Neth. J. Path. 93 (1987) 261-272

Detection of viral antigen in semi-thin sections of plant tissue by immunogold-silver staining and light microscopy

J.W.M. VAN LENT and B.J.M. VERDUIN[1]

Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, the Netherlands

Accepted 14 July 1987

Abstract

The immunogold-silver staining technique was developed for the light microscopical localization of viral antigen in plant tissue. Semi-thin sections of LR White-embedded plant tissue were immunologically labelled with primary antiserum and protein A-gold. Individual gold particles were covered with a silver precipitate using a physical developer. This precipitate could be seen as black spots in a conventional light microscope with brightfield and as brilliant white spots with darkfield illumination. Maximal sensitivity and low background was obtained when immunogold-labelled sections were fixed in glutaraldehyde prior to silver enhancement. Simultaneous observation of the silver coated gold label and cell morphology was achieved by epipolarization microscopy. Using this technique cowpea chlorotic mottle virus coat protein was detected in cowpea plants as function of the infection period. Virus translocation and multiplication was monitored in systemically inoculated tissue, showing viral antigen in phloem parenchyma of petiolules 6 h after systemic inoculation and subsequent spreading from the phloem to the neighbouring bundle sheath and cortex cells.

Additional keywords: LR White, epipolarization, cowpea chlorotic mottle virus, bromovirus, electron microscopy.

Introduction

Colloidal gold is now a commonly used marker in immunocytochemistry. Various antigens are specifically localized by immunogold labelling on thin sections of resin-embedded animal and plant tissue (Roth, 1983; De Mey, 1983; Patterson and Verduin, 1987). This labelling also proved to be useful in light microscopical localization of antigens (Geoghegan et al., 1978; De Mey, 1983; Roth, 1983), and gold particles were visualized as red stain in brightfield illumination.

It is possible to cover the gold particles with silver precipitate improving visualization, by using a physical developer containing silver lactate and hydroquinone (Danscher, 1981). Then the grains of gold catalyze the reduction of silver ions to metallic silver. As a result, invisible traces of gold forming a latent image are surrounded by a growing shell of metallic silver which reveals the localization of the gold in the tissue. The silver precipitate is observed as black stain in brightfield microscopy. This method is called immunogold-silver staining (IGSS).

[1] To whom correspondence should be addressed.

A further improvement to observe gold particles or silver precipitate can be achieved by epipolarization microscopy, using an epi-illumination microscope with an epipolarization filterblock (De Mey, 1983; De Mey et al., 1986). De Mey et al. (1986) found that colloidal gold bound to protein A or to antibodies can be detected with high sensitivity by polarized light epi-illumination microscopy. The dark granules, visible with brightfield microscopy strongly back-scatter incident polarized light. Since polarization of the back-scattered light is lost, it will pass the analyzer, while other excitation light will be extinguished. Use of a mercury-arc lamp in combination with silver staining of the gold particles yields an extremely bright blue-coloured signal, that is still visible against a low background of transmitted non-polarized light, which allows identification of cytochemically stained cells.

In this paper we present technical details for the application of IGSS in plant tissue containing viral antigens. Using cowpea chlorotic mottle virus (CCMV) and cowpea plants as a model system, we were able to localize CCMV multiplication and translocation in infected plant tissue. The same preparation can be examined with both light and electron microscopes. The method has potentials for research on other antigens, a.o. in the field of plant pathology.

Materials and methods

Virus and antiserum. CCMV was maintained, purified and used as antigen to raise antiserum as described by Van Lent and Verduin (1986). Gammaglobulin (IgG) was purified by affinity chromatography. One ml of antiserum was mixed with 1 ml phosphate buffered saline (PBS: 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 3.1 mM NaN_3) pH 7.4. The mixture was applied to a column of sepharose CL-4B-protein A (1 g preswollen for 1 h at room temperature in PBS). The column was washed with PBS to remove all unbound proteins. Gammaglobulins were then eluted from the column with 0.2 M glycine buffered with HCl at pH 2.8, dialysed against PBS and stored at −20 °C.

Inoculation of cowpea plants with CCMV. The primary leaves of cowpea plants (*Vigna unguiculata* cv. California Blackeye) with leaflets of the first trifoliate leaves that were 2-4 cm long were inoculated with purified CCMV (1 mg ml^{-1} in 0.01 M NaH_2PO_4, 5 mM $MgCl_2$, adjusted with NaOH to pH 6.0) and submitted to a differential temperature treatment as described by Dawson and Schlegel (1976) who claimed with this procedure systemic inoculation of the secondary leaves. Briefly, the primary leaves were kept in light at 27-30 °C, and the secondary leaves were kept in the dark at 10 °C. After three days the plants were transferred to a growth cabinet at 25 °C and continuous light (fluorescent tubes, 25 kW m^{-2} at the height of the primary leaves). The transfer was defined as time zero (t = 0) of systemic inoculation. Samples were taken from the petiolule at different times and further processed for light and electron microscopic examination.

