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Assembly and accumulation sites of *Maize mosaic virus* (*Rhabdoviridae*) in plant host and insect vector using transmission electron and confocal laser scanning microscopy

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Maize mosaic virus (MMV, *Rhabdoviridae*) infects maize and some other gramineous plants worldwide. The only means of MMV spread between plants in nature is through transmission by its insect (planthopper) vector *Peregrinus maidis* (Delphacidae, Hemiptera). The assembly and accumulation sites of MMV in maize leaf cells and in various tissues of the insect vector were studied using transmission electron microscopy (TEM) of thin sections, and immunofluorescence confocal laser scanning microscopy (iCLSM) of thick sections of plants and insects as well as whole mounts of insect organs. In maize leaf cells, virions of MMV assemble by budding through nuclear or cytoplasmic membranes, and accumulate in perinuclear space or in cytoplasmic cisternae connected to the endoplasmic reticulum. In most tissues of *P. maidis*, MMV virions bud mainly through nuclear membranes and accumulate in perinuclear space. However, in secretory cells of the salivary glands, MMV virions bud through the plasma membrane accumulating in intercellular and extracellular spaces, apparently facilitating release of virions into the salivary ductules and ducts, which is necessary for virus transmission by the vector. Although TEM provides higher resolution to study MMV assembly and accumulation sites at the cellular and subcellular levels, iCLSM allows studying MMV infection at the tissue and organ levels, in much larger and more numerous samples, at a relatively shorter time. The methodology and merits of using these three techniques (thin-sectioning TEM, and iCLSM of thick sections and whole mounts of dissected organs) in localization studies of viruses and other pathogens in their hosts and insect vectors are discussed.

Keywords *Maize mosaic virus*, *Rhabdoviridae*, planthopper, *Peregrinus maidis*, maize, assembly, accumulation sites, transmission, electron microscopy, immunofluorescent labeling, confocal laser scanning microscopy

1. Introduction

Insects are important vectors of human, animal and plant diseases. Sap-feeding insects of the order Hemiptera that includes aphids, whiteflies, leafhoppers and planthoppers transmit more than half of the approximate 800 plant viruses described [1,2]. In addition, they transmit several economically important bacterial pathogens of plants, including the mollicutes spiroplasmas and phytoplasmas, as well as *Xylella fastidiosa* [3,4,5,6].

Interactions of plant pathogens with their insect vectors are quite diverse. Non- and semi-persistently transmitted viruses remain attached to cuticular lining of the stylets and/or foregut, whereas the persistently transmitted viruses and mollicutes cross the insect's gut and salivary glands before they can be transmitted [1]. Luteo- and poleroviruses invade the gut and salivary glands of their insect vectors but apparently do not replicate in them [7], whereas other pathogens, such as the rhabdoviruses, reoviruses

