Detection of Viral Antigen by Immunogold Cytochemistry in Ovules, Pollen, and Anthers of Alfalfa Infected with Alfalfa Mosaic Virus

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ABSTRACT

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Alfalfa mosaic virus (AMV) was localized in alfalfa gametes by immunogold cytochemistry. AMV antigen was detected by immunogold labeling in the cytoplasm, and vacuoles of ovule integuments, microspores, mature pollen grains, and anther tapetum cells. Raftlike aggregates of virus particles and large crystalline bodies were observed in the cytoplasm of pollen grains and anther tapetum cells, while nonaggregated virions were detected in the vacuoles and the cytoplasm of ovule integument cells. This indicates that a mechanism exists for the transmission of AMV to seed through infected gametes. The immunogold technique was more sensitive than heavy metal straining in detecting the viral antigen in ultrathin sections of plant tissue. Staining with gold-labeled-goat anti-rabbit IgG was higher than with protein A-gold complex in purified virus preparations.

Although alfalfa mosaic virus (AMV) is transmitted to alfalfa seed through both male and female gametes, the mechanism of virus transmission is still unknown. A previous electron microscope study on the localization and aggregation forms of two strains of AMV in alfalfa gametes revealed the presence of rafts, and starlike aggregates, of virus in the cytoplasm of another parenchyma cells and pollen grains (25). However, the virus was not detected in thin sections of alfalfa ovules using a heavy-metal staining method. Recently, we detected AMV by enzyme-linked immunosorbent assay (ELISA) in the seed coat and embryo of alfalfa seed (19). In individual seeds, AMV was detected in either the seed coat or the embryo, or in both of them simultaneously. These results can be interpreted as a random distribution of AMV in alfalfa seed and suggest that virus distribution within infected pollen and ovules and the mode of virus transmission influence the type of seed infection.

Immunogold cytochemistry has been introduced as an alternative to the heavy metal staining for virus identification in leaf-dip preparations and in situ localization of intact virus particles or viral antigen by electron and light microscopy (6,14–16,23). It is particularly advantageous for detection of small polyhedral viruses in thin sections of plant tissue where virus particles can not be readily distinguished from cytoplasmic ribosomes (7,13,25). Digestion of ribosomes with ribonuclease is another method employed for a similar purpose (8,9).

The present immunocytochemical investigation was undertaken to detect the distribution of AMV in pollen, ovule integuments, and embryo sacs of alfalfa and to ascertain their role in AMV transmission to alfalfa seed.

MATERIALS AND METHODS

Virus and virus assay. AMV isolate A-515 (11) was propagated in *Nicotiana tabacum* L. 'White Burley' in the greenhouse at 23 C and a 13-hr photoperiod. AMV was purified from leaves with symptoms harvested 10 days after inoculation (24). Direct double antibody sandwich ELISA (1) was used for virus detection in alfalfa plants.

Plants. Three clones from Beaver alfalfa, B-19, B-24, and B-39 (18), were inoculated at the six- to eight-leaf stage with a purified AMV preparation, 1 mg/ml of 0.025 phosphate buffer, pH 7.0. Inoculated and virus-free clonal plants were maintained on separate benches in the same greenhouse at 18 C with a 16-hr photoperiod. At 4-wk intervals plants were assayed by ELISA for the presence of AMV.

Antiserum. Antiserum against AMV isolate A-515 was produced in rabbits using purified virion preparations in a combination of intravenous and intramuscular injections. After the first intravenous injection (1 mg/ml in 0.01 M phosphate buffer, pH 7.0), a series of intramuscular booster injections was given at weekly intervals with a mixture (1:1, v/v) of AMV and Freund's incomplete adjuvant. Rabbits were exsanguinated when the titer of antiserum determined by double diffusion tests reached 1/256. To remove the antibodies to host proteins, the antiserum was absorbed with acetone-extracted powder of virus-free tobacco plants (3). The powder, obtained from 10 g of frozen tobacco leaves, was washed with 0.01 M phosphate-buffered saline (PBS), pH 7.4, and incubated with 10 ml of antiserum for 2.5 hr at 40 C in a water bath. The antiserum was separated from the precipitate by low-speed centrifugation. The immunoglobulin fraction of the antiserum was precipitated by mixing equal volumes of 4 M $(NH_4)_2SO_4$ and antiserum for 2 hr at room temperature. After centrifugation for 10 min at 3,000 rpm, the precipitate was resuspended in half-strength PBS, pH 7.4, and dialyzed overnight at 4 C against three changes of PBS. The concentration of purified immunoglobulin was determined by measuring the absorbance at 280 nm using the extinction coefficient of 1.35 $(mg/ml)^{-1}cm^{-1}$ (12). The immunoglobulin was stored at −20 C.

