In Situ Reaction of Clover Yellow Mosaic Virus (CYMV) Inclusion Bodies with Fluorescent Antibodies to CYMV

D. V. Rao, P. Shukla, and C. Hiruki

Former Graduate Research Assistant, Former Postdoctoral Fellow, and Professor, respectively. Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada, T6G 2E3. Present address of the second author: Division of Plant Pathology, C. S. Azad University of Agriculture and Technology, Kanpur-2, India.

Supported in part by the Canadian Department of Agriculture (Grant 9006). Thanks are due to T. Tribe for preparing the illustrations.

Accepted for publication 5 January 1978.

ABSTRACT

RAO, D. V., P. SHUKLA, and C. HIRUKI. 1978. In situ reaction of clover yellow mosaic virus (CYMV) inclusion bodies with fluorescent antibodies to CYMV. Phytopathology 68: 1156-1159.

Amorphous inclusions were observed as diffuse masses of yellowish-green fluorescence in clover yellow mosaic virus (CYMV)-infected cells of the primary leaves of cowpea (*Vigna sinensis* 'Black Eye'). The CYMV inclusions identified by immunofluorescence microscopy corresponded to those found by light microscopy and to virus aggregates found by electron microscopy. The immunofluorescence technique should be useful in the identification of virus inclusions in situ.

Additional key words: cytology, potexvirus.

Inclusion bodies are produced by many plant viruses in their host tissues (6, 8, 9). Some of these inclusions are specific for a given virus or a virus group and are of significant diagnostic value in plant virology (3, 7, 8). The demonstration of the antigenicity of these inclusions in situ is necessary either to differentiate them from inclusions of nonviral origin or to identify different types of inclusions induced by the same virus (14). The present paper reports the demonstration of the antigenicity of the inclusions occurring in the cells of legume plants infected with clover yellow mosaic virus (CYMV, cryptogram */* : */* : E/E : S/*, potexvirus group) by fluorescence microscopy.

MATERIALS AND METHODS

Virus and host plants.—The vetch isolate of clover yellow mosaic virus (CYMV) (5) multiplied in *Pisum sativum* L. 'Alaska' was used throughout this investigation. Legume hosts used in this study were field vetch (*Vicia sativa* L.), Alaska pea, broad bean (*Vicia faba* L. 'Broad Windsor'), and cowpea (*Vigna sinensis* Savi 'Black Eye'). The leaves were inoculated with crude sap of infected pea tissue approximately 2 wk after germination. Sampling was done 1 and 2 wk after inoculation. Epidermal strips obtained from the undersides of systemically infected leaves were used except in the case of cowpea, epidermal strips from that plant were taken from the inoculated primary leaves.

Light microscopy.—The stains employed to differentiate virus inclusions by light microscopy were Phloxine B, Azure B, and iodine-potassium iodide. Phloxine B and Azure B were prepared at 1% concentration in the solvent previously described (2). Iodine-potassium iodide was prepared by dissolving 2 g of potassium iodide and 0.2 g iodine in 100 ml of distilled water. Of the stains mentioned above, Phloxine B was most commonly used.

The epidermal strips were immersed in the stain solutions for 10-15 min, rinsed in absolute ethanol (30-40 sec), and mounted directly in Euparal (3). Sometimes the stained tissues also were mounted in distilled water. Epidermal strips from leaves of noninoculated plants served as controls.

Immunofluorescence microscopy.—Anti-CYMV rabbit serum with antibody titres of 1/2,048-1/4,096 was prepared against CYMV. The immunoglobulin fraction of the antiserum was precipitated with ammonium sulfate (1). After the third precipitation, the precipitate was dissolved in and dialyzed against phosphate-buffered saline (PBS) for 24 hr. Conjugation with fluorescein isothiocyanate (FITC, ICN Pharmaceuticals Inc., Irvine, CA 92715) was performed (11) after adjusting the protein concentration to 1%. The conjugated preparation had a staining titre of 1/32 and the ratio of fluorescein to protein, determined by absorbancies at 495 nm and 280 nm, was 1.7. The preparation was stored at −20 C in 1-ml lots until use.

The conjugated globulin was absorbed with acetone powder of healthy pea leaves at a rate of 50 mg/2 ml for 2 hr at room temperature. The mixture then was centrifuged at 2,000 *g* for 15 min to obtain the supernatant liquid. Conjugated normal rabbit globulin, prepared as described for anti-CYMV globulin, served as a control.