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and mollicutes, replicate in various tissues of their insect vectors, and induce several cytological and other pathological changes [8,9,10]. These propagative plant pathogens are of particular interest as they are capable of invading and replicating in insects and plants, organisms belonging to different kingdoms, and hence may interact with proteins conserved among these organisms.
Additionally, mollicutes and rhabdoviruses infect a broad range of organisms. Within the Class Mollicutes, mycoplasmas are common pathogens of humans and livestock, and three *Spiroplasma* spp. and all phytoplasmas are insect-transmitted plant pathogens that infect several hundreds of plant species including economically important crops [3,4]. Similarly, the family *Rhabdoviridae* includes the human and animal pathogen *Rabies virus*, the insect-transmitted livestock pathogenic vesicular stomatitis viruses and *Bovine ephemeral fever virus*, and an estimated 70 insect-transmitted plant pathogens, many of which infect economically important crops [11,12,13].
In spite of the fact that mollicutes and rhabdoviruses infect a broad range of organisms, insect transmission of these pathogens appears specific, i.e. one or a few related insect species usually transmit one or a few pathogens efficiently. For example, the planthopper *Peregrinus maidis* transmits *Maize mosaic virus* (MMV) but not Maize fine streak virus (MFSV), whereas the leafhopper *Graminella nigrifrons* transmits MFSV but not MMV [14,15]. It remains unclear what determines this specificity. It is clear, however, that the mollicute or rhabdovirus pathogens have to replicate and/or cross several barriers in the insect vector, including epithelial cells of the intestinal tract, the hemolymph or other tissues, and the salivary gland cells, before being translocated into the insect saliva and introduced into plant cells during insect feeding [15,16]. Thus far, the localization of such pathogens in their insect vectors has been primarily investigated with transmission electron microscopy (TEM) of thin sections, sometimes combined with immunogold labeling techniques [17]. These techniques are valuable tools for determining assembly and/or accumulation sites of a pathogen at the cellular and subcellular levels [8,10]. However, because thin sections are used, projects involving TEM are usually targeted to particular tissues or organs of the vector. Additionally, time course studies using TEM are laborious and time consuming, particularly if the transmission rate of the pathogen by the vector is fairly low. As a consequence, for many insect-transmitted plant pathogens, the primary route of infection in their vectors is still largely unknown.
Data presented herein demonstrate the development of immunofluorescence confocal laser scanning microscopy (iCLSM) to investigate the distribution of MMV in maize leaves and in its planthopper vector *P. maidis*. Thick sections of plants and insects, as well as whole mounts of dissected insect organs, were studied with iCLSM, and compared with TEM of thin sections. It is hoped that these methods can be integrated and used in future studies of vector and host relations of various pathogens including viruses and mollicutes.

2. Materials and methods

2.1 Virus acquisition and processing of vector insects and plants for TEM

A Hawaiian isolate of MMV [18] was maintained on sweet corn (cv. Aristogold Bantam Evergreen) through transmission by the insect (planthopper) vector *Peregrinus maidis*. To obtain MMV-infective p1anthoppers, young nymphs of *P. maidis* were fed on MMV-infected plants for 7 days. One week later, these planthoppers were transferred singly to healthy maize seedlings for an infectivity-bioassay test for 3 weeks (1 insect/plant/week). Surviving (adult) planthoppers were anesthetized individually for 2 minutes each with $C0_2$, then carefully dissected under a stereo-microscope, using fine forceps (no.5, Dumont & Fils, Switzerland), in 0.1 M potassium phosphate buffer (pH 7.4). The head, salivary glands, alimentary canal, male and female reproductive organs were dissected out and immediately fixed for 3 h in cold 2.5% glutaraldehyde in the same buffer, postfixed for 3 hrs in cold 1% Os 0_4, dehydrated in ethanol-acetone series, and embedded in Spurr's medium. Throughout these steps, before embedding, dissected organs from each planthopper were kept separately, by placing them (with an i.d. number on paper label) in polyethylene specimen-processing holders, 14 mm wide and 18 mm high, perforated at the bottom

(Electron Microscopy Sciences, Fort Washington, PA, USA) or in (home-made) small, perforated stainless steel baskets [17]. Thus, only organs from planthoppers that later were proven infective in the previously mentioned bioassay tests were sectioned for LM and TEM. This was done because, under similar experimental conditions, MMV transmission rate by *P. maidis* was only 19-35% [19]. Semi-thin sections (1-2 μm thick) were stained with toluidine blue to identify the insect tissues before thin sectioning, whereas ultrathin sections were stained with uranyl acetate and lead citrate, and examined on a Philips 201 or a Hitachi H-7500 electron microscope. Sections from organs of *P. maidis* planthoppers, which were of similar age but never exposed to MMV-infected plants, were examined as controls. Pieces of maize leaves, healthy or infected with MMV, were similarly processed for TEM.

