Immunogold silver staining associated with epi-fluorescence for cucumber mosaic virus localisation on semi-thin sections of banana tissues

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The immunogold-silver staining (IGSS) technique in combination with epi-fluorescence detection was used to localise cucumber mosaic virus (CMV) particles within banana infected tissues. For this purpose, tissue samples (2 mm^3) were excised from CMV-infected and highly proliferating meristem cultures of Williams BSJ banana (ITC. 0570, AAA, Cavendish subgroup). These samples were immediately fixed in a 2% paraformaldehyde/0.25% glutaraldehyde mixture, dehydrated in ethanol, and finally embedded in L.R.White resin. Semi-thin sections were cut, mounted on clean treated glass slides and immunostained for CMV particles using gold-labelled secondary antibodies and silver enhancement. Sections were counterstained with basic fuchsin and examined using laser scanning confocal microscopy. Negative controls included immuno-stained samples excised from non-virus infected material as well as infected material on which primary or secondary antibodies were not applied. Images of autofluorescence (in red) and of epi-reflectance of silver-enhanced immunogold particles (in green) were recorded separately and merged, allowing the specific localisation of CMV particles at the cellular level on semi-thin sections of aldehyde-fixed banana tissues. The main advantage of this analytical approach compared to previously published protocols is that it combines a fast staining procedure, stable preparation, a high resolution, and a narrow plane of focus with the flexibility in generation, processing and analysis of images offered by laser scanning confocal microscopy. Finally, the presence of numerous CMV particles within banana meristems constitutes a clear explanation of the very low CMV elimination efficiency when using meristem-tip culture alone.

Key words: Cucumber Mosaic Virus (CMV), immunogold-silver staining, immunolocalisation, Musa.

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Musa production and germplasm exchange is hampered by different viruses including BBTV (Banana Bunchy Top Virus), BSV (Banana Streak Virus), BBrMV (Banana Bract Mosaic Virus) and CMV (Sharman *et al.,* 2000). These viruses are transmitted during traditional vegetative propagation using side-shoots (or suckers) or by modern methods of *in vitro* multiplication. Management of viral diseases in banana (*Musa* spp.) is currently mainly based on the use of virus-free planting material. The CMV pathogen belongs to the genus *Cucumovirus* in the family *Bromoviridae* and causes diseases in more than 800 different plant species, including bananas (Gallitelli, 1998, Yot-Dauthy and Bové, 1966). This virus is transmitted by numerous aphid vectors (Yu *et al.,* 2000) from weeds (Aebig *et al.,* 2005) or other crops such as tomato, melon and pepper. Hence, the availability of virus-free planting material strongly depends on the development of efficient virus eradication techniques.

The eradication rate in *Musa* infected by CMV depends on the performed treatment. Recently, we reported CMV eradication from banana by different *in vitro* techniques such as meristem culture alone or in combination with thermo-, chemo-, electro-, or cryo-therapy (Helliot *et al.,* 2001, 2002, 2003, 2004). A low frequency of CMV eradication was achieved (1% for *in vivo* plants and 7% for *in vitro* plants) following the culture of single meristems. However, eradication frequency increased with more severe treatments. For instance, thermotherapy combined with meristem culture led to an eradication frequency of 38% when meristems were excised from *in vivo* plants and 70% when meristems were excised from *in vitro* plants. Cryotherapy resulted in a CMV eradication frequency reaching up to 30%.

In the context of germplasm preservation and distribution, Faccioli and Marani (1998) advised the

determination of the viral distribution for each individual virus-host combination before starting mass plant production through *in vitro* meristem culture. The authors also recommended direct virus detection in meristem tips to facilitate the choice of the correct explant size, which is presumably virus free. Thus, there is a need to develop a reliable and sensitive technique for virus localisation in plants. In banana, the exact localisation of CMV particles within infected tissues would also allow a better understanding of the mode of action of different virus eradication methods such as meristem-tip culture.