Tissue preparation. Anthers and ovaries were collected from selected flowers of infected and virus-free plants by tripping the flower and dissecting it under a stereomicroscope. Tissues were vacuum infiltrated with 0.01 M PBS, pH 7.4, to remove air and to assist in fixative penetration. They were fixed with 1% glutaraldehyde in 0.01 M PBS, pH 7.4, at 4 C overnight, rinsed in three changes of PBS, 1 hr each, and postfixed in 2% OsO_4 for 2 hr at room temperature. After a rinsing in distilled water, anthers and ovaries were dehydrated in a graded series of acetone (70–100%), with three changes, 20 min each, in absolute acetone. Anthers and ovaries were then passed through a mixture of equal parts of acetone and low-viscosity Spurr's resin for 2 hr, then Spurr's resin overnight and cured at 65 C for 10 hr. Thin sections were stained with 2% uranyl acetate for 60 min and lead citrate for 2 min. For immunogold cytochemisty, low-acid, water-soluble GMA

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embedding mixture consisting of glycol methacrylate (2-hydroxyethyl methacrylate), benzoil peroxide, and butyl methacrylate was used for dehydration, infiltration, and embedding of plant tissue. After primary fixation in 1% glutaraldehyde in 0.01 M PBS, pH 7.4, and rinsing in PBS, the samples were dehydrated and infiltrated simultaneously in aqueous solutions of GMA (85 and 97% GMA) and kept in the final nonprepolymerized mixture at 4 C overnight. For final embedding, the samples were placed in prepolymerized GMA in size 00 gelatin capsules (J.B.EM. Services Inc., St. Lawrence, Quebec, Canada), and polymerized at 4 C for 18–24 hr in an ultraviolet light apparatus equipped with long wavelength (3150 A) UV lamps (G.E. no. F6T5, BL) at a distance of 1.5 cm from the light source.

Chemicals and immunogold reagents. SPI-CHEM low-acid GMA water-soluble embedding kit for transmission electron microscopy (SPI Supplies Division of Structural Probe Inc., West Chester, PA), Spurr's low-viscosity embedding resin (J.B.EM. Services Inc., St. Lawrence, Quebec, Canada), and protein A (Pharmacia, Dorval, Quebec, Canada) were used in this study. Gold-labeled-goat anti-rabbit IgG with colloidal gold particles of the nominal size of 15 mm (Jansen Life Sciences Products, a