Staining with fluorescent antibody was done as follows. Epidermal strips from inoculated leaves of cowpea were cut into 2-mm squares and placed on glass slides smeared with Haupt's adhesive. These slides then were immersed in 95% ethanol for 15 min and washed in PBS. A drop of fluorescent antibody was applied to the

00032-949X/78/000 204$03.00/0

sample on a glass slide and incubated in a petri dish for 2 hr at 37 C by placing the dish on a rack standing in a water bath. The slide then was washed with two changes of PBS for 90 min, mounted in PBS containing 50% glycerol, and examined with a Leitz Ortholux I microscope equipped with a 200-W ultra high pressure mercury lamp. The exciter filters UG 1 (1 mm), BG 3 (1 mm) and a suppression filter BG 38 (4 mm) were used with an eyepiece barrier filter K 490. Observations were recorded with an automatic camera using a Kodak Tri-X film.

Electron microscopy.—Systemically infected leaves of field vetch were sampled 2 wk after inoculation. The sample tissues, cut in 2-mm squares, were fixed in an equivolume mixture of 3% formalin and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 at 4 C for 7 hr. The fixed materials then were washed and postfixed in 2% osmium tetroxide for 5 hr, dehydrated through a graded ethanol series, taken to propylene oxide, and embedded in Araldite. Sections were cut on a Reichert ultramicrotome using a diamond knife and stained with 2% aqueous uranyl acetate for 2 hr followed by lead citrate for 2-5 min.

RESULTS

Identical amorphous inclusions were seen in all hosts examined and were absent in the comparable, healthy controls.

Light microscopy.—Amorphous inclusions were observed in infected leaves 1 and 2 wk after inoculation. They appeared as large aggregates mostly deposited along the cell wall. That portion of the inclusion against the wall was shaped to the contour of the wall (Fig. 1, 2). Wide variations in inclusion size were noted both between hosts and between cells of the same host.

Immunofluorescence microscopy.—After the preliminary tests, cowpea was chosen as a test sample host, mainly because of relative ease in obtaining epidermal tissue in addition to the fact that it produced abundant amorphus inclusions similar to those in other hosts. Epidermal tissue from the primary leaves was used 1 and 2 wk after inoculation. The amorphous inclusions were observed as diffuse masses of yellowish green fluorescence, distributed throughout the cytoplasm of the infected cells (Fig. 3, 4).

Electron microscopy.—Figure 5 represents the general view of the infected leaf tissue sectioned longitudinally. Large amorphous aggregates of virus particles were observed in the infected cells (Fig. 6). These were similar to the amorphous bodies observed in the light

