Detection of Virus Infection and Spread by Immunofluorescent Staining of Enzyme Treated Leaves

J. D. Lei and G. N. Agrios

Graduate assistant and professor, respectively, Department of Plant Pathology, University of Massachusetts, Amherst 01003.
Accepted for publication 3 April 1986.

ABSTRACT

Lei, J. D., and Agrios, G. N. 1986. Detection of virus infection and spread by immunofluorescent staining of enzyme treated leaves. Phytopathology 76:1031-1033.

A fluorescent antibody staining technique, which allows precise presymptomatic location of virus infected cells in a leaf area, was developed during investigation of maize dwarf mosaic virus infection of corn. Inoculated leaves were brushed with Carborundum and briefly treated with cellulolytic enzymes. The partially digested leaves were fixed with acetone and sequentially treated with virus antibody produced in rabbits and then with fluorescein isothiocyanate-conjugated anti-rabbit antibody produced in goats. Epifluorescent microscopy was used to locate the fluorescent, antibody-stained infected cells. The epidermal cells and one to two layers of mesophyll cells could be clearly observed with this method. The effectiveness of the method was demonstrated.

The immunofluorescent technique developed by Coons et al (1) is readily applicable in most animal systems and has been used extensively in various aspects of virus research. However, because of the difficulty with which antibodies penetrate plant tissues and the interfering autofluorescence of plant materials, the technique must be modified for application in plant tissues.

Thin sectioning of tissues is a general practice in most histological studies employing fluorescent antibody staining of plant cells. Both frozen sections (9) and paraffin sections (5) have been used with success. Nagaraj (4) developed an immunofluorescent technique that used tobacco cells dissociated by pectinase. Tobacco mosaic virus (TMV) antigen in the dissociated cells could then be stained with fluorescent antibody. The use of single cells eliminated the interference of autofluorescence of plant materials and allowed good penetration of antibody into the cells.

Hosokawa and Mori (2) found that in peeled epidermal cells of cucumber leaves treated with cellulolytic enzymes, the intracellular viral antigen can be stained with fluorescent antibody. Nishiguchi et al (6) adopted a similar method to analyze the profile of TMV spread in epidermis of tomato.

In this report we present a method of preparing virus-inoculated leaf tissues of monocotyledonous plants for immunofluorescent staining of the viral antigen in the cells. The method uses leaves that are partially digested with cellulolytic enzymes. After treating the tissues first with virus antibody produced in rabbits and then with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody produced in goats, the entire leaf section is observed with an epifluorescent microscope. The behavior of maize dwarf mosaic virus strain B (MDMV-B) in corn (*Zea mays* L.) varieties exhibiting susceptibility or resistance to MDMV-B was investigated to demonstrate the method.

MATERIALS AND METHODS

The B strain of MDMV, supplied by R. E. Ford, University of Illinois, Urbana, was used throughout these studies. Two MDMV-resistant inbred varieties, Pa405, a field corn, and Bsq, a sweet corn, and the MDMV-susceptible sweet corn inbred Ma5125, were used in all experiments. The initial seeds of all corn lines and hybrids were provided by David MacKenzie, Harris Seed Co., Rochester, NY. Plants were grown in the greenhouse in 15-cm-diameter plastic pots in a 1:1:1 sand, peat, and soil mixture amended with lime, at a temperature maintained generally near 25 C, but occasionally reaching 30 C during the day and 20 C during the night. All plants were kept in the greenhouse until use. For several experiments, the plants were transferred to the growth chamber and were kept at 27 C and a 16-hr day period. MDMV-B was maintained in young Golden Cross Bantam sweet corn plants. Young leaves with mosaic symptoms were used as inoculum after grinding in inoculation buffer (0.05 M sodium phosphate, 10 mM sodium diethyldithiocarbamate, pH 7.4) at a ratio of 1:10 (w/v). Carborundum was added to plant sap, which was then rubbed onto plants with cotton swabs. The first fully opened leaves of plants at the four-leaf stage were inoculated in all the experiments unless otherwise stated. The plants were randomized during inoculation.

Preparation of antibody. MDMV-B was purified by the procedure of von Baumgarten and Ford (8). At weekly intervals, rabbits were given three subcutaneous injections, then one intravenous injection of 0.5 mg of purified MDMV-B. The rabbits were bled 1 wk after the last injection. The serum had a titer of 1:256 as determined by the microprecipitin test. The antiserum was routinely adsorbed with acetone-treated healthy corn leaf powder before use. The antiserum was precipitated with 50% ammonium sulfate, centrifuged for 5 min, and the pellet was dissolved in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.2% sodium azide, and dialyzed against three changes of 500 ml of PBS.