Since the first report of Coons (1941), immunofluorescence has become a standard technique for indirect detection of antigens because of its rapidity, sensitivity and reliability. Its major disadvantages, however, are (a) the requirement of a microscope equipped with fluorescence illumination and filters, (b) the difficulty in observing the morphology of a tissue, (c) the necessity of an aqueous mounting medium (Hacker, 1989) and (d) fluorescence fading during exposure to UV light (Tsien and Waggoner, 1995). Although more time consuming and complex, indirect immunoenzymatic methods leading to permanent preparations were later developed. These preparations, which can be counterstained with conventional procedures, have been increasingly used, particularly with horseradish peroxidase (Nakane and Pierce, 1966) or with alkaline phosphatase (Avraemas, 1972). However, high background staining, resulting from endogenous cell enzyme activity, is often observed (Thorpe and Kerr, 1994). Additionally, the diffusion of reaction products from the source of antigen often reduces resolution because of the possibility of masking the targeted cell structure, thereby compromising the subcellular localisation of the antigen (Spector *et al.,* 1998). Immunogold staining (IGS) (Geoghegan *et al.,* 1978) appears as an interesting alternative given the discrete nature of gold particles and their tight absorption by proteins resulting in high-resolution detection of the antigens (Hacker, 1989). Gold particles are inert and stable (Lackie, 1996). When gold particles accumulate sufficiently at target sites in cells or tissue sections, the red colour of colloidal gold particles is visible in bright-field light microscopy. Although somewhat less sensitive than most of the immunocytochemical techniques, the main advantage of IGS methods is that no revealing procedure is needed (Hacker, 1989). Nevertheless, IGS methods are not widely used in light microscopy because high concentrations of gold particles are needed, making these methods rather expensive (Hacker, 1989). Several attempts have been made to increase the sensitivity of IGS using bright field microscopy. The immunogold-silver staining (IGSS) technique provides a sensitive amplification system by depositing metallic silver around the gold particles (Danscher, 1981), thereby increasing their size and producing a black label that is visible through bright field light microscopy (Holgate *et al.*, 1983, Lackie, 1996). IGSS appears to be a valuable and therefore widely used technique for the localisation of mammalian viruses (Magar *et al.,* 1993). Other advantages include the rapidity of the staining procedure and the fact that hazardous reagents are avoided (Hacker, 1989).

A combination of the IGSS technique with laser scanning confocal microscopy offers a higher resolution and a narrower plane of focus than traditional fluorescence microscopy. Moreover, it provides more flexibility in image generation, processing and analysis (Howard, 2001), and constitutes a powerful analytical tool to localise antigens in sub-cellular compartments.

This paper describes an optimised protocol combining the immunogold silver staining system with epifluorescence using confocal microscopy. This technique allows the localisation of CMV particles in banana infected tissues, detected by epireflectance on silver-enhanced immunogold particles, while the tissue structure is revealed by autofluorescence.

Materials and Methods

Plant materials

Starting material

The dessert banana cultivar Williams BSJ (ITC. 0570, AAA Cavendish subgroup) (Daniells *et al.,* 2001) was provided by the INIBAP Transit Center (ITC, Leuven, Belgium). The CMV isolate (subgroup DTL), originating from Colombia, was mechanically transmitted to the banana plants.

Production of proliferating meristem clumps

In vitro shoots were placed on p4 medium which is the Murashige and Skoog (1962) semi-solid medium supplemented with 100 µM BA, 1 µM IAA and 3 % sucrose. Every 1 to 2 months, the materi-

al was subcultured and only small white clumps of highly proliferating meristems were selected and transferred to fresh medium.

