Localization of viral antigens in leaf protoplasts and plants by immunogold labelling

STELLINGEN

1. De waarnemingen van Plaskitt en medewerkers met betrekking tot de locatie van de TMV-ontmanteling en initiële genexpressie in beschadigde epidermiscellen, rechtvaardigen niet het gebruik van de term "in vivo" in hun conclusies.
   Plaskitt, K.A., Watkins, P.A.C., Sleat, D.E., Gallie, D.R., Shaw, J.G. & Wilson, T.M.A., 1988. Molecular Plant-Microbe Interactions 1, 10-16.

2. Ontmanteling van enkelstrengige RNA-virussen in planten via intracellulaire ribosomen is waarschijnlijker dan via de extracellulaire celwand.
   Wilson, T.M.A., 1985. Journal of general Virology 66, 1201-1207.
   Shaw, J.G., Plaskitt, K.A. & Wilson, T.M.A., 1986. Virology 148, 326-336.
   Gaard, G. & De Zoeten, G.A., 1979. Virology 96, 21-31.
   De Zoeten, G.A. & Gaard, G., 1984. Virus Research 1, 713-725.

3. De conclusie dat "pea enation mosaic virus" zich verspreidt in de plant via cytopathische structuren is voorbarig.
   De Zoeten, G.A. & Gaard, G., 1983. Intervirology 19, 85-94.

4. De conclusie van Piazolla en medewerkers, dat satellietachtige RNA's van "chicory yellow mottle virus" een rol spelen in de ontwikkeling van de ziekte wordt onvoldoende bewezen door hun resultaten.
   Piazolla, P., Vovlas, C. & Rubino, L., 1986. Journal of Phytopathology 115, 124-129.

5. De engelse term "cross-protection" en het in Nederland ingeburgerde cross-protectie zijn verkeerde benamingen voor het verschijnsel dat met de Nederlandse term premunitie wordt aangeduid.
   Fulton, R.W., 1986. Annual Review of Phytopathology 24, 67-81.
   Thung, T.H., 1949. Grondbeginselen der plantenvirologie.

6. Het zoeken naar het startpunt van de replicatie en de signalen voor de assemblage op het genoom van "Autographa californica nuclear polyhedrosis virus" kan beperkt worden tot het gebied tussen de posities 46% en 2,6%.
   Carstens, E.B., 1982. Journal of Virology 43, 809-818.
   Carstens, E.B., 1987. Virology 161, 8-17.

7. Voor een doeltreffende biologische bestrijding van insekten met baculovirusrecombinanten, is de inbouw van een homoloog insekte-gen te verkiezen boven de inbouw van het heteroloog toxine-gen van Bacillus thuringiensis.

8. Het falen van internationale onderzoekscentra, welke zijn opgericht met het doel de landbouw in de derde wereld te verbeteren, is inherent aan de tot nu toe gekozen werkwijze.

9. Voor een succesvolle invoering van het concept van de "zorgzame samenleving" is heropvoeding van oudere generaties in de Nederlandse samenleving noodzakelijk.

10. Een objectievere weergave van de wereldgeschiedenis in het onderwijs is doeltreffender voor het bestrijden van discriminatie en racisme dan elke groots opgezette informatiecampagne van de overheid.

11. Het gastheerbereik van computervirussen beperkt zich niet uitsluitend tot programmatuur.

12. De duur van een redevoering is helaas maar al te vaak omgekeerd evenredig met het retorisch talent van de redenaar.

Stellingen behorend bij het proefschrift:

"Localization of viral antigens in leaf protoplasts and plants by immunogold labelling"

22 april 1988 Jan W.M. van Lent

Jan W.M. van Lent

LOCALIZATION OF VIRAL ANTIGENS IN LEAF PROTOPLASTS AND PLANTS BY IMMUNOGOLD LABELLING

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 22 april 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Promotor: dr.ir. J.P.H. van der Want
emeritus-hoogleraar in de Virologie

Co-promotor: dr.ir. B.J.M. Verduin
universitair hoofddocent bij de vakgroep Virologie

CONTENTS

VOORWOORD

Na voltooiing van dit proefschrift wil ik graag een aantal mensen bedanken voor hun bijdrage en interesse tijdens het onderzoek en het tot stand komen van dit proefschrift.
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GENERAL INTRODUCTION

Infection of a cell by virus is marked by a sequence of events: entry of the viral particle, uncoating, translation and replication of the viral genome, assembly of virus particles and transport. The involvement of both viral and host factors in mechanisms underlying these events has become obvious, but our knowledge of these complex virus-host interactions is still limited.

Early events in plant virus infection (entry and uncoating) have been subject of numerous studies using different experimental systems (Watts *et al.*, 1981; De Zoeten, 1981; Shaw, 1985; Zaitlin and Hull, 1987). Upon inoculation virus probably enters the cell through membrane lesions, because there is no evidence as yet for a virus-specific mechanism. The uncoating of virus may be extracellular (De Zoeten and Gaard, 1984) or at the cytoplasmic ribosomes (co-translational disassembly; Wilson, 1985).

Advanced knowledge of the plant virus genome and *in vitro* and *in vivo* biochemical studies (Dougherty and Hiebert, 1985) have implicated virus-encoded proteins, but also host proteins in virus replication. In this respect, elaborate *in situ* studies of the cytopathology of plant viruses have shown that virus infection is accompanied by cytological changes in the cell (review see Francki *et al.*, 1985), suggesting the involvement of cytopathic structures, in particular membranes, in virus replication and assembly.

Finally, the release of virus for transport within the plant concludes the infection cycle in a cell. Two forms of transport are involved in the spread of virus within plants: long distance transport and short distance (cell-to-cell) transport. It is generally accepted that viruses move in the phloem and follow the route of assimilate transport (Gibbs, 1976; De Zoeten, 1981; Matthews, 1981), although conclusive evidence on this has not yet been presented. The form in which virus is transported is not certain, but may be another 'infective entity' than the complete virus particle (Atabekov and Dorokhov, 1984). Cell-to-cell transport probably involves plasmodesmata through which the 'infective entity' can pass (Gibbs, 1976; Gunning and Overall, 1983) and a virus-encoded 'transport function' may be essential for systemic spread (Atabekov and Dorokhov, 1984; Stussi-Garaud *et al.*, 1987).

Obviously, a major problem in the study of the infection process is constituted by the fact that it is difficult to link findings obtained from *in vitro* studies to phenomena observed *in situ*. Sensitive immunocytochemical techniques enabling the localization of antigens in light and electron microscopy may

partly bridge this gap and provide evidence on the association of cell structures with virus infection.

The aim of this study is to investigate whether specific cell structures are involved in virus multiplication. Cowpea chlorotic mottle virus (CCMV)-infection of cowpea cells was chosen as a model system. Kim 1977 showed the formation of membranous vesicles and amorphous and filamentous inclusions in cells infected with CCMV and suggested their involvement in viral replication. Furthermore, studies on the in vitro dissociation and association processes as imitations of in vivo uncoating and assembly have shown that the specificity of assembly and the encapsidation of nucleic acid in vitro is clearly different from assembly in vivo (Verduin, 1978; Krüse, 1979). It is expected that cell structures, possibly membranes, or compartmentalization play a role not only in the replication but also in the assembly of the virus in vivo.

The investigation was carried out by in situ study of the CCMV-infection of cowpea protoplasts and plants and localization of viral antigen as function of the infection time. Immunogold labelling with conjugates of colloidal gold and protein A (Romano and Romano, 1977) was adapted as a marker system in electron and light microscopy.

CHAPTER 1

REVIEW OF RELEVANT LITERATURE

Cytopathology and replication

Viruses only multiply in living cells and this fact already implies the involvement of host factors in virus infection. In vivo studies of infected plant tissues and individual cells have revealed evidence for the participation of host components in viral infection. Such components may be enzymes or cellular structures. For example for several viruses it was shown that actinomycin D (AMD; inhibitor of DNA-dependent RNA synthesis), when administered early after inoculation, inhibited viral replication (Otsuki et al., 1974; Dawson and Schlegel, 1976; Rottier et al., 1979; Renaudin and Bové, 1984). The inhibitory effect of AMD on viral replication suggested the involvement of a virus-induced but host-encoded component. Goldbach and Van Kammen (1985) speculated that this host-specified component might be an essential factor in the process of viral replication, or that the formation of sites for cowpea mosaic virus (CPMV)-RNA replication requires host-directed protein synthesis.

So far (ultra)structural studies of infected cells have mainly lead to the description of pathological phenomena occurring in the cell upon viral infection, but have failed to provide evidence concerning the function of the structural alterations and cytopathic structures in the infection process. The cytopathic structures as observed in light microscopy are often called inclusion bodies. The diversity of inclusion bodies and cytological alterations in cells infected with different viruses is obvious and has been used for diagnosis of viral diseases (Christie and Edwardson, 1977; Martelli and Russo, 1977; 1984; 1985). Some of the cytological changes relate to the modifications of cell organelles leading, for instance, to alterations in respiration and photosynthesis rates, and contribute to symptom development. Apart from the apparent cytological changes which may be attributed to defense reactions of the plant cell upon infection (Martelli, 1980), it seems likely that at least some of the cytopathic structures are involved in viral replication and assembly of virus particles.

A typical and common cytopathological feature of many plant viruses seems to be the formation of membranous vesicles or membranous bodies. In some cases

evidence for the involvement of such membranes in viral replication has been presented either directly by in situ localization of viral RNA (De Zoeten et al., 1974; Hatta and Francki, 1978; 1981; Russo et al., 1983; Lin and Langenberg, 1985;) or the replicase (Garnier et al., 1986; Saito et al., 1987; Hills et al., 1987a,b), or indirectly by isolation of viral replicase from membranous fractions of cells infected with for example CPMV (Zabel et al., 1974), CCMV (White and Dawson, 1978), turnip yellow mosaic virus (TYMV: Lafl$\grave{e}$che et al., 1972; Mouches et al., 1974), alfalfa mosaic virus (AlMV: Bol et al., 1976), cucumber mosaic virus (CMV: Clark et al., 1974; May et al., 1970), tobacco necrosis virus (TNV: Fraenkel-Conrat, 1976), tobacco mosaic virus (TMV: Ralph et al., 1971), and brome mosaic virus (BMV: Semal et al., 1974). It is, therefore, suggested that membranes are possible intracellular sites of virus replication.

The origin and location of such vesicles or bodies may differ with different viruses. In cells infected with TYMV (tymovirus group) chloroplast membranes develop small peripheral vesicles early in infection (Chalcroft and Matthews, 1966; Ushiyama and Matthews, 1970; Hatta et al., 1973). These vesicles are formed by invagination of the outher chloroplast membrane and contain fibrillar material reminiscent of nucleic acid. Evidence has been provided that the vesicles are the site of RNA replication (Lafl$\grave{e}$che and Bov$\grave{e}$, 1968; Lafl$\grave{e}$che and Bov$\grave{e}$, 1971; Lafl$\grave{e}$che et al., 1972). Recently Garnier et al. (1986) localized the TYMV replicase both near the chloroplast vesicles and scattered throughout the cytoplasm.

CPMV and other viruses of the comovirus group induce large vesiculate inclusions in the cytoplasm, consisting of membrane-bound vesicles. Next to these vesicles, patches of amorphous electron-dense material are observed (Van der Scheer and Groenewegen, 1971; Langenberg and Schroeder, 1975). The membranous vesicles contain fibrils which may represent the double-stranded replicative form RNA (Assink et al., 1973; De Zoeten et al., 1974; Hatta and Francki, 1978), thus implicating the vesicles in viral replication.

With bromovirus infection vesicles have also been implicated in viral replication. In cells infected with broad bean mottle virus (BBMV), virus-dependent RNA-synthesis was located in small areas near the Golgi bodies (De Zoeten and Schlegel, 1967b). These areas may correspond to patches of vesicles which were also observed in BBMV-infected cells (Francki et al., 1985). Similar vesicles containing fibrils, derived from the endoplasmic reticulum (ER), and amorphous electron-dense inclusions have been observed in BMV- and CCMV-infected cells. Seven hours after infection of tobacco protoplasts Burgess et

al. (1974) observed a clumping of the ER and vacuolation of the nuclear membrane. At 15 h after inoculation the first virus particles were observed throughout the cytoplasm. The ER consisted of clumps of distended membranes and circular profiles of small vacuoles bounded by ER. The distended membranes contained vesicles often with a central dot of electron dense material. The vacuoles frequently contained fibrillar material. Similar vacuoles seemed to arise from the nuclear membrane by a blebbing process. Virus was found randomly throughout the cytoplasm and showed no association with any of the other cytoplasmic organelles. In plants of French bean and cowpea Kim (1977) found similar structures as a result of infection with CCMV. Additionally, Kim described the appearance of amorphous inclusions (AI) and filamentous inclusions (FI). AI appeared in the cytoplasm of infected cell as granular masses sandwiched between the ER and the nuclear envelope at early stages of infection. Furthermore, AI appeared prior to the FI which consisted of long, unbranched, flexuous, rod-like structures of 17 nm in diameter. As FI increased in amount, AI decreased. FI were observed in the nucleus often associated with the nucleolus. Kim suggested that the configurations of AI and FI reflected different developmental stages of similar materials. Although the significance of the inclusion bodies is not known, Kim suggested their involvement in the replication of virus particles within the nucleus. However, this may be disputed as Burgess et al. (1974) did not observe any of these inclusion bodies in tobacco protoplasts infected with CCMV. Apparently, the only consistent series of structural changes following infection with CCMV is the proliferation of the ER and the formation of vesicles containing fibrillar material and patches of amorphous electron-dense material.

Characteristic small vesicles associated with the tonoplast were the only consistent cytological changes observed in cells infected with CMV (cucumovirus group). Hatta and Francki (1981) showed that the fibrillary content of these vesicles consisted of double-stranded RNA and suggested that these vesicles may be the sites of viral RNA replication.

TMV-infection (tobamovirus group) is accompanied by a variety of cytological changes depending on the type of virus. Prominent are the formation of membrane-containing X-bodies (Esau and Cronshaw, 1967) and the formation of small vesicles at the tonoplast, similar to cucumovirus infection. Different sites for RNA-replication have been suggested. Betto et al. (1972) presented evidence for the involvement of the chloroplast membrane in TMV-replication. The mitochondrial vesicles observed with other tobamoviruses may also be sites of viral replication (Hatta et al., 1971; Hatta and Ushiyama, 1973). Langenberg

and Schlegel (1967) and Ralph et al. (1971) suggested that the replication of TMV occurred at cytopasmic membranes. Recently, Saito et al. (1987) localized components of the viral replicase in granular inclusion bodies present in the cytoplasm of TMV-infected cells. The authors suggested these inclusion bodies to be the sites of viral replication. Hills et al. (1987) localized TMV-replicase in viroplasms, but also in clusters of virus particles at the edge of these viroplasms. Both the granular inclusion bodies and the viroplasms may be immature forms of the so-called X-bodies, which are inclusion bodies formed in the cytoplasm consisting of membranes, tubules and virus particles (Esau and Cronshaw, 1967). Hills et al. (1987) suggested the edges of the viroplasms to be the possible sites of TMV replication, while the replicase detected inside the viroplasm represents a metabolically inert form of the replicase.

It is obvious that ultrastructural studies of virus-infected cells alone, will not elucidate the mechanisms involved in the viral infection cycle. Ultrastructural studies using conventional electron microscopic techniques do not reveal much information about the nature and function of observed (virus-induced) structures. Already the identification of virus particles, like the small isometric plant viruses, which have an appearance similar to ribosomes, has proven difficult or impossible with conventional electron microscopy. As a consequence, our knowledge of intracellular locations of such small isometric viruses is very limited. In some cases viruses can be located because they occupy locations or assume arrays unlike those of the ribosomes. In other cases investigators have tried to induce virus particles to form crystalline aggregates (Milne, 1967), or dissolved ribosomes to facilitate virus localization (Honda and Matsui, 1974) by subjecting infected tissue to abnormal physiological conditions prior to fixation. However, manipulation of the specimen prior to fixation may induce artefacts and the observations made do not reflect the actual situation and are, therefore, of limited value.

Cytochemical, immunochemical and autoradiographic techniques will reveal more accurate information about the location of viral particles and proteins, about the chemical composition of observed cytological structures and about associations of viral components with such structures. However, there is not much information from these sources about virus-infected plant cells, at least partly because of the presence of impermeable walls. Techniques for the characterization of cellular components have been available, but only incidently applied in plant virology. Kim et al. (1978) used an electron staining method to differentiate between ribonucleoprotein and deoxyribonucleoprotein structures in the nuclei of cells infected with bean golden mosaic virus.

Enzyme staining for intracellular identification and localization of catalase, peroxidase or glycolate oxidase was applied by De Zoeten et al. (1973) and Russo et al. (1983). Hatta and Francki (1978) have developed an enzyme digestion technique for intracellular localization of RNA and were able to distinguish between single-stranded (ss) and double-stranded (ds) RNA by RNAse digestion in the presence of low and high salt concentrations. With this technique ds-RNA was identified in virus-induced vesicles in cells infected with viruses belonging to different taxonomic groups (Hatta and Francki, 1978; 1981; Russo et al., 1983). Enzyme digestion of ribosomal RNA was used to distinguish between isometric virus particles and ribosomes (Hatta and Francki, 1979). However, these cytochemical techniques have not been widely used, as they can be applied only for the identification of a small group of substances and reveal limited information.

Electron microscopic immunocytochemical methods for the localization of antigens after reaction with specific antibodies seems to offer a great potential in the localization of viral structural and non-structural proteins. Antibody-antigen complexes can be observed in free virus preparations (Milne and Luisoni, 1977), but without appropriate markers they cannot be spotted in ultrathin sections. Ferritin as an electron dense marker (Shalla and Amici, 1964) and ^{125}I as a marker for autoradiography (Langenberg and Schlegel, 1967; Schlegel and De Lisle, 1971) have been applied occasionally for the detection of plant viral antigens. The most promising marker for in situ immunocytochemical localization of antigens seems to be colloidal gold particles. The so-called immunogold labelling is briefly discussed in the next section.

Immunogold labelling

An immunocolloid method for the localization of antigens was first reported by Faulk and Taylor (1971) and has since proven to be a very useful marker system in electron microscopic and light microscopic immunocytochemistry (Horisberger, 1979; 1981; De Mey, 1983a; Roth, 1982; 1983; De Mey et al., 1986).

Colloidal gold as marker in immunocytochemistry has several advantages over established markers like ferritin and isotopes (autoradiography). The extreme electron density of colloidal gold particles makes them readily visible in the electron microscope enabling quantification of labelling density. They can be easily prepared in a range of sizes from 5 to 80 nm by the reduction of

chloroauric acid with a suitable agent like trisodium citrate, tannic acid and white phosphorus (Frens, 1973; Mühlpfordt, 1982; Slot and Geuze, 1981; De Mey, 1983b). The different sizes make them suitable for labelling two or even three antigens in the same specimen. Several biologically active proteins (e.g. protein A, immunoglobulins, (strept)avidin, nuclease, enzymes) can be adsorbed to the surface of the gold particles (Roth, 1983) and the markers are therefore applicable in detection systems other than the immunocytochemical system, like for instance in situ hybridization (Hutchison et al., 1982). The same gold markers used for localization of antigens in electron microscopy may be used in light microscopy, with or without enhancement by silver staining (De Mey et al., 1986). Hence, the immunogold marker system is suitable for the localization of antigens at different levels of cytological studies.

Gold markers are most commonly used in post-embedding immunolabelling of antigens. Tissue is fixed, dehydrated and embedded, and ultrathin sections are cut from the specimen. Subsequently, the sections are first incubated on a solution containing excess protein (often bovine serum albumin (BSA)) to block sites for aspecific protein attachment. Then the sections are incubated on the antigen-specific antiserum, washed and further incubated on a suspension of gold particles to which an appropriate protein is adsorbed (e.g. protein A or a secondary antibody). The sections are finally washed and stained. Fig. 1.1 schematically shows a typical procedure used for post-embedding labelling of antigens.

Gold label can be visualized in the light microscope by a simple silver staining method, immunogold-silver staining (IGSS) as described by Holgate et al. (1983). When applying a physical developer containing silver lactate and hydroquinone (Danscher, 1981), the gold catalyzes the reduction of silver ions to metallic silver. This metallic silver precipitates around the gold particles and is visible in brightfield light microscopy as a black silver precipitate. Alternatively, the silver can be observed with polarized light in an epipolarization microscope as a bright blue-coloured signal (De Mey, 1986).

An essential requirement for the detection of antigens in thin sections of biological tissue is the retention of antigenic properties of the polypeptides to be detected, and at the same time a satisfying preservation of cellular structures. Conventional fixation, dehydration and embedding schedules including osmium tetroxide and uranyl acetate fixation and apolar embedding media like methacrylate, spurr or epon, result in good structural preservation. However, antigenic properties of proteins are often partially or completely lost with these procedures. Especially post-fixation with osmium tetroxide

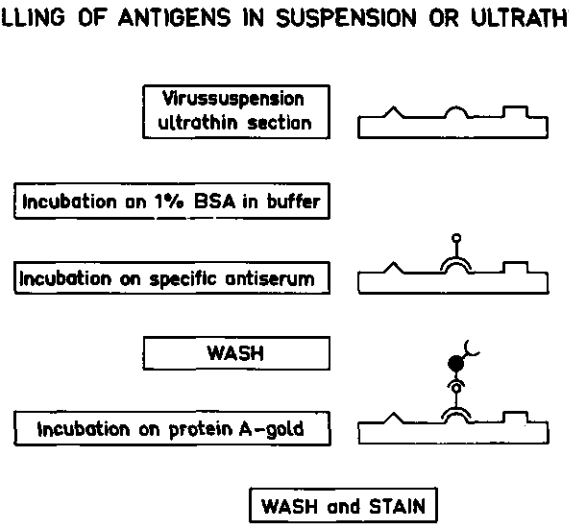


Fig. 1.1. Schematic representation of the immunogold labelling of antigens in preparations of virus suspensions or in thin sections of biological material (post-embedding immunogold labelling). The specimens are first incubated with a buffer containing bovine serum albumin (BSA) to block possible sites for aspecific protein attachment. Subsequent incubation with antigen-specific immunoglobulins results in the formation of antigen-antibody complexes. Finally, protein A-gold particles are bound to these complexes. The electron-dense gold particles can easily be traced in the electron microscope, where they appear as black dots.

severely decreases the antigenicity of many proteins, reducing the intensity of labelling by 60-80% (Roth, 1982; Bendayan and Zollinger, 1983). Part of the success of the immunogold marker system is undoubtedly due to the simultaneous development and/or improvement of alternative methods for fixation, dehydration and embedding of tissues. Cryo-fixation, cryosectioning (Tokuyasu, 1980; Slot and Geuze, 1983) and recently developed polar embedding resins like Lowicryl K4M (Roth et al., 1981b; Carlemalm et al., 1982) and London Resin white and gold (LR white; LR gold), have improved preservation of cellular structures and retention of antigenicity. Low temperature embedding after only a mild fixation with aldehydes, enabled the in situ immunodetection on thin sections with light- and electron microscopy.

The described characteristics of immunogold labelling and new developments in the preparation of tissue specimens for electron microscopy have lead by now to an extensive use of the gold marker system in animal and plant virology (for a recent review see Patterson and Verduin, 1987). For the first time a reasonably easy and reliable technique is available for the detection of plant viral antigens. Using specific antisera against virus particles Lin and Langenberg (1982; 1983; 1984a; 1984b) were the first to report on the electron and light microscopic localization of barley stripe mosaic virus (BSMV) in thin sections of infected wheat plants. Tomenius et al. (1983) localized the spherical comovirus, red clover mottle virus (RCMV), in the cytoplasm of systemically infected pea cells. Using antisera against polyinosinic:polycytidylic acid Lin and Langenberg (1985) were able to detect ds-RNA in vesicles occurring in proplastids of BSMV-infected wheat cells.