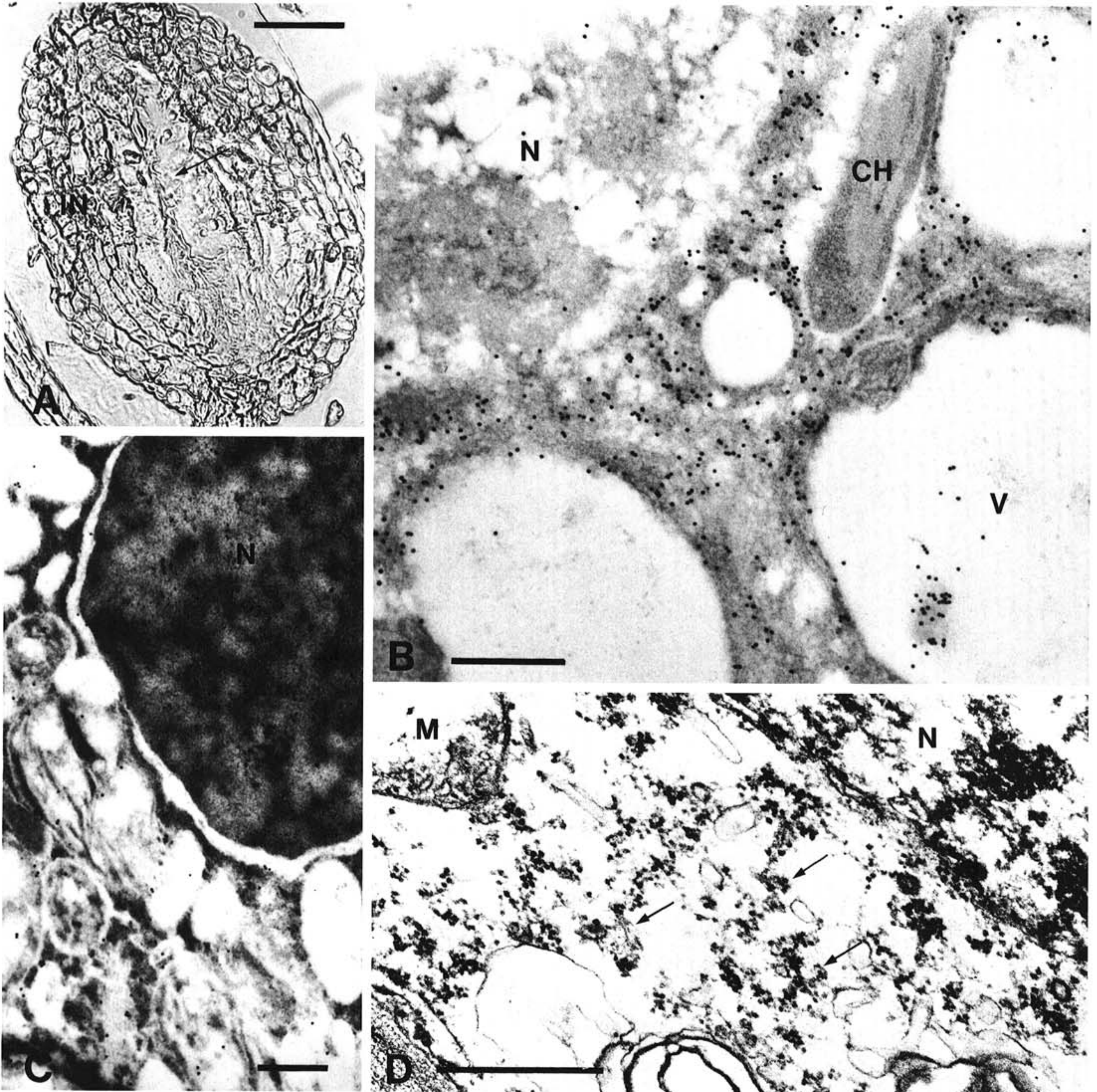

Fig. 1. Alfalfa mosaic virus (AMV)-infected ovules of alfalfa clone B-24. **A,** Thick section of alfalfa ovule. IN = integuments surrounding embryo sac and nucellus (arrow) (150×). Bar represents 100 μm. **B,** Localization of AMV in ovule integument cell stained with gold-goat anti-rabbit IgG. Gold-labelled viral antigen is found in the cytoplasm and vacuoles (V), N = nucleus, CH = chloroplast (37,700×). **C,** AMV-infected integument cell exposed to normal rabbit serum and stained with gold-IgG complex. Nonspecific staining is present in low concentrations over the cytoplasm and nucleus (N) (22,100×). **D,** Localization of AMV in thin sections of the integument cell stained with uranyl acetate and lead citrate. Nonaggregated virions are located in the cytoplasm (arrows). M = mitochondrion, N = nucleus, CW = cell wall, (57,000×). Bar represents 0.5 μm unless otherwise indicated.

division of Jansen Pharmaceutica, B-2340, Beerse, Belgium) and protein A-gold were used for immuno-cytochemical staining of plant tissue. Protein A was coupled to colloidal gold particles of the nominal size 10–15 nm (20,21). The final solution of gold-IgG and protein A-gold was prepared in 0.5% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.4, containing 0.05% Tween 20.