Tissue preparation for immunocytochemistry

Pieces (2 mm^3) of tissue were sampled with a razor blade from CMV-infected highly proliferating meristems of banana. The samples were fixed at 4±1°C for 60 h in a 2% paraformaldehyde (Agar Scientific Ltd.) – 0.25% glutaraldehyde (70%, Agar Scientific Ltd.) solution in a phosphate buffer (0.1 M, pH 7.4). After three washes in the same buffer (3x20 min), samples were dehydrated in 30% and 50% ethanol for one hour each and in 70% ethanol overnight at 4±1°C on a rotamix. The tissue was then infiltrated with mixtures (1:1, 1:2, 2:1) of ethanol 90% / L.R.White resin (Sigma-Aldrich, Inc., USA) for 2 hours each at 4±1°C on a rotamix and then in pure resin for 48 h at 4±1°C. The tissue samples were transferred into individual Beem capsules, completely filled with cold resin, tightly capped with a cover-glass and anaerobically polymerised at 55±1°C for 20 h. Serial 0.3 µm thick semi-thin sections were cut on a Pyramitome® 11800 (LKB Bromma) with glass knives.

Immunogold labelling

The semi-thin sections were mounted on 3-amino-propyl-trietoxisilane (2% in acetone, TESPA, Sigma-Aldrich, Inc., USA) pre-coated Super Frost glass slides. The samples were treated for 1 h at 20°C with 50 µL of 5% commercial milk powder in 0.47 µm-filtered PBS buffer (137 mM NaCl, 1.5 mM Na_2HPO_4, 8 mM KH_2PO_4, 2.7 mM KCl, 0.2 g $NaNO_3$, pH 7.4) to quench aldehyde groups induced during fixation. Sections were carefully dried and incubated overnight at 4±1°C with 50 µL of the primary immunoglobulin G (polyclonal rabbit anti-CMV-IgG, Loewe kit, Biochemica GmbH, Germany) diluted (1/100) in PBS / (1%) commercial milk powder / (0.1%) Tween 20. After incubation with the primary antibody solution, sections were washed 5 times for 5 min each on a drop of PBS / (1%) commercial milk powder / (0.1%) Tween 20. Sections were then incubated for 1 h at room temperature with the conjugated secondary goat anti-rabbit (GAR) immunoglobulin G antibodies (BioCell Research Laboratories, Cardiff, UK), labelled with 5 nm gold particles and diluted (1/100) in PBS / (1%) commercial milk powder / (0.1%) Tween 20. The sections were washed 3 times for 5 min each on a drop of PBS / (1%) commercial milk powder / (0.1%) Tween 20, twice for 5 min on a PBS drop and once in a PBS bath. Antibody complexes were cross-linked for 10 min at 20 ±1°C in 50 µL of 1% glutaraldehyde in PBS buffer to preserve labelling. Subsequently, sections were washed 3 times for 5 min each on a drop of distilled water and once in a water bath. Silver enhancement was performed by incubating sections with 40 µL of silver enhancing solution (BioCell Research Laboratories, Cardiff, UK) for 12 min at 20±1°C in the dark. Sections were then washed 2 times for 5 min each in a gently stirred bath of distilled water. Sections were counterstained for 5 min at 20±1°C with 0.5% of basic fuchsin in distilled water, washed with distilled water, dried carefully, covered with a droplet of Mowiol warmed up to 55°C and protected with a cover-glass (13 mm in diameter, 0.13-0.17 mm in thickness, Assistent).

The two control treatments in this work were assays performed on sections taken from healthy highly proliferating meristems of banana (cv. Williams BSJ, ITC. 0570) and treatments carried out on infected specimens but during which the primary or the secondary antibody incubation steps were omitted.

Light microscopy

For light microscopy observations, sections were collected at 15 serial section intervals on glass slides and stained with toluidine blue. Thin sections were observed with an Olympus AX 70 light microscope.

Confocal microscopy

For confocal microscopy, sections were examined with a Leica TCS NT laser scanning confocal microscope, with excitation and emission wavelengths of 488 and >515 nm respectively, using a RT 30/70 mirror (Bush and McCann, 1999). Images of autofluorescence and epi-reflectance were recorded separately, merged with the Confocal Assistant 4.02 software and processed with Adobe Photoshop 4.0.