2.2 Preparing vector insects for immunofluorescence confocal laser scanning microscopy (iCLSM)

Young nymphs of *P. maidis* were fed on healthy or MMV-diseased plants for 3 weeks during which they became adults. These planthoppers were starved, 1-2 weeks later, for 2-3 hr (to clear their guts from plant tissue), and anesthetized with CO_2 for 2 min. They were then processed in two different ways for iCLSM:
(A) Some planthoppers were dissected in 0.1 M potassium phosphate buffer (pH 7.4), and their heads (with attached salivary glands), alimentary canal, and male or female reproductive organs, were immediately immersed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 + 0.1% Triton X 100) placed in the specimen processing holders or baskets described above, overnight at 4° C.
(B) Other planthoppers were cut in the above fixative, using a sharp razor blade, into 2 pieces: the head with anterior half of the thorax, and the rest of the thorax with the abdomen that had the posterior tip removed to improve penetration of fixative and other reagents into the specimens. These pieces were immediately fixed as described above.

2.3 Processing of whole mounts of dissected insect organs for iCLSM

Fixed planthopper organs, prepared as described under (A) above, were washed (3x30 min) in PBS-T (phosphate buffered saline, pH 7.4 + 0.1 % Triton X 100), then permeablized overnight (1-3 hrs were tested but apparently were not enough) in PBS containing 0.5% Triton X 100. Organs were then immersed in blocking buffer (PBS-T + 10% normal goat serum) for 30 min at room temperature (RT) and incubated for 2-3 hrs at RT with rabbit antiserum to MMV (Hawaiian isolate), diluted 1/300-1/400 in incubation buffer (PBS-T + 1% normal goat serum). They were washed (5 x 5 min) in PBS-T, incubated for 1 hr at RT with secondary antibodies (goat anti-rabbit IgG labeled with Cy5 or Alexa Fluor 488 (Molecular Probes, OR, USA) diluted 1/600 in incubation buffer. Specimens were then washed in PBS-T (4 x 5 min); those treated with Alexa Fluor 488 were incubated for 5 min with 3 nM solution of the nucleic acid stain propidium iodide (Molecular Probes), before the final wash (4 x 5 min) in PBS- T. Specimens were mounted on microscope slides under cover slips in Gel-Mount (Biomeda, Foster, CA.). Starting with the secondary antibody step, specimens were always protected from light as much as possible to minimize fading/photobleaching. They were examined as soon as possible by epifluorescence or confocal laser scanning microscope (Leica TCS SP). When necessary, however, mounted specimens can be stored up to 2 weeks in the dark at 4° C before examination.

2.4 Processing of paraffin-embedded insects and maize leaves for iCLSM

Fixed pieces of planthoppers, prepared as described under (B) above, and similarly fixed pieces of healthy or MMV-infected maize leaves, were washed (3x 30 min) in 0.1 M phosphate buffer pH 7.4, dehydrated in a gradient of ethanol up to 100% (2 x 30 min), followed by 100% xylene (2 x 30 min), infiltrated with paraffin at 60ºC, then oriented and embedded in 60°C paraffin in Flat Embedding Molds with single tapered ends (Electron Microscopy Sciences, PA, USA). RT-cooled paraffin blocks were

sectioned (6-8 µm thick sections) with glass knives, on a Sorvall Porter-Blum (MT2-B) microtome. Sections, picked up on "Tissue Tack" microscope slides (Polysciences, Inc., PA., USA), were stored at 4°C until further processing to preserve antigenicity. Sections were deparaffinized in xylene, and dehydrated in graded ethanol series and air-dried. Using a PAP pen (EMS, Electron Microscopy Sciences), a circle was made around each group of sections to minimize the quantity of antibodies and other reagents used. Sections were then processed for iCSLM as described above for planthopper organs, except that the permeabilization step was omitted.