Immunocytochemical staining. Grids with thin sections attached were floated face down on 0.5% BSA-PBS-Tween at room temperature for 5 min to block the nonspecific binding sites. The grids were transferred without rinsing to a Falcon 30340 microtest plate (Falcon Plastics, Oxnard, CA) containing antiviral immunoglobulin and incubated at 37 C for 2 hr. The concentration of immunoglobulin was adjusted to 5, 10, and 20 μg/ml 0.05% BSA-PBS-Tween. The sections were rinsed with 1% BSA in 0.01 M PBS, pH 7.4, for 20 sec using a plastic spray bottle, then placed in PBS for 5 min, rinsed with PBS, and blotted on filter paper. The grids were placed on drops of immunoglobulin-gold complex (20-fold dilution of the stock solution) for 1 hr at room temperature. The grids were rinsed with PBS for 30 sec, immersed in PBS for 10 min, rinsed a second time with PBS, and finally rinsed with distilled water. The sections were subsequently stained with 2% uranyl acetate for 20 min and lead citrate for 5 min.

Electron and light microscopy. Thin sections (70–100 nm) of anthers and ovules embedded in Spurr's resin of GMA were made on a Reichert Om U2 microtome with a glass knife. The sections mounted on Formvar-coated nickel grids were examined in a Phillips EM 201 electron microscope at 60 kV. Thick sections of ovules (500 nm) embedded in GMA were mounted in a drop of water on a glass slide and examined in a Leitz Ortholux microscope at 100× magnification.

Comparison of antigen-antibody reaction. To compare staining with gold-labeled-goat anti-rabbit IgG and protein A-gold complexes for detection of AMV isolate A-515, grids coated with purified virus preparation were treated with antiviral immunoglobulin or normal serum before staining with gold conjugates and 1% phosphotungstic acid, pH 3.5. The comparison of staining was expressed as the number of virus particles labeled vs. the number of virus particles without a label. For evaluation, 15 counts were made at random from different areas of the grid, each consisting of 15 cm^2, on a photograph (140,000×).

RESULTS

Immunoglobulin concentration. The preliminary testing of three immunoglobulin concentrations, 5, 10, and 20 μg/ml, was conducted to determine the optimum concentration of first antibody for cytochemical staining of AMV antigen in thin sections. A concentration of 10 μg/ml was optimum for gold labeling of viral antigen. Nonspecific labeling occurred with the higher concentration, while only a few virus particles were labeled with gold at a concentration of 5 μg/ml.

AMV detection in alfalfa ovules. AMV was detected in sections of integuments (Fig. 1A) stained with gold-goat anti-rabbit IgG and by the heavy metals. Viral antigen was detected by colloidal gold in the cytoplasm, and in vacuoles of the integument cells (Fig. 1B). The intensity of gold labeling was high throughout the cytoplasm and in areas surrounding nuclear and vacuolar membranes (Fig. 1B). In ultrathin sections of integument cells, stained with heavy metals, nonaggregated, randomly distributed virus particles were observed in the cytoplasm (Fig. 1D). Virus particles were distinguishable from ribosomes by the lower density of staining and bacilliform shape. AMV was not detected by either method in the embryo sac. In ultrathin sections of virus-free ovules and sections of infected tissue treated with normal rabbit serum, the gold background labeling was low (Fig. 1C).

AMV detection in alfalfa anthers. AMV was detected in ultrathin sections of alfalfa anthers poststained with gold-IgG or protein A-gold complex. Large crystalline bodies and short and long rafts of virus particles were observed in the cytoplasm of anther tapetum cells (Fig. 2A). In anther tapetum cells stained with the protein A-gold complex, the intensity of antigen labeling was lower than with the gold-IgG complex (Fig. 2B). In the majority of cells examined, aggregates of virus particles were densely distributed throughout the cytoplasm. A noticeably high intensity of labeling was associated with virus particles in raftlike aggregates and in crystalline bodies (Fig. 2C).

AMV detection in alfalfa pollen. AMV was detected in microspores stained with the gold-IgG complex (Fig. 3A) and mature pollen grains stained with heavy metals (Fig. 3B). In microspores, the differentiation of the wall of the pollen grain was noticeable, as well as the large cytoplasmic bridges connecting microspores to one another in the early stages of development. Long rafts and crystalline aggregates of virus particles similar to those in anther tapetum cells were observed in the cytoplasm (Fig. 3A and B). AMV was also present on the surface of pollen exine and in the cytoplasmic bridges (Fig. 3A). The aggregation and distribution of virus particles in mature pollen grains were similar to those observed in microspores (Fig. 3B).