Fixation and embedding of plant tissue. Relatively large pieces (1-2 mm) of the petiolule were fixed under vacuum (55-66 mbar) in 1% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 1.5 mM $CaCl_2$ in phosphate/citrate buffer pH 7.2 (0.1 M Na_2HPO_4 and 2.7 mM citric acid) for 1 h at room temperature. After infiltration of

the fixative, fixation proceeded for at least 12 h at 4 °C. The tissue was then washed six times for 10 min in phosphate/citrate buffer and dehydrated in a graded series of ethanol. The ethanol was replaced with London Resin White embedding resin (a polar medium) and infiltration was allowed for 16 h at 4 °C. Tissue pieces were transferred to gelatin capsules and the resin was polymerised at 50 °C for 24 h. Semi-thin sections (approx. 3 μm) were cut on a LKB Ultrotome V using dry glass knives. Sections were spread on a drop of 40% (v/v) acetone in distilled water and dried onto a microscope slide using a hot plate with a temperature of 60-70 °C. Attachment of the sections was improved when the slides were coated with poly-L-lysine (Huang et al., 1983).

Immunogold labelling. Protein A-gold (pAg) complexes with gold particle diameter of 7 nm were prepared as described previously (Van Lent and Verduin, 1986). Semi-thin sections were incubated for 30 min in PBS containing 1% (v/v) bovine serum albumin (PBS-BSA). Drops of 0.01 mg anti-CCMV IgG per ml in PBS-BSA containing 0.05% (v/v) Tween 20 (PBS-BSA-Tween) were placed onto the sections and the mixture was incubated for 2 h. The slides were washed three times for 5 min in PBS-tween and drops of pAg in PBS-BSA-Tween (A_{520nm} = 0.1) were placed on the sections and incubated for 90 min. The slides were then washed 3 × 5 min in PBS-Tween, fixed for 15 min in 1% (w/v) glutaraldehyde in PBS and washed 3 × 5 min in double distilled water. All incubations were carried out at room temperature. The slides were either dried and stored or immediately subjected to silver staining. Immunogold labelling of ultrathin sections for electron microscopy was done as described by Van Lent and Verduin (1986).

Silver staining. Silver staining was essentially carried out as originally described by Danscher (1981) omitting the addition of gum arabic as reported by Moeremans et al. (1984). The developer with final concentrations of 77 mM hydroquinone and 5.5 mM silver lactate in 200 mM citrate buffer, pH 3.85, was prepared as follows: 2.0 M citrate buffer was made by dissolving 25.5 g trisodium citrate.$2H_2O$ and 23.5 g citric acid.H_2O in 100 ml double distilled water. Ten ml of 2.0 M citrate buffer was mixed with 60 ml double distilled water (solution A) and 0.85 g hydroquinone or 0.11 g silver lactate were each dissolved in 15 ml double distilled water (solution B and C). All solutions were kept at 20 °C. As solutions B and C are photosensitive, the flasks were covered with aluminium foil. Both preparation of the final developing solution and of the silver staining were carried out in a darkroom under red safety light. Prior to silver enhancement, the slides were incubated for 5 min in a tenfold dilution of 2.0 M citrate buffer. The developer was prepared immediately before use by mixing solutions A and B followed by the addition of solution C. Incubation of the slides in the developer was for 7 to 14 min at 20 °C. The enhancement was stopped by transferring the slides to a fixing solution (tenfold dilution of Agefix) for 3 min, after which they were washed for 3 × 5 min in distilled or tap water and air dried. Sections were stained with 1% (w/v) toluidine blue in 1% (w/v) sodium tetraborate in distilled water. Unstained sections were examined with a Wild light microscope with either brightfield, darkfield or phase-contrast illumination. Stained sections were examined with epi-illumination in a Leitz Orthoplan microscope equipped with water-immersion objective lenses (25, 50 and 100 ×) and a polarization filterblock (epipolarization microscopy), obtained from Leitz. Silver enhancement of gold labelled ultrathin sections for electron microscopy was

done as described above, but enhancement time was 3-4 min at 20 °C. Sections were then stained for 15 min with 4% (w/v) uranylacetate in double distilled water.

Results

Semi-thin sections of LR White-embedded plant tissue were easy to cut on dry glass knives, but were difficult to stretch on water. It was therefore difficult to mount them flat onto the microscope slide. Floating on 40% acetone and drying on a hot plate circumvented this problem.