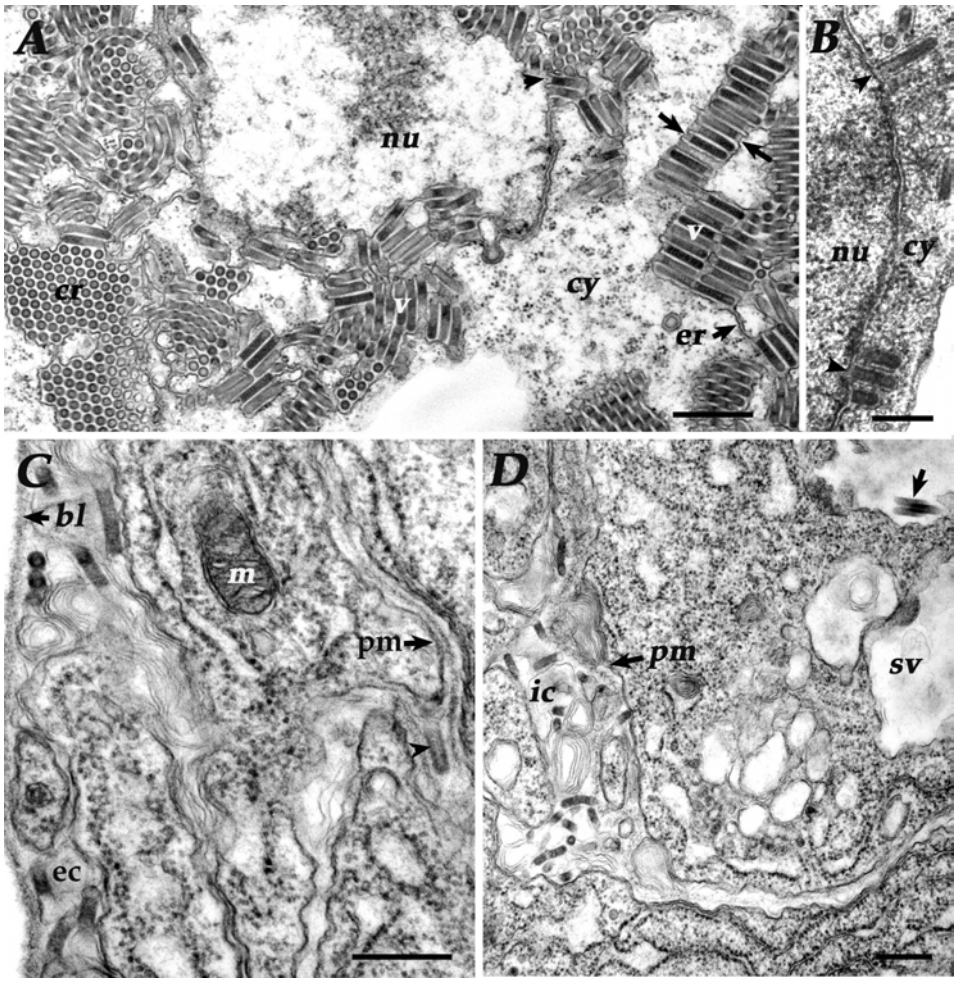

Fig. 1 TEM of *Maize mosaic virus* (MMV) in a maize leaf cell (A) and in cells of the planthopper vector Peregrinus maidis (B-D). A, Virions (v) budding through nuclear (arrowhead) and cytoplasmic (arrows) membranes in a parenchyma leaf cell. B, Virions budding through nuclear membrane (arrowheads) in an epithelial cell of the vector's esophagus. C & D, Virions budding through plasma membrane (arrowhead in C) in secretory cells of the principal salivary gland, accumulating in extracellular (ec) and intercellular (ic) spaces and in secretory vesicles (arrow in D). bl, basal lamina; cr, crystalline virion aggregates; cy, cytoplasm; er, endoplasmic reticulum; m, mitochondrion; nu, nucleus; pm, plasma membrane; sv, secretory vesicle. Scale bars: A, C & D, 500 nm; B, 250 nm.