Most promising in the in situ study of plant viral infection is undoubtedly the localization of viral non-structural proteins involved in replication (Garnier et al., 1986; Saito et al., 1987; Hills et al., 1987a,1987b) and transport of viruses (Stussi-Garaud et al., 1987; Tomenius et al., 1987). The nucleotide sequencing of plant viral genomes has allowed selection of specific amino acid sequences which can then be synthesized in vitro and used as an antigen for the production of antiserum (Stussi-Garaud et al., 1987; Tomenius et al., 1987). Alternatively, plant viral genes coding for specific proteins can be manipulated by recombinant DNA techniques to obtain expressed proteins as antigens. Saito et al. (1986) prepared antisera against the 130K and 180K TMV-proteins (putative replicase components), using fusion proteins expressed in Eschericia coli as antigens. Immunogold labelling on thin sections of TMV-infected cells, using these antisera, showed the localization of the proteins in granular inclusion bodies present in the cytoplasm (Saito et al., 1987). The authors suggested these inclusion bodies to be the sites of viral replication.

Immunogold labelling as a method for in situ localization of structural and non-structural viral proteins seems to be the first (immuno)cytochemical technique with potential for routine application in the light and electron microscopical study of plant virus-infected cells. Due to its simplicity and flexibility there seems to be little limitation for the improvement of existing assays and the development of new ones. Undoubtedly the technique will contribute in the future to a better understanding of the mechanisms involved in the viral infection process.

Cowpea chlorotic mottle virus

Cowpea chlorotic mottle virus (CCMV) is a small spherical plant virus belonging to the bromovirus group. This virus group comprises three definite members: the type member BMV, CCMV and BBMV. The properties of these viruses are similar and have been extensively reviewed (Lane, 1974; 1979; Bancroft, 1970; Bancroft and Horne, 1977). The coat proteins of the three viruses are serologically more closely related than native virus. Antisera prepared against the viruses react with both native virus and coat protein preparations, but also with formalinized virus and coat protein preparations (Von Wechmar and Van Regenmortel, 1968; Rybicki and Von Wechmar, 1981).

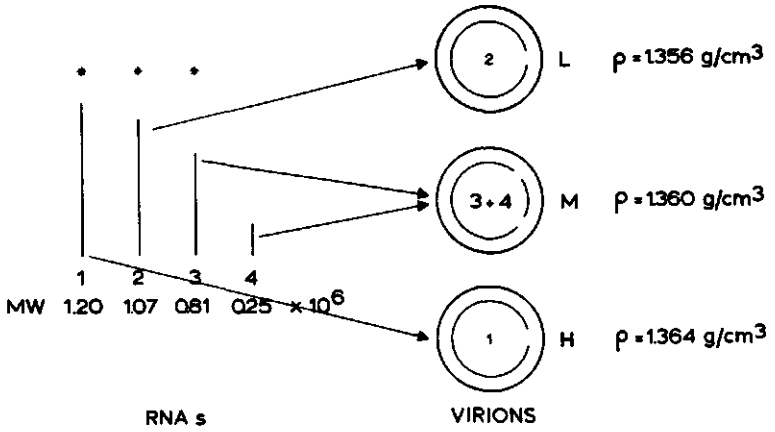


Fig. 1.2. Schematic representation of the distribution of CCMV RNA molecules over virions of different buoyant density. The RNAs marked with an asteriks constitute the genome of the virus, and RNA-4 arises during infection from RNA-3 and encodes for the coat protein. Three types of nucleoprotein particles are present, called light (L), medium dense (M) and heavy (H) particles according to their density (ρ) in CsCl.

CCMV consists of three types of nucleoprotein particles, composed of RNA which is encapsidated in a coat of 180 identical protein subunits with M_r 19,400. Natural isolates of the virus do not contain empty protein shells (Lane, 1974). Four species of single-stranded (ss), positive-sense RNA molecules (RNA-1, -2, -3, and -4) with respective molecular masses of 1.20, 1.07, 0.81 and 0.25 x 10^3 (Reijnders *et al.*, 1974) are encapsidated to form three virus particles. RNA-1 and RNA-2 are encapsidated separately and RNA-3 and

RNA-4 are packed together in polyhedral particles of about 26 nm in diameter. On the basis of their buoyant density, the three nucleoprotein particles are denoted as heavy (H; RNA-1), medium dense (M; RNA-3 and -4) and light (L; RNA-2) particles (Fig. 1.2) (Bancroft and Flack, 1972). Both RNA-1 and RNA-2 code for polypeptides with an estimated molecular mass of 105,000 kDa. RNA-3 codes for a 32,000 M_r product. RNA-4 is a subgenomic messenger RNA, derived from RNA-3, for the 19,400 M_r capsid protein (Davies and Verduin, 1979). Similar products are detected in CCMV-infected tobacco protoplasts (Sakai *et al.*, 1977). For successful infection of plants with CCMV, all three nucleoprotein particles are required or, when inoculating with extracted RNA, infection is obtained with a mixture of RNAs-1, -2 and -3. The function of the RNA-1, -2 and 3 products in CCMV replication is not known. However, in similarity to BMV it can be suggested that the polypeptides coded for by the two largest RNA molecules (1 and 2) are involved in viral replication (Kiberstis *et al.*, 1981) and may represent the viral component of the replicase complex.

The *in vitro* dissociation and association processes of CCMV as a model for *in vivo* uncoating and assembly respectively have been extensively reviewed and studied by Verduin (1978b). In this study it was found that the specificity of assembly and the encapsidation of nucleic acid *in vitro* is different from the assembly *in vivo* in that RNA-3 and -4 are not consequently encapsidated in the same particle. Furthermore, RNA molecules other than those which comprise the CCMV genome may be encapsidated *in vitro* (Bancroft *et al.*, 1969; Adolph and Butler, 1977), while such phenomena are not recorded *in vivo*. An explanation for the discrepancy between *in vitro* and *in vivo* assembly of virus particles may be found in an involvement of host-specified factors (e.g. membranes) in the regulation of the assembly process.

CHAPTER 2

SPECIFIC GOLD-LABELLING OF ANTIBODIES BOUND TO PLANT VIRUSES IN MIXED SUSPENSIONS

SUMMARY

Protein A-gold complexes with gold particle diameters of 7 and 16 nm were prepared and could be stored at 4°C for at least 5 months without loosing activity. The complexes were used to detect antibodies bound to two plant viruses in mixed suspensions. Depending on the antibodies used, each virus could be labelled specifically with protein A-gold complexes with a gold particle diameter of either 7 nm or 16 nm.

A double-labelling technique was developed by which the viruses in suspension could be labelled specifically with protein A-gold complexes with gold particle diameters of either of the two sizes mentioned. Using this technique it was possible to distinguish and identify two viruses with a similar spherical appearance in the electron microscope in mixed preparations.

INTRODUCTION

Gold labelling of virus particles in purified suspensions and extracts of infected plant tissue has shown to be a useful technique in addition to other immuno-electron microscopical techniques as described by Derrick (1973) and Milne and Luisoni (1975). Labelling virus particles with protein A-gold (pAg) (Pares and Whitecross, 1982) and gammaglobulin (IgG)-gold (Lin, 1984) complexes for positive identification appeared to have advantages over the decoration technique described by Milne and Luisoni (1975). The pAg technique makes use of the two binding properties of IgG. Each IgG molecule consists of one region (F_c-fragment), which binds to protein A and two other identical regions ($F(ab')_2$-fragments), which bind to the antigen. Pares and Whitecross (1982) described the gold-labelled antibody decoration (GLAD) with protein A-gold complexes using different strains of tobacco mosaic virus as antigens. With this serological technique distantly related viruses could be distinguished by quantitative analysis of the number of adsorbed gold particles, but such an analysis failed to distinguish between closely related viruses. Lin (1984)

showed the merit of IgG-gold complexes for the rapid detection and identification of viruses in leaf-dip preparations of infected plant tissue. A combination of the immunosorbent electron microscopical (ISEM) technique, by which viruses in suspension are trapped on grids precoated with antibodies (Derrick, 1973) and subsequent gold labelling resulted in an increased background labelling. Louro and Lesemann (1984) showed that this aspecific adsorption of protein A-gold complexes to precoated grids could be eliminated almost entirely by using specific antibodies from which the F_c fragment had been removed, leaving the $F(ab')_2$ fragments intact. This technique combines the sensitivity of the ISEM technique and the discrimination of the GLAD technique. In the present study we investigated the possibility of labelling two different antigens in mixed preparations with protein A-gold complexes consisting of gold particles with different diameters.

MATERIAL AND METHODS

Viruses. Cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (SBMV), cowpea strain, were maintained in cowpea (Vigna unguiculata cv. Blackeye Early Ramshorn). Tobacco mosaic virus (Wageningen strain) was maintained in Nicotiana tabacum var. White Burley.

CCMV was purified as described by Verduin, 1978a. Essentially the same purification protocol was used for SBMV but the buffers differed. As homogenization buffer 0.25 M citrate buffer (pH 4.8) containing 0.02 M $MgCl_2$ and 5 mM disodium EDTA was used. Pellets were resuspended in 0.2 M citrate buffer (pH 5.5) containing 0.02 M $MgCl_2$, 0.001 M disodium EDTA and 0.001 M sodium azide. TMV was purified according to procedure 5 as described in Noordam (1973). Mixed suspensions of CCMV and TMV or CCMV and SBMV were prepared in 0.1 M sodium acetate buffer (pH 5.0). Each virus was used in a final concentration of 0.01 mg/ml.

Antisera. Antibodies against CCMV (anti-CCMV), SBMV (anti-SBMV) and TMV (anti-TMV) were elicited in rabbits by one intravenal injection of 1 mg of purified virus followed by two intramuscular injections of 2 mg purified virus in Freund's complete adjuvant with a two-week interval. In double diffusion tests titres of 1/32, 1/512 and 1/64 for CCMV, SBMV and TMV respectively were reached against virus at 1 mg/ml 8 weeks after the initial injection. Antisera of the three viruses were purified by passing the whole serum through a column of

Affigel Blue (1 ml serum per 4.7 ml Affigel Blue; Biorad). Gammaglobulin was then eluted with 0.02 M Tris/HCl buffer (pH 8.0) containing 0.028 M NaCl and 3 mM NaN_3. The obtained purified gammaglobulin (IgG) suspensions were diluted in 0.1 M sodium acetate buffer (pH 5.0) containing 1% (w/v) bovine serum albumin (BSA).

Preparation of 7 nm and 16 nm gold particles. Colloidal gold was prepared by reduction of hydrochloroauric acid ($HAuCl_4$) with sodium citrate (Frens, 1973; Slot and Geuze, 1981) or a mixture of tannic acid and sodium citrate (Mühlpfordt, 1982), resulting in suspensions of colloidal gold particles with respective average diameters of 16 and 7 nm. For the production of the colloidal gold suspensions, glassware was cleaned extremely well by boiling in distilled water. Solutions were made in freshly prepared distilled water and filtered through 0.2 µm nitrocellulose filters prior to use. Colloidal gold particles with a diameter of 16 nm were prepared as follows: 247.5 ml of distilled water was boiled in a clean flask under reflux and 2.5 ml of a 1% (w/v) $HAuCl_4$ solution was added. Then 7.5 ml of 1% (w/v) tri-sodium citrate was quickly added to the boiling $HAuCl_4$ solution while stirring. The mixture was boiled for 15-30 min under reflux.

Colloidal gold particles with a diameter of 7 nm were prepared by adding a mixture of 2 ml 1% (w/v) tri-sodium citrate and 0.45 ml 1% (w/v) tannic acid quickly to 100 ml of a boiling 0.01% (w/v) $HAuCl_4$ solution. The mixture was then boiled for another 15 min under reflux. The colloidal gold suspensions were allowed to cool, divided in 30 ml portions and stored at 4°C until use. To determine the diameters of the gold particles in the suspensions thus prepared, the gold particles were allowed to adsorb to grids covered with formvar/carbon and previously incubated for 10 min on a solution of 0.01% (w/v) poly-L-lysin ($\underline{M}_r$ 70,000). Diameters of gold particles were measured from micrographs taken of each suspension.

Preparation of the protein A-gold complexes. Lyophilized protein A (pA) was dissolved in distilled water to a concentration of 2 mg/ml. The colloidal gold suspensions were, if necessary, adjusted to a pH between 5 and 6 by adding drops of 0.1 M K_2CO_3 in double distilled water. The optimal amount of pA necessary to stabilize the colloidal gold suspensions was determined by adding increasing amounts of pA to samples of the gold suspensions as described by Horisberger and Rosset (1977). When colloidal gold was completely stabilized by pA, the color of the suspension remained red after addition of NaCl. The

minimum amounts of pA necessary to stabilize 1 ml of the suspensions of 7 nm and 16 nm particles were 10 and 5 μg ml/ml, respectively.

The appropriate amount of pA, plus 10% excess, was added to 30 ml of gold suspension and the mixture was stirred for 5 minutes. Then 0.3 ml of 5% (w/v) polyethylene-glycol (M_r 20,000) was added. The suspension was centrifuged (16 nm gold: 1 h at 15,000 *g*; 7 nm gold: 45 min at 104,000 *g*; all *g* values are given at R_{av} and all centrifugations were done at 4°C) and the supernatant removed. The loose pellet containing the pAg complexes was resuspended in the residual supernatant and left for 2 h at 4°C. The concentrated suspension was diluted in phosphate buffered saline (PBS: 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot H_2O$, 2.7 mM KCl, 3.1 mM NaN_3), pH 7.4 and centrifuged for 15 min at 250 *g*. Then the supernatant was again centrifuged at high speed, the resulting supernatant carefully removed and the loose pellet of pAg particles was resuspended in PBS containing 1% (w/v) BSA (PBS-BSA). The absorption at 520 nm was determined and the pAg suspension was diluted to an A_{520} = 5 in PBS-BSA containing 3mM sodium azide. Protein A-gold suspensions were stored at 4°C.

Single and double gold labelling of virus. Preparations were made on 150 mesh nickel grids covered with formvar and coated with a *c*. 3 nm carbon layer. Prior to use the grids were exposed to a glow discharge in air for 10 s. For single gold labelling, grids were incubated on drops of a mixed virus suspension for 10 min, followed by an incubation for 10 min on 1% (w/v) BSA in 0.1 M sodium acetate (pH 5.0) and 10 min on 0.01 mg IgG/ml. The grids were then washed with 30 drops of 0.1 M sodium acetate buffer (pH 5.0). Grids were further incubated for 10 min on a suspension of 7 nm pAg (pAg-7) particles diluted to A_{520} = 0.5 in 1% (w/v) BSA in 0.1 M sodium acetate buffer (pH 5.0). Grids were washed with 30 drops of buffer, 10 drops of distilled water and stained with 2% (w/v) uranyl acetate in water.

For double gold labelling, grids were treated as described above. After incubation on pAg-7 the grids were washed with 30 drops of buffer and incubated on the second antibody suspension for 10 min, washed with 30 drops of buffer and incubated for 10 min on pAg-16 (A_{520} = 0.5). Finally the grids were washed with 30 drops of buffer, 10 drops of water and stained with uranyl acetate. All incubations were at room temperature. Preparations were observed with a Siemens Elmiskop 101 or a Zeiss EM109 electron microscope.

RESULTS

Colloidal gold suspensions prepared by reduction of chloroauric acid with sodium citrate or tannic acid/citrate using the described methods, repeatedly yielded suspensions containing gold particles with average diameters of 16 nm and 7 nm, respectively. The distribution of particle diameters in suspensions is shown in Fig. 2.1A (7 nm particles) and Fig. 2.1B (16 nm particles). As there was no overlap of particle diameters between the 7 nm and 16 nm suspensions, no attempt was made to isolate fractions of more homodisperse particles by centrifugation of pAg complexes on glycerol gradients (as described by Slot and Geuze, 1981). Seven nm and 16 nm gold suspensions stabilized with pA were used in single- and double-labelling experiments.

The pAg complexes remained sufficiently active over a period of at least 3-5 months when kept at 4°C. Labelling of CCMV-particles with pAg-7 gave an average binding of seven gold particles to one virus particle and this number did not decrease over the period in which the pAg-complex was stored. By contrast, labelling with pAg-16 resulted in an average binding of three gold particles per virus particle. Both single- and double-labelling were tested on a mixture of TMV and CCMV. Preparations of this mixture were adsorbed to grids, incubated on specific antibody solutions against TMV or CCMV followed by incubation with pAg-7. The results of these experiments are shown in Figs 2.2A and

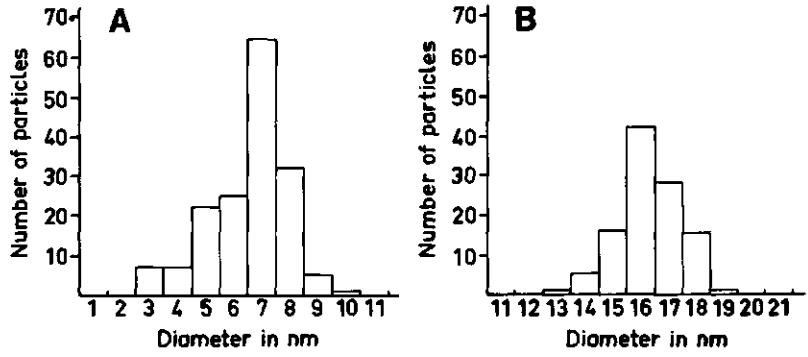


Fig. 2.1. Histograms illustrating the diameters of colloidal gold particles prepared by the reduction of $HAuCl_4$ with a mixture of tannic acid and sodium citrate (A) and with sodium citrate alone (B) .

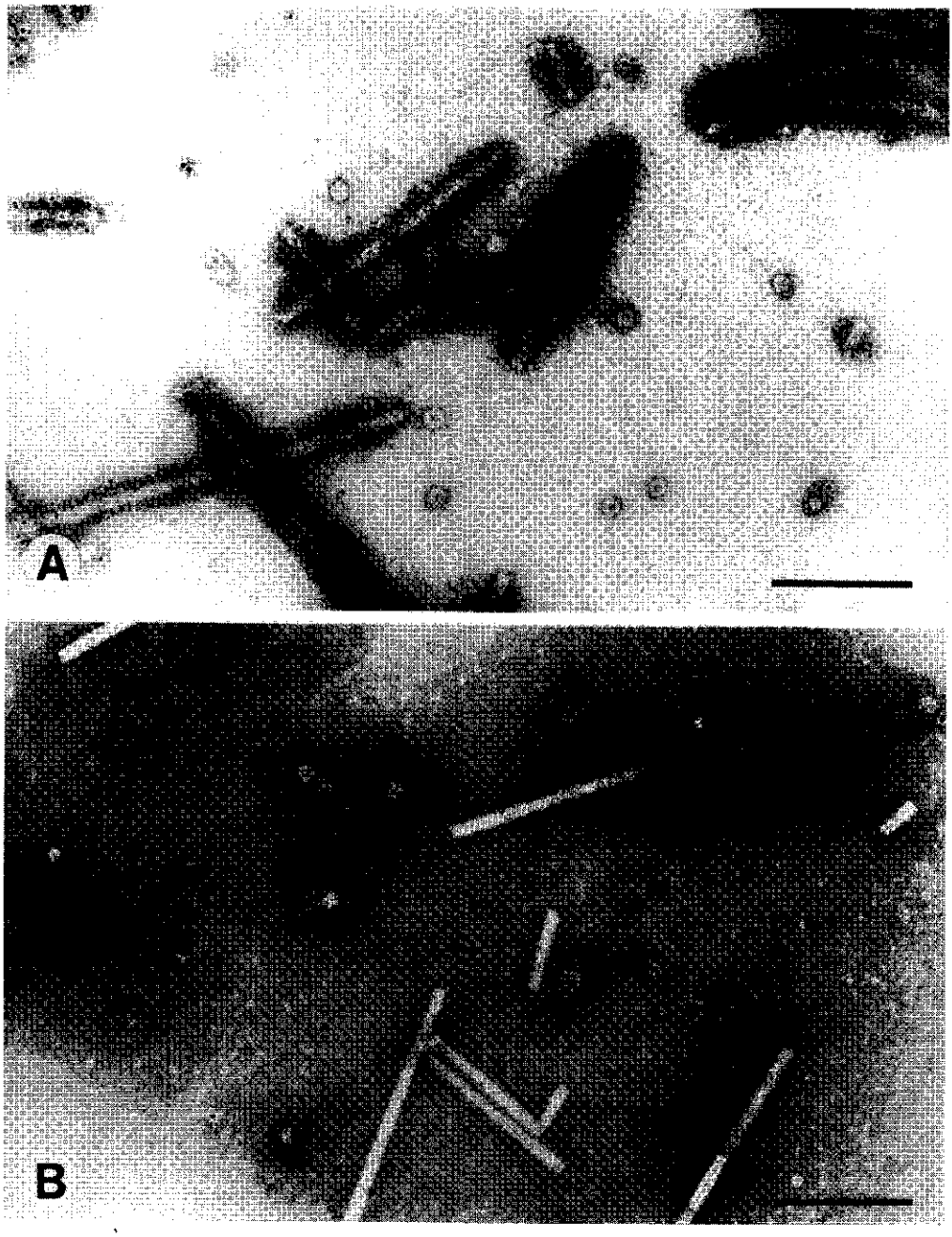


Fig. 2.2. Specific labelling of TMV (A) and CCMV (B) with protein A-gold complexes with a gold particle diameter of 7 nm (bar represents 200 nm).

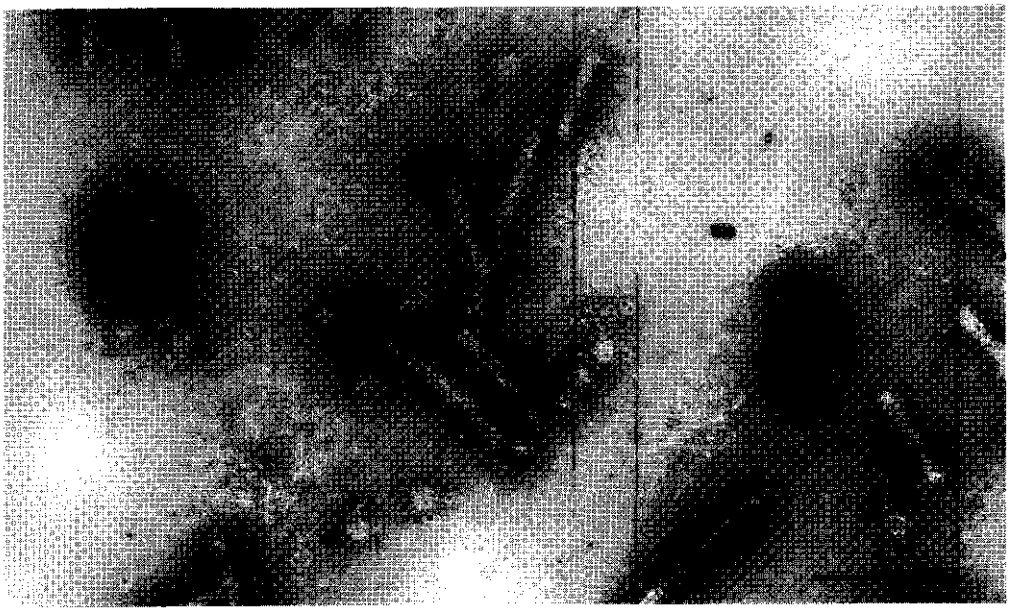


Fig. 2.3. Double-labelling of two viruses in suspension. TMV is specifically labelled with protein A-gold complexes with a gold particle diameter of 16 nm and CCMV with particles of 7 nm in diameter (bar represents 200 nm).