In sections, immunogold-labelled with 7-nm particles no label was seen. In order to observe label in the light microscope, silver enhancement of the 7-nm gold label using a mixture of silver lactate and hydroquinone in citrate buffer was essential. For visualization of the silver enhanced label with brightfield, darkfield or phase-contrast illumination enhancement time should be prolonged as long as possible. As the silver-staining solution remained stable for a maximum of 15-17 min at 20 °C before self-nucleation started, enhancement was carried out for 14 min at this temperature. In Fig. 1 labelling of CCMV is demonstrated on a single longitudinal section of the petiolule of cowpea leaves at t = 24 h with different types of illumination. Label was observed as a black precipitate on the surface of the tissue sections with brightfield illumination (Fig. 1A) and as a brilliant white precipitate with darkfield illumination (Fig. 1B). Phase-contrast illumination was applied when both black precipitate and cell structures had to be observed (Fig. 1C). The silver-coated gold label was detected in phloem tissue, the bundle sheath and cortex cells. The specificity of the label was tested on sections of 24-h infected tissue incubated with anti-TMV (Fig. 1D, E and F) and sections of healthy tissue incubated with anti-CCMV. In both controls only background levels of silver stain were observed. When sections were stained with toluidine blue for simultaneous observation of cell structures and specific labelling with brightfield illumination, the black silver precipitate could only be recognized in densily labelled areas, whereas in cells with less silver staining (less CCMV) the appearance of the silver precipitate was masked by the blue colour of the tissue. With darkfield illumination of cytochemically unstained and stained sections reflections from the plant tissue may interfere with reflections from the silver precipitate, thus masking minute silverstaining.

For obtaining maximal sensitivity and low background sections had to be fixed with glutaraldehyde after immunogold labelling and prior to washing in distilled water and silver enhancement as shown in Fig. 2. Serial cross sections of a petiolule 24 h after systemic inoculation were immunogold-labelled with anti-CCMV and protein A-gold followed by silver enhancement (Fig. 2A and B) and glutaraldehyde fixation prior to silver enhancement (Fig. 2C and D).

Simultaneous observation of cell morphology and specific silverstaining in stained sections was achieved with brightfield transillumination combined with epi-illumination through a polarizing filterblock (epipolarization microscopy) (Fig. 3B and 4B). With this technique details of cell morphology could be observed due to staining with toluidine blue, whereas the specific silver precipitate was observed as a brilliant blue deposit on the surface of the section. Because the silver precipitate was recognized by its brilliance and blue color, even low densities of specific labelling could be detected against a background of stained tissue. For observation with epipolarization illumination it was found that silver enhancement time had to be reduced. Long enhancement time