Immunofluorescent staining. The lower epidermis of 4-cm sections of virus-inoculated leaves was brushed with an artist's watercolor brush dipped in a slurry of Carborundum in distilled water. The leaf sections were brushed parallel to the veins 50–200 times in each direction. Older leaves required more brushing to permit sufficient penetration and partial digestion of cell walls by the enzymes. The brushed leaves were rinsed with distilled water to remove the Carborundum. Three leaf pieces were incubated with the brushed side down in 5 ml of enzyme solution [2% Cellulycin (Calbiochem, La Jolla, CA), 1% Driselase (Kogyo, Tokyo, Japan), 0.5 M mannitol, and 10 mM $CaCl$, pH 5.5] in a petri dish. This and all subsequent incubations were carried out on a rotary shaker (20 rpm) at room temperature. A uniform water-soaked appearance over the entire leaf section signaled satisfactory completion of cell wall digestion for the purpose of immunofluorescent staining. Digestion was usually completed in about 40–60 min. The enzyme solution was removed and the leaves were washed with two changes of PBS at 5-min intervals. The PBS was drained and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

©1986 The American Phytopathological Society

acetone was added to the dish for 40–60 min to fix and decolorize the tissues. The fixed, decolorized leaf pieces were washed with three changes of PBS at 30-min intervals to remove the acetone and were stored in PBS at 4 C until further processing. Two milliliters of MDMV-B antibody of $A_{280} = 0.01$ were added per dish of leaf sections. After 12–24-hr incubation, the leaves were washed with two hourly changes of PBS and the third wash overnight. Three milliliters of 1:50 diluted FITC-conjugated anti-rabbit antibody produced in goats (Sigma Chemical Co., St. Louis, MO) was added to the leaves, which, after 12–24-hr incubation at room temperature, were washed with PBS as before. After the last wash the leaves were floated in PBS and were lifted out and placed flat on microscope slides. Excess PBS was allowed to run off and the leaves were mounted in 10% glycerin in PBS. The stained leaves were observed with a Leitz Dialux 20 epifluorescent microscope supplied with a mercury HBO 100W light source and Leitz K2 and L2 filter systems, which allow passage of an extremely narrow band of blue light at 495 nm and a selective barrier at 525 nm, specific for FITC and a few other stains. The correct concentrations of antibodies were determined previously by