3. Results

3.1 MMV assembly and accumulation sites in maize leaf cells

Using both TEM of thin sections and iCLSM of thick sections, MMV was found to assemble and accumulate in almost all cell types of infected maize leaves, including the epidermis, mesophyll, vascular parenchyma, and phloem elements (Figs. 1A and 2A). TEM results indicated that MMV virions bud through the inner membrane of the nuclear envelope, and through cytoplasmic membranes connected to the endoplasmic reticulum (Fig. lA), as reported earlier by McDaniel et al. [18]. Virions accumulate in the perinuclear space and in dilated cisternae connected to the outer nuclear membrane or to the endoplasmic reticulum. Frequently, massive accumulations of virions, sometimes in crystalline or paracrystalline arrays (Fig. lA), were found in various tissues of infected leaves (Fig. 2A). No virions or green fluorescence were observed in sections of similarly treated healthy control maize leaves (Fig. 2B). Some cell walls exhibited light green or light red fluorescence that was, however, much weaker than the green fluorescence associated with MMV infection or the red fluorescence associated with propidium iodide-stained nuclei (Figs. 2A and 2B).

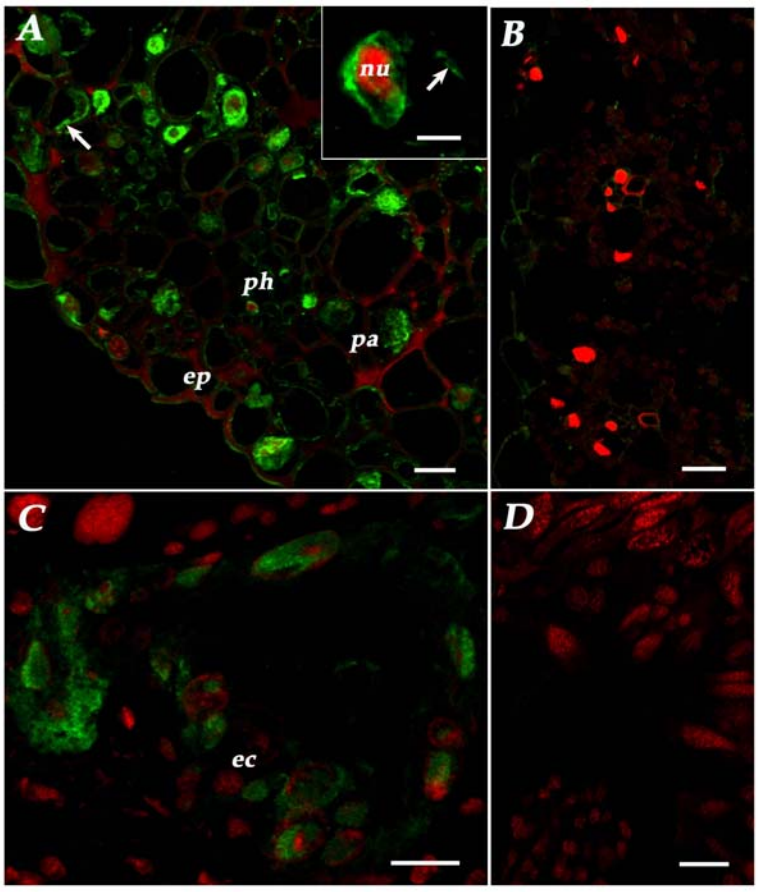

Fig. 2 Confocal images of sections of maize leaves (A & B) or midguts of the planthopper vector (C & D), immunolabeled for MMV with Alexa Fluor (green) and stained for nuclei with propidium iodide (red). A, MMV accumulations, mainly perinuclear but some cytoplasmic (arrows), in the epidermis (ep), parenchyma (pa), and phloem (ph) leaf cells; nu, nucleus. B, Healthy control maize leaf. C, Perinuclear accumulation of MMV in epithelial cells (ec) of the vector's midgut. D, Healthy control midgut. Scale bars: 20 µm (inset in A, 5 µm).

