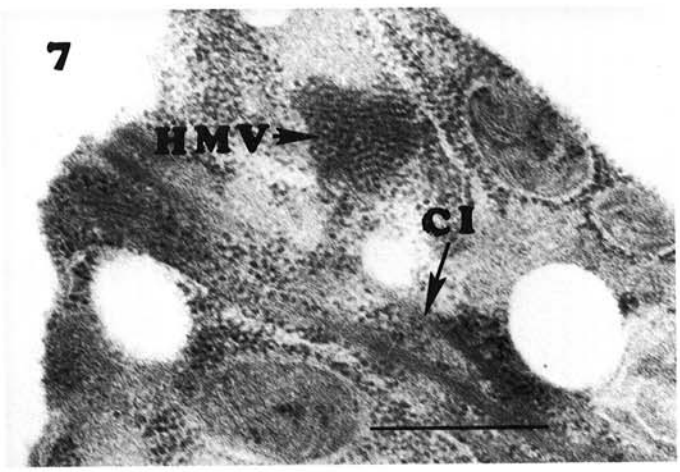

Fig. 7. Section of Hordeum mosaic virus (HMV)-only infected tissue treated with rabbit antiserum to soilborne wheat mosaic virus and then immunogold. No label is visible over cylindrical inclusion (CI) or the HMV aggregate. Bar represents 500 nm.

HMV and SBWMV. The same controls as with AMV and SBWMV were made. Figure 6 shows a thin section of SBWMV-only infected tissue labeled with rabbit anti-HMV. Only a few scattered background gold particles were seen. Figure 7 is a thin section of HMV-only infected wheat showing an HMV aggregate and CI not labeled by rabbit antiserum to SBWMV. Only a few background gold particles were randomly scattered over the cell. Also for HMV and SBWMV, controls were regularly performed to guard against mislabeling of tissues or vials with diluted specific antiserum. No mixups were experienced and each antiserum was specific for its homologous antigen only.

Figure 8A is the first of a series of three serial sections of a region of a cell doubly infected by HMV and SBWMV. The section was labeled with diluted rabbit anti-HMV antiserum as the primary label. Cylindrical inclusions of HMV and scattered aggregates of HMV (arrows) were labeled but SBWMV aggregates (arrows) were not. Light label over the CIs of HMV with antiserum of HMV again was expected because young tissue was sectioned. In young tissue infected with other potyviruses, viral antigen is associated with CIs (13). Compare the position of HMV virions in Figure 8A and B with that in Figure 8C where they are most clearly visible. Figure 8B is a serial section of the one shown in Figure 8A but labeled with antiserum to SBWMV. Moderate label shows that SBWMV antigen also is attached to HMV-CIs. SBWMV aggregates label but HMV aggregates do not. In Figure 8C, the third serial section is shown labeled with diluted antiserum to SBWMV but absorbed with intact SBWMV virion aggregates leaving antibodies with specificity for capsid proteins of SBWMV. HMV CIs are lightly labeled, indicating that SBWMV capsid protein was attached in addition to SBWMV virions.

For HMV + SBWMV-infected cells, the results were no different than those for AMV + SBWMV. Again, no leaf cells were seen in all three independent fixations and embedding experiments that were infected with one virus and not the other. Both viruses occupied all cells of the leaf except mature tracheal cells. Label was always seen over CIs of HMV with antibody to SBWMV as the primary label. Mature fully expanded leaves were not examined. Samples were taken of doubly infected tissues until plants entered the booth stage.

Generally, the label density over a CI after labeling double infections with antiserum to SBWMV varied from heavy (Fig. 5B) to moderate (Fig. 8B) but was always heavier than after labeling with antiserum to the homologous potyvirus. Several different forms of antigen were thus associated with cylindrical inclusions, from intact virions of either virus to capsid proteins.

DISCUSSION

This report shows that attachment of antigens of viruses to a CI is not limited to WSMV and WSSMV (13) or double infections of these viruses with SBWMV (11,12). CIs of two other potyviruses of wheat, AMV and HMV, showed similar characteristics. Attachment of SBWMV virions or capsid protein to a CI was again specific because no other cell organelles had associated antigen. It is unlikely, therefore, that attachment of virions or their capsid proteins to CIs was the result of simple charge effects. However, both SBWMV and BSMV have been shown attached to concentric membrane systems and endoplasmic reticulum, respectively (8,18), and BSMV also has been shown attached to chloroplast membranes (7). Whereas SBWMV capsid protein attaches to CIs of WSMV, WSSMV (11,12), AMV, and HMV, those of BSMV or BMV do not (15). Only intact BSMV could be found associated with CIs of WSMV. Icosahedral virions of BMV were not. Possibly attachment of rod-shaped viruses and their capsid proteins or the viruses only and not their capsid proteins may reflect the presence or absence of capsid proteins in the cytoplasm. BSMV capsid protein first appeared at the cytoplasmic side of chloroplast or plastid invaginations. It also was here that intact virions first were detected (18). No free or aggregated BSMV capsid protein was present in the cytoplasm.