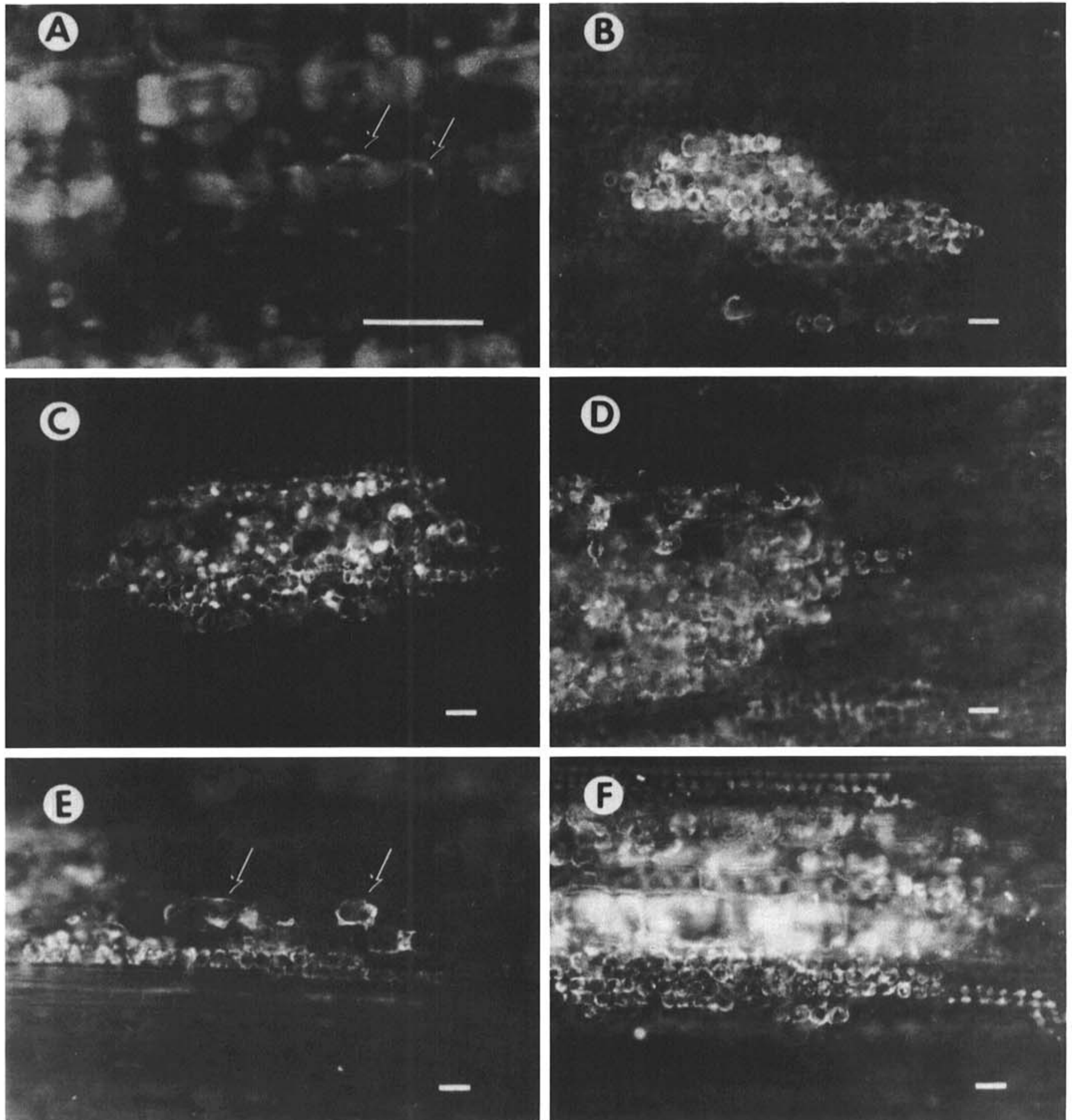

Fig. 1. Fluorescent antibody staining of MDMV-B infected cells in enzyme-treated leaves of a susceptible (Ma5125) and two resistant (Pa405 and Bsq) corn varieties as observed by epifluorescent microscopy. **A,** Fluorescing virus infected Pa405 cells (arrows) were first detected at 24 hr after inoculation at 27 C. **B and C,** Groups of virus infected cells in Bsq and Ma5125 leaves, respectively, each originating from a single infection locus (3 days after inoculation). **D,** Pointed front of fluorescing virus infected cells indicating the direction and extent of virus spread in Ma5125 (9 days after inoculation). **E,** Continuous rows of infected cells bordering leaf veins, and occasional infected epidermal cells (arrows) in Pa405 (4 days after inoculation). **F,** Numerous infected mesophyll cells and mostly uninfected (nonfluorescing) epidermal cells in Ma5125 (6 days after inoculation). Bar = 33.3 μm.

indirect immunofluorescent staining of infected protoplasts isolated from symptomatic leaves. Twofold diluted MDMV-B and FITC-conjugated anti-rabbit antibodies were tested in combination and the highest dilutions that yielded satisfactory staining of the infected cells were used.

RESULTS

Immunofluorescent staining technique. Under ultraviolet light, the acetone-fixed, decolorized, and antiserum-treated healthy leaves produced a red fluorescence of low intensity, whereas similarly treated virus infected cells produced a bright apple-green fluorescence. All treated mesophyll cells were in a state of plasmolysis and thus appeared to be spherical (Fig. 1A–F). The epidermal cells and one to two layers of mesophyll cells could be clearly observed with epifluorescent microscopy. The first signs of fluorescence appeared as small bright spots in the periphery of cells 24 hr after leaf inoculation (Fig. 1A). Two to three days after inoculation, bright fluorescence in infected cells was observed near the periphery of the rounded protoplasts (Fig. 1B). Sometimes specks of bright green fluorescence developed irregularly in the cytoplasm 3 days after inoculation (Fig. 1C). Front edges of infected areas were often pointed (Fig. 1D). Infected areas often bordered veins (Fig. 1E). At 3–4 days after inoculation, large numbers of infected mesophyll cells were present (Fig. 1C), whereas only a few infected nearby epidermal cells were detected (Fig. 1E). In Ma5125, numerous infected mesophyll cells and mostly uninfected (nonfluorescing) epidermal cells appeared by the sixth day after inoculation (Fig. 1F). At 9–12 days after inoculation more infected epidermal cells appeared and interfered with the observation of the mesophyll cells in lower layers. Cells of the bundle sheath and of the vascular system were beyond the resolution of this method. We have run the immunofluorescent staining test on corn leaf tissues more than 10 times with satisfactory results every time.