Comparison of alfalfa clones. There was no difference in detection of AMV in ovules, anthers, and pollen of three alfalfa clones (B-19, B-24, and B-39) of cultivar Beaver (data not shown).

Comparison of antigen-antibody reaction. In purified AMV preparations stained with gold-goat anti-rabbit IgG (Fig. 4B) or protein A-gold complex (Fig. 4C), labeling of virus particles with gold-IgG was higher than with protein A-gold complex (Table 1). Only a few particles exposed to normal rabbit serum before immunogold staining were labelled with colloidal gold (Fig. 4A).

DISCUSSION

For the first time in the study of seed transmission of AMV, virus particles were localized in alfalfa ovules. Thus far, AMV transmission through female gametes has been demonstrated only in cross-pollination experiments between infected and virus-free alfalfa plants (5,10). The mechanism of virus transmission to seed, however, has been unknown mainly because of the scarcity of any direct evidence of virus distribution in infected ovules. Localization of AMV in the integuments of alfalfa ovules in this study and previous detection of AMV in the seed coat of alfalfa by ELISA strongly indicate that seed coat infection results from ovule transmission of AMV (19). The fact that AMV was not detected in the embryo sac of alfalfa ovules by either the immunocytochemistry or by the heavy-metal staining method in this investigation but was previously found in the embryo of alfalfa seed (19) suggests that embryo infection occurs solely through infected pollen during fertilization. A similar mechanism of virus transmission to seed through gametes has been reported recently for cherry leaf roll in birch (2). Cross-pollination experiments with various combinations of infected and virus-free gametes are needed for a more comprehensive understanding of the mechanism of seed transmission of AMV.

Viral antigen and AMV virions were detected in the cytoplasm and vacuoles of infected gametes and anther tapetum cells. Two strains of AMV, F2 and U21, were found previously in the cytoplasm of anther parenchyma cells and pollen grains but were not associated with any of the cell organelles (25). However, an isolate of AMV from pepper was detected in aggregated form in vacuoles of pepper mesophyll cells (4). The results obtained in this study and those previously reported suggest that virus distribution within the cell is both strain- and host-specific. Localization of AMV in the cytoplasmic bridges indicated that movement of virions might occur between microspores in the early stages of development of pollen grains (17).

Consistent differences in aggregation forms of virus particles were observed between pollen and ovules in each of three alfalfa clones. Nonaggregated virus particles were detected in the cytoplasm and vacuoles of ovule integument cells. Large crystalline bodies and rafts of virus particles randomly distributed in the cytoplasm of anther tapetum cells, microspores, and mature pollen grains morphologically resemble those of strains F1 and U21 in the cytoplasm of pollen grains and anther parenchyma cells (25). Differences in aggregation in various alfalfa organs including pollen and anthers were also reported for AMV strains F1 and U21 (25).

Although AMV was localized in the ovules, pollen, and anthers by both immunogold cytochemisty and heavy-metal staining, the advantage of using the immunogold technique was in detecting viral antigen and complete virions. Fixation of plant tissue in 1% glutaraldehyde, dehydration in glycol methacrylate, and low-temperature embedding preserved cellular organization and the antigenic properties of virus particles as shown by the high intensity of gold-IgG labeling and the formation of antigen-antibody complex. These results are comparable to those previously reported for AMV (22) and barley stripe mosaic virus (15). The specificity of the immunogold method for detection of viral antigen was also demonstrated by the absence of gold labeling in ultrathin sections of virus-free tissue subjected to anti-A-515 immunoglobulin and in infected tissue treated with normal rabbit serum. The results obtained in this study and those previously reported for in situ localization of cauliflower mosaic virus in turnip (6) and red clover mottle virus in pea (23) clearly indicated that the antigen specific and sensitive immunogold technique can be successfully applied for identification and localization of plant viruses in infected tissue.