3.2 MMV assembly and accumulation sites in the vector

TEM of thin sections of the planthopper vector *P. maidis*, indicated that MMV buds mainly through nuclear membranes in most of the vector's tissues examined (Fig. 1B), as reported earlier by Ammar and Nault [8]. ICLSM confirmed perinuclear accumulation of MMV in various tissues including the midgut and anterior diverticulum (Figs. 2C and 4C), brain and other nerve ganglia (Fig. 3C), epidermis, muscles and fat tissue (Figs. 3C and 3E), epithelial cells of the male accessory gland (Fig. 4D) and trachea (Fig. 4E). In secretory cells of the principal and accessory salivary glands, in addition to budding through nuclear membranes, MMV virions were found to bud through the plasma membrane, accumulating in intercellular (between cells) and extracellular (peripheral, close to the basal lamina) spaces and in secretory vesicles (Figs. 1C, 1D, 3A, and 4A) as well as in perinuclear areas (Figs. 3A and 4A). No virions or immunofluorescence were detected in thin or thick sections or whole organs of control planthoppers that have not been exposed to MMV (Figs. 2D, 3B, 3D, and 4B). However, plenty of yeast-like organisms (possibly symbionts), the cell walls of which showed light green fluorescence (Fig. 3F), were found mainly in fat tissues of the planthopper vector, whether infected or uninfected with MMV.

In the TEM experiment, all seven planthoppers that proved infective in bioassay tests (23% of 30 insects exposed to MMV) had MMV virions in their tissues. In the iCLSM whole-mount organs experiment, MMV accumulations were detected in 13 out of 45 MMV-exposed insects (29%); whereas with the iCLSM thick sections experiment, MMV accumulations were detected in 5 out of 16 MMV-exposed insects (31%). The number of healthy control planthoppers examined, that have not been exposed to MMV-diseased plants, was 15-16 in each case.

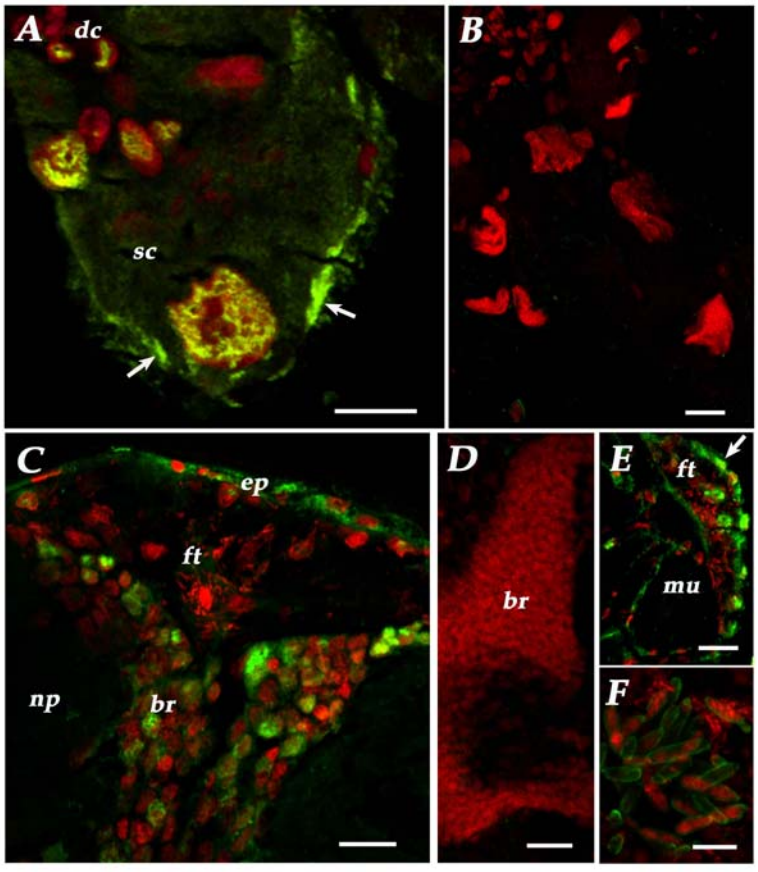

Fig. 3 Confocal images of sections of the planthopper vector, immunolabeled for MMV with Alexa Fluor (green) and stained for nuclei with propidium iodide (red). Peripheral (arrows) and perinuclear accumulations of MMV in secretory cells (sc) and duct cells (dc) of the principal salivary gland. B, Healthy control salivary gland. C, Perinuclear accumulation of MMV in the brain (br) and epidermal (ep) cells. ft, fat tissue; np, neuropile. D, Brain cells from a healthy control insect. E, MMV accumulations in the epidermis (arrow), fat tissue (ft) and muscle (mu) cells. F, Yeast-like symbiotes in fat tissue. Scale bars: A-E, 20 µm; F, 10 µm.