Younger and older plants having from two to seven leaves have also been used with success. Carborundum must be applied uniformly over the entire leaf surface, because uneven brushing results in some areas of the leaf being digested faster than others. Uneven digestion is indicated by nonuniform discoloration of leaves during fixation. The number of brushings needed to achieve efficient digestion of older leaves may be obtained in a few trials. Usually, digestion was completed in about 40–90 min.

Early detection of infection loci. The inoculated plants were incubated in the growth chamber. The inoculated leaves were sampled at 12-hr intervals after inoculation and checked with the fluorescent antibody method for the presence of virus infected cells. Virus infected cells were first detected 24 hr after inoculation. The fluorescent cells were present in groups of 3–50 cells (Fig. 1A). The intensity of fluorescence at first was weak but quite obvious. The intensity of fluorescence at 36 hr was stronger and easily observed.

DISCUSSION

Although the pectinase digestion method of Nagaraj (4) and the protoplast method of Otsuki and Takebe (7) can be used to monitor the increase in number of infected cells, the spatial relationship of the infected cells cannot be analyzed. The method of Hosokawa and Mori (2) is capable of detecting and analyzing the relationship of infected cells but only in the epidermis. The epidermis of most plants, however, and particularly of monocotyledonous plants, is difficult if not impossible to peel. In the method presented here, large leaf pieces are used, which allows uninterrupted observation of large areas of leaf tissue. Epifluorescent microscopy of infected leaves partially digested with enzymes and indirectly stained with fluorescent antibody enables observation of the first few layers of cells at and below the surface of leaves without sectioning or peeling of the leaves. Epidermal cells as well as 1–2 layers of mesophyll cells may be observed with this method.

Infection loci could be detected in inoculated leaves of asymptomatic resistant or tolerant inbreds (Pa405 and Bsq) as well as in varieties producing local or systemic symptoms (Ma5125). In Ma5125 the immunofluorescent technique allowed detection of infection loci early, when only a few cells had become infected, and these were detected several days before symptoms visible to the naked eye became apparent. The method can be used to determine whether a variety is immune or resistant to infection by a virus and to determine the relative frequency of infection sites and relative rate of spread of the virus in asymptomatic resistant varieties. It may also be used as a "local lesion assay" if a local lesion-responding host is not available or if the desired host does not produce local lesions. The method has been successfully tried on MDMV-inoculated corn and sorghum. It is expected that the technique will be applicable to studies with other plant-virus systems that involve similar leaf structures. More experimentation done with the immunofluorescent staining technique, and other methods, to investigate the mechanisms of resistance in corn against MDMV are reported in a separate paper (3).

LITERATURE CITED

1. Coons, A. H., Creech, H. J., Jones, R. N., and Berlimer, E. 1942. The determination of Pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 45:159-170.
2. Hosokawa, D., and Mori, K. 1974. Investigation of CMV multiplication in tobacco epidermal cells by fluorescent antibody technique. Ann. Phytopathol. Soc. Jpn. 40:210.
3. Lei, J. D., and Agrios, G. N. 1986. Mechanisms of resistance in corn to maize dwarf mosaic virus. Phytopathology 76:1034-1040.
4. Nagaraj, A. N. 1962. Fluorescent antibody staining of viral antigen in dissociated plant cells. Virology 18:329-332.
5. Nagaraj, A. N. 1965. Immunofluorescence studies on synthesis and distribution of tobacco mosaic virus antigen in tobacco. Virology 25:133-142.
6. Nishiguchi, M., Motoyoshi, F., and Oshima, N. 1980. Further investigation of a temperature-sensitive strain of tobacco mosaic virus: Its behaviour in tomato leaf epidermis. J. Gen. Virol. 46:497-500.
7. Otsuki, Y., and Takebe, I. 1969. Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. Virology 38:497-499.
8. von Baumgarten, G., and Ford, R. E. 1981. Purification and partial characterization of maize dwarf mosaic virus strain A. Phytopathology 71:36-41.
9. Worley, J. F., and Schneider, I. R. 1963. Progressive distribution of southern bean mosaic virus antigen in bean leaves determined with a fluorescent antibody stain. Phytopathology 53:1255-1257.