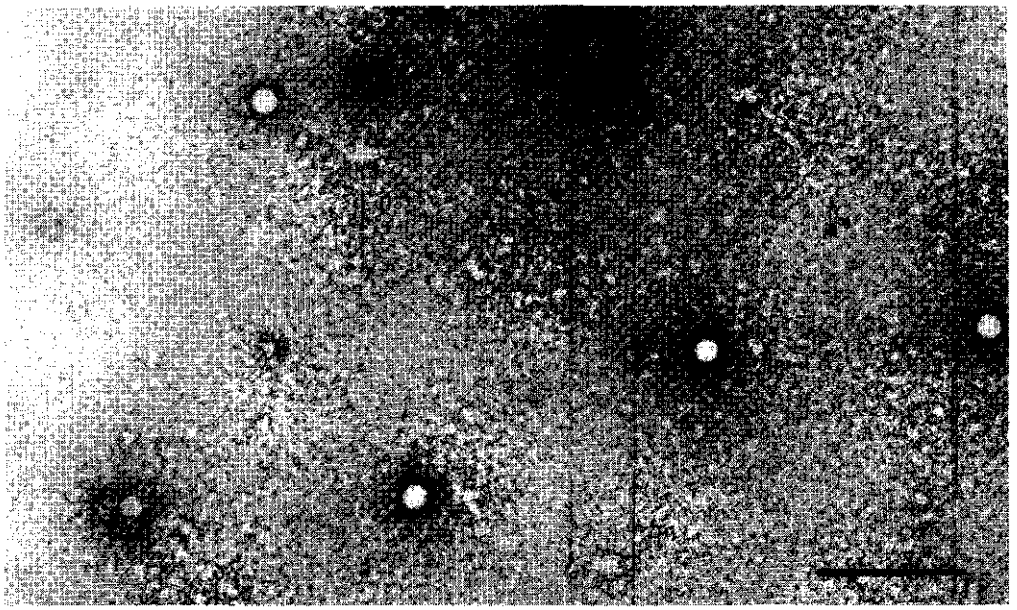


Fig. 2.4. Specific labelling of CCMV with protein A-gold complexes with a gold diameter of 7 nm, in a mixed suspension of CCMV and SBMV (bar represents 200 nm).

2.2B. In Fig. 2.2A the virus preparation was incubated with specific antibodies against TMV. TMV particles were covered with 7 nm gold particles, whereas the CCMV particles were not labelled at all. Fig. 2.2B demonstrates the reverse situation where the virus preparation was incubated with specific antibodies against CCMV and only CCMV particles were labelled with 7 nm gold particles. Identical results were obtained when labelling was done with pAg-16 instead of pAg-7.

When virus preparations labelled for CCMV with pAg-7 were incubated further with specific antibodies to TMV followed by pAg-16, double-labelling was achieved. CCMV particles were surrounded with 7 nm gold particles and TMV with 16 nm particles (Fig. 2.3). Cross-adsorption of 16 nm particles to CCMV particles rarely occurred.

These experiments were repeated with a mixture of two spherical viruses, CCMV and SBMV. Due to their similar morphological appearance individual particles of CCMV and SBMV cannot be identified with the electron microscope when

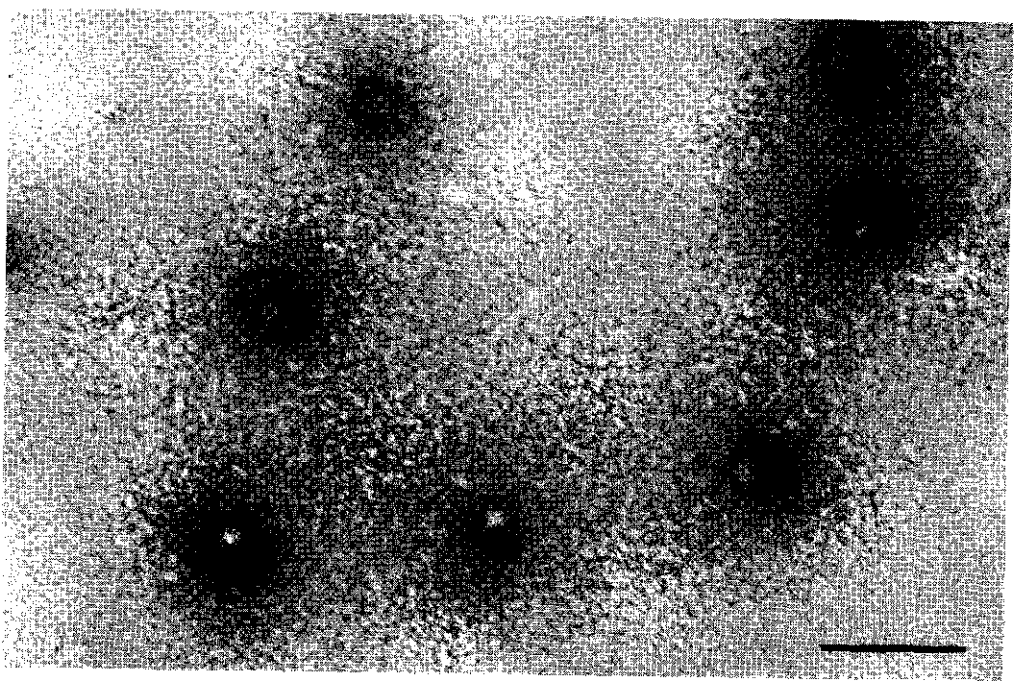


Fig. 2.5. Double-labelling of CCMV and SBMV. CCMV is specifically labelled with protein A-gold complexes with a gold particle of 7 nm and SBMV with particles of 16 nm in diameter (bar represents 200 nm).

occurring in a mixture. However, Fig. 2.4 demonstrates specific labelling of CCMV with pAg-7 in a CCMV/SBMV mixture incubated with specific antibodies to CCMV. Some spherical particles are labelled (CCMV) and some have no label (SBMV). In Fig. 2.5 double-labelling is shown with pAg-7 bound to CCMV particles and pAg-16 to SBMV particles. Cross-adsorption of pAg-16 to the first specific antibodies present on CCMV occurred only rarely. Apparently the pAg-7 blocked almost all available binding sites for pA. Incubation of the preparations with 0.01 mg/ml pA in 0.1 M sodium acetate buffer (pH 5.0) containing 1% (w/v) BSA, in between the first labelling with pAg-7 and subsequent incubation with the second antibody, reduced cross-adsorption, but was not used routinely.

DISCUSSION

Preparation of suspensions of gold particles with defined diameters according to the described methods appeared to be easy and reliable. As our studies involve several antigenically different plant viruses we have chosen to work with pAg complexes instead of immunoglobulin-gold complexes. Absorption of pA to these gold particles was easy and resulted in active and stable electron-dense markers which could be used for the detection of antigen-antibody complexes in the electron microscopy.

Slot and Geuze (1981) and Geuze et al. (1981) demonstrated the use of pA complexes of two different sizes for double-labelling of two antigens in sections of frozen tissue. We have demonstrated here the use of double-labelling for two antigens in mixed plant virus suspensions. Labelling one antigen with pAg-7 and the other with pAg-16 appeared to be possible with minimal cross-adsorption. Not only could CCMV be distinguished from TMV by labelling the first with 7 nm gold particles and the latter with 16 nm particles, but also could CCMV and SBMV particles be identified in mixed preparations of the two viruses. Both viruses could not be clearly identified in mixed preparations due to a similar morphological appearance in the electron microscope. Immunogold labelling has been used to distinguish between serologically related viruses (Pares and Whitecross, 1982; Alexander and Toler, 1985; 1986), by quantitative comparison of the number of gold particles per heterologous or homologous virus particle. Additionally, double-labelling of antigens could be a powerful technique in the detection and identification of viruses in suspensions and in dip-preparations of plant tissue from mixed infections. Continuing the idea of Pares and Whitecross (1982) to distinguish serologically more closely-related

viruses, it might be possible to identify and quantify this relation by double-labelling of a single virus. This virus, incubated with mixtures of heterologous and homologous antibodies respectively adsorbed to 7 and 16 nm gold particles will be labelled with 7 and 16 nm gold particles. The ratio of 7 nm of 16 nm gold particles should be a measure to the relationship between these two viruses. The limitations of this method will depend on the maximum number of gold particles which can bind to one virus particle. Further investigations will be needed to demonstrate the usefulness of this technique.

CHAPTER 3

DETECTION OF VIRAL PROTEIN AND PARTICLES IN THIN SECTIONS OF VIRUS INOCULATED PLANT PROTOPLASTS USING IMMUNOGOLD LABELLING

SUMMARY

Protein A-gold complexes with gold particle diameters of 7 nm and 16 nm were prepared and could be stored at 4°C for at least 5 months without noticeable decrease in labelling capacity. The complexes were used successfully to detect antibodies bound to cowpea chlorotic mottle virus (CCMV) coat protein antigen in thin sections of infected cowpea protoplasts. Specific gold labelling of viral antigen was obtained both in thin sections of protoplasts fixed with aldehydes and osmium tetroxide and embedded in methacrylate, and in sections of aldehyde-fixed protoplasts embedded in Lowicryl K4M. In general, gold labelling appeared to be more dense on sections embedded in Lowicryl. In both types of sections, one gold particle was associated with less than 10 virus particles.

Virus particles could be detected in protoplasts from zero time of infection (end of inoculation period) up to 48 h after inoculation. At zero time they were present as aggregates outside the plasmalemma and within vesicles resulting from invaginations of the plasmalemma membrane; all these particles were identified as inoculum. Between 6 and 9 h after inoculation gold label was found to be associated with certain areas of the cytoplasm where no virus particle morphology could be distinguished. Such gold labelling of viral protein was found consistently in Lowicryl-embedded protoplasts but was rarely seen in sections of infected protoplasts embedded in methacrylate. At this stage of infection it was difficult to distinguish between inoculated virus and newly-synthesized coat protein.

INTRODUCTION

Our understanding of the mode of infection of plants by viruses is still rather limited and mostly confined to knowledge of some biochemical processes in the replication cycle of single-stranded (ss) RNA-containing viruses. A number of different steps can be distinguished in the infection process:

adsorption of virus to cell wall or plasmalemma, penetration, release of the viral genome or uncoating, followed by transcription and translation of the genome, and assembly of new virus particles and transport of. The transport may be intra- or intercellular over long distances within the plant. Uncoating and assembly were the subjects of studies in the late sixties and early seventies (Leberman, 1968; Fraenkel-Conrat, 1970), while in the last decade transcription and translation of ssRNA-containing plant viruses was investigated (Strauss and Strauss, 1983; Joshi and Haenni, 1984). However, the initial interactions between the virus and the plant cell, its entry into the cell and transport within the plant are poorly understood (De Zoeten, 1981).

The development of sensitive techniques to detect viruses, viral proteins and nucleic acids either in solution (Taylor et al., 1976; Clark and Adams, 1977) or in situ (Pardue and Gall, 1975) made possible the localization of viral components within the plant cell, from the entry of viral genomic material to the transport of infectious entity from cell to cell or over long distances. The immunogold labelling technique offers great potential for the detection of antigen-antibody complexes. Protein A adsorbed to gold particles specifically binds to such complexes which in turn are easily observed in the electron microscope (EM) because the gold particles are electron dense (Roth et al., 1978). In this chapter, we describe procedures for fixing and embedding infected protoplasts suitable for use in detection of viral protein by the immunogold labelling technique. The ultimate aim of the investigation was to localize cowpea chlorotic mottle virus (CCMV) in infected leaf tissue of cowpea. We used protoplasts as a model system (Takebe, 1983; Murakishi et al., 1984) because the high percentage of synchronously infected cells that can be obtained facilitates both the detection of virus and the development of sensitive and specific detection systems.

MATERIALS AND METHODS

Virus isolate and antiserum. The virus isolate of CCMV (originally obtained from J.B. Bancroft) was maintained in cowpea (Vigna unguiculata cv. California Blackeye). It was purified from cowpea as described by Verduin (1978). Antibodies against CCMV were prepared as described in Chapter 2.

Preparation and inoculation of protoplasts. Cowpea plants were grown and mesophyll protoplasts isolated from primary leaves of 10 day old plants essen-

tially as described by Hibi et al. (1975) and later modified by Van Beek et al. (1985). All media contained 0.6 M mannitol and 2.5 mM 2(N-morpholino)ethane sulphonic acid, and were adjusted to pH 5.7. Inoculation with CCMV was done essentially as described by Dawson et al. (1978) and later modified by Maule et al. (1980). Protoplasts (3 x 10^6) were centrifuged for 3 min at 60 g, and 10.8 µl containing 150 µg CCMV in 0.1 M sodium acetate buffer, pH 5.0 was mixed with the protoplast pellet. Then, 1 ml of 40% (w/v) polyethylene glycol (PEG; mol. wt 6,000) containing 3 mM calcium chloride was added, mixed with the protoplast-virus suspension for c. 10 s and diluted with 9 ml 0.6 M mannitol containing 0.1 M calcium chloride. The protoplast-virus suspension was incubated for 15 min at 25°C, centrifuged and washed three times with 2 ml 0.6 M mannitol containing 0.1 mM calcium chloride. Then, the inoculated protoplasts were resuspended in culture medium and incubated as described by Van Beek et al. (1985). A similar procedure was used to inoculate protoplasts with CCMV-RNA. Infection of the protoplasts with CCMV was scored 44 h after incubation by determining the percentage of fluorescent protoplasts as described by Van Beek et al. (1985). For this, purified anti-CCMV IgG was used as the primary antibody at a concentration of 0.01 mg/ml.

Fixation and embedding of the protoplasts. Protoplasts were fixed at different times after inoculation with CCMV by adding glutaraldehyde (10% w/v) to the suspension to a final concentration of 1%. The protoplasts were left for 3-17 h at 0°C and then embedded in 10% (w/v) gelatin in phosphate/citrate buffer pH 7.2 (0.1 M $Na_2HPO_4 \cdot 2H_2O$ and 2.7 mM citric acid). Small pieces of gelatin containing protoplasts (the specimens) were hardened by fixation for 1 h at 0°C in 1% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde in phosphate/citrate buffer and then washed six times for 10 min in buffer.

Before embedding in methacrylate resin, specimens were fixed for 1 h at room temperature in 1% (w/v) osmium tetroxide in phosphate/citrate buffer. After washing in buffer and distilled water, they were fixed in 1% (w/v) uranyl acetate, dehydrated in ethanol and infiltrated with the resin, which consisted of a mixture of 80 ml n-butyl methacrylate, 20 ml methyl methacrylate, 5 ml divinyl benzene and 1 g benzoyl peroxide (Kushida, 1961). Infiltration was allowed to proceed for 18 h at 4°C followed by 2 h at room temperature. Finally, the specimens were transferred to gelatin capsules and the resin polymerized at 50°C for 48 h. Before embedding in Lowicryl K4M (Lowicryl; Carlemalm et al., 1982), the specimens were dehydrated in ethanol following the glutaraldehyde/parformaldehyde fixation. Dehydration, infiltration and polymer-

isation were done essentially as described by Roth et al. (1981b) and modified by Fryer and Wells (1983). The specimens were infiltrated with resin at -30°C for 3 days. Then, the resin was polymerized under ultraviolet light (Philips TLAD 15W/05) for 24 h at -25°C followed by 5 days at room temperature. Specimens were stored under vacuum over a desiccant at room temperature. These prolonged infiltration and polymerization times as well as storage under vacuum seemed essential to improve the sectioning properties of the specimens.

Ultrathin sections were cut with a LKB Ultrotome III using glass or diamond knives. Sections were collected on 150 mesh nickel grids with a formvar film with a c. 2 nm carbon layer.

Immunogold labelling of antigens. Protein A-gold (pAg) complexes with gold particles of 7 and 16 nm were prepared as described in Chapter 2. Thin sections of specimens fixed with glutaraldehyde/paraformaldehyde and osmium tetroxide and embedded in methacrylate were incubated for 60 min at room temperature on a saturated aqueous solution of sodium metaperiodate (Bendayan and Zollinger, 1983), thoroughly washed in distilled water and then processed for pAg labelling. Thin sections of specimens embedded in Lowicryl were processed for pAg labelling without pre-treatment with sodium metaperiodate. For processing, grids with ultrathin sections were incubated for 30 min at room temperature on 15 µl drops of PBS-BSA. They were then transferred to drops of 0.01 mg/ml anti-CCMV IgG in PBS-BSA and incubated for 1 h. After washing the grids with 30 drops of PBS, they were incubated for 1 h on pAg which had been diluted in PBS-BSA to give a suspension with A_{520} = 0.5. After washing with 30 drops of PBS and 10 drops of distilled water, the sections were stained. Lowicryl sections were stained for 20 min with 4% (w/v) uranyl acetate in distilled water. Methacrylate sections were stained with 2% (w/v) uranyl acetate for 5 min and subsequently with lead citrate for 5 min (Reynolds, 1963). Controls consisted of sections of protoplasts which were mock-inoculated with 0.1 M sodium acetate buffer, pH 5.0. Specimens were observed with a Siemens Elmiskop 101 or a Zeiss EM109 electron microscope.

RESULTS

Protoplasts. Mesophyll protoplast suspensions usually contained 85-90% of spherical cells. Inoculation of these protoplasts with purified CCMV consistently resulted in *c.* 70% being infected with virus as determined by the percentage of fluorescent protoplasts..

Fixation and embedding. Protoplasts were fixed at different times after inoculation with CCMV. Time zero (t=0) was defined as the end of the 15 min incubation period at 25°C, just before the protoplasts were washed and resuspended in culture medium. At times t=0, 0.5, 3, 6, 9, 12, 18, 24, 36 and 48 h after inoculation, samples of the inoculated protoplasts were pelleted, fixed, dehydrated and embedded in methacrylate or Lowicryl. Ultrathin sections with a thickness of 40-50 nm were cut using a diamond knife from specimens embedded in methacrylate. Ultrathin sections with a thickness of 60-70 nm were cut with a glass knife from the specimens embedded in Lowicryl.

The ultrastructure of the protoplasts was preserved well in both methacrylate- and Lowicryl-embedded material. Membranes, individual ribosomes and virus particles could clearly be seen in thin sections embedded in methacrylate. Specimens embedded in Lowicryl also showed these structures but their appearance was less distinct. This was probably partly due to the absence of osmium tetroxide fixation, but also the sections in Lowicryl were thicker than sections in methacrylate, resulting in poor resolution in the EM. When the thickness of sections of Lowicryl-embedded material was decreased to 40-50 nm, poor contrast was obtained even after staining with uranyl acetate. Additional staining of the sections with lead citrate did not improve contrast.

Detection of virus particles and coat protein antigen in thin sections of infected protoplasts. Because 16 nm gold particles were more easily observed in the EM even at low magnifications, pAg-16 was used for labelling antigen-antibody complexes in ultrathin sections. Virus particles could be labelled specifically in sections of inoculated protoplasts which had been embedded in methacrylate. This type of embedding is used routinely in our laboratory to provide electron micrographs of sectioned plant material with high resolution and a well defined appearance of the membrane structure.

Fig. 3.1A shows specific labelling of an aggregate of CCMV particles enclosed within a vesicle of a 6 h-infected protoplast. When pre-treatment with sodium metaperiodate prior to labelling with pAg was omitted, labelling still

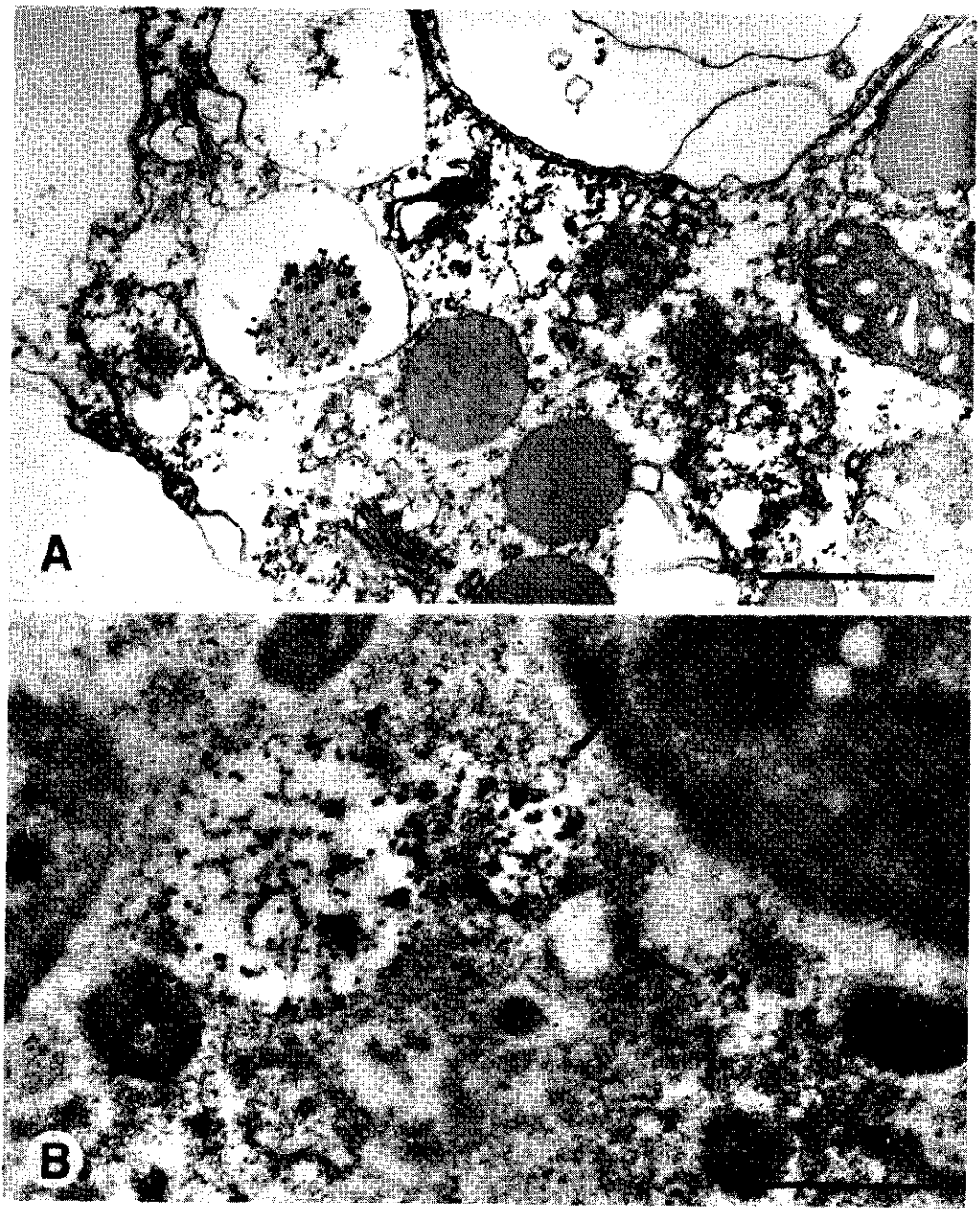


Fig. 3.1. Specific labelling of cowpea chlorotic mottle virus in thin sections of inoculated protoplasts with pAg-16. Effect of embedding material: A) protoplast 6 h after inoculation fixed with aldehyde and osmium tetroxide, and embedded in methacrylate; B) protoplast 9 h after inoculation fixed with aldehyde and embedded in Lowicryl at low temperature. (Bar represents 500nm).

Table 3.1. The relationship between the number of cowpea chlorotic mottle virus and gold particles in sections of methacrylate- and Lowicryl-embedded protoplasts.

Experiments*	Number of virus particles	Number of gold particles	Ratio of virus particles/ gold particles
1	1971	795	2.5
2	541	110	4.9
3	1032	129	8.0
4	724	368	2.0
5	110	23	4.8
6	486	62	7.8

* Each set of experiments (1 and 4, 2 and 5, 3 and 6) was done with a different batch of pAg; 1-3 were with methacrylate sections and 4-6 were parallel experiments with Lowicryl sections.

occurred but with fewer gold particles per virus particle. Labelling density also decreased in specimens which were fixed in 1% (w/v) uranyl acetate in water to immobilize lipids and improve contrast following the osmium tetroxide fixation. Treatment of the sections with sodium metaperiodate did not compensate for the effect of uranyl acetate. In metaperiodate-treated sections contrast was less than in untreated sections and staining with uranyl acetate and lead citrate did not enhance it.

Lowicryl is a water compatible, polar embedding material which preserves biologically active structures in the embedded section sufficiently to facilitate binding of antibody molecules (Roth *et al.*, 1981a,b). Specific gold labelling of virus could be achieved in sections of Lowicryl-embedded, inoculated protoplasts. In Fig. 3.1B specific gold labelling of virus particles in vesicles near the nucleus is shown in an infected protoplast 9 h after inoculation, but as mentioned above, the resolution and structure of the organelles was poor compared to that in the methacrylate-embedded sections. Background labelling was very low with both embedding materials, as confirmed by inspection of mock-inoculated protoplasts treated with anti-CCMV and pAg, or treatment with preimmune serum and pAg.