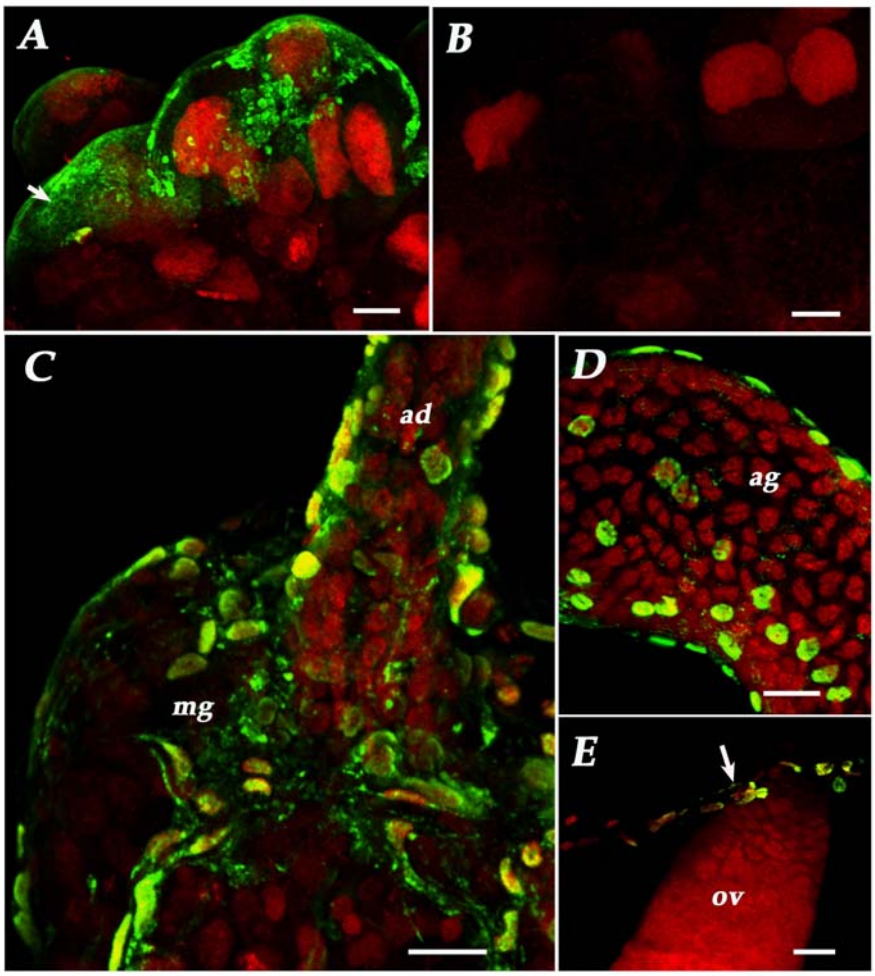

Fig. 4 Confocal images of whole-mount organs of the planthopper vector, immunolabeled for MMV with Alexa Fluor (green) and stained for nuclei with propidium iodide (red). A, Part of the principal salivary gland, showing accumulation of MMV in cell periphery. B, Healthy control salivary gland. C-E, Perinuclear accumulations of MMV in the midgut (mg), anterior diverticulum (ad), epithelial cells of the male accessory gland (ag) and tracheae (arrow in E) close to an ovariole (ov). Scale bars: 20 µm.