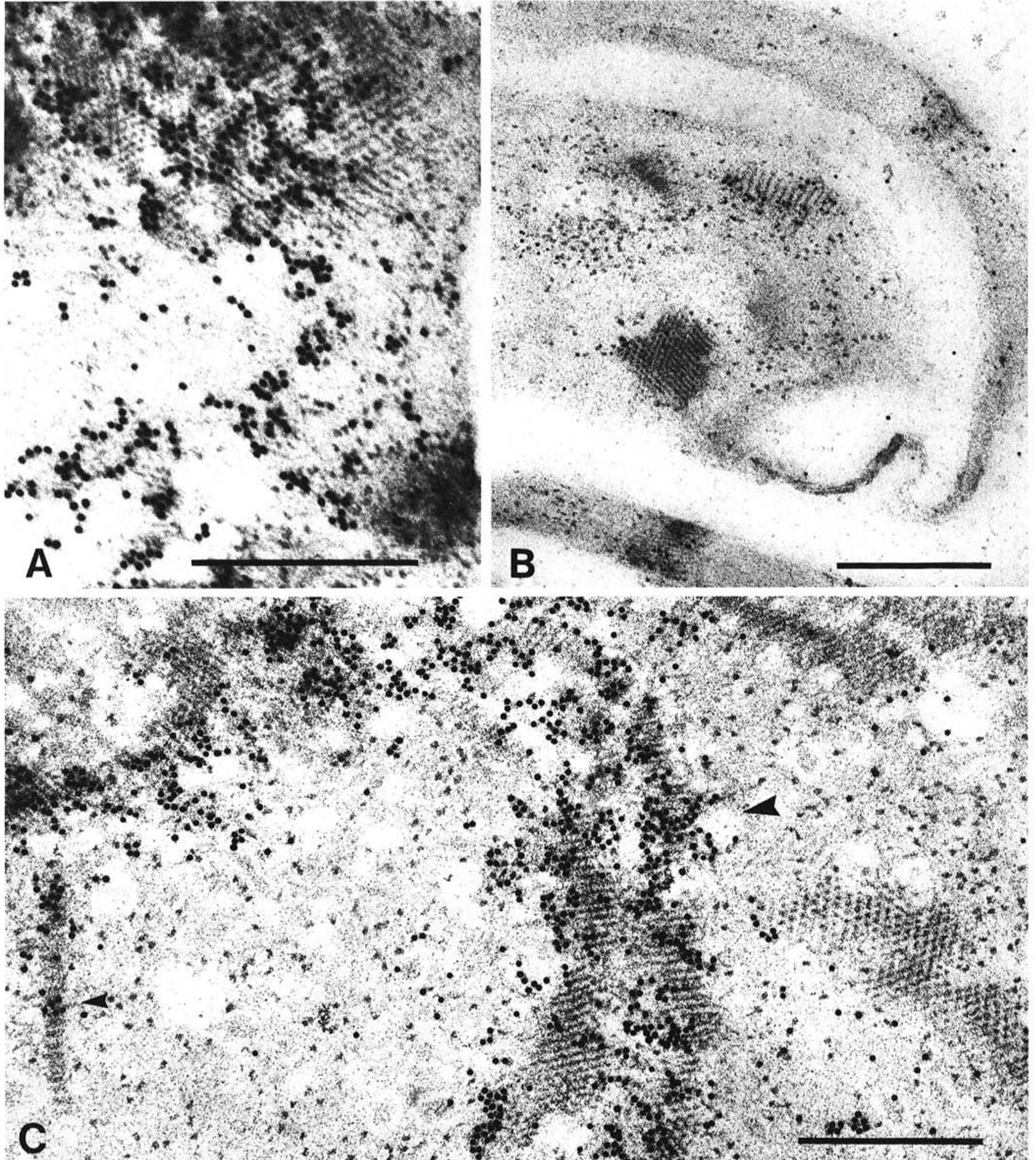

Fig. 2. Localization of alfalfa mosaic virus (AMV) in anthers of alfalfa clone B-24 infected with isolate A-515. **A,** Anther tapetum cells stained with gold-IgGj complex (78,000×). **B,** Anther tapetum cells stained with protein A-gold complex (53,000×). **C,** A raft aggregate of AMV particles (small arrow head) and large crystalline bodies (large arrow head) in the cytoplasm of anther tapetum cells stained with gold-IgG complex (62,000×). Bar represents 0.5 μm.