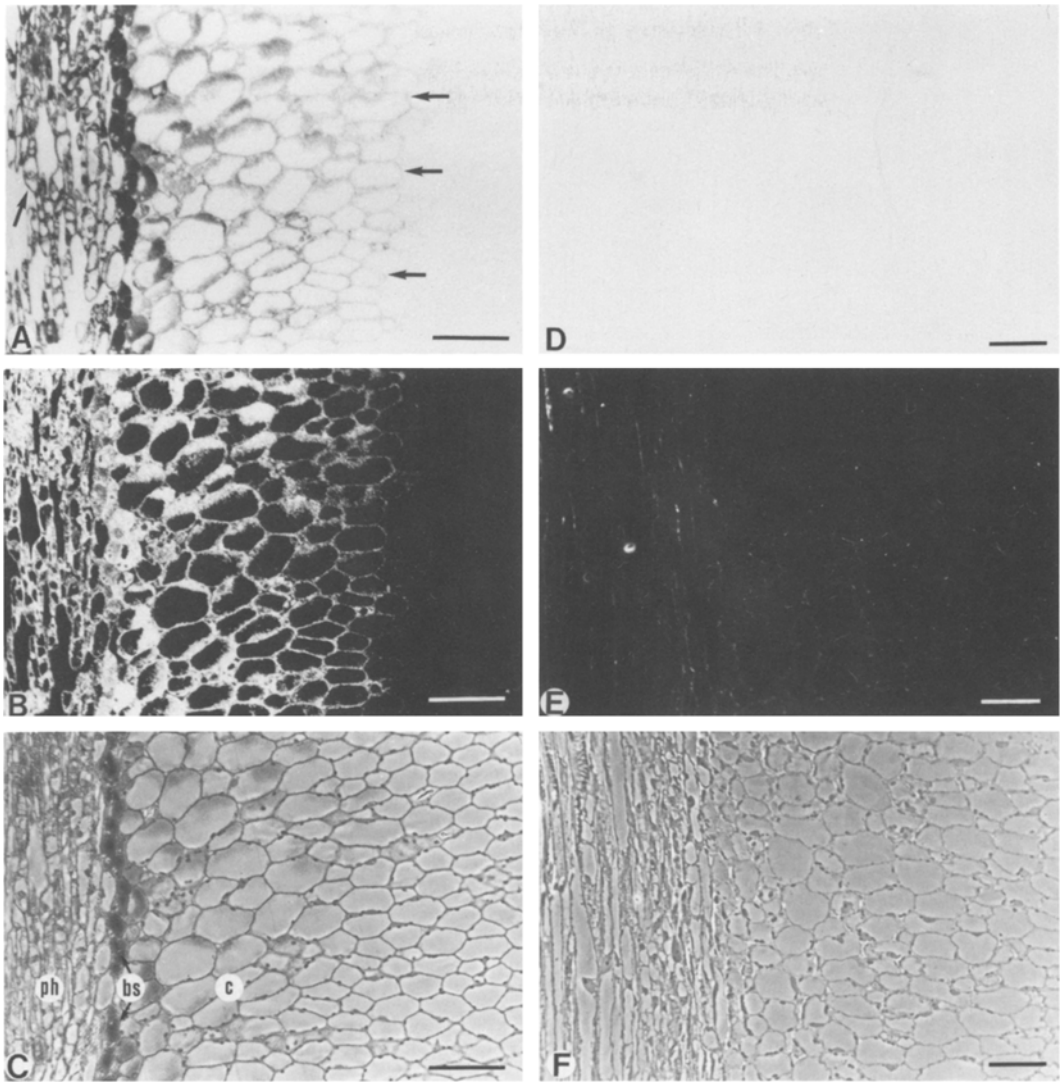


Fig. 1. Longitudinal section of petiolule 24 h after systemic inoculation with CCMV. Sections were incubated with anti-CCMV (A, B, C) or anti-TMV (D, E, F), followed by protein A-gold and subsequent enhancement with silver for 14 min at 20 °C. Silver stain (arrows) is observed as a black precipitate with brightfield illumination (A) and as a brilliant white precipitate with darkfield illumination (B) on cells of the phloem (ph), bundle sheath (bs) and cortex (c) tissue. Cell morphology is visualized by phase-contrast illumination (C). Appropriate control sections, also 24 h infected with CCMV, were incubated with anti-TMV, protein A-gold and silver and viewed with different illuminations (D, E and F respectively). Bar represents 40 μm.

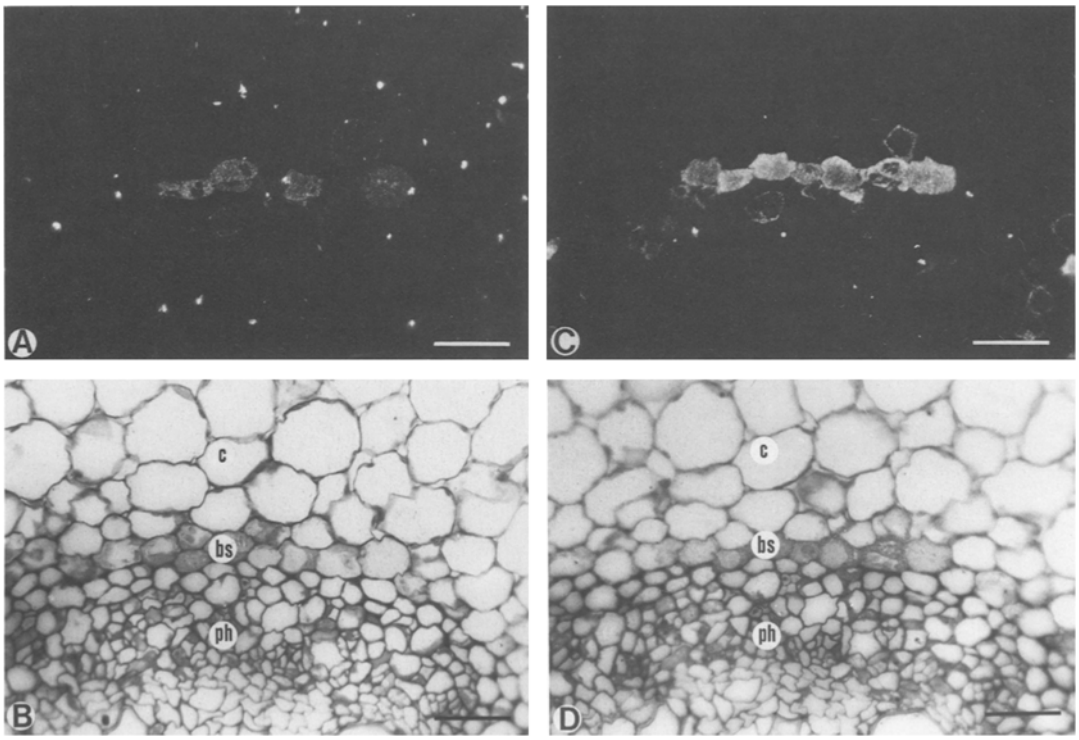


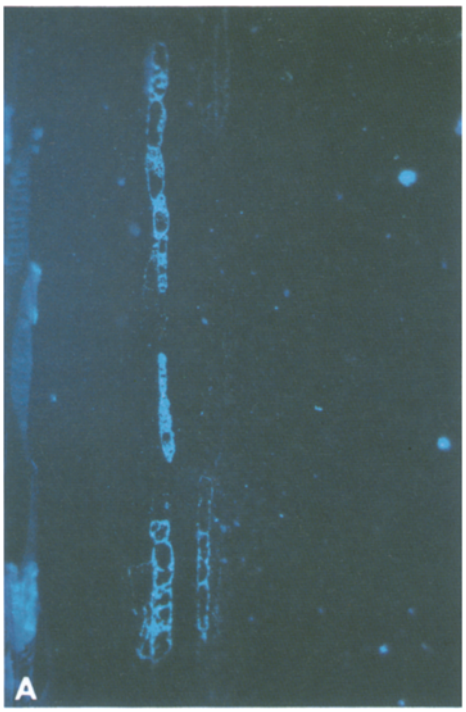
Fig. 2. Serial cross sections of a petiolule 24 h after systemic inoculation. Sections were incubated with anti-CCMV, protein A-gold, and subsequently enhanced with silver for 14 min at 20 °C (A, B) or fixed with glutaraldehyde prior to silver enhancement (C, D). Sections were cytochemically stained with toluidine blue. Silver stain is observed as a brightly shining precipitate with epi-illumination (A and C), while cell morphology is visualized with brightfield transillumination (B and D). Bar represents 10 μm.