Results and Discussion

As shown in Figure 1 (parts 1-d and 1-e), CMV particles could be localised within banana tissues. Structural and ultrastructural studies of highly proliferating meristems in banana (Figure 1-a), previously performed by Helliot *et al.* (2002), showed

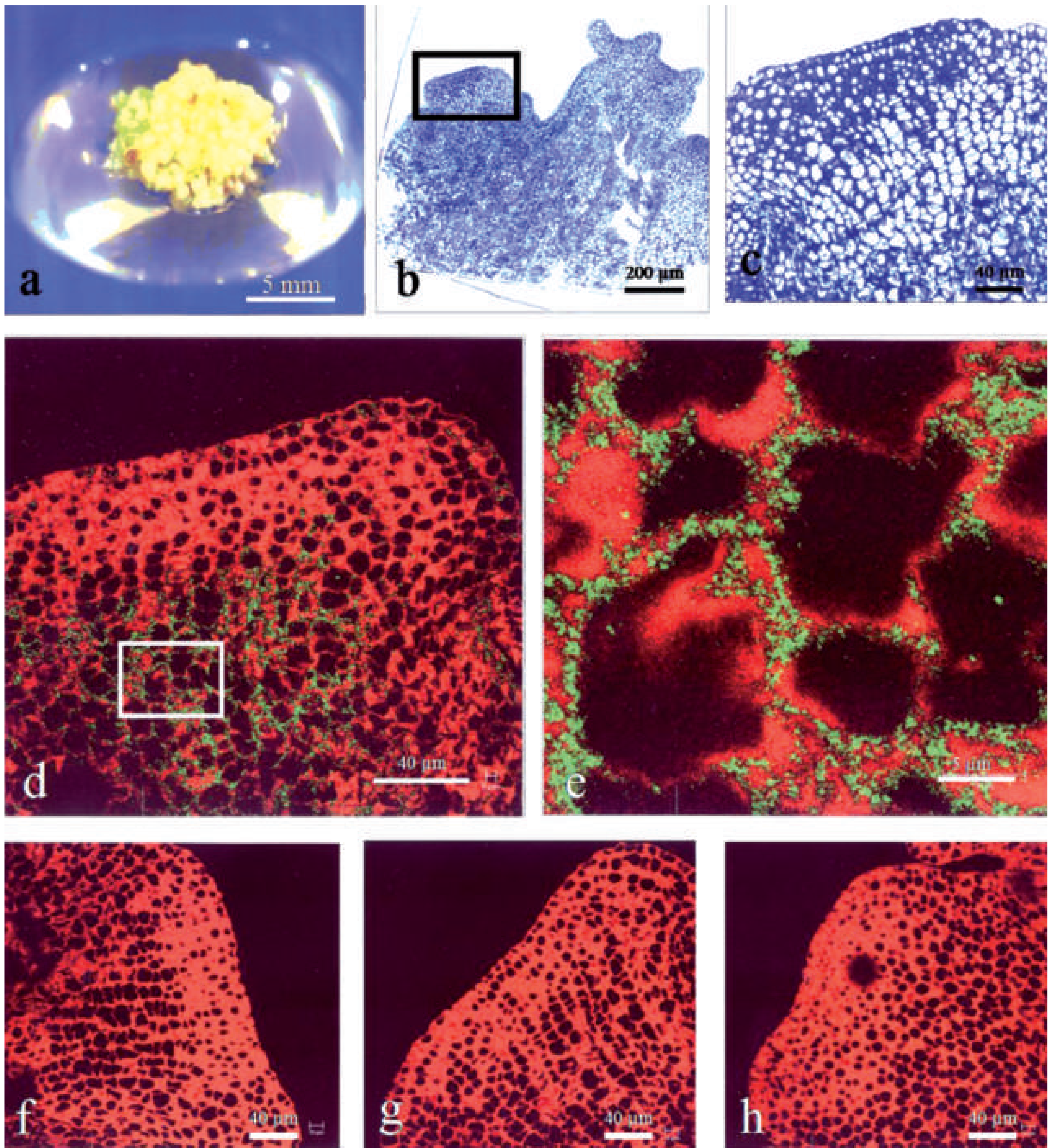

Figure 1. Immunolocalisation of CMV particles within highly proliferating banana meristem sections gold-labeled with commercial anti-CMV primary IgG and commercial GAR (5 nm) secondary IgG. The preparation was silver-enhanced and visualised by confocal microscopy. Part a corresponds to global view of highly proliferating meristems, part b to a section of highly proliferating tissue showing several meristematic domes, part c to a meristematic dome of highly proliferating cells exhibiting the typical cubic shape of meristematic cells, part d to the immunolocalisation of CMV particles in highly proliferating meristems (the autofluorescence of the cell wall appeared in red while the specific labeling appeared in green), part e to the magnification of the area framed in section d , part f to a negative control obtained without the primary antibody incubation step, part g to a negative control obtained without the primary and secondary antibody incubation steps and part h to a healthy plant gold-labeled and silver-enhanced.

that meristematic cells, located on top of a wide and flat apical dome (Banerjee *et al.,* 1986), are characterised by a small size (?15 µm length) and a high nucleo-cytoplasmic ratio typical for actively dividing cells. This ratio decreased progressively with increasing distance from the meristematic dome (Figure 1-b and 1-c). Immunolocalisation carried out on this material revealed the exact localisation and distribution of CMV particles in the meristem and in the primordia. Specifically, strong CMV-labelling was found into the corpus of the infected meristem (Figure 1-d) and in the primordia (*data not shown*) while no or only weak labelling was observed in the tunica of the meristem (Figure 1-d). Higher magnification revealed the presence of virus particles in the cytoplasm (Figure 1-e) pressed between the plasmalemma and the tonoplast surrounding the vacuole as illustrated by the toluidine blue counterstained sections (Figure 1-c). No virus labelling was observed in meristems used as negative controls (Figure 1-f and 1-g) or in meristems excised from healthy plantlets (Figure 1-h). Localisation of CMV within banana meristems constitutes an innovative observation revealing that the concept of meristem immunity towards viruses (Morel, 1948) is not completely true in the banana-CMV pathosystem. The observed distribution of CMV particles in the meristematic dome of banana provides an explanation for the very low efficiency of CMV eradication through banana meristem-tip culture (Helliot *et al.,* 2002).