As virus particles located within vesicles could be seen individually, an attempt was made to determine the relationship between the ratio of virus to gold particles. This ratio reflects the variability in the individual pAg preparations and in the fixation and embedding procedures. It therefore

provides information about the sensitivity of the labelling. Counts were made of the number of 16 nm gold particles associated with virus particles (Table 3.1). Experiments 1-3 were counts of labelling on methacrylate sections, while 4-6 were from Lowicryl-embedded material; counts were taken from several micrographs of different sections. Each set of experiments (1 and 4, 2 and 5, 3 and 6) was labelled with a different batch of pAg. Although there was some variation in the number of virus particles associated with one gold particle, this number never exceeded 10.

Localization of CCMV-coat protein antigen in inoculated protoplasts. After inoculation of protoplasts with CCMV using PEG, aggregates of virus-like particles were seen attached to the outer side of the plasmamembrane. In some instances, these aggregates were found penetrating the protoplast (Fig. 3.2A). Using pAg with methacrylate-embedded protoplasts, they could be identified unambiguously as CCMV particles (Fig. 3.2B). When isolated by conventional methods, protoplasts show invagination of the plasmalemma (Mahlberg et al., 1971). The PEG inoculation method increased the number of invaginations resulting in many vesicles containing virus particles (Fig. 3.3A). Usually the virus particles were attached to the inside of the vesicle-membrane (Fig. 3.3A,C). In some cases the membrane surrounding the virus particles was not closed (Fig. 3.3B, arrow) or not visible suggesting release of virus particles into the cytoplasm (Fig. 3.3A, arrow). A large number of vesicles in the cytoplasm was also found in mock-infected protoplasts at t=0. Observations on protoplasts inoculated with CCMV and fixed at t=0.5, 3 and 6 h confirmed that the vesicles near the plasmalemma observed at t=0 were true vesicles and not incomplete invaginations of the plasmalemma because they were observed throughout the cytoplasm, indicating that those formed during inoculation migrated inside the protoplast.

At t=6 h after inoculation aggregates of virus particles were seen in the center of the vesicle free from the membrane, and these were still found at later times (t=12 (Fig 3.4A); t=18 h (Fig. 3.4B)) in vacuoles which apparently increased in size. That these originated from the inoculum and were not newly-synthesized was shown by inoculation with glutaraldehyde-fixed CCMV and with ultraviolet light-irradiated CCMV. Although vesicles with virus particles were still present, protoplasts inoculated with these virus preparations did not become infected and did not show virus multiplication when tested by fluorescent-antibody staining 44 h after inoculation. The vesicles with virus particles were not observed in protoplasts infected with CCMV-RNA (Chapter 4).

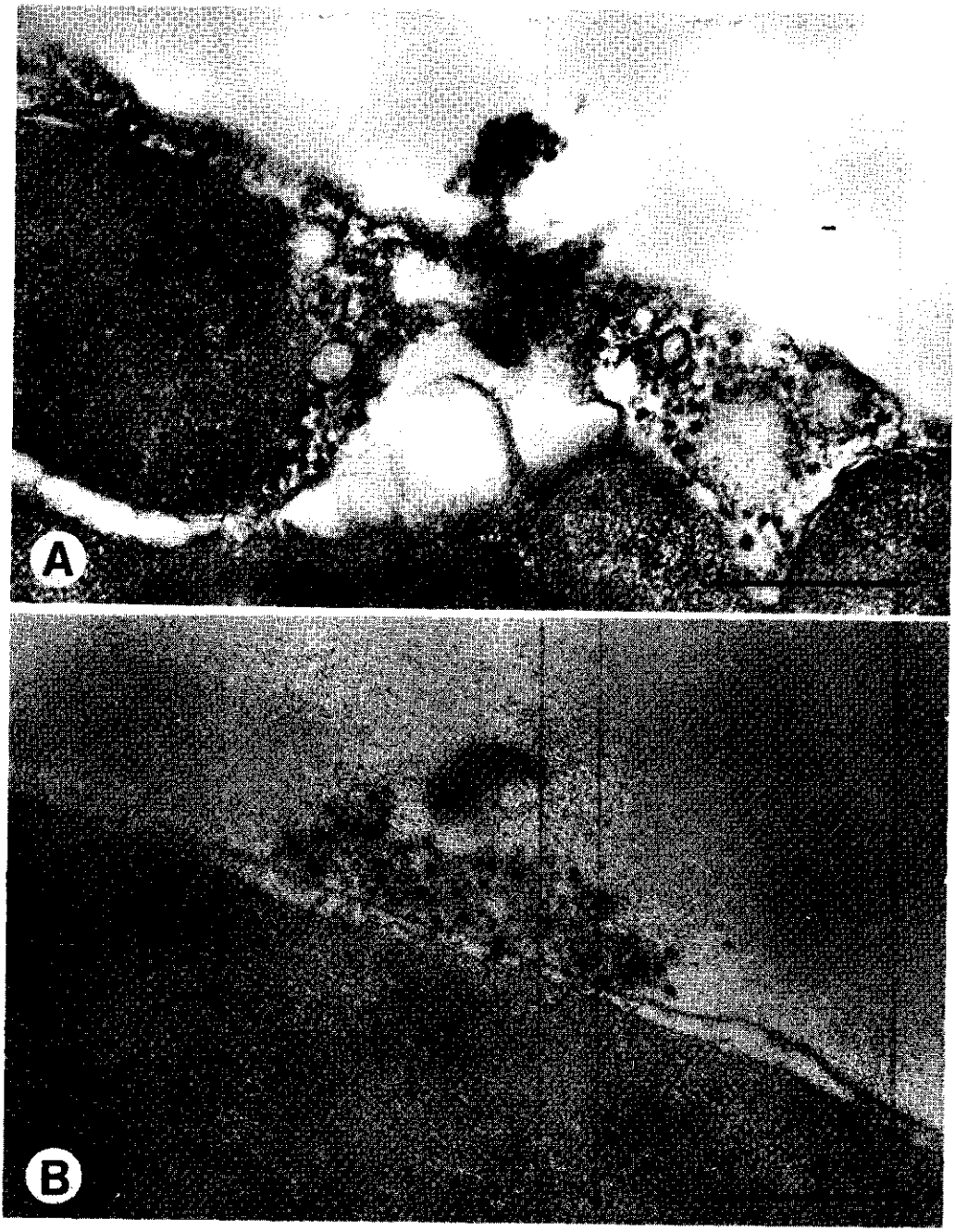


Fig. 3.2. Methacrylate sections of protoplasts immediately after inoculation with cowpea chlorotic mottle virus. A) Aggregate of virus particles penetrating the surface of a protoplast. B) Aggregate of virus particles attached to the plasmalemma membrane and labelled with protein A-gold. (Bar represents 300 nm).

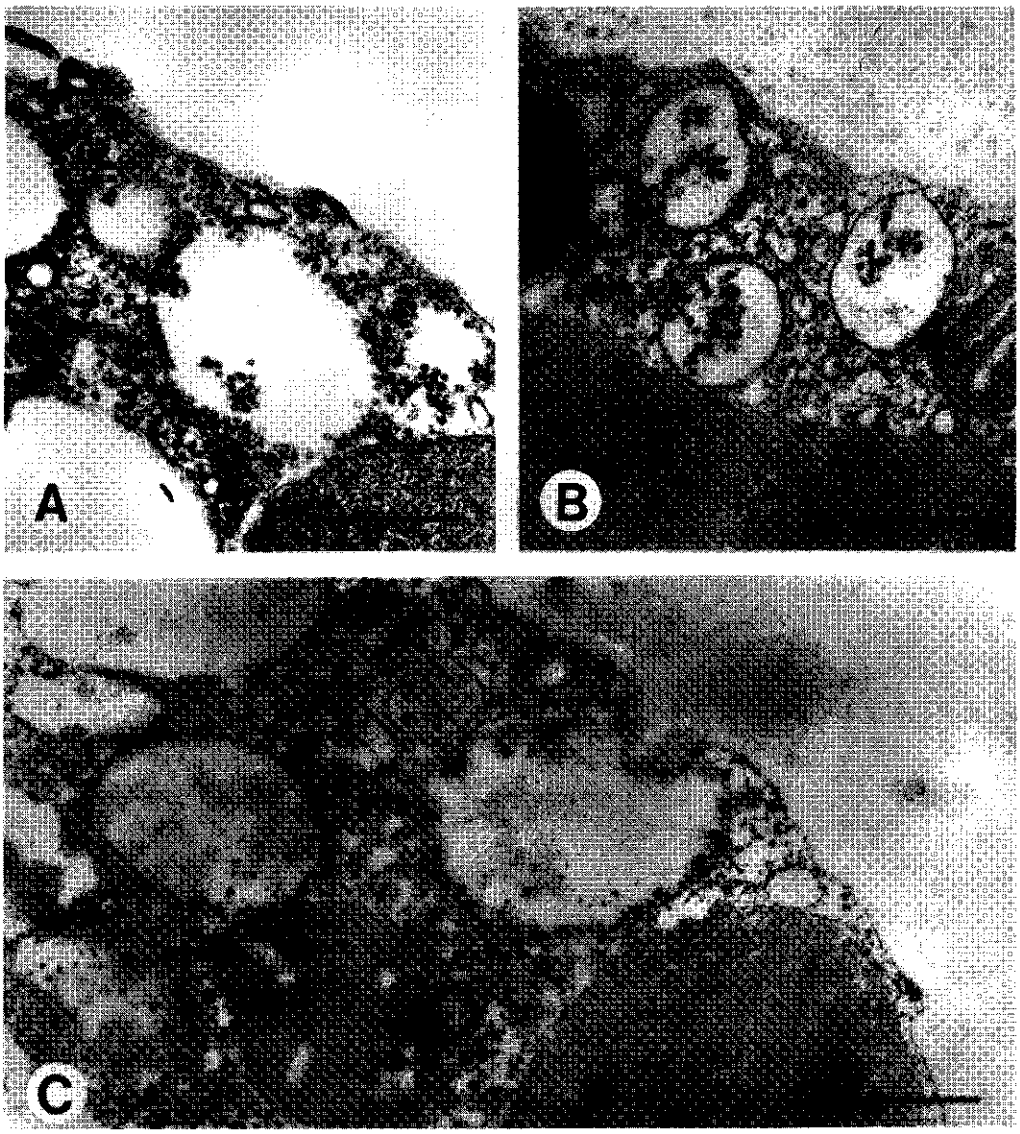


Fig. 3.3. Methacrylate sections of protoplasts immediately after inoculation with cowpea chlorotic mottle virus. A) Several large vesicles containing individual as well as aggregates of virus particles in the cytoplasm, some which are not surrounded by a membrane (arrow). B) Incomplete invagination of the plasmalemma membrane with the open end still visible (arrow). (C) Complete invagination of the plasmalemma membrane and vesicles containing virus particles. (Bar represents 300 nm).

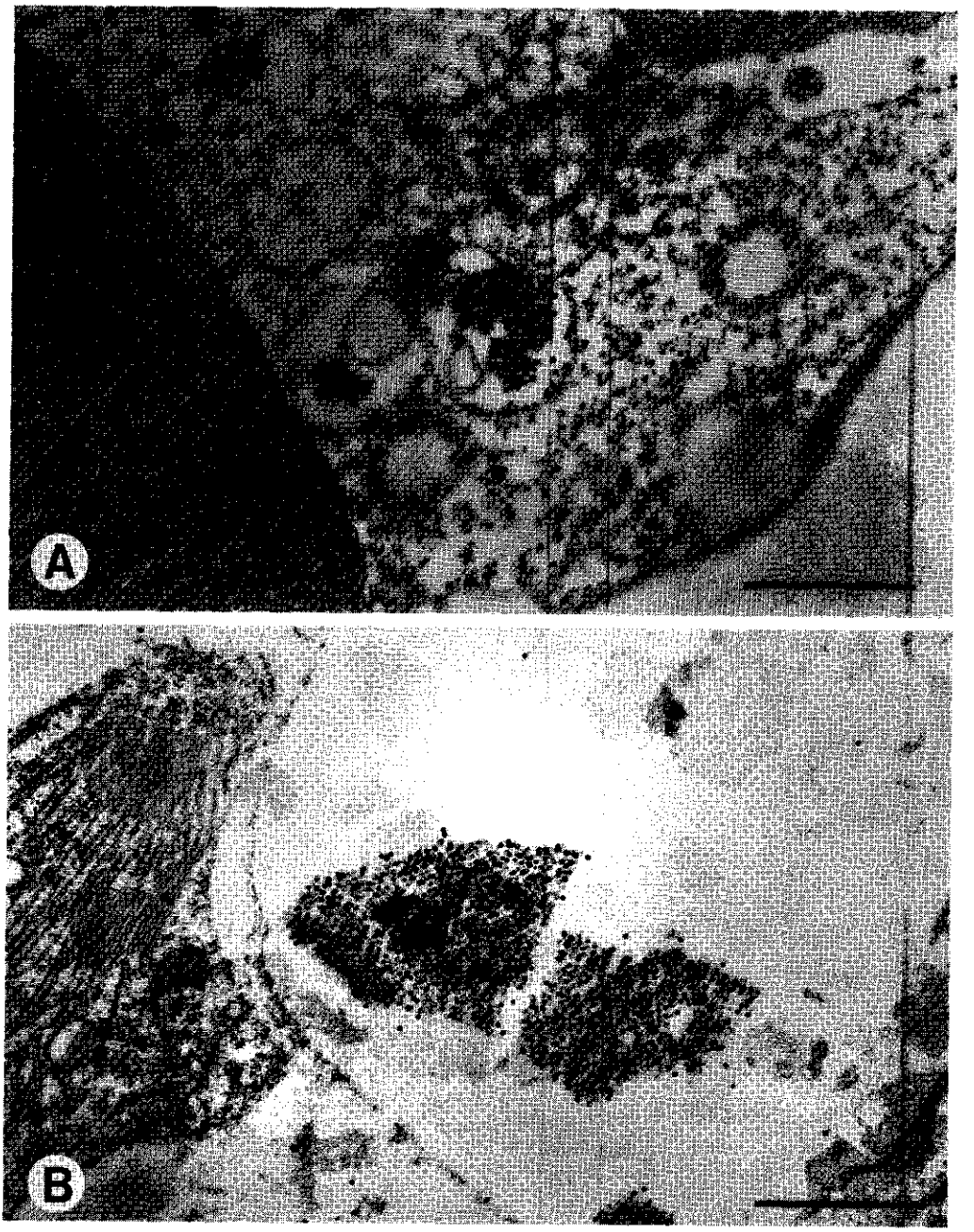
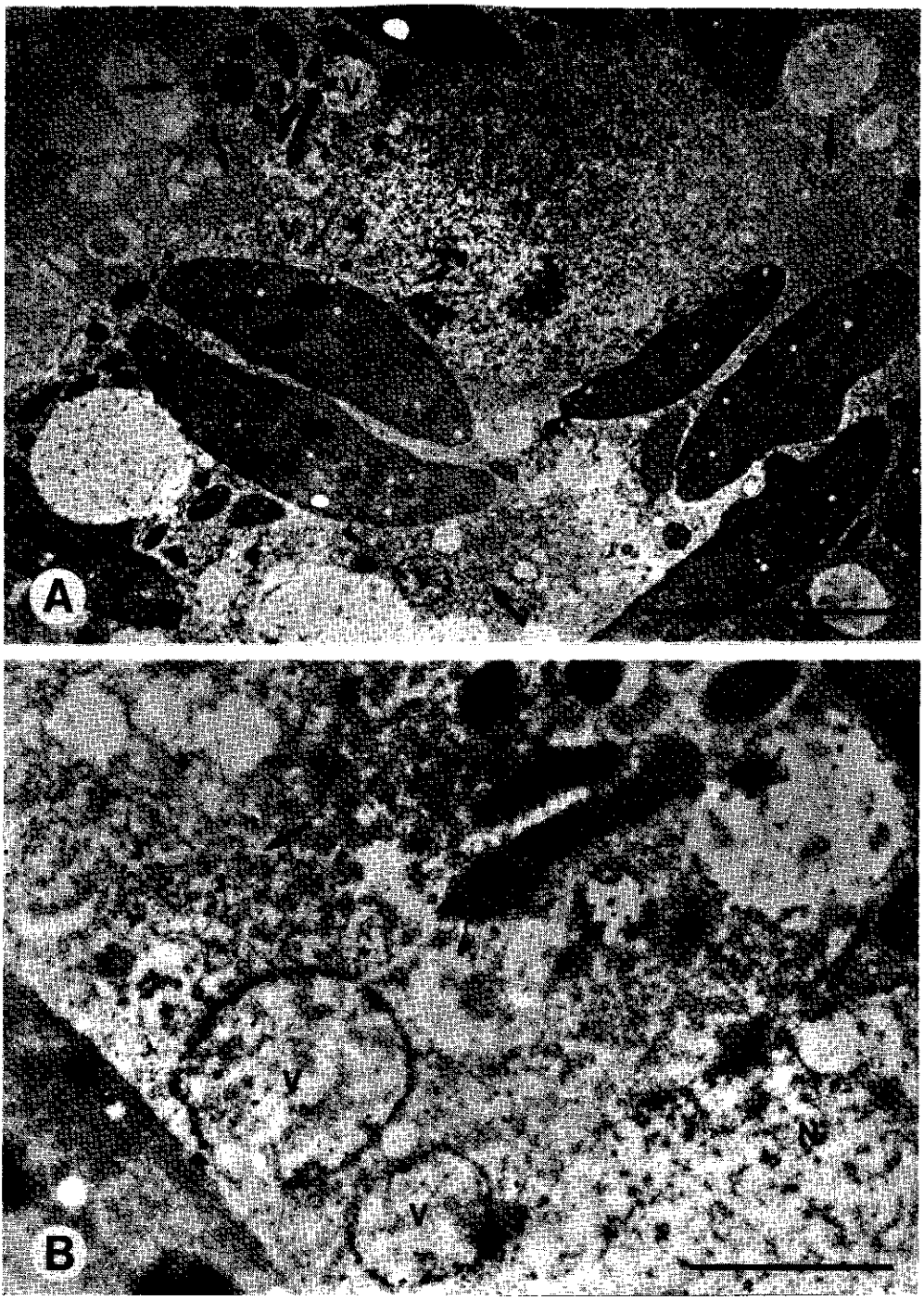


Fig. 3.4. Methacrylate sections of protoplasts inoculated with cowpea chlorotic mottle virus. Virus particles labelled with protein A-gold complexes are present within vesicles in the cytoplasm singly and aggregated at t=12 h (A) and mainly as large clumps at t=18 h (B). (Bar represents 500 nm).


A
B
V
V
V

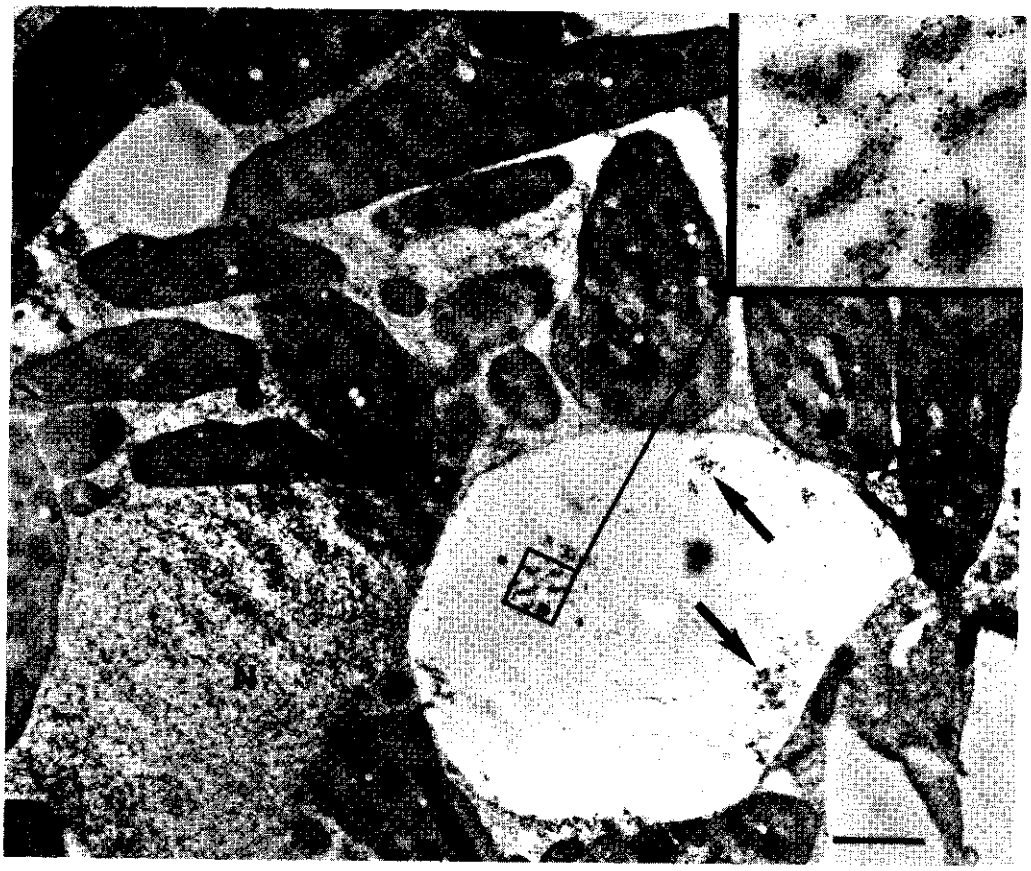


Fig. 3.6. Lowicryl section of a protoplast at 36 h post-inoculation with cowpea chlorotic mottle virus. Gold label was only observed on amorphous material in a large vacuole (arrow; inset), but not in the cytoplasm. C, chloroplast. N, nucleus. Bar represents 1 μm.

←

Fig. 3.5. Lowicryl sections of protoplasts at t=9 h after inoculation with cowpea chlorotic mottle virus. A) Specific labelling of virus in vesicles (V) can be observed as well as labelling of some areas in the cytoplasm (arrows) indicating the synthesis of new viral coat protein. (Bar represents 5 μm.) B) Enlargement of an area with gold label associated with virus particles in vesicles (V) and viral coat protein in the cytoplasm. N, nucleus. C, chloroplast. (Bar represents 1 μm.).

At times t=0, 0.5 and 3 h after inoculation, pAg was only associated with virus particles in vesicles. No pAg could ever be found in other parts of the protoplasts after these incubation times. However, from t=6 h onwards pAg was observed in some other areas of the cytoplasm, where no virus particles were observed. Fig. 3.5A shows a section of a Lowicryl-embedded protoplasts at t=9 h. Arrows indicate the areas in the cytoplasm where pAg was found in large numbers above the background. Some of these areas also contained vesicles (V) with virus particles and pAg. One of the areas where pAg was found both inside and outside the vesicles is shown magnified in Fig. 3.5B. With time the gold labelled area as well as the density of labelling of the cytoplasm increased. However, as demonstrated in Fig. 3.6 protoplasts could be found as late as 36 h after inoculation showing gold labelling of antigens in large vacuoles, but no gold label on the cytoplasm. These protoplasts were apparently not infected, but still contained some of the inoculum virus.

DISCUSSION

The immunogold labelling technique using pAg has great potential for detection of virus particles and viral coat protein in ultrathin sections of infected plant tissue. Specific labelling of virus particles was satisfactory both with glutaraldehyde/paraformaldehyde- and osmium tetroxide-fixed specimens embedded in methacrylate, and with glutaraldehyde/paraformaldehyde-fixed specimens embedded in Lowicryl K4M. Treatment of sections of methacrylate-embedded specimens with sodium metaperiodate seemed essential to obtain good labelling. Sodium metaperiodate is a strong oxidizing agent which partly removes the osmium from the macromolecules in the specimens, thereby unmasking some of their antigenic sites (Bendayan and Zollinger, 1983). In contrast to the findings of Bendayan and Zollinger, we have observed some bleaching of the structures in thin sections treated with sodium metaperiodate; contrast was less than in untreated sections despite the staining with uranyl acetate and lead citrate. In general, gold labelling was found to be more dense on sections of specimens embedded in Lowicryl. This apparent higher sensitivity of the immunogold labelling on Lowicryl sections may explain why the gold particles were also found in restricted areas of the cytoplasm at t=6, 9 and 12 h after inoculation of the protoplasts. These areas probably contain newly-synthezised viral proteins resulting from infection of the protoplasts.