necessary for brightfield observation resulted in too bright a signal. Silver enhancement of the 7-nm gold particles for 2-4 min at 20 °C appeared to be sufficient to observe each individual silver-coated gold particle with epipolarization microscopy using a high magnification objective lens ($\times$ 100). However, silver enhancement for 7-8 min was

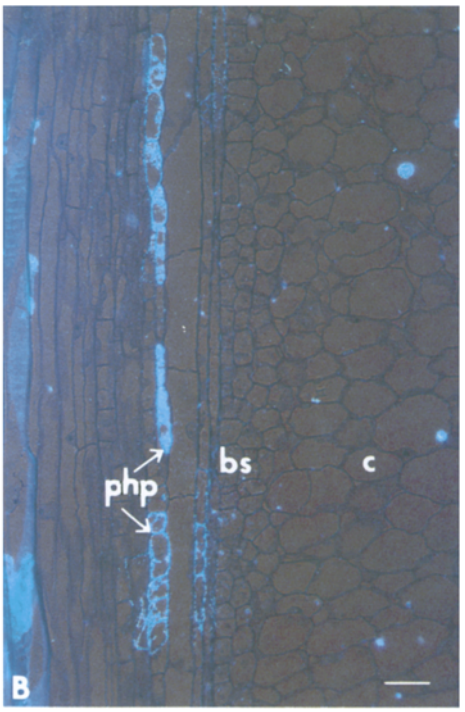
→

Fig. 3. Longitudinal section of a petiolule 6 h after systemic inoculation, incubated with anti-CCMV, protein A-gold, fixed and subsequently enhanced with silver for 8 min at 20 °C. Silver stain is observed as a brilliant blue precipitate against a dark background with epi-illumination (A) and together with cell morphology by combining epi-illumination with brightfield transillumination (B). Sections were stained with toluidine blue. php, phloem parenchyma cells; bs, bundle sheath; c, cortex. Bar represents 10 μm.

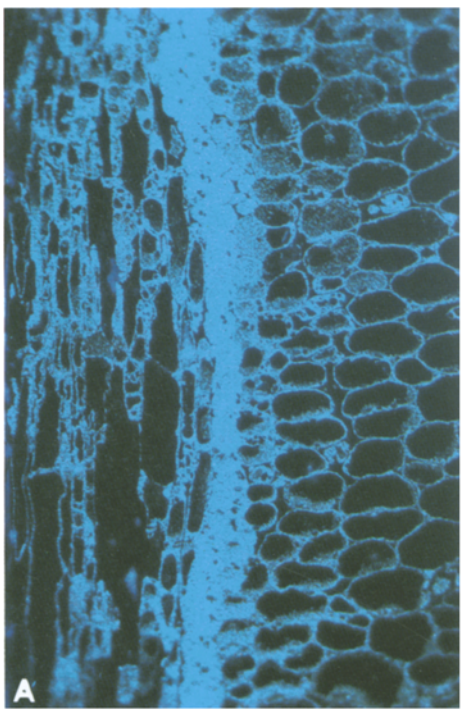
Fig. 4. Longitudinal section of a petiolule at 24 h after systemic inoculation, incubated with anti-CCMV, protein A-gold, fixed and subsequently enhanced with silver for 8 min at 20 °C. Silver stain is observed with epi-illumination (A) and a combination of epi-illumination and brightfield transillumination (B). Sections were stained with toluidine blue. ph, phloem; bs, bundle sheath; c, cortex. Bar represents 10 μm.