4. Discussion

We used iCLSM and TEM to study the distribution of the rhabdovirus MMV in its host plant and in the insect vector *P. maidis*. We developed novel procedures for fluorescent immunolabeling of MMV using either thick sections of paraffin embedded insects and plant leaves, or whole mounts of dissected insect organs. Examination of samples by iCLSM allowed us to positively identify MMV in insect and plant tissues, examine a large number of plant and insect samples for MMV presence, and identify sites of MMV infection at the organ and tissue levels. TEM results, which correspond with those previously reported by McDaniel et al. [18] and Ammar and Nault, [8], allowed the examination of MMV assembly and accumulation sites at the cellular and subcellular levels.

Preliminary results using Cy5 (649/670) as a secondary antibody with iCLSM (data not shown) were similar to those using Alexa Fluor 488 (Figs. 2-4). However, the latter antibody was preferred and used more extensively, because it is visualized more readily on the epifluorescent microscope, and this made it easier and faster to select areas for confocal laser scanning microscopy. Additionally, the green fluo-

rescence of Alexa Fluor 488, was better suited for double labeling with the nucleic acid stain propidium iodide (535/617), because of their more distinct spectra. This helped identify the tissues observed and indicated the perinuclear localization of MMV in plant and insect cells. Similar results to those obtained with iCLSM, but with some loss in resolution, could be obtained by examining the immunolabeled samples on a regular epifluorescent microscope equipped with the appropriate emission and excitation filters. Although TEM provides a much higher resolution for studying viruses and other pathogens in their host plants and insect vectors at the cellular level, iCLSM allows studying much larger and more samples at a relatively shorter time. Indeed, in most TEM studies, only a small fraction of each insect or insect organ is sampled by thin-sectioning, whereas in our iCLSM study most of the insect body was examined either in thick sections or whole-mount organs. Furthermore, each of the (6-8 μm) thick sections examined was approximately 10 times the area and more than 60-80 times as thick as most of the TEM thin sections. Thus, each thick section represents more than 600-800 times the volume of tissue found in a thin section. This is especially important for tracking infection when the distribution of the pathogen is not uniform in various organs or tissues. This advantage becomes even more valuable if the rate of transmission by the vector is fairly low, as is the case with MMV, with infectivity rates of planthoppers between 3.7 and 35.3% [19]. Thus, particularly for TEM studies, the use of ELISA or infectivity bioassay tests, as was done here and previously [8] might be necessary to screen insects before their processing for and examination with TEM.

ICLSM of whole-mount organs is faster and less labor intensive than iCLSM of thick sections of paraffin embedded samples, because the embedding and sectioning steps are eliminated. However, permeability of whole organs to the primary and/or secondary antibodies can be a problem. In our study, even after extensive permeabilization of the dissected insect organs for 24 hr (see Material and Methods), apparently MMV was labeled mainly at the periphery of the salivary glands (Fig. 4A), while it was labeled around most of the nuclei in thick sections of salivary glands (Fig. 3A). Thus, whereas iCLSM of thick sections may be more laborious and time consuming than iCLSM of whole-mount organs, the former might be necessary in some cases to reach deeper cells layers.

In conclusion, each of the methods described here has some advantages and disadvantages in studying pathogen-host and vector relations that can be summarized as follows:

1. TEM of thin sections provides higher resolution for the localization of viruses and mollicutes at the cellular and subcellular levels, but is more laborious and time consuming, thereby allowing examination of a limited number of samples and much smaller tissue pieces.
2. ICLSM of whole mounts of dissected organs is much faster, allowing the study of more samples as well as the examination of infection at the organ and tissue levels. However, permeability of certain organs and tissues can be a problem.
3. ICLSM of thick sections provides some advantages of each of the above two methods, allowing examination of more samples at the tissue level, with better permeability than in processing whole organs.

Finally, a combination of two or three of the above described methods might be necessary for elucidating various aspects of the transmission mechanisms of viruses, mollicutes or other pathogens in their insect vectors and hosts.

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