While interpreting the results of the counts of the number of gold particles which are associated with virus particles (Table 3.1), it should be kept in mind that in many cases the sections in Lowicryl were about 1.5 times thicker than the sections in methacrylate. Therefore Lowicryl sections will contain more visible virus particles per section surface area, but these particles have less chance to be at the section surface. The ratios found for sections of methacrylate and Lowicryl can therefore not be compared directly.

The immunogold labelling technique has been found to be very helpful in detecting virus particles and proteins, and also in identifying spherical structures as virus particles (Tomenius et al., 1983; Lin and Langenberg, 1984; Giband et al., 1984). We have shown that large numbers of positively-identified virus particles enter the protoplasts by endocytosis after inoculation of cowpea protoplasts with CCMV using PEG as polymer to enhance infection. As shown previously by Motoyoshi et al. (1973), these virus particles were observed in vesicles at t=0, 0.5, 3 and 6 h after inoculation. The vesicles with virus particles were apparently quite stable within the cytoplasm as they were still observed after 12 and 18 h. Using glutaraldehyde-fixed and ultraviolet light-irradiated CCMV, we found that the virus in the vesicles originated from the inoculum and was not newly-synthesized. Inoculation with CCMV-RNA did not reveal vesicles with virus particles within 12 h of inoculation.

On the outside of the plasmalemma virus was always seen as particle aggregates. Sometimes these aggregates were seen penetrating the plasmalemma. The virus particles found in the cytoplasm at t=0 after inoculation (Fig. 3.4A) and not surrounded by a membrane, may have been separated from an aggregate of virus particles which had entered previously. These data do not show conclusively whether PEG stimulates endocytosis or induces plasmalemma lesions which presumably serve as binding sites for virus. However, when 40% (w/v) PEG was added and subsequently diluted to 4% (w/v), there was an increase in conductivity of a protoplast suspension, indicating alteration of the cell membrane (W. van Dis, personal communication). Parallel experiments have shown that CCMV precipitates at high and redissolves at low PEG concentration. These data therefore support a wounding mechanism for the entry of virus into plant cells as suggested by Watts et al. (1981). However, the recently suggested co-translational disassembly of rod-shaped and spherical plant viruses does not favour any of the proposed mechanism of entry (Wilson, 1984a,b). Further studies are needed to determine the significance of endocytosis in virus entry and concomitant infection of the protoplast and the relationship of the endocytotic vesicles with the vesicles seen by Burgess et al. (1974) in tobacco

protoplasts infected with CCMV for 15 h.

CHAPTER 4

LOCALIZATION OF CCMV IN PROTOPLASTS INFECTED WITH CCMV-RNA

SUMMARY

In osmium-fixed cowpea protoplasts structural changes of the cytoplasmic membrane system were observed as early as 9 h after inoculation with CCMV-RNA. The endoplasmic reticulum appeared dilated and membranous vesicles were formed, which often contained smaller vesicles and dots of electron-dense material. With time the cytoplasmic area showing dilated ER and vesicles increased, apparently forming membranous masses. Such cytopathic structures were not observed in mock-inoculated protoplasts. In thin sections of aldehyde-fixed and Lowicryl K4M-embedded protoplasts, membranestructure was poorly preserved and modifications of the ER could not be clearly identified. However, CCMV coat protein antigen was localized in restricted areas of the cytoplasm as early as 6 to 9 h post-inoculation. The typical rough appearance of these areas suggested the presence of membranous structures. With time the cytoplasmic area showing gold labelling increased. Quantification of the gold labelling, by determining the number of gold particles per μm^2, showed no specific labelling of chloroplasts, mitochondria, vacuoles and microbodies. However, from 12 h post-inoculation onwards the nucleus and in particular the nucleolus showed increasing densities of gold label, indicating the presence of viral coat protein.

INTRODUCTION

In Chapter 3 observations with regard to initial events in virus infection of protoplasts were described and discussed. Immunogold labelling was used to identify and localize CCMV in thin sections of cowpea protoplasts. Accurate study of the infection process by localization of viral antigen was disturbed by the presence of large amounts of inoculum virus entering the protoplasts upon inoculation and remaining in vesicles for long periods of time. Newly synthesized virus and inoculum virus could, therefore, not be distinguished by immunogold labelling.

In this study we report on the ultrastructure of CCMV-infected protoplasts

and the in situ localization of CCMV coat protein. Protoplasts were inoculated with CCMV-RNA, thus omitting the introduction of coat protein antigen during inoculation.

MATERIALS AND METHODS

Virus isolate, RNA and antiserum. Maintainance of CCMV isolate, purification of the virus and preparation of antiserum were as described in Chapter 2. Gammaglobulin (IgG) was purified by affinity-chromatography. The whole serum was passed over a column of sepharose CL-4B-protein A (Sigma). The column was washed with PBS to remove all proteins which did not bind to protein A. Gammaglobulins were then eluted from the column with 0.2 M glycin/HCl, pH 2.8. The absorption of the eluent was monitored at 280 nm and the fraction containing protein was collected, dialysed against PBS and stored at -20°C.

Protoplasts. Preparation and inoculation of protoplasts with CCMV, RNA and buffer were done as described in Chapter 3. Infection of the protoplasts was scored 40 h after inoculation with immunofluorescent labelling using anti-CCMV IgG. Immuno-electron microscopy (IEM) was used to detect virus particles in protoplasts at 17 h after inoculation. For this 1.5×10^6 protoplasts were pelleted and homogenized in 100 µl of 0.1 M sodium acetate buffer pH 5.0. The homogenate was centrifuged for 1 min in an Eppendorf centrifuge and the supernatant was removed. Grids with a formvar film and a c. 5 nm carbon layer were exposed to a glow discharge in air for 15 s. The grids were then incubated for 20 min on 0.1 mg/ml anti-CCMV IgG in 0.1 M sodium acetate buffer. Grids were transferred to drops of the supernatant and incubated for 20 min, washed and stained with 2% (w/v) uranyl acetate in distilled water. The protoplasts were fixed and embedded in methacrylate or Lowicryl K4M at 0, 6, 9, 12, 24 and 40 h after inoculation as described in Chapter 3.

Immunogold labelling and quantification of the labelling. Immunogold labelling of CCMV antigen on thin sections of methacrylate or Lowicryl-embedded protoplasts was as described in Chapter 3. For a quantitative evaluation of the gold labelling, sections of protoplasts fixed at 9, 12, 24 and 40 h after inoculation were immunogold-labelled by treatment with specific antiserum and protein A-gold (pAg-7). For quantification of the density of labelling, cross-sections of protoplasts containing part of the nucleus were selected, in which

specific labelling of antigen was observed as judged by visual inspection of the sections. Controls consisted of sections of mock-inoculated protoplasts. Parts of the protoplasts present in the sections were photographed at a magnification of 7.000 times. The micrographs were enlarged 4 times and the surface of chloroplasts, mitochondria, vacuoles, microbodies and nucleus were measured from the prints. The remainder of the cell, defined as "the cytoplasm" was calculated from the difference between the total surface of the cell and the sum of the surfaces of all organelles. The total number of pAg particles on each organelle was counted and the labelling density (pAg/ m^2) was calculated.

RESULTS

Protoplasts. Mesophyll protoplast suspensions usually contained 85-90% of spherical intact cells. Inoculation of these protoplasts with CCMV-RNA consistently resulted in c. 70% being infected with virus as determined by the percentage of fluorescent protoplasts at 40h after inoculation. In homogenates of protoplasts prepared at 17 h after inoculation with RNA, virus particles could be detected using IEM indicating the synthesis of complete progeny virus.

Ultrastructure of CCMV-infected protoplasts. In sections of protoplasts which were fixed with aldehydes and osmium tetroxide, dehydrated in ethanol and embedded in methacrylate, the ultrastructure of cell organelles and membranes was preserved well. Protoplasts showed numerous invaginations of the plasmalemma and the presence of large numbers of small vacuoles (vesicles) in the cytoplasm immediately after inoculation with RNA or mock-inoculation with buffer as a result of the PEG-inoculation method. The vacuoles were formed by invagination of the plasmalemma and subsequent fusion of the membrane. The presence of the small vacuoles as a result of the inoculation conditions appeared to be only temporary. In time (between 12 and 24 h) their number decreased while the size increased, probably as a result of fusion with other vacuoles or the large central vacuole. Due to the chaotic appearance of the cytoplasm in early hours after inoculation, caused by the presence of the membrane-bound vacuoles, ultrastructural comparison of RNA-inoculated and mock-inoculated protoplasts was difficult. However, a consistent change in the ultrastructural appearance of the endoplasmic reticulum (ER) was observed only in RNA-inoculated protoplasts (Figs 4.1 and 4.3), and not in healthy protoplasts (Fig. 4.2). As early as 9 h after inoculation some swelling and

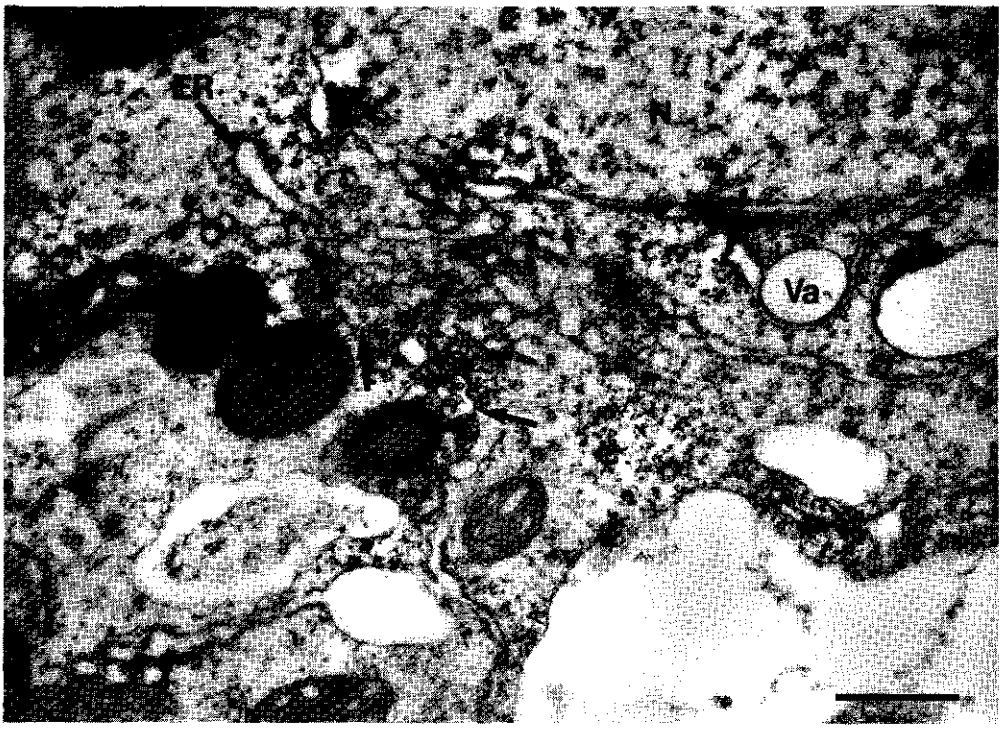


Fig. 4.1. Thin section of an osmium-fixed protoplast at 9 h post-inoculation. Dilation of the endoplasmic reticulum (ER) and formation of vesicles (Vs), some of which contain small vesicles (arrows), in the cytoplasm near the nucleus (N). The vacuole (Va) possibly originates from endocytotic activity of the plasmalemma during PEG-inoculation. Bar represents 500nm.

clumping of the ER was observed in areas of the cytoplasm, accompanied by the formation of small membranous vesicles often containing another small vesicle and a central dot of electron-dense material (Fig. 4.1 A). At 12 h (Fig. 4.3) and later times after inoculation similar disturbances of the ER-system were observed, only more frequently and the vesiculated area appeared larger (Fig. 4.3A and B). No additional cytopathic structures were observed at any time after inoculation. Virus particles could not be identified in the protoplasts probably due to their appearance in thin sections which is similar to ribosomes. No crystalline aggregates of virus particles were observed in the cytoplasm or vacuoles of protoplasts.

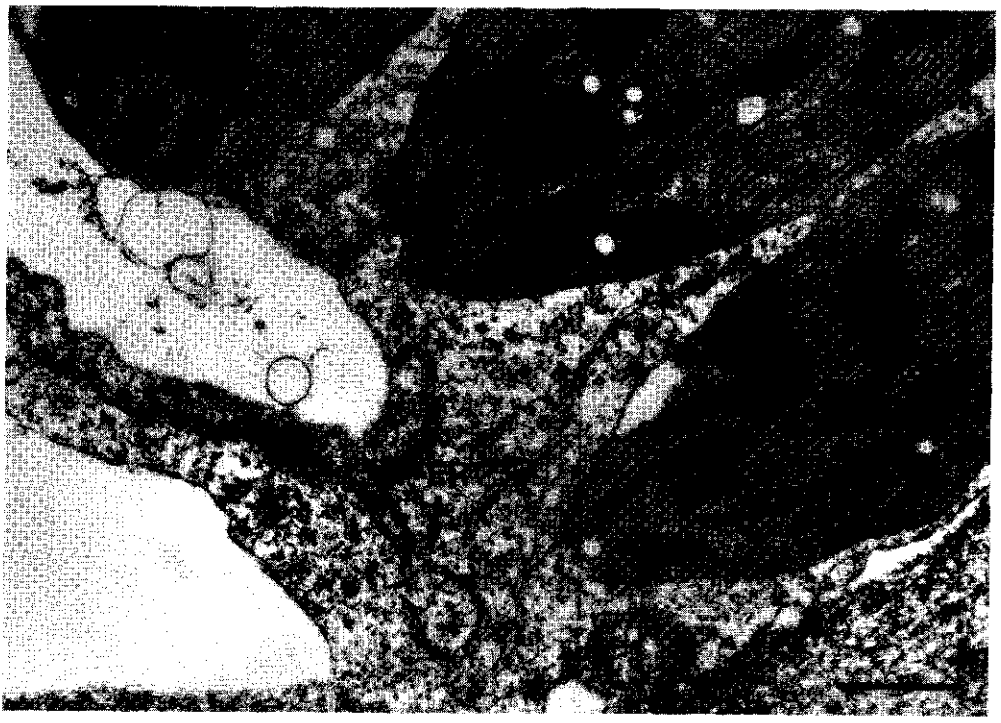


Fig. 4.2. Thin section of an osmium-fixed protoplast at 12 h after mock-inoculation with buffer. No disturbances in the endoplasmic reticulum (ER) are observed. C, chloroplast. Bar represents 500 nm.

Localization of CCMV coat protein antigen. In sections of protoplasts inoculated with CCMV-RNA and fixed with aldehydes and osmium tetroxide, no viral antigen could be detected by immunogold labelling. The damaging effect of osmium tetroxide on the antigenicity of proteins has been reported previously (see Chapter 3).

In sections of the same protoplast suspensions which were fixed with aldehydes, dehydrated and embedded in Lowicryl K4M at low temperature, the ultrastructure of cell organelles was preserved reasonably well. In most of the sections individual ribosomes could be observed. However, the preservation of membranes varied considerably between individual protoplasts. In most of the sections the membrane structure had disappeared, leaving a white band around the membrane-bound organelles. As a result, dictyosomes were only rarely observed. The ER could be observed more readily including the ribosomes at the

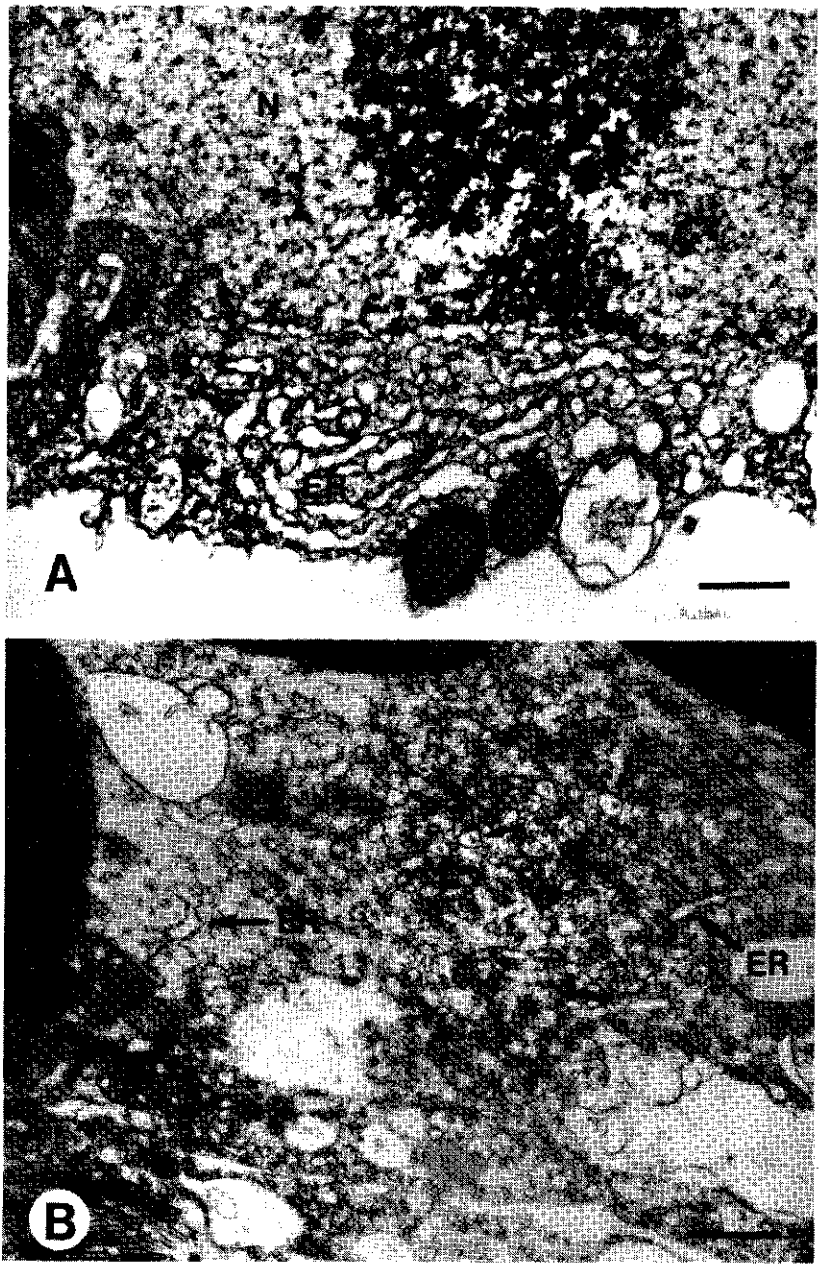


Fig. 4.3. Thin sections of osmium-fixed protoplasts at 12 h post-inoculation. A) Dilation and stacking of the endoplasmic reticulum (ER) in the cytoplasm near the nucleus (N) and B) cytoplasmic areas containing numerous small vesicles (Vs, arrows) and distended ER are consistent features of CCMV-infection. Bar represents 500nm.

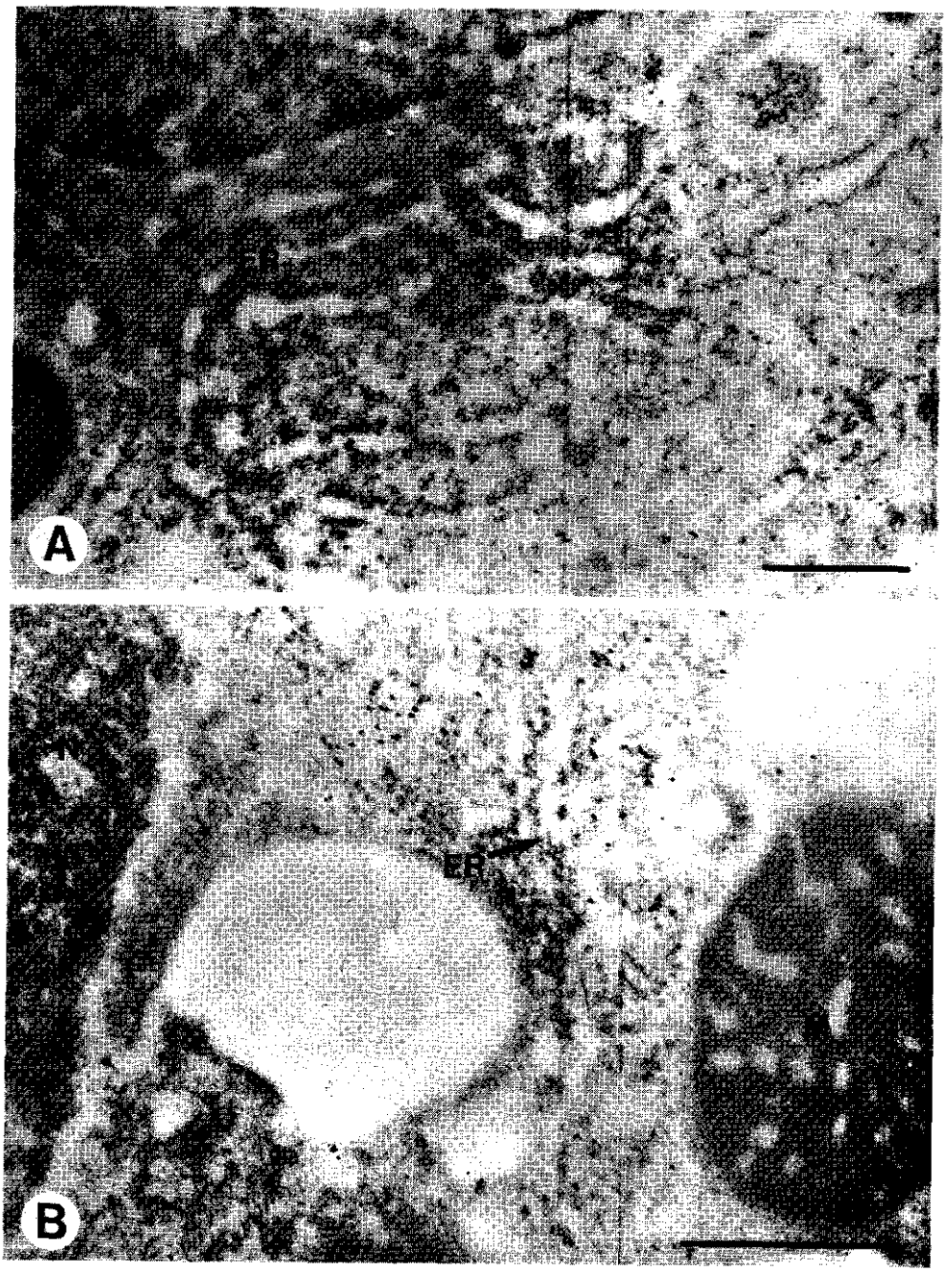


Fig. 4.4 A and B. Thin sections of Lowicryl embedded protoplasts at 6 h post-inoculation, treated with anti-CCMV and pAg-7. Gold label is found in the cytoplasm near the nucleus (N) in between and around ER which appears to be somewhat dilated. M, mitochondria. Bar represents 500nm

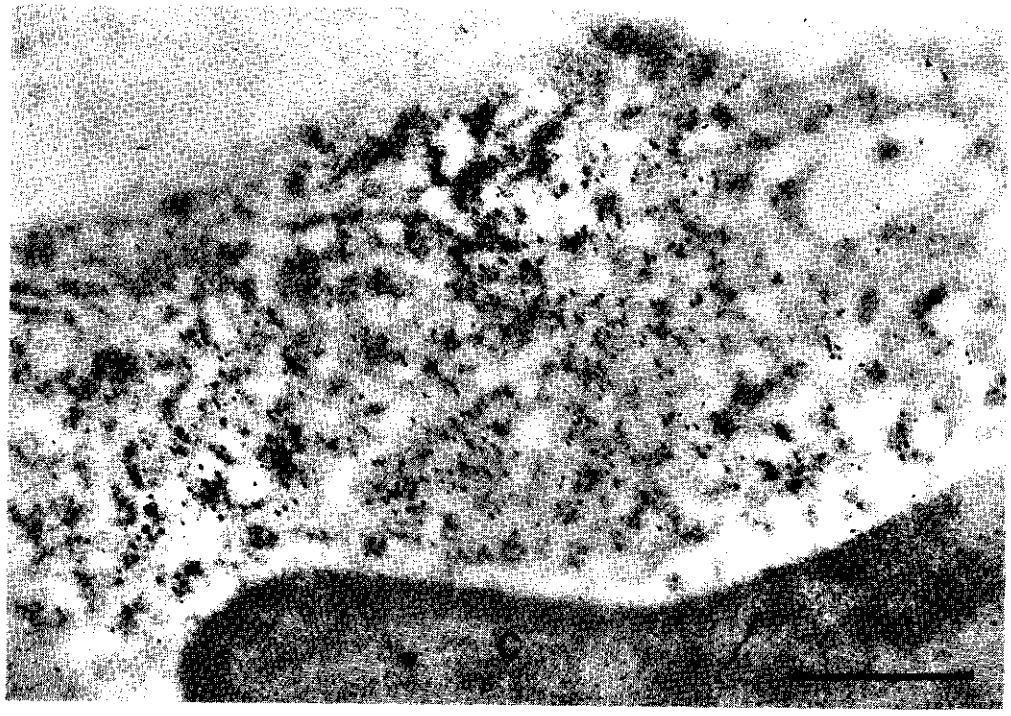


Fig. 4.5. Thin section of a Lowicryl embedded protoplast at 9 h post-inoculation, treated with anti-CCMV and pAg-7. Gold label is found in a large but restricted area of the cytoplasm. C, chloroplast. Bar represents 500nm.

surface of the membranes.