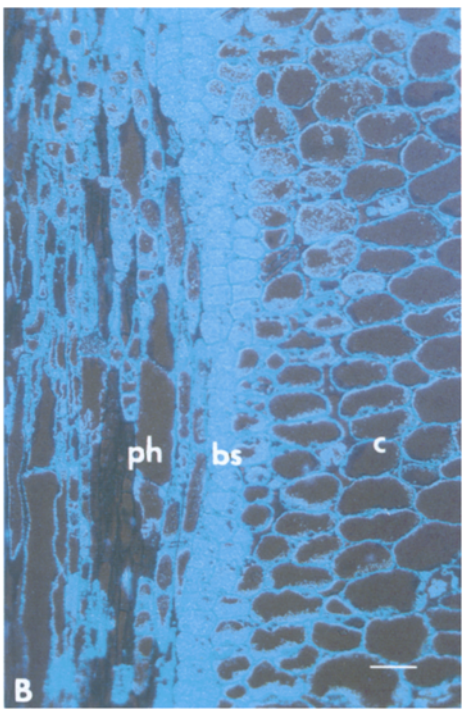


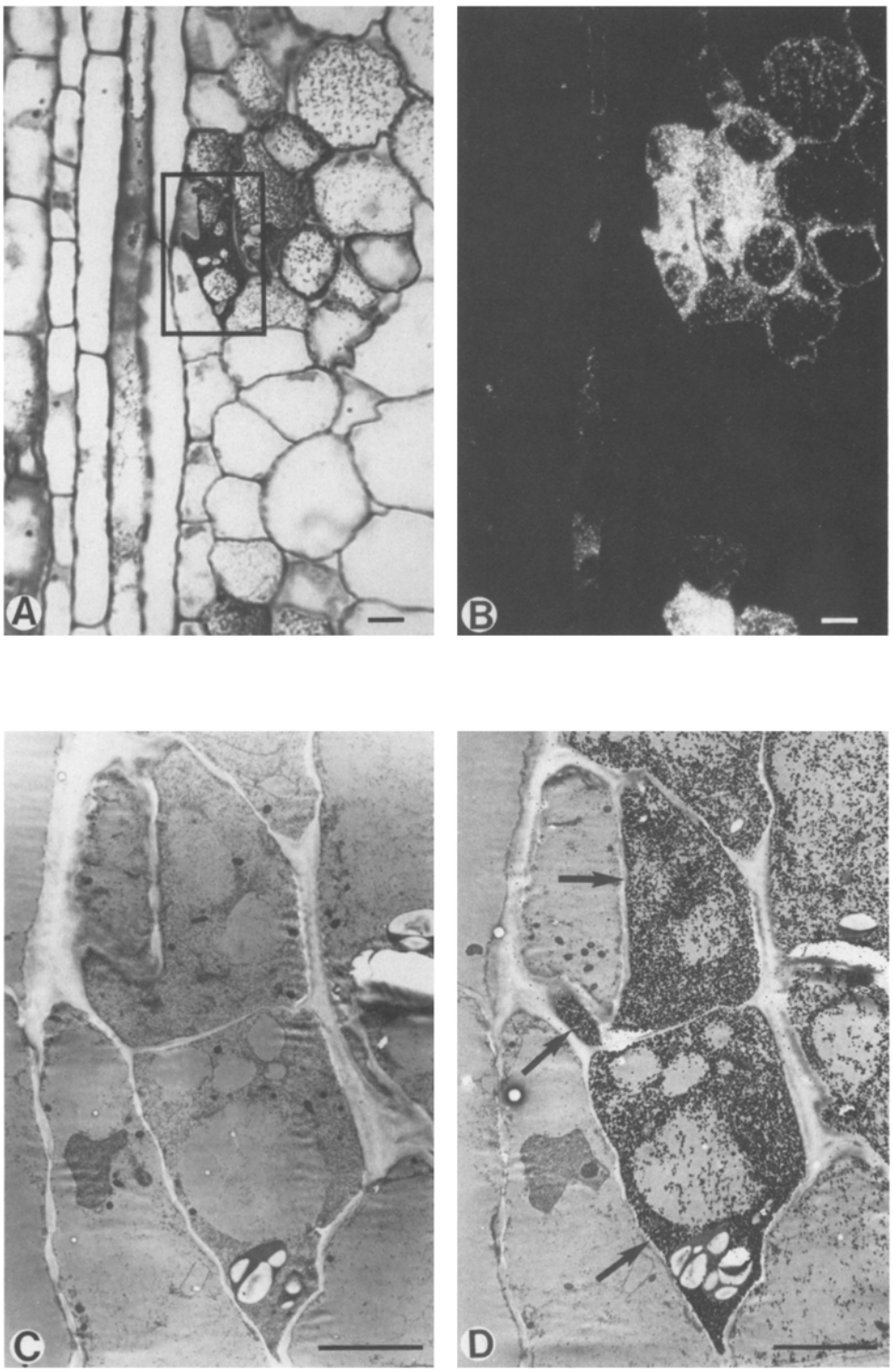
▲ 3



4 ▼






A
B
C
D

convenient to detect low levels of specific labelling even at low magnification. To illustrate the potential of IGSS with epipolarization microscopy CCMV was immunogold-labelled in longitudinal sections of an infected petiolule, fixed, developed and observed with epipolarized light (Fig. 3A and 4A) or with epipolarized light in combination with brightfield transillumination (Fig. 3B and 4B). Petiolules of trifoliate cowpea leaves were collected 6 h (Fig. 3) and 24 h (Fig. 4) after systemic inoculation. Label was not detected in the petiolule at t = 0. First specific label was found in phloem parenchyma cells at 6 h. In time label was observed in higher densities and spread to cells of adjoining tissue. At t = 24 h label was detected in almost all phloem, bundle sheath and cortex cells.

The immunogold-silver staining as demonstrated for light microscopy may be used also in electron microscopy. On one hand it offers the possibility to compare preparations of light (Fig. 5A and B) and electron (Fig. 5C and D) microscopy directly. On the other hand silver enhancement of gold label in electron microscopy allows viewing of label on sections at low magnification (compare Figs. 5C and D, without and with silver enhancement respectively).