The binding of colloidal gold to AMV particles in purified preparations was much higher with gold-IgG than with protein A-gold complex (Table 1). Two factors might be involved in the resulting differences in the specificity of antigen-antibody complex in this investigation, namely 1) the indirect method of immunogold labeling and 2) use of colloidal gold-labeled-goat anti-rabbit IgG that will react specifically with rabbit IgG to AMV antigen (15).

In summary, the results of this investigation indicated that AMV can be effectively localized in alfalfa ovules, pollen, and anthers by immunogold cytochemistry using gold-IgG and protein A-gold

Fig. 3. Alfalfa mosaic virus (AMV)-infected pollen of alfalfa clone B-24. **A,** Localization of AMV in the microspores by immunogold staining with gold-IgG complex. An arrow head indicates the area depicted in an inserted micrograph (58,000×). Aggregates of virus particles are located in the cytoplasm. Viral antigen is also present in the cytoplasmic bridges (arrow) and on the surface of developing pollen exine (17,600×). Bar represents 0.3 μm. **B,** Aggregates of virions in the cytoplasm of mature pollen grain stained with uranyl acetate and lead citrate (123,600×). Bar represents 0.25 μm.

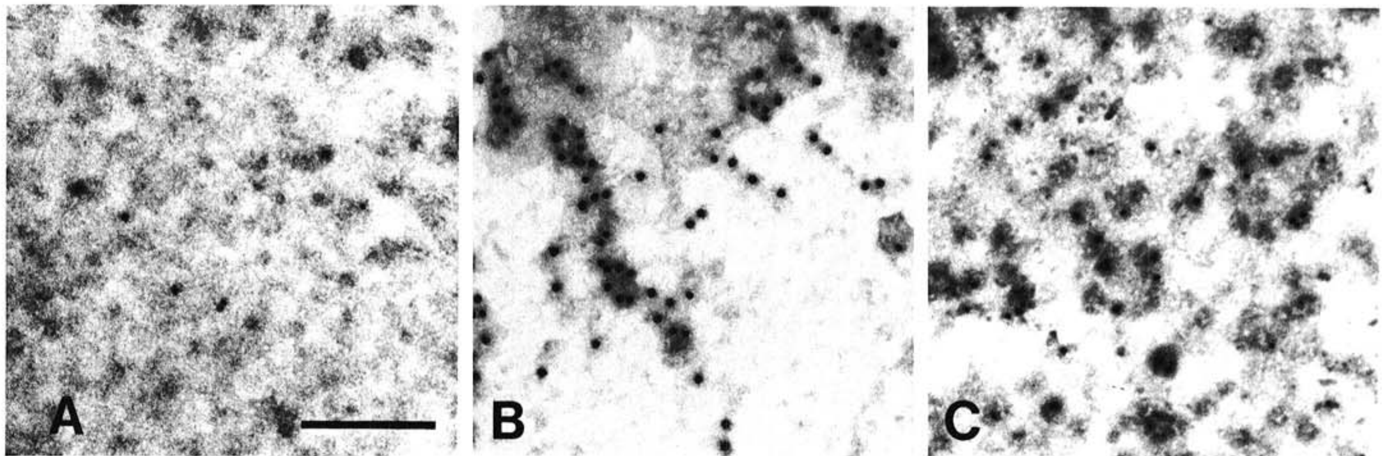

Fig. 4. Detection of purified alfalfa mosaic virus (AMV) by immunogold staining. **A,** AMV particles exposed to normal rabbit serum before staining with protein A-gold complex. **B,** Labelling of viral antigen with gold-IgG, and, **C,** protein A-gold complex (70,000×). Bar represents 0.25 μm.

TABLE 1. Detection of alfalfa mosaic virus by immunogold staining in purified preparations

First antibody	Secondary antibody	Number of virions		% labelled
		Total	Labelled	
Anti-A-515 immunoglobulin	Gold-IgG	504	395	78.4 ± 5.6
	Protein A-gold	437	218	49.8 ± 6.0
Normal rabbit serum	Gold-IgG	356	10	2.8 ± 2.9
	Protein A-gold	425	10	2.3 ± 2.3

complexes as markers of viral antigen, and provided additional evidence toward a better understanding of seed transmission of AMV.

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