On sections of Lowicryl-embedded protoplasts specific gold labelling of coat protein antigen could be first observed at low density in some areas of the cytoplasm at 6 h post-inoculation (Fig. 4.4A and B). In Fig. 4.4A gold label is shown in the cytoplasm of a protoplast in between ER which appears somewhat dilated. A consistent association between gold labelling and (dilated) ER was not observed. Gold labelling was also found in areas of the cytoplasm without clear ER morphology, although the typical rough appearance of the cytoplasm in these labelled areas at later times (Fig. 4.5 and 4.6A) suggested the presence of membranous structures as observed in osmium-fixed protoplasts. The density of labelling and the area of the cytoplasm which was labelled increased from 6h post-inoculation to 9 h post-inoculation. In most of the protoplasts at t=9 h several of these labelled cytoplasmic areas could be observed at different locations in the cell. Specific labelling in these areas

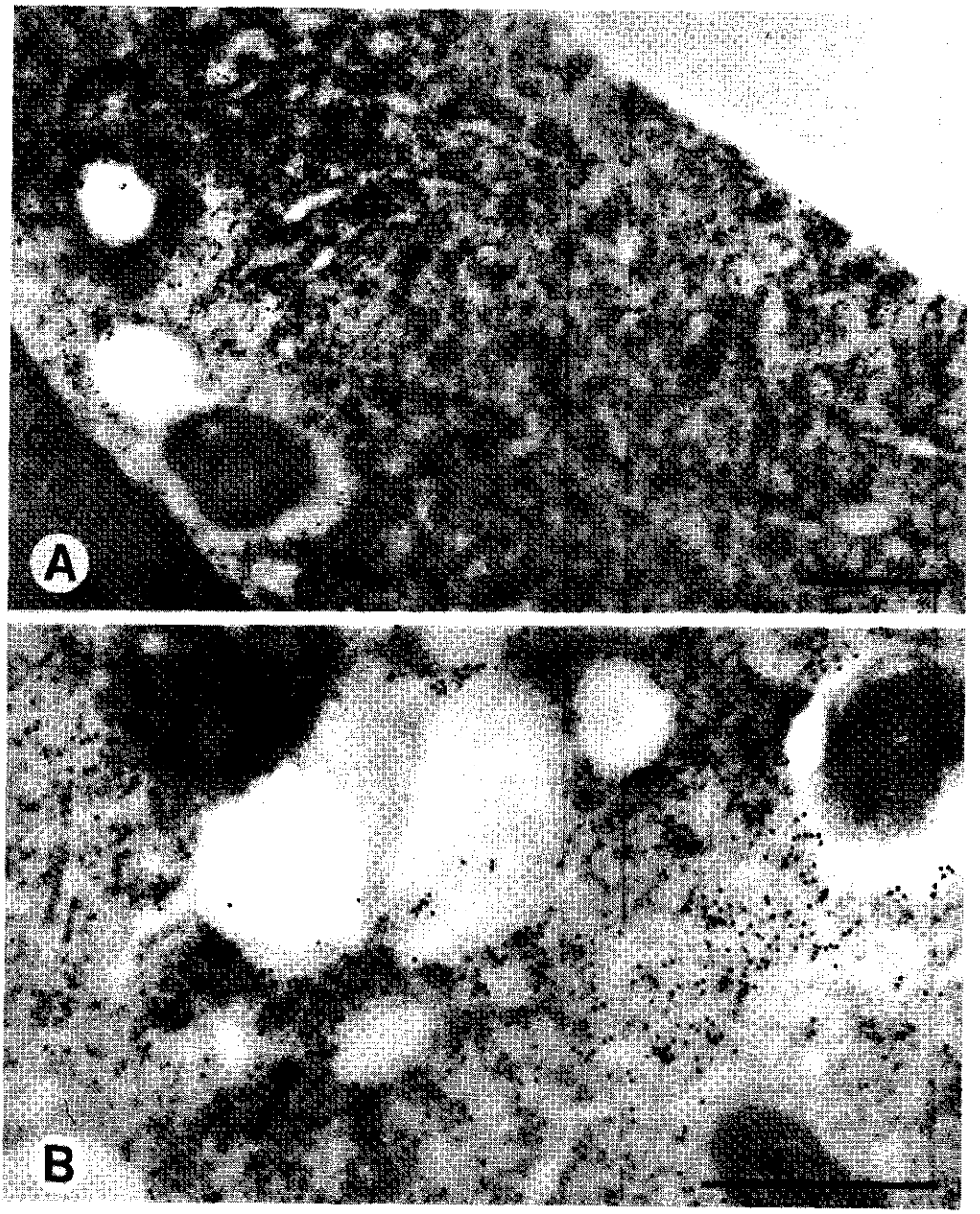


Fig. 4.6. Thin sections of Lowicryl embedded protoplasts at 24 h post-inoculation, treated with anti-CCMV and pAg-7. Gold label is located throughout the cytoplasm. A) In the cytoplasm areas with a rough structure are observed adjoining ER. B) Viral antigen appears not to accumulate equally in the entire cytoplasm. Certain areas of the cytoplasm are more densily labelled than others. C, chloroplast. M, mitochondrion. Bar represents 500nm.

Table 4.1. Quantification of gold labelling in six distinct areas of the cytoplasm identified by the presence of high densities of gold label. The area observed represents c. 1/6 of the total section of a protoplast at 9 h post-inoculation. Furthermore, the labelling density of the remaining cytoplasm is given as well as the density of labelling in the entire cytoplasm.

area (μm^2)	surface (μm^2)	pAg/μm^2
1	0.3	67
2	1.4	39
3	0.3	143
4	0.3	200
5	0.2	90
6	0.2	90
remaining cytoplasm	16.7	7
total cytoplasm	19.4	18

pAg/μm^2= protein A-gold particles per μm^2

was demonstrated by determining the density of labelling. In one micrograph of a protoplast at t=9 h, representing c. 1/6 of the total section, six labelled cytoplasmic areas could be identified by visual inspection. The surface of each area was measured and the pAg particles were counted. Table 4.1 shows the results of these countings. Labelling of these areas varied from 39 to 200 pAg/μm^2, while background labelling of cytoplasm was up to 4 pAg/μm^2. (Table 4.3). Labelling of the remaining cytoplasm was low (7 pAg/μm^2) and did not clearly exceed the background labelling. The labelling of the total cytoplasm (including the densily labelled areas) was 18 pAg/μm^2, which is about 4 times background labelling.

At t=12 h after inoculation CCMV coat protein antigen could be detected in nearly the entire cytoplasm. Cytoplasmic areas with higher labelling density were still present. Table 4.2 shows the results of quantification of the labelling density in complete sections of three infected and three mock-inoculated protoplasts at 12 h. The results show that at this time of infection labelling of the organelles in the infected cells does not exceed labelling of the same organelles in not-infected cells. Background labelling of the different organelles was between 2 and 7 pAg/μm^2. Labelling density of microbodies was higher than the labelling density of other organelles, but this occurred both

Table 4.2. Labelling densities of the organelles and cytoplasm in sections of three infected and three mock-inoculated protoplasts at 12 h post-inoculation. The protoplasts-sections were selected for the presence of the nucleus and (infected protoplasts) for the presence of specific labelling.

	CHL		MIT		VAC		MIC		NUC		CYT	
protopl.	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2
infected												
1	94	6	7.3	9	88	5	2.3	45	13	7	73	96
2	216	4	5.7	4	39	2	5.2	31	11	3	59	39
3	209	4	9.6	3	54	3	5.3	45	11	4	66	57
control												
1	109	3	1.9	4	34	3	2.6	21	21	3	49	3
2	91	4	4.8	4	50	4	2.8	39	13	3	55	4
3	77	4	6.7	4	37	3	2.0	47	3.2	4	26	4

A = area in μm^2; pAg/μm^2= protein A-gold particles per μm^2.
CHL, chloroplasts; MIT, mitochondria; VAC, vacuoles; MIC, microbodies; NUC, nucleus; CYT, cytoplasm.

Table 4.3. Quantification of the density of gold label on the different organelles in sections of infected and mock-inoculated protoplasts at 9, 12, 24 and 40 h post-inoculation. At each time one protoplast-section was selected for the presence of the nucleus and (infected protoplasts) for the presence of specific labelling.

	CHL		MIT		VAC		MIC		NUC		CYT	
inoc. time	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2	A	pAg$_2$ /μm^2
infected												
9 h	170	4	7	4	25	3	5	15	3	7	77	19
12 h	520	5	23	5	180	3	13	39	36	5	197	66
24 h	67	5	3	7	6	4	0.7	17	8	18	41	382
40 h	104	4	5	9	13	8	2	28	34	10	101	237
control												
9 h	62	2	4	4	5	4	5	20	15	4	42	2
12 h	277	4	13	4	121	3	7	37	37	3	130	4
24 h	48	5	3	1	7	3	2	19	21	2	40	2
40 h	37	4	2	3	8	3	1	39	36	3	43	2

A = area in μm^2; pAg/μm^2= protein A-gold particles per μm^2.
CHL, chloroplasts; MIT, mitochondria; VAC, vacuoles; MIC, microbodies; NUC, nucleus; CYT, cytoplasm.

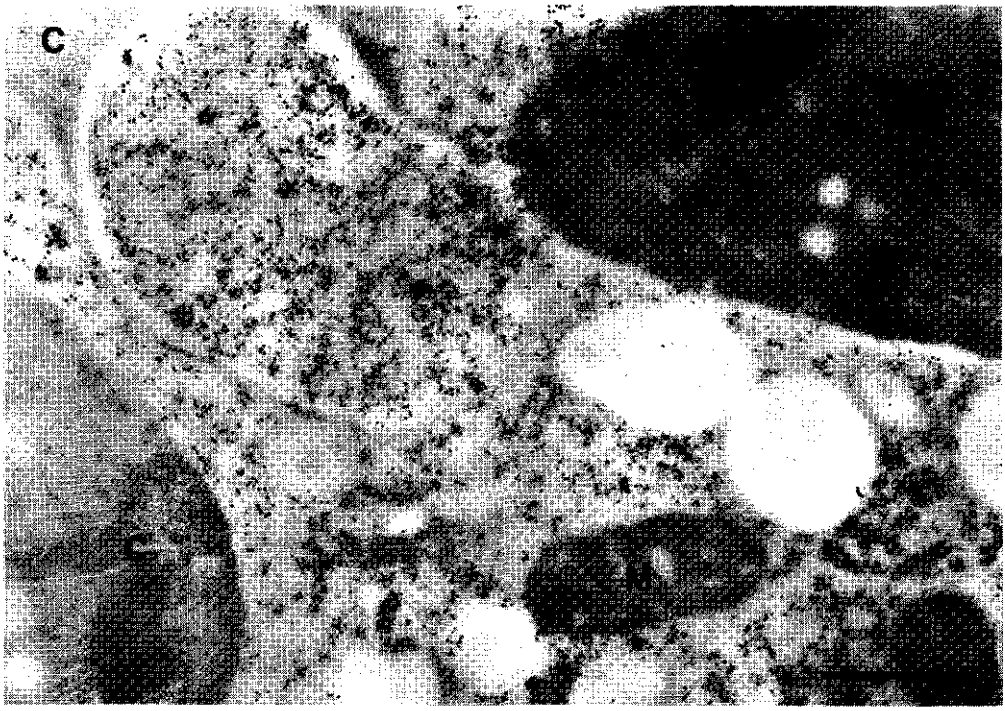


Fig. 4.7. Thin section of a Lowicryl embedded protoplast at 40 h post-inoculation, treated with anti-CCMV and pAg-7. Gold label only occurs on the cytoplasm, but not on chloroplasts (C) and mitochondrion (M). Bar represents 500nm.

in sections of infected and mock-inoculated cells. Only the cytoplasm was specifically labelled with gold and the density of labelling varied between the three protoplasts (96, 39 and 75 pAg/μm^2).

With time the density of labelling of the cytoplasm increased. Figs. 4.6A and 4.6B show parts of infected protoplasts at t=24 h and Fig. 4.7 represents a protoplast at 40 h post-inoculation. Gold label is present on the entire cytoplasm, not evenly spread (Fig. 4.6B), but at some locations the density of labelling was higher than elsewhere in the cytoplasm. Table 4.3 shows the

Fig. 4.8. Thin sections of Lowicryl embedded protoplasts at 24 h after inoculation with CCMV-RNA (A) and buffer (B), treated with anti-CCMV and pAg-7 showing part of the nucleus (N) and the nucleolus. Gold label is found on the nucleus and especially the nucleolus of the infected protoplast (A), while absent on the nucleus of a healthy protoplast. Bar represents 500nm.

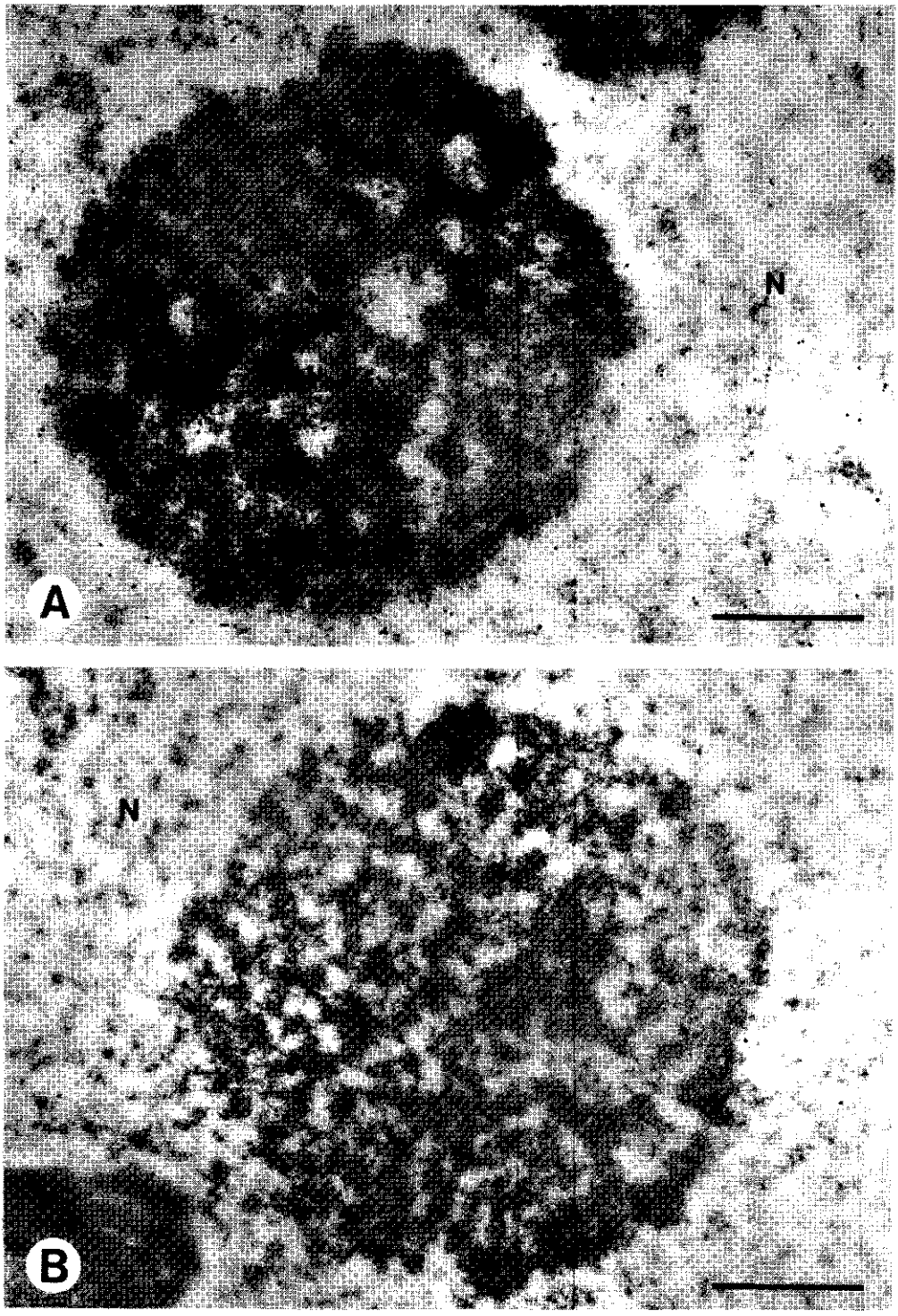

N
A
N
B

Table 4.4. Quantification of the labelling densities on three nuclei of both RNA- and mock-inoculated protoplasts at 9,12 and 24 h post-inoculation.

time after inoc.	no.	infected protoplast		control protoplast	
		nucleus**	nucleolus	nucleus**	nucleolus
9 h	1	3*	6	5	4
	2	5	2	5	6
	3	7	3	4	4
12 h	1	5	27	3	6
	2	6	15	4	2
	3	6	33	6	7
24 h	1	38	185	6	8
	2	18	150	5	4
	3	25	136	7	5

* figures indicate the number of protein A-gold (pAg) particles per μm^2.
** area of the nucleus minus the area of the nucleolus.

results of quantification of the labelling densities on complete sections of protoplasts at t=9, 12, 24 and 40 h after inoculation. The densities presented for t=12 h are averages of the densities in Tabel 4.2. The densities presented for t=9, 24 and 40 h were obtained from one complete section of a protoplast at each period. Labelling densities on the chloroplasts, mitochondria, vacuoles and microbodies in infected cells did not exceed the densities found on the same organelles in mock-inoculated protoplasts. The labelling density in the area of the nucleus, in particularly the nucleolus, appeared to increase in time. Figs. 4.8A and 4.8B show part of a nucleus and the nucleolus of an infected and not-infected protoplast, respectively, at 24 h post-inoculation. Table 4.4 presents labelling densities on three nuclei of protoplasts infected with CCMV (judged by detection of coat protein antigen in the cytoplasm) and three nuclei of mock-inoculated protoplasts at 9, 12 and 24 h respectively. Labelling of the nucleoli appears to be specific at 12 and 24 h, indicating the presence of coat protein antigen and the density of labelling increases in time.

DISCUSSION

CCMV-infection of cowpea protoplasts is accompanied by the formation of cytopathic membranous structures in the cytoplasm. Dilation of the ER and formation of vesicles were observed as consistent changes of the infected cell as early as 9-12 h post-inoculation. With time the area in the cytoplasm with distended ER and vesicles became larger. The distended ER and vesicles of different sizes seem to form a type of membranous body in the cytoplasm. Similar structural changes were reported for CCMV-infected tobacco protoplasts (Burgess et al., 1974; Motoyoshi et al., 1973) and such disturbances of the cytoplasmic membrane system seem to be a common feature of the bromovirus infection (BMV: Burgess et al., 1974; BBMV: Martelli and Russo, 1985). In addition, Kim (1977) observed amorphous inclusions in the cytoplasm and filamentous inclusions both in the cytoplasm and nucleus of CCMV-infected cowpea cells. The filamentous inclusions in the nucleus were often associated with the nucleolus. These types of inclusions were not found in the present study and were also not reported by Burgess et al. (1974) and Motoyoshi et al. (1973).

At the same time when abnormalities in the cytoplasmic membrane system are observed, newly synthesized coat protein antigen was localized in Lowicryl embedded sections of protoplasts. As an alternative to the description of a visual observation, quantification of the density of labelling on different organelles of both infected and not-infected cells represents another, but laborious way of demonstrating specific and background gold labelling. Coat protein antigen was localized in several restricted areas of the cytoplasm as early as 6-9 h post-inoculation, while no labelling above background was observed in the area of chloroplasts, mitochindria, vacuoles, microbodies and nucleus. These results indicate that coat protein synthesis takes place at the cytoplasmic ribosomes. For BBMV, Gibbs and MacDonald (1974) also demonstrated that translation of viral RNA only occurred at the cytoplasmic ribosomes. Furthermore, the synthesis of coat protein apparently starts at multiple locations in the cytoplasm as a result of translation and transcription of inoculum RNA.

The restricted areas containing gold label, indicating the presence of coat protein, may be the same as the membranous cytoplasmic areas with distended ER and vesicles, which were observed in well preserved protoplasts. The involvement of membranes in viral replication has been suggested for bromoviruses and many other viruses from different groups and seems to be a common feature of

plant virus infection (Francki et al., 1985). With respect to the involvement of membranes in bromovirus replication, De Zoeten and Schlegel (1967b) established by autoradiographic experiments that virus-dependent RNA synthesis of BBMV occurred in small areas in the cytoplasm, often near Golgi bodies. RNA-dependent RNA polymerase activity was shown in BBMV-infected (Jacquemin and Lopez, 1974) and CCMV-infected (White and Dawson, 1978) plants. Virus encoded enzymes were also detected and isolated from membrane fractions of BMV-infected plants (Semal et al., 1974; Bujarski et al., 1982). It seems, therefore, likely that CCMV-replication is located at the membranous structures. Localized replication of viral RNA, in particular coat protein messenger RNA, could also explain the apparently associated coat protein accumulation in cytoplasmic areas early in infection. Furthermore, the membranous structures may present suitable cellular conditions for virus particle assembly.

As CCMV infection progresses, coat protein antigen is detected in increasing areas of the cytoplasm. Already at 12 h post-inoculation gold labelling was observed over the entire cytoplasm of some protoplasts. These observations indicate the migration and accumulation of virus throughout the cytoplasm. With time the density of gold labelling of the cytoplasm increases as more virus accumulates. No coat protein antigen was detected at any time in the chloroplasts, mitochondria and vacuoles. Microbodies contained a relative high density of labelling as compared to other organelles, but a quantitative comparison of the labelling of microbodies in virus-infected and healthy protoplasts showed no specific gold labelling. Microbodies accumulate different enzymes, some of which may have some affinity for either antibodies or protein A. Microbodies, but also chloroplasts, mitochondria and vacuoles are probably not involved in CCMV replication, assembly and accumulation. The only organelle showing specific gold labelling was the nucleus, and in particular the nucleolus. Coat protein antigen was first detected in the nucleolus at 12 h after inoculation and gold labelling rapidly increased in the next 12 h to densities of labelling similar to those found on cytoplasm. The function of coat protein accumulation in the nucleolus of infected cells cannot be explained. The coat protein may have an affinity for ribosomal proteins and perhaps an infection-related function in the process of ribosome assembly, which takes place in the nucleolus (Perry, 1976; Jordan, 1978). The amount of antigen in the nucleolus is relatively high, as judged by the density of labelling. As immunogold labelling does not discriminate between virus particles or coat protein subunits, the nature of the antigen was not established. Bromovirus particles have been observed in the nuclei of infected plant cells

(BMV: Hills and Plaskit, 1968; Moline and Ford, 1974; CCMV: Kim, 1977; Martelli and Russo, 1985). With immunoautoradiography, Lastra and Schlegel (1975) detected BBMV antigen in the nucleus and cytoplasm of cells early in the infection. The authors suggested viral protein synthesis to occur in the nucleus. Protein synthesis, however, does not occur in the nucleus. CCMV or its coat protein apparently migrate from the cytoplasm to the nucleus and accumulate in the nucleolus. In this respect, the filamentous inclusions near the nucleolus described by Kim (1977) may be aggregates of CCMV-coat protein. Bancroft et al. (1969) showed the arrangement of CCMV-coat protein into narrow tubules under suitable conditions.