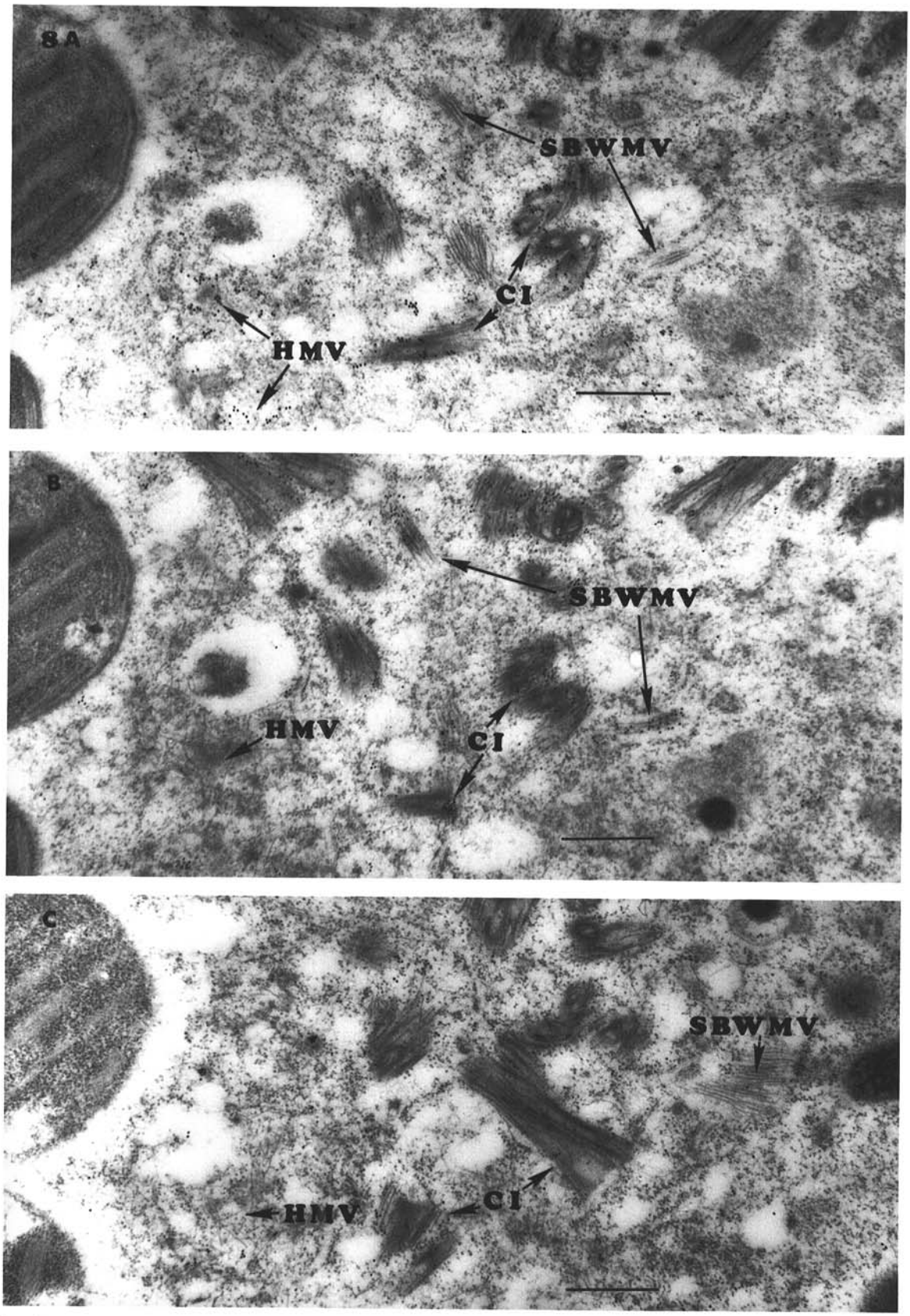

Fig. 8. A, Ultrathin section of Hordeum mosaic virus (HMV) and soilborne wheat mosaic virus (SBWMV) doubly infected tissue labeled with rabbit antiserum to HMV and then immunogold. HMV virions (arrows) label positively whereas SBWMV aggregates (arrows) do not. Some HMV antigen also is associated with cylindrical inclusions (CIs) in this area of mixed HMV and SBWMV infection products. Bar represents 500 nm. **B,** Serial section of the same block as that in A labeled with rabbit antiserum to SBWMV and immunogold. SBWMV aggregates (arrows) label positively; HMV (arrows) does not. Labels over CIs indicate the presence of SBWMV antigen on HMV-CIs. Bar represents 500 nm. **C,** Serial section of the same tissue block as that in A and B labeled with rabbit antiserum to SBWMV which was absorbed with intact virion aggregates of SBWMV, thus removing all antibodies specific for virions only of SBWMV. Immunogold was used to detect the rabbit IgG as in A and B. HMV again does not label (arrow). The SBWMV aggregate (arrow) can be identified from its position in A and B and does not label but CIs still label lightly, indicating that capsid proteins of SBWMV also are attached to HMV-CIs. Bar represents 500 nm.

In the absence of a pool of cytoplasmic BSMV capsid protein, it is possible that it was not available for reaction with CIs of the co-infecting WSMV.

Although small aggregates or even individual virions can be resolved in thin sections, resolution was not sufficient to identify SBWMV virions with certainty as being associated with AMV or HMV CIs, especially not when these were cut lengthwise. The colloidal gold-labeling technique has proven to be invaluable in detecting the attachment of virions and capsid proteins to CIs.

An expanded investigation with other rod-shaped viruses and potyviruses, in dicotyledonous hosts, may further clarify if capsid protein and virion binding is a general property of potyviral CIs.

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