These findings confirm the putative localisation of CMV particles in infected tobacco mesophyll protoplasts, as previously reported by Otsuki and Takebe (1973). They observed fluorescent masses scattered in the cytoplasm, associated with the plasmalemma and tonoplast. Mori and Hosokawa (1977) used immunofluorescence to localize CMV-antigens in shoot apices of CMV-infected tobacco, tomato, petunia and cucumber. In these samples, viral particles were also located near the apical meristem but neither in the meristematic dome including tunica and corpus, nor in the very small leaf primordia. However, CMV particles were observed in abundance in the larger leaf primordia or near the phloem.

CMV can be detected in plant tissues using different techniques including electron microscopy, serological tests based on the ELISA technology and molecular amplification by RT-PCR (De Breuil *et al.,* 2005; Yu *et al.,* 2005). However these techniques do not allow a precise localization of the viral antigens in plant tissues. In contrast, immunolocalisation techniques allow both the detection of viral particles and their localisation. Several authors have reported on virus immunolocalisation within infected tissues. For example, Wege *et al.* (2001) established the relationship between symptom expression and virus localisation in *Nicotiana benthamiana* tissues infected with different species of bipartite geminiviruses. Immunolocalisation of the tomato mosaic tobamovirus (ToMV) revealed the presence of this virus in the vascular tissues and meristematic cells in seedlings of *Picea rebens* (Bachand and Castello, 2001).

In this paper, we demonstrate for the first time that IGSS combined with confocal microscopy is a powerful tool for CMV localisation in banana plants. Indeed, the main advantage of this analytical approach compared to previously published protocols is that it combines a fast staining procedure, stable preparation, a high resolution, and a narrow plane of focus with the flexibility in generation, processing and analysis of images offered by laser scanning confocal microscopy. Localisation of other important banana viruses such as BBTV, BSV, BBrMV also needs to be undertaken. The approach developed in this paper could provide a new mean of monitoring virus presence and understanding the efficiency of different viral eradication treatments used.

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