CHAPTER 5

LOCALIZATION OF STRUCTURAL AND NON-STRUCTURAL PROTEINS OF COWPEA MOSAIC VIRUS

SUMMARY

Infection of cowpea cells or protoplasts with cowpea mosaic virus (CPMV) is accompanied by the appearance of characteristic cytopathic structures. A major constituent of these cytopathic structures represented in thin sections by electron-dense material, was shown to contain viral non-structural proteins by its reaction with antisera to non-structural proteins and their detection with protein A-gold. With the same technique virus particles were found to accumulate mainly in the cytoplasm.

INTRODUCTION

The in situ localization of virus using antiserum against purified virus exposing its structural (coat) protein as reported in Chapters 3 and 4 for CCMV, has provided information on the initial events in virus infection (adsorption, penetration) and on the possible sites of virus multiplication (coat protein synthesis, virus assembly and virus accumulation). Conclusive evidence for the association between virus multiplication and cellular structures was not demonstrated for CCMV. However, the sites of capsid synthesis, virus assembly and virus accumulation do not necessarily have to be also the sites of viral RNA replication. More details about the sites of RNA replication could be obtained by localization of non-structural proteins and RNA.

Localization of non-structural proteins was not possible for CCMV, because antisera against such proteins were not yet available. Therefore, we used CPMV, a comovirus which also infects cowpea and of which antisera to three different non-structural proteins have been proven active with their respective proteins blotted on nitrocellulose (Wellink, 1987). Its genome is different from the CCMV genome in that it consists of only two positive strand RNAs which are separately encapsidated in identical protein shells. Both RNAs (denoted B and M RNA) have a small protein (VPg) linked at their 5' end, are polyadenylated and are expressed via the production of large, so-called polyproteins (for a review

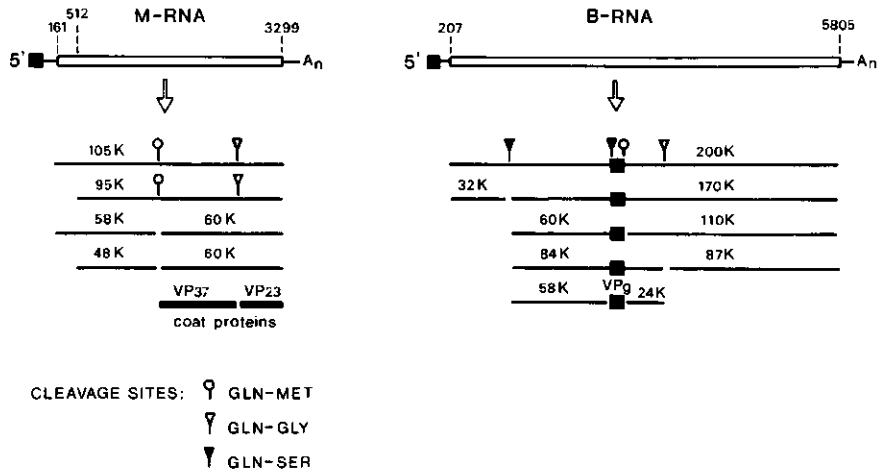


Fig. 5.1. Model for the expression of the CPMV RNAs. The open reading frames in both RNAs are indicated by white bars. Proteins are represented as single lines and VPg as a black square. All proteins shown are produced upon translation in vitro of the CPMV RNAs. With the exception of the 105K, 95K and 58K proteins, these proteins are also found in CPMV-infected protoplasts. The positions of the translational start and stop codons are indicated.

see Goldbach and Van Kammen, 1985). Figure 5.1 schematically shows how these polyproteins are processed into functional polypeptides.

Infection with CPMV is accompanied by the appearance of characteristic cytopathic structures (Assink et al., 1973; De Zoeten et al., 1974). These structures consist of amorphous electron-dense material and numerous membranous vesicles. Autoradiography performed on sections of isolated cytopathic structures treated with [^{3}H] uridine provided evidence that the replication of the CPMV RNAs was associated with these vesicles (De Zoeten et al., 1974), but the nature of the electron-dense material has remained unknown. Rezelman et al. (1982) showed that cytopathic structures were induced by infection with the B-component alone. These structures are, therefore, not related to progeny virus accumulation, since M-RNA encodes for the coat proteins.

In CPMV infected cells large amounts of virus particles are produced (up to 1 mg per g leaves). Besides, most of the non-structural proteins are also easily detectable in infected protoplasts by labelling with [^{35}S] methionine (Rezelman et al., 1980). We have used the immunogold labelling technique to localize virus particles and non-structural proteins within the infected cell.

MATERIALS AND METHODS

The antisera used for the experiments have all been characterized for their specificity and reactivity with CPMV-specific proteins. The anti-24K (kiloDalton) serum recognizes the 24K protein and all proteins containing the amino acid sequence of the 24K protein, namely the 170, 110 and 84K proteins (Fig. 5.1; Wellink et al., 1987). Anti-170K serum recognizes the 170, 110 and 87K proteins (Dorssers et al., 1984). Anti-VPg serum, obtained after injection of a rabbit with synthetic VPg (Jaegle et al., 1987), reacted with 170, 84 and 60K proteins on a protein blot (J. Wellink, unpublished results). The anti-CPMV serum was obtained by injecting a rabbit with purified CPMV components. Gammaglobulins (IgG) were purified from the antisera by affinity-chromatography on columns of sepharose CL-4B-protein A as described in Chapter 4.

Cowpea mesophyll protoplasts were isolated, inoculated with CPMV components or RNA and cultured under conditions of continuous light as described in Chapter 3. At different times after inoculation (t=0, 12, 24 and 48 h) samples of these cells were fixed, dehydrated and embedded at low temperature in Lowicryl K4M or methacrylate as described in Chapter 3. Ultrathin sections were subjected to immunogold labelling of antigens according to the method described in the same Chapter. The double-labelling was done essentially as described for virus suspensions on grids in Chapter 2.

RESULTS

In thin sections of inoculated protoplasts, adsorption of virus to the plasmalemma, formation of endocytotic vesicles containing virus and the occurrence of such vesicles in the cytoplasm up to 48 h were observed, similar to the phenomena reported for CCMV (Chapter 3). Small electron-dense structures were observed at 12h after inoculation with virus or RNA in the cytoplasm. Virus-induced vesicles were not observed at this time. Cytopathic structures consisting of both electron-dense material and an increasing number of membranous vesicles had developed in the cytoplasm after 24 (Fig. 5.2A and B) and 48h.

With anti-CPMV IgG and protein A-gold, newly synthesized virus was first detected at t=24 in protoplasts inoculated with RNA. The label was found throughout the cytoplasm of infected protoplasts and in between the vesicles

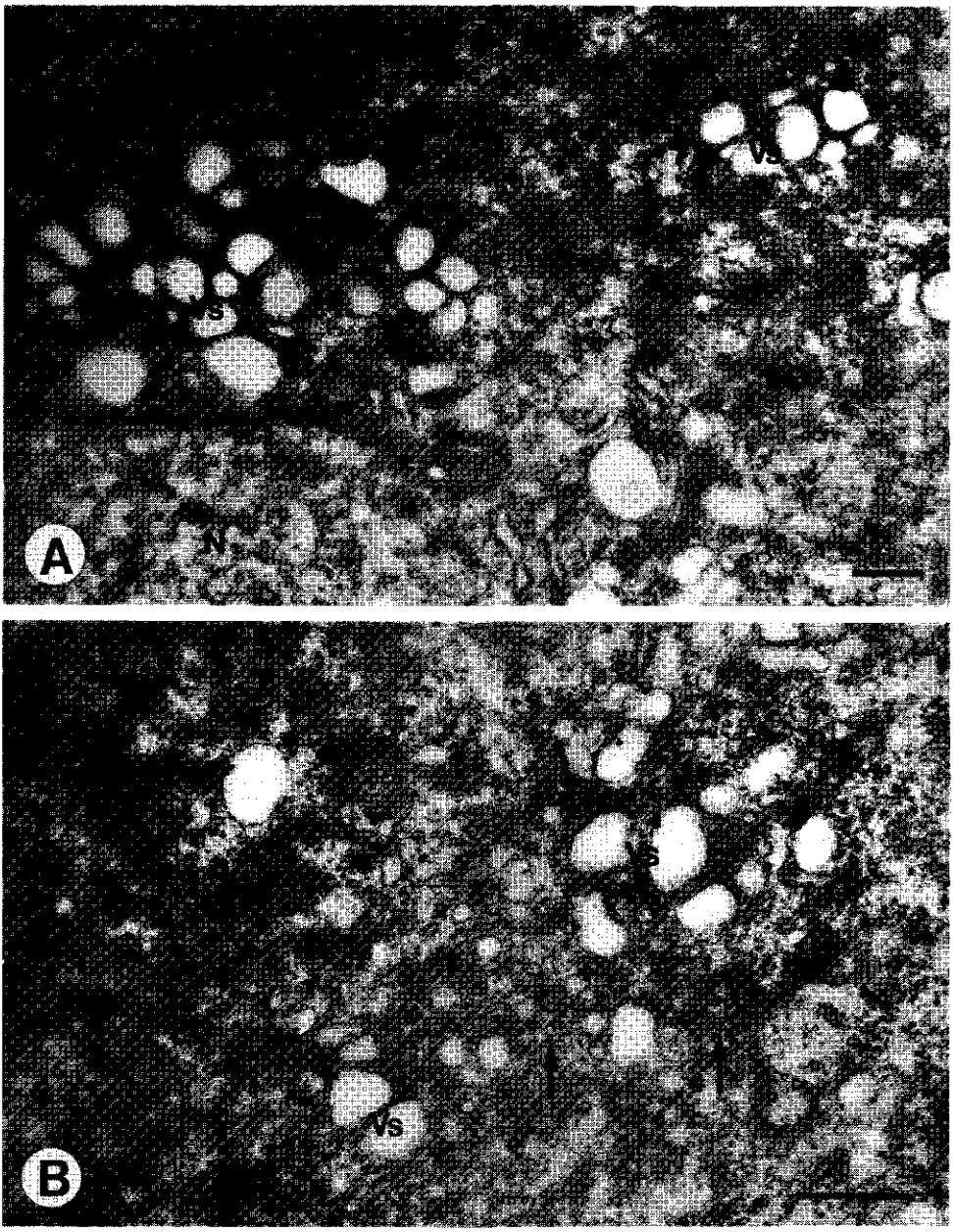


Fig. 5.2 A, B. Sections of a cowpea mesophyll protoplast, fixed with aldehydes and osmium tetroxide at 24 h after inoculation with CPMV. Cytopathic structures consisting of amorphous electron-dense material (ED) and membranous vesicles (Vs) are present in the cytoplasm. Arrows indicate fine membrane-like structures. N, nucleus. Bar represents 300nm.

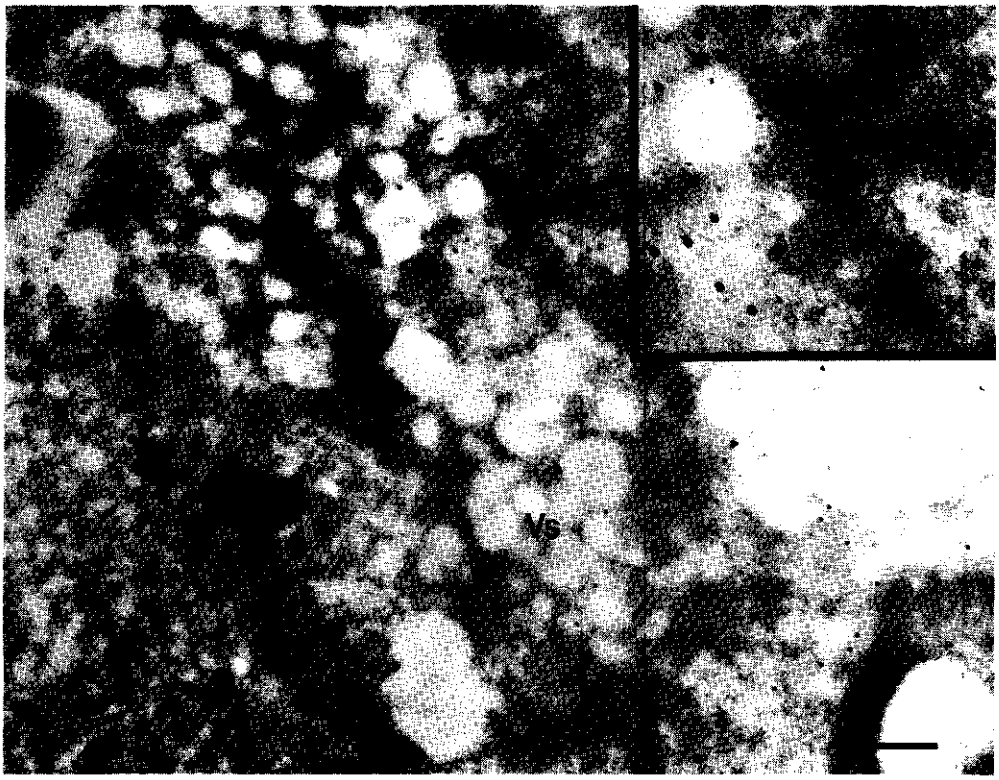


Fig. 5.3. Section of a cowpea mesophyll protoplast, embedded in Lowicryl K4M at 48 h after inoculation with CPMV. Both CPMV coat protein antigen and 24K-antigen are simultanously detected by double-labelling with 16 nm and 7 nm protein A-gold particles respectively. CPMV antigen is localized in the cytoplasm between the vesicles (Vs) and electron-dense structures (ED), while 24K-antigen is exclusively localized in the electron-dense material. Inset shows a detail of the electron-dense material. N, nucleus. Bar represents 500nm.

and electron-dense structures of the cytopathic complex. Fig. 5.3 shows the localization of virus and the 24K protein in the cytopathic complex of a protoplast at t=48 h. In this section CPMV and 24K protein are labelled using the double-labelling procedure. CPMV being labelled by 16-nm gold particles and the 24K protein by 7-nm gold particles. The 24K protein is localized only in the electron-dense material, while virus is present in between the vesicles and in the electron-dense material. In sections of infected protoplasts at t=12, 24 and 48 h after inoculation, incubated with anti-24K IgG and protein A-gold, gold label was exclusively located on the electron-dense material of the cytopathic complex (Fig. 5.4). Similar results were obtained with anti-VPg

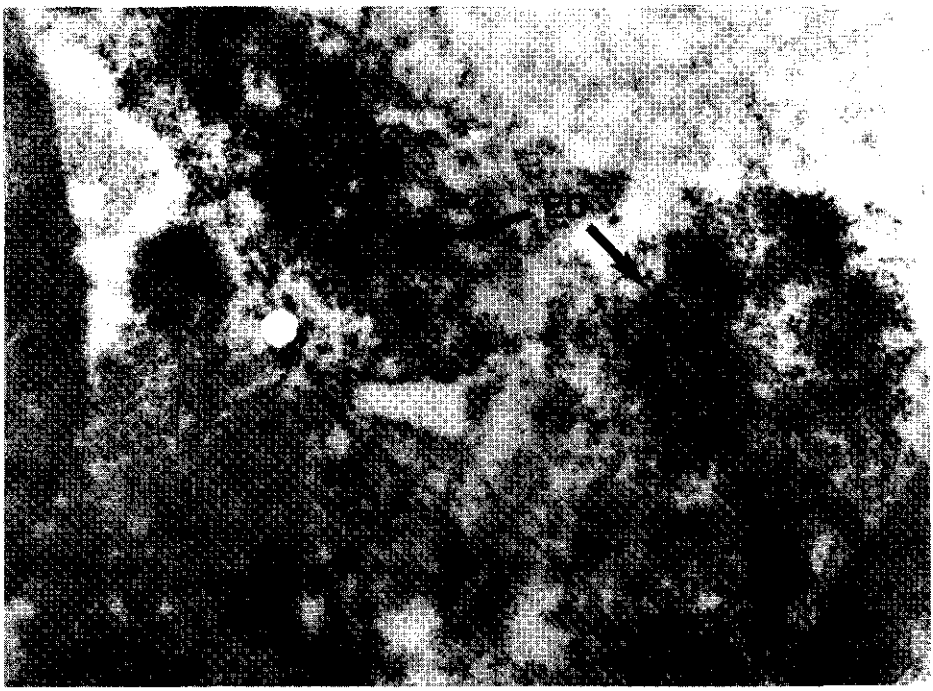


Fig. 5.4. Section of a cowpea mesophyll protoplast, embedded in Lowicryl K4M at 48 h after inoculation with CPMV and incubated with anti-24K IgG and protein A-gold (7 nm). Gold label is mainly present over the electron-dense material (ED). C, chloroplast. Bar represents 300nm.

serum (data not shown) and anti-170K serum. (Fig. 5.5). Gold label was not detected in or near the vesicles of the cytopathic complex, the cytoplasm or in cell organelles when antisera against non-structural proteins were used.

DISCUSSION

The initial events occurring after inoculation of protoplasts with CPMV were similar to those reported for CCMV (Chapter 3). Upon inoculation virus adsorbed to the plasmalemma, virus particles were occluded in vesicles formed by the plasmalemma and such vesicles were found scattered throughout the cytoplasm at later times. The location of newly synthesized virus in infected protoplasts was also similar to CCMV. Virus was detected in the cytoplasm only at 24 h and 48 h. These findings agree with the results obtained with red

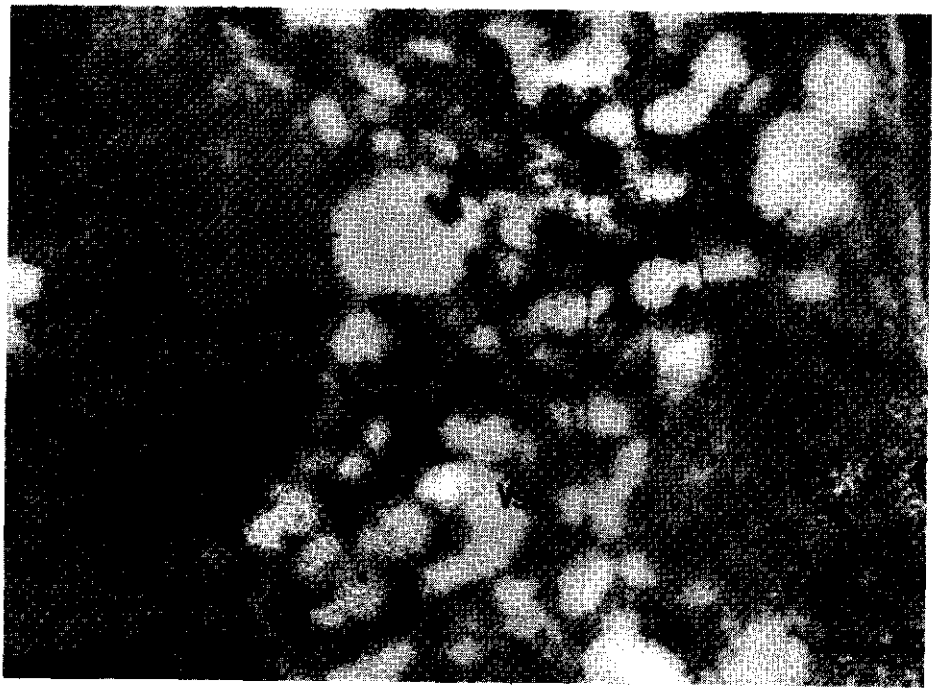


Fig. 5.5. Section of a cowpea mesophyll protoplast, embedded in Lowicryl K4M at 48 h after inoculation with CPMV, and incubated with anti-170K IgG and protein A-gold (7nm). Gold label is mainly present over the electron-dense material (ED) but not over and between the vesicles (Vs). C, chloroplast. Bar represents 300 nm.

clover mottle virus, another comovirus, in pea leaf cells (Tomenius et al., 1983).

The cytopathology of CPMV in cowpea protoplasts is, however, distinct from that of CCMV. In cells infected with CPMV cytopathic structures consisting of electron-dense material and membranous vesicles are prominent. At first the electron-dense material is found in protoplasts at t=12 h after inoculation and later an increasing amount of vesicles is formed. Similar results were reported by Rezelman et al. (1982). The results obtained with the antisera against the 24K and 170K proteins and VPg show that the electron-dense material contains (CPMV B RNA-encoded) non-structural proteins. So far we have not been able to determine whether all proteins, coded for by B-RNA, are present in this structure, since the three antisera used for these experiments each react with a set of overlapping proteins (Fig. 5.1). The non-structural proteins were not found in or near the vesicles of the cytopathic complex, although these

vesicles have been implicated in viral RNA replication (De Zoeten et al., 1974). The absence of label may be due to low concentrations of non-structural proteins being associated with these vesicles. Further investigations using cryofixation and subsequent immunogold labelling of frozen thin sections may increase the sensitivity of detection. It remains to be established whether the electron dense material also fulfils an active function in the viral RNA replication. It has been proposed that the viral proteins active in the replication of the CPMV RNAs are able to synthesize only one RNA strand (Van Kammen and Eggen, 1986). Hence, it is possible that these 'used up' proteins accumulate as electron-dense material.

Recently, Saito et al. (1987) localized the putative replicase components of tobacco mosaic virus (TMV), the 130 and 180K viral proteins, in granular inclusion bodies found in TMV-infected cells. The authors suggested these to be the possible sites of RNA-replication. Hills et al. (1987) localized the TMV-replicase in viroplasms and in clusters of virus particles at the edge of or within these viroplasms. The granular inclusion bodies described by Saito et al. and the viroplasms reported by Hills et al. are probably immature forms of the so-called X-bodies which consist of membranous vesicles, ribosomes, X-tubules and virus particles (Esau and Cronshaw, 1967). In analogy of the electron-dense structures of CPMV, these bodies may in part represent accumulations of wasted or metabolically inert proteins, as suggested by Hills et al. (1987), while viral replication takes place elsewhere.

CHAPTER 6

DETECTION OF VIRAL ANTIGEN IN SEMI-THIN SECTIONS OF PLANT TISSUE BY IMMUNOGOLD-SILVER STAINING AND LIGHT MICROSCOPY

SUMMARY

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 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 were 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.

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 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).

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 in Chapter 2. Gammaglobulin (IgG) was purified by affinity chromatography as described in Chapter 4.

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 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 ^{o}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 microscopical 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 polymerized at 50 °C for 24 h. Semi-thin sections (approx. 3 um) 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 in Chapter 2. Semi-thin sections were incubated for 30 min in PBS containing 1% (w/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 x 5 min in PBS-Tween, fixed for 15 min in 1% (w/v) glutaraldehyde in PBS and washed 3 x 5 min in double distilled water. Controls consisted of sections incubated with anti-TMV IgG instead of anti-CCMV IgG and sections of healthy tissue treated likewise. 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 in Chapter 3.