Discussion

The immunogold-silver staining (IGSS) descibed by Holgate et al. (1983) and Danscher and Nörgaard (1983) appeared to be a specific and reliable technique for the light microscopical localization of viral antigen in plant tissue. Silver enhancement intensified the gold label by depositing metallic silver around the small 7-nm gold particles. The black silver precipitate became visible in brightfield light microscopy. Under our conditions 14 min of enhancement at 20 °C was sufficient to obtain such a signal.

However, epipolarization microscopy (De Mey, 1983; De Brabander et al., 1985; De Mey et al., 1986) seemed to be the best option for examining the silver stain. With this technique individual silver-coated gold particles were already seen after 2-4 min of enhancement at 20 °C, but optimal staining was obtained after enhancement for 7-8 min. De Brabander et al. (1985) reported that individual gold particles were visible in epipolarization light microscopy when the size of the particles was 20 nm or more. Lackie et al. (1985) demonstrated that the average diameter of 5 nm gold particles increased up to 25, 70 and 127 nm after silver enhancement for resp. 2, 4 and 7 min at 20 °C. These results confirm our observation that the silver precipitate can be observed after 2 min of silver enhancement.

CCMV could be specifically detected with IGSS and we were able to show multiplication and translocation of CCMV in systemically infected cowpea plants. In conclusion, the viral genome was translocated from the primary to the secondary leaves through phloem tissue from where it spread to bundle sheath and cortex cells following the transport route of assimilates.

←

Fig. 5. Serial longitudinal sections of a petiolule at 24 h after systemic inoculation, incubated with anti-CCMV, protein A-gold (C) and subsequently fixed and enhanced with silver for 8 min at 20 °C (A, B) or 4 min at 20 °C (D). Semi-thin sections were cytochemically stained with toluidine blue and viewed in the light microscope with brightfield illumination (A) or epi-illumination (B) and ultrathin sections (C and D) in the electron microscope. Square (A) indicates the group of bundle sheath cells shown in electron micrographs C and D. Arrows (D) indicate the cells with silver precipitate. Bar represents 5 μm.

Light microscopic immunodetection of plant viral antigens has not been used extensively in the study of viral infections in plant tissue. The reason for this might be that available techniques like indirect immunoenzyme and peroxidase-antiperoxidase (PAP) methods are difficult to apply to plant tissue because of low specificity and high background staining. With immunofluorescence (Lei and Agrios, 1986) loss of signal due to quenching and fading of fluorescence is a disadvantage. IGSS appears to be a suitable technique with great potential for the light microscopic localization of viral antigens in plant tissue. The technique is specific and highly sensitive as compared to other immunodetection methods (Holgate et al., 1983; Hacker et al., 1985). The label can be observed with a normal light microscope or even better with a microscope equipped with epi-illuminination optics and a polarizing filterblock. The labelled specimens can be re-examined at will without loosing signal and the labeling can be easily recorded by photographic means. Another advantage of the IGSS method is its application on sections of resin embedded tissue for both light and electron microscopy. We have confirmed the observations of Lackie et al. (1985) that silver enhancement is applicable also in electron microscopy enabling the microscopist to view gold label on sections at low magnification. With IGSS optimal immunogold labelling with small gold particles is combined with subsequent controlled silver enhancement to obtain silver-coated gold particles of a size desired for light or electron microscopy.

Acknowledgement

We thank Hanke Bloksma for the preparation of virus and antisera and J.P.H. van der Want for his constant interest and support. The investigations were supported by the Netherlands Foundation for Fundamental Biological Research, which is subsidised by the Netherlands Organization for the Advancement of Pure Research.

Samenvatting

Toepassing van een immunologische techniek (IGSS) voor het aantonen van viraal antigeen in semi-dunne plakjes planteweefsel met de lichtmicroscoop

De immunologische techniek (IGSS), waarbij complexen van antilichamen met proteïne A geadsorbeerd aan kolloïdaal goud (pAg) worden bedekt met zilver, werd met succes toegepast voor het aantonen van viraal antigeen in geïnfecteerd planteweefsel met behulp van de lichtmicroscoop. Semi-dunne plakjes weefsel werden ingebed in LR White en behandeld met antiserum tegen het 'cowpea chlorotic mottle' virus (CCMV). Aan dit antigeen-antilichaam complex werd pAg gehecht. Vervolgens werd op de individuele gouddeeltjes zilver neergeslagen met een ontwikkelaar bestaande uit een mensel van zilverlactaat en hydroquinone. De gouddeeltjes katalyseren de reductie van de zilverionen in oplossing tot metallisch zilver, dat neerslaat op de gouddeeltjes. Het zilverprecipitaat is waarneembaar als zwarting in een lichtmicroscoop met doorvallend licht en licht wit op bij donkerveld belichting. Maximale gevoeligheid van detectie en lage achtergrondkleuring werden bereikt door fixatie van het antigeen-antilichaam-pAg complex met glutaaraldehyde vóór de zilverkleuring.