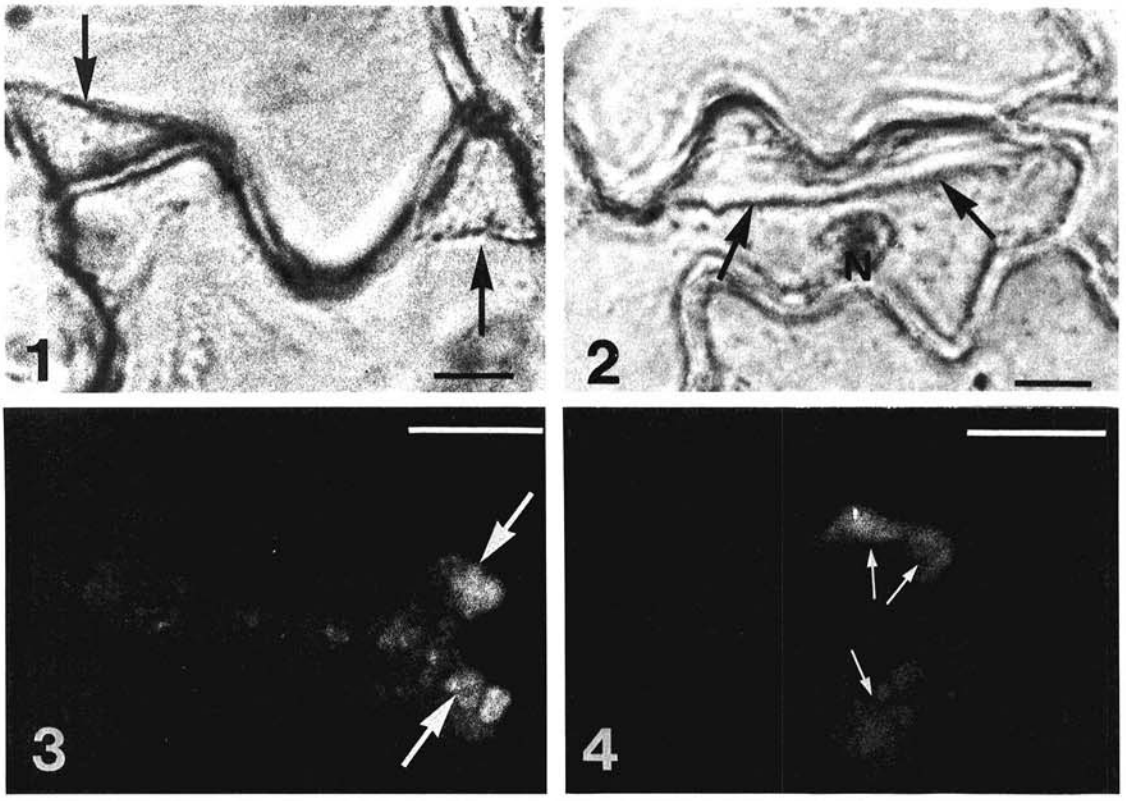
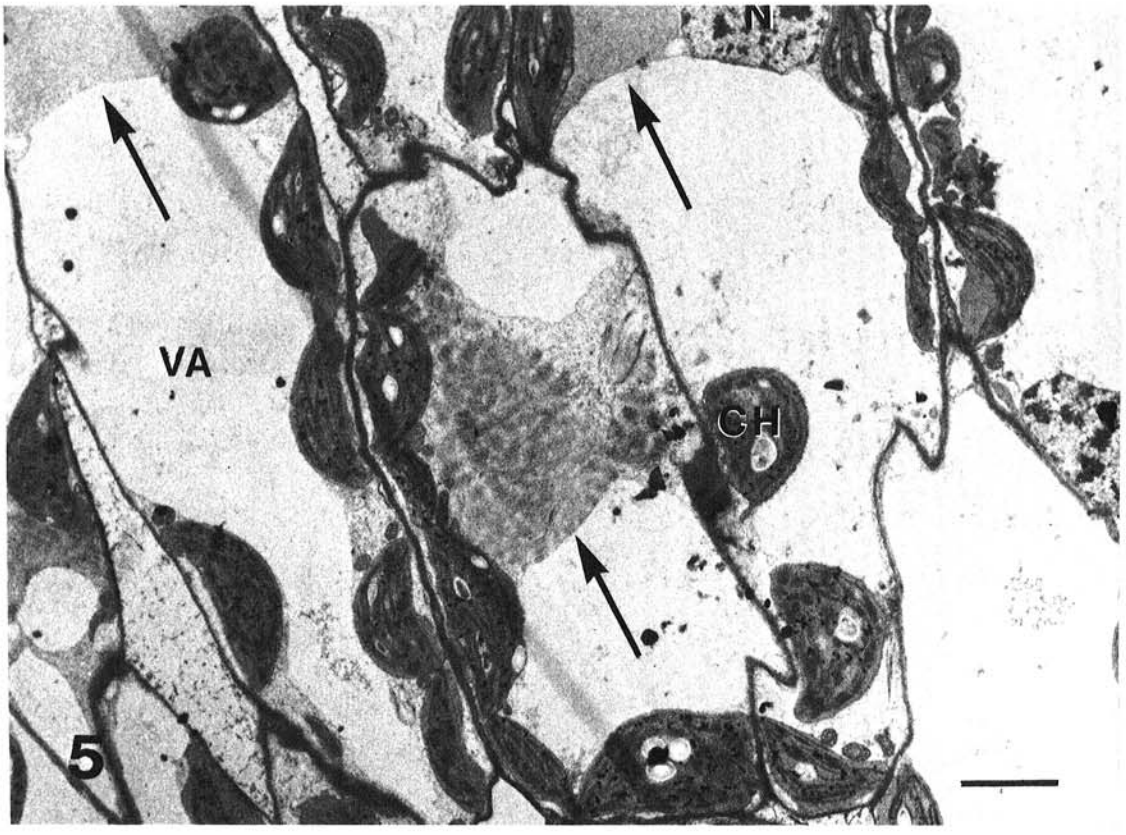