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 3.8, 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. Preparation of the final developing solution as well as 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 x 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 (x25, x50 and x100) and a polarization filter-block (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

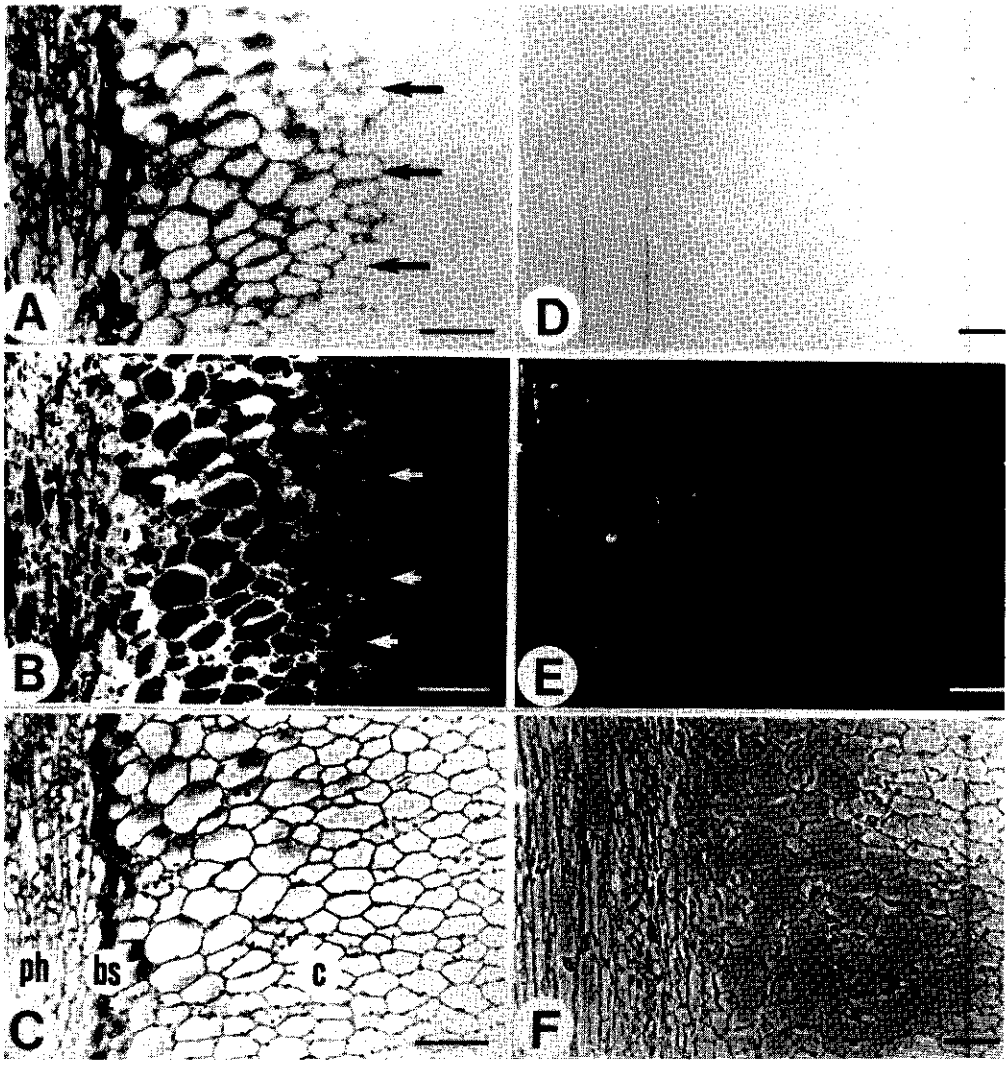


Fig. 6.1. Longitudinal section of a petiolule 24 h after systemic inoculation with CCMV. Sections were incubated with anti-CCMV (A, B, C) or anti-TMV (D, E, F), followed by protein A-gold and subsequent enhancement with silver for 14 min at 20°C. Silver stain (arrows) is observed as a black precipitate with brightfield illumination (A) and as a brilliant white precipitate with dark-field 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.

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 silverstaining 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. 6.1 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. 6.1A) and as a brilliant white precipitate with darkfield illumination (Fig. 6.1B). Phase-contrast illumination was applied when both black precipitate and cell structures had to be observed (Fig. 6.1C). 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. 6.1D, 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 color 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. 6.2. 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. 6.2A and B) and glutaraldehyde fixation prior to silver enhancement (Fig. 6.2C 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. 6.3B and 6.4B). 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

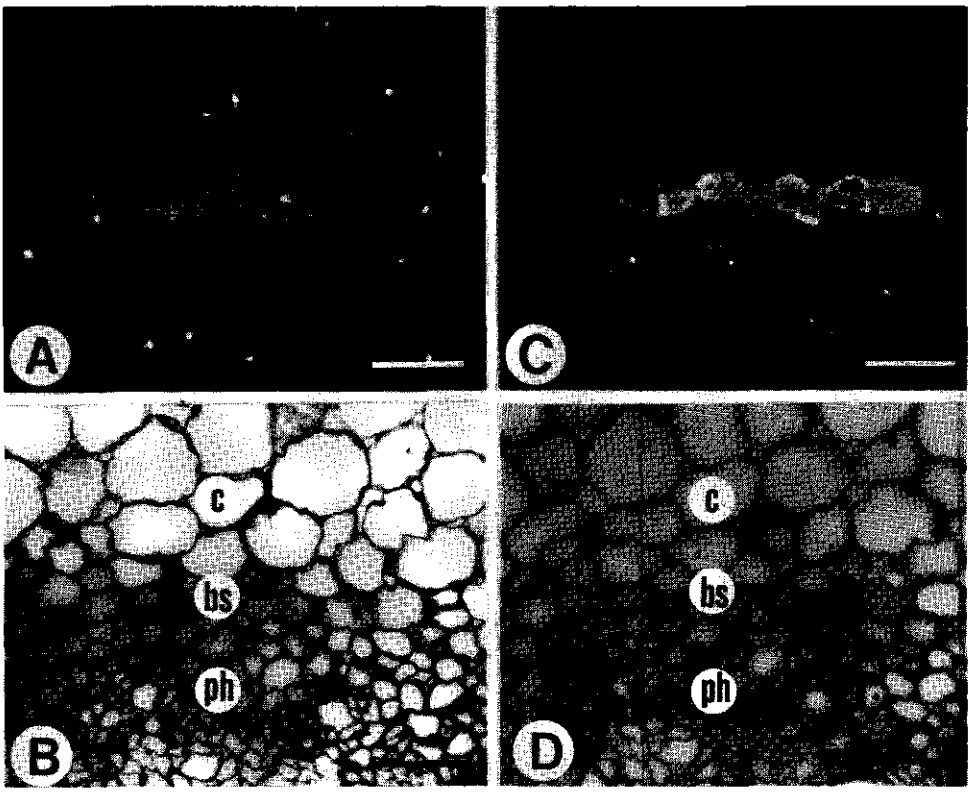
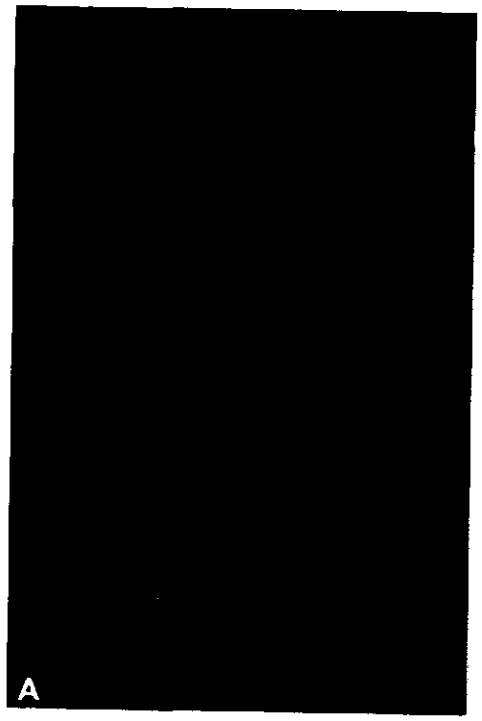


Fig. 6.2. Serial cross sections of a petiolule 24 h after systemic inoculation. Sections were incubated with anti-CCMV, protein A-gold and subsequently enhanced with silver for 14 min at 20 °C (A, B) or fixed with glutaraldehyde prior to silver enhancement (C, D). Sections were cytochemically stained with toluidine blue. Silver stain is observed as a brightly shining precipitate with epi-illumination (A and C), while cell morphology is visualized with bright-field transillumination (B and D). Bar represents 10 μm

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 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 (x100). However, silver enhancement for 7-8 min was convenient to detect low levels of specific labelling even at low magnification.



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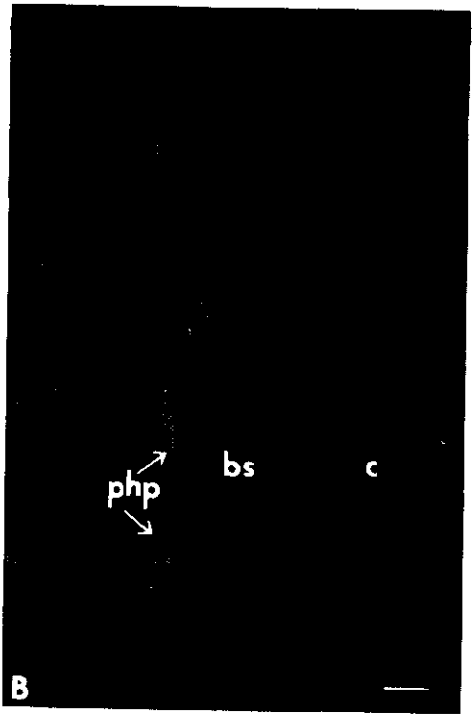

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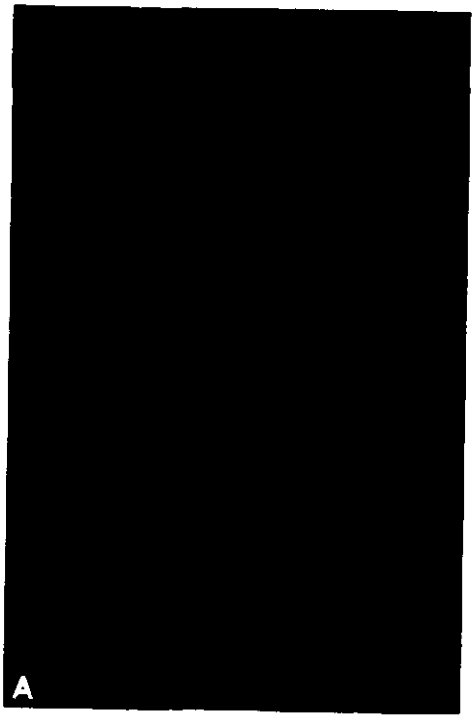
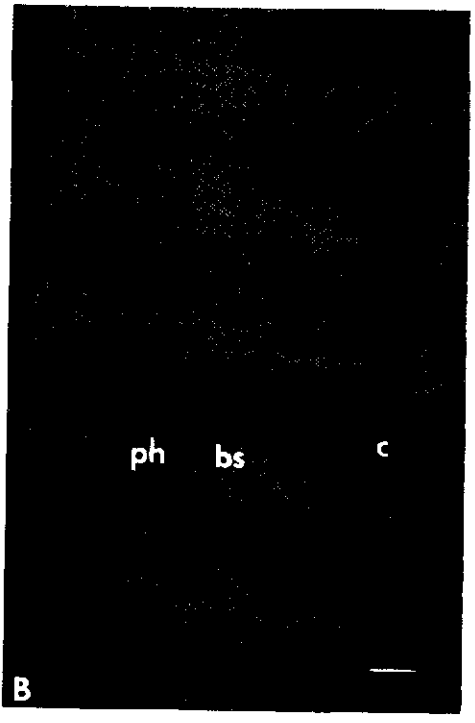




To illustrate the potential of IGSS with epipolarization microscopy CCMV was immunogold-labelled in longitudinal sections of an infected petiolule, fixed, developed and observed with epipolarized light (Fig. 6.3A and 6.4A) or with epipolarized light in combination with brightfield transillumination (Fig. 6.3B and 6.4B). Petiolules of trifoliate cowpea leaves were collected 6 h (Fig. 6.3) and 24 h (Fig. 6.4) 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.

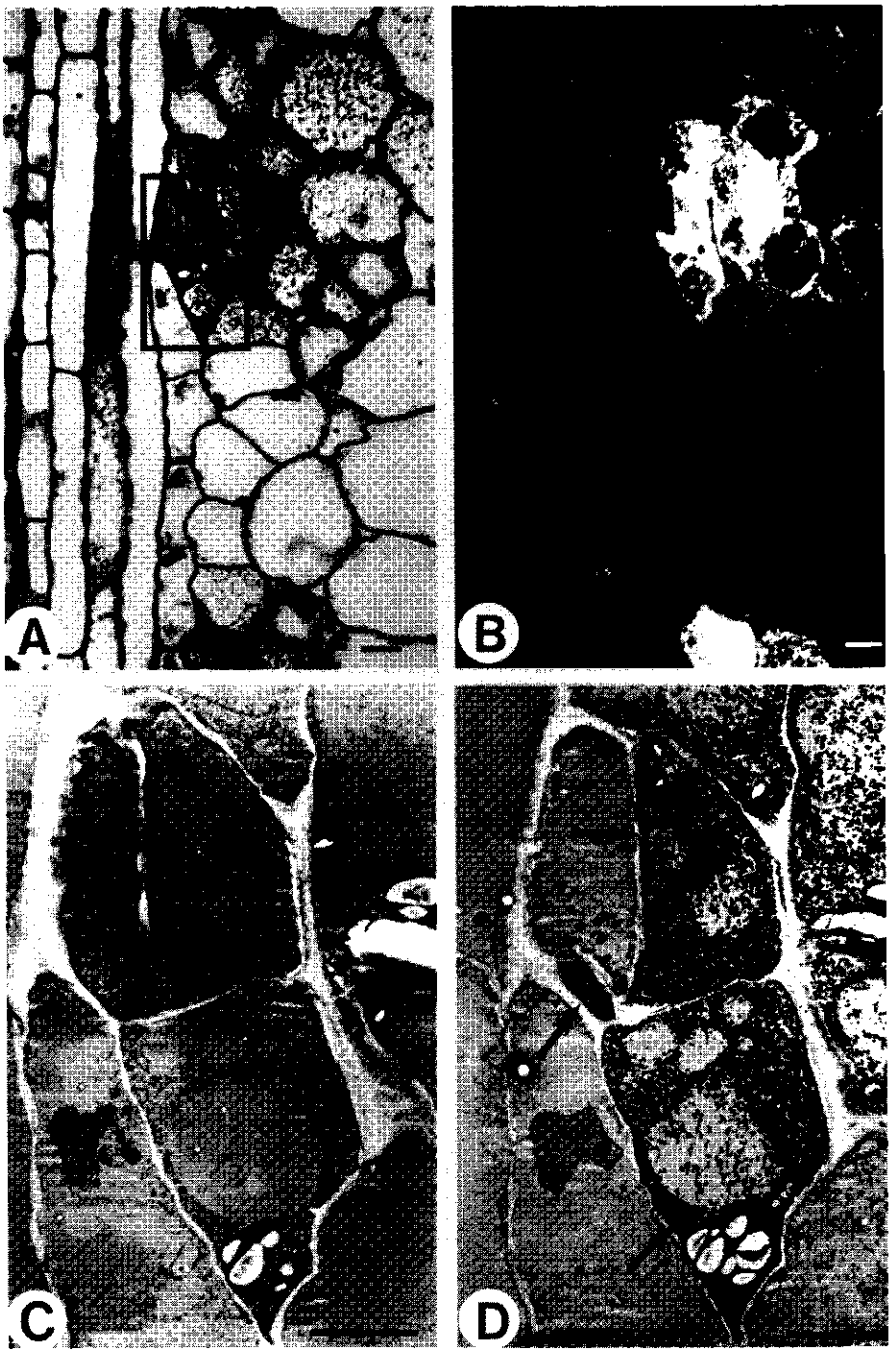
The immunogold-silver staining as demonstrated for light microscopy may be used also in electron microscopy. On one hand it offers the possibility to compare preparations of light (Fig. 6.5A and B) and electron (Fig. 6.5C and D) microscopy directly. On the other hand silver enhancement of gold label in electron microscopy allows viewing of label on sections at low magnification (compare Figs. 6.5C and D, without and with silver enhancement respectively)

DISCUSSION

The immunogold-silver staining (IGSS) described 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

Fig. 6.3. 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. 6.4. 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.


A
B
C
D

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 lightmicroscopy 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. 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-illumination optics and a polarizing filterblock. The labelled specimens can be re-examined at will without loosing signal and

Fig. 6.5. 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). Semithin 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.

the labelling 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.

CHAPTER 7

LIGHT MICROSCOPICAL STUDIES ON THE DISTRIBUTION OF VIRAL ANTIGEN IN INFECTED PLANTS

SUMMARY

Translocation of CCMV was studied cowpea plants which were systemically inoculated with CCMV by differential temperature treatment. Time zero (t=0) of systemic inoculation was defined as the moment when plants were transferred to conditions permissive for virus multiplication. Up to t=6 h after systemic inoculation none or only small quantities of virus were detected in the secondary leaves by the enzyme-linked immunosorbent assay (ELISA). Virus concentration rapidly increased between 6 and 24 h, indicating some synchrony of virus multiplication. CCMV coat protein antigen was detected by immunogold-silver staining (IGSS) and epipolarization microscopy in semi-thin sections of aldehyde fixed and LR White embedded samples of the stems, petioles, petiolules and leaves of systemically inoculated plants. At t=0 the antigen could only be detected in the phloem and bundle sheath of stems and petioles, but not in the petiolules and leaves. In the petiolule coat protein antigen was first detected in phloem parenchyma cells at 3 h after systemic inoculation. From t=3 h onwards the antigen was detected progressively in the neighbouring tissues, first in the bundle sheath, followed by the cortex en finally at t=24 h also in the procambium and xylem. Similar patterns were observed in mayor veins of the secondary leaves and in the leaf blade tissue, where coat protein antigen was first detected at t=3 h in the phloem of minor veins and subsequently in the mesophyll and epidermal cells. CCMV is apparently transported through the phloem, following the routes of assimilate transport. No viral antigen was detected in mature sieve elements or companion cells at any time after systemical inoculation, indicating that the virus is possibly transported in a form dissimilar to virus particles.

INTRODUCTION

Protoplasts supply a suitable model system for the study of different steps in the viral replication cycle, because of the high percentage of synchronously infected cells that can be obtained (Takebe, 1983; Murakishi et al., 1984). However, these systems lack cell-to-cell contacts and are therefore not suited to study cell-to-cell transport and long distance transport. Dawson and Schlegel (1973; 1976) developed a differential temperature inoculation technique, which enabled the investigator to infect leaf tissue more or less synchronously (also referred to as: systemic inoculation).

Here we report on the light microscopic distribution of CCMV in systemically inoculated cowpea plants as function of the infection time. Viral antigen was localized in semi-thin sections of resin embedded stem, petiole, petiolule and leaf tissue, by the immunogold silverstaining (IGSS) and epipolarization microscopy (Chapter 6).

MATERIALS AND METHODS

Virus and antiserum. CCMV was maintained, purified and used as antigen to raise antiserum as described in Chapter 2. Gammaglobulin (IgG) was purified by affinity chromatography on a column of sepharose CL-4B-protein A as described in Chapter 4.

Systemic 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 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. During this treatment, the primary leaves were kept in light at 27-30 ^{o}C, and the secondary leaves were kept in the dark at 10 ^{o}C. After three or four days the plants were transferred to a growth cabinet at 25 ^{o}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, consisting of whole trifoliate leaves, single leaflets or discs, were taken from the leaflets at different times after systemic inoculation. When leaves were separated from the plant at t=0, the petiole was wrapped in paper

tissue soaked in Hoagland's nutrient solution and the whole was placed in a petridish. Alternatively, discs were cut out of the base of the leaf (one on each side of the major vein) at t=0 using a 5 mm diameter cork bore and floated on Hoagland's nutrient solution. Virus concentration in the leaf samples was estimated using the direct double antibody sandwich procedure of the enzym-linked immunosorbent assay (ELISA). Infectivity of the samples was tested by local lesion assay on Chenopodium hybridum.

ELISA. Preparation of the alkaline phosphatase and anti-CCMV-IgG conjugate and performance of the ELISA test were carried out as described by Clark and Adams (1977). The sample buffer consisted of phosphate buffered saline (PBS) pH 7.4, containing 0.05% (v/v) Tween 20 and 1% (w/v) polyvinylpyrrollidone (PVP). Samples of the systemically inoculated leaflets were homogenized in sample buffer in a ratio of 0.1 g leaf material to 1 ml buffer, centrifuged for 5 min at 10.000 g and the supernatant was further diluted ten times. Controls consisted of samples taken from healthy leaflets prepared as described above. The reaction of the substrate was quantified by measuring the absorption at 405 nm (A_{405}) in a Titertek Multiscan at different times after addition of the substrate. Dilutions of purified CCMV in an extract of healthy leaves were used as standard samples to estimate virus concentration.

Fixation and embedding of plant tissue. Relatively large pieces of the stem, petiole, petiolule (1-2 mm) and leaf (2 x 3 mm) were fixed in glutaraldehyde/-paraformaldehyde, dehydrated in ethanol and subsequently embedded in LR White as previously described (Chapter 6). Semi-thin sections were cut and mounted onto microscope slides as described.

Immunogold-silver staining (IGSS). CCMV was localized in semi-thin sections of embedded tissue samples by IgSS as described in Chapter 6. Sections were cytochemically stained with 1% (w/v) toluidine blue in 1% (w/v) sodium tetraborate in distilled water. Cytochemically stained sections were examined with epi-illumination in a Leitz Orthoplan microscope equipped with water-immersion objective lenses (x25, x50 and x100) and a Leitz polarization filter-block (epipolarization microscopy). Unstained sections were examined with a Wild light microscope with either brightfield, darkfield or phase-contrast illumination.

RESULTS

CCMV multiplication in the secondary leaves of cowpea plants was monitored by estimating the virus concentration in leaf samples with ELISA at different times after systemic inoculation. The primary leaves of 7 cowpea plants were inoculated with CCMV and the plants were subjected to a differential temperature treatment for 4 days. Of five out of seven plants (1-5) the trifoliate leaf was left attached to the plant at t=0, while the trifoliate leaves of the other two plants (6 and 7) were removed from the stem and separately incubated (see materials and methods section). At t=0, 3, 6, 12 and 24 h the trifoliate leaf was taken from plants 1 to 5 respectively and analyzed for virus content. From each of plants 6 and 7 a leaflet of the trifoliate leaf was sampled at t=0, 12 and 24 resp. and analyzed for virus content. Table 7.1 lists the CCMV concentrations in the different leaf samples as determined by ELISA. No CCMV could be detected with ELISA in the leaves of 7 plants at t=0. Low concentrations of virus were detected at t=3 and t=6 h, and virus concentration rapidly increased between 6 and 24 h after systemic inoculation. Both in leaves left attached to the plant and in leaves separated from the inoculum source (primary leaves) at t=0, virus concentration increased to about 0.5 - 1 mg virus per gram of leaf tissue at 24 h.

In a second experiment cowpea plants were subjected to three days of differential temperature treatment. Samples for ELISA consisted of a total of 6 discs taken from each trifoliate leaf of three plants (1-3) at t=0. At t= 0, 3, 6, 12 and 24 h from each plant a disc was tested in ELISA. The remainder of the leaf of plants 1 and 2 was left attached to the stem and at t=0, 12 and 24 h a leaflet was sampled. Table 7.2 lists the virus concentration in the discs and leaf samples. No virus or could be detected with ELISA in any of the samples at t=0. When testing the same samples by mechanical inoculation on Chenopodium hybridum no infectivity was found. Virus was first detected by ELISA in one plant (3) at t=3 h and in the other two plants (1 and 2) at t=6 h and virus concentration rapidly increased in the following 18 h. The average virus concentration was considerably lower in leaf tissue of the discs compared to leaf tissue sampled from the remainder of the leaves.

In both experiments also samples were taken at t=0, 3, 6, 12 and 24 h from the stem, petiole, petiolule and leaf and these samples were processed for light microscopical study. In Fig. 7.1 a schematic drawing is shown of the cowpea plant and locations are indicated from where samples were taken. Leaf samples were taken from the base of the leaf and included a part of the first

Table 7.1. Concentration of CCMV in leaf tissue estimated by ELISA at different times after 4 days of differential temperature treatment.

	µg CCMV/g leaf		
t	1-5	6	7
0[1]	<<[2]	<<	<<
3	1.2	ND[3]	ND
6	1.2	ND	ND
12	800	33	142
24	1052	1005	565

1) At each indicated time a trifoliate leaf was sampled from one of five plants with the trifoliate leaves left attached to the plant (1-5; each value represents a different plant). From each of two trifoliate leaves, separated from plants 6 and 7 at t=0, a leaflet was sampled at 0, 12 and 24 h.
2) << = virus not detectable.