Gelijktijdige waarneming van het zilver label en de morfologie van de cellen was mogelijk door toepassing van gepolariseerd licht in een microscoop met opvallende

belichting (epipolarisatiemicroscopie) in combinatie met doorvallend licht. Het zilverprecipitaat is hierbij waarneembaar als een helder blauwe kleur door de weerkaatsing en verstrooiing van het gefiltreerde gepolariseerde licht, terwijl de morfologie van de cytochemisch gekleurde cellen zichtbaar is met doorvallende belichting. Met IGSS in combinatie met epipolarisatiemicroscopie werd het CCMV gelokaliseerd in cowpea planten als functie van de infectieduur. De translocatie en vermenigvuldiging van het virus werden gevolgd in planteweefsel dat systemisch was geïnoculeerd volgens de differentiële-temperatuur-inoculatietechniek. Zes uur na systemische inoculatie werd het virus voor het eerst waargenomen in enkele floeemparenchymcellen en de infectie breidde zich daarna snel uit. Vierentwintig uur na inoculatie kon virus worden aangetoond in grote delen van het floeem, in de bundelschede en in de aangrenzende delen van de schors. Concluderend kan worden gesteld dat het virus vanuit de primaire bladeren naar de secundaire bladeren werd getransporteerd via het floeem, analoog aan het transport van assimilaten.

Tot slot werden dunne plakjes geïnfecteerd weefsel, geïncubeerd met antiserum en proteïne A-goud, na zilverbehandeling vergeleken in licht- en elektronenmicroscoop.

References

Danscher, G., 1981. Localization of gold in biological tissue. A photochemical method for light and electron microscopy. Histochemistry 71: 81-88.

Danscher, G. & Nörgaard, J.O.R., 1983. Light microscopic visualization of colloidal gold on resin-embedded tissue. The Journal of Histochemistry and Cytochemistry 31: 1394-1398.

Dawson, W.O. & SchlegeL, D.E., 1976. Synchronization of cowpea chlorotic mottle virus replication in cowpea leaves. Intervirology 7: 284-291.

De Brabander, M., Geuens, G., Nuydens, R., Moeremans, M. & De Mey, J., 1985. Probing microtubule-dependent intracellular motility with nanometer particle video ultramicroscopy (Nanovid ultramicroscopy). Cytobios 43, 273-283.

De Mey, J., 1983. Colloidal gold probes in immunocytochemistry. In: J.M. Polak and S. van Noorden (Eds), Immunocytochemistry. Practical applications in pathology and biology. Wright. PSG, Bristol, 82-112.

De Mey, J., Hacker, G.W., De Waele, M. & Springall, D.R., 1986. Gold probes in light microscopy. In: J.M. Polak and S. van Noorden (Eds), Immunocytochemistry. Modern methods and applications. 2nd edn., Wright.PSG, Bristol, 71-88.

Geoghegan, W.D., Scillian, J.J. & Ackerman, G.A., 1978. The detection of human B-lymphocytes by both light and electron microscopy utilizing colloidal gold-labelled anti-immunoglobulin. Immunological Communications 7: 1-12.

Hacker, G.W., Springall, D.R., Van Noorden, S., Bishop, A.E., Grimelius, L. & Polak, J.M., 1985. The immunogold-silver staining method. A powerfull tool in histopathology. Virchows Archives [Pathol. Anat.] 406: 449-461.

Holgate, C.S., Jackson, P., Cowen, P.N. & Bird, C.C., 1983. Immunogold-silver staining: New method of immunostaining with enhanced sensitivity. The Journal of Histochemistry and Cytochemistry 31: 938-944.

Huang, W.M., Gibson, S.J., Facer, P., Gu, J. & Polak, J.M., 1983. Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. Histochemistry 77: 275-279.

Lackie, P.M., Hennessy, R.J., Hacker, G.W. & Polak J.M., 1985. Investigation of immunogold-silver staining by electron microscopy. Histochemistry 83: 545-550.

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

Lent, J.W.M. van & Verduin, B.J.M., 1986. Detection of viral protein and particles in thin sections of infected plant tissue using immunogold labelling. In: R.A.C. Jones and L. Torrance (Eds), Developments in applied biology. 1. Developments and applications in virus testing. Association of Applied Biologists, Wellesbourne, U.K., 193-211.

Moeremans, M., Daneels, G., Van Dijck, A., Langanger, G. & De Mey, J., 1984. Sensitive visualization of antigen-antibody reactions in dot and blot immune overlay assays with immunogold and immunogold/silver staining. Journal of Immunological Methods 74: 353-360.

Patterson, S. & Verduin, B.J.M., 1987. Applications of immunogold labelling in animal and plant virology. Archives of Virology, in press.

Roth, J., 1983. The colloidal gold marker system for light and electron microscopic cytochemistry. In: G.R. Bullock and P. Petrusz (Eds), Techniques in immunocytochemistry, vol. II. Academic Press, London, 217-284.