Fig. 1-4. Photomicrographs of inclusion bodies produced in the epidermal cells of the primary host leaves after inoculation with clover yellow mosaic virus. **1)** Two amorphous inclusions, seen as corner deposits (arrows) in the infected epidermal cells of broad bean (*Vicia faba* 'Broad Windsor') 1 wk after inoculation. Stained with Phloxine B. Bar represents 5 μm. **2)** A large amorphous inclusion body (arrows) in the infected epidermal cells of Pea (*Pisum sativum* 'Alaska') 1 wk after inoculation. Stained with Phloxine B. N = nucleus. Bar represents 5 μm. **3-4)** Fluorescence micrographs of infected epidermal cells of cowpea (*Vigna sinensis* 'Black Eye') 2 wk after inoculation. The amorphous inclusions (arrows) in the corners fluoresced as yellowish green masses after having been stained with anti-CYMV globulin labelled with fluorescein isothiocyanate. Bars represent 10 μm.



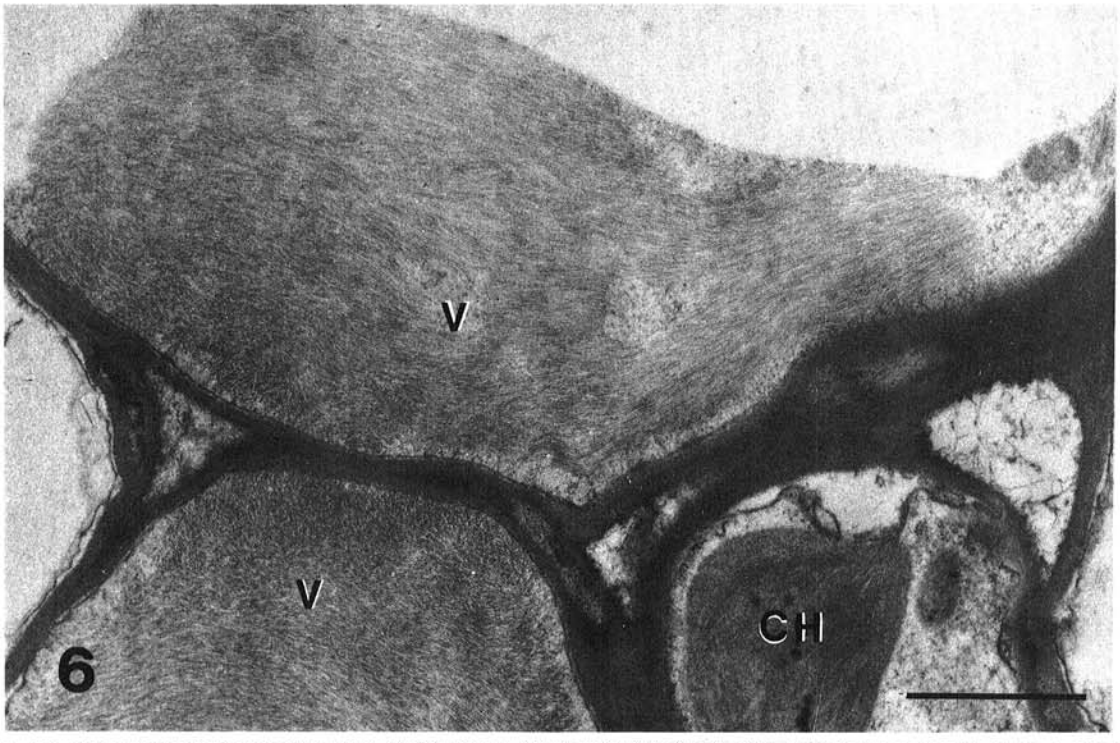


Fig. 5-6. Thin-sections of mesophyll cells of field vetch (*Vicia sativa*) infected with clover yellow mosaic virus, 2 wk after inoculation. **5)** A low magnification micrograph of a longitudinal section of mesophyll cells showing virus aggregates (arrows). Bar represents 3 μm. **6)** A transverse section of mesophyll cells containing large virus aggregates (V). Legend: CH = chloroplast, N = nucleus, and VA = vacuole. Bar represents 1 μm.

microscope. The tissue from noninoculated plants did not contain these aggregates.

DISCUSSION

Amorphous virus aggregates of CYMV, as observed in the thin sections by electron microscopy, were similar to those seen previously (5, 12), and lacked uniformity in size or shape or in arrangements of virus particles. The CYMV inclusions, identified by immunofluorescence microscopy, corresponded to those found by both light and electron microscopy.

Antigenicity of inclusion bodies formed after infection with CYMV was reported previously using an immunoautoradiography technique (13). Other workers (10, 15, 16) also demonstrated viral antigenicity in situ with other virus infections. However, the method employed to demonstrate the antigenicity of CYMV in the present work is simple and efficient for two reasons. First, there is no need to section the material since epidermal cells are thin walled; this allows the fluorescent antibody to penetrate them. Second, the fluorescence can be observed easily with the light microscope. In this study, fluorescence was observed throughout the body of amorphous inclusions, which appeared as large aggregates in the infected cells.

The fluorescent antibody technique had only limited usage in plant virology until it was applied to detection of tobacco mosaic virus infection of tobacco protoplasts (17). The technique is not only useful in a study such as the above, but has an important utility in the identification of viral inclusions in situ. There are mainly two categories of inclusion bodies, namely (i) inclusions consisting entirely or almost entirely of virus particles, and (ii) virus-associated inclusions which do not consist of virus particles (4, 14). Fluorescent antibodies may be used to detect inclusions composed of virus particles, and distinguish these from nonviral protein inclusions or other types of inclusion bodies that occur in host cells in association with virus infection.

The application of immunofluorescence microscopy, as in this study, may prove useful in future studies related to the identification of inclusions.

LITERATURE CITED

1. CAMPBELL, D. H., H. S. GARVEY, N. E. CREMER, and D. H. SUSSDORF. 1970. Isolation of rabbit antibodies and their subunits. Pages 183-234 *in* Methods in immunology. W. A. Benjamin, New York. 454 p.
2. CHRISTIE, R. G. 1967. Rapid staining procedures for differentiating plant virus inclusions in epidermal strips. Virology 31:268-271.
3. CHRISTIE, R. G., and J. R. EDWARDSON. 1977. Light and electron microscopy of plant virus inclusions. Fla. Agric. Exp. Stn. Monogr. Ser. 9. 150 p.
4. HIEBERT, E., D. E. PURCIFULL, R. G. CHRISTIE, and S. R. CHRISTIE. 1971. Partial purification of inclusions induced by tobacco etch virus and potato virus Y. Virology 43:638-646.
5. HIRUKI, C., P. SHUKLA, and D. V. RAO. 1976. Occurrence of clover yellow mosaic virus aggregates in transfer cells of Pisum sativum. Phytopathology 66:594-597.
6. HOOKER, W. J. 1964. Cytological aspects of virus multiplication–potato virus X. Pages 29-36 *in* Z. Kiraly and G. Ubrizsy, eds. Host-parasite relations in plant pathology. Budapest, Hungary. 257 p.
7. MARTELLI, G. P., and M. RUSSO. 1977. Plant virus inclusion bodies. Adv. Virus Res. 21:175-266.
8. MATSUI, C., and A. YAMAGUCHI. 1966. Some aspects of plant viruses in situ. Adv. Virus Res. 12:127-174.
9. MC WHORTER, F. P. 1965. Plant virus inclusions. Annu. Rev. Phytopathol. 3:287-312.
10. NAGARAJ, A. N., and L. M. BLACK. 1961. Localization of wound-tumor virus antigen in plant tumors by the use of fluorescent antibodies. Virology 15:289-294.
11. OTSUKI, Y., and I. TAKEBE. 1969. Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. Virology 38:497-499.
12. PURCIFULL, D. E., J. R. EDWARDSON, and R. G. CHRISTIE. 1966. Electron microscopy of intracellular aggregates in pea (Pisum sativum) infected with clover yellow mosaic virus. Virology 29:276-284.
13. SCHLEGEL, D. E., and D. E. DELISLE. 1971. Viral protein in the early stages of clover yellow mosaic virus infection of Vicia faba. Virology 45:747-754.
14. SHEPARD, J. F., and T. A. SHALLA. 1969. Tobacco etch virus cylindrical inclusions: Antigenically unrelated to the casual virus. Virology 38:185-188.
15. SINHA, R. C., and L. M. BLACK. 1963. Wound-tumor virus antigens in the internal organs of an insect vector. Virology 21:183-187.
16. SINHA, R. C., and Y. C. PALIWAL. 1977. Detection of wheat streak mosaic virus antigens in vector mites with fluorescent antibodies. Phytopathology 67:570–572.
17. TAKEBE, I., and Y. OTSUKI. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Proc. Nat. Acad. Sci. USA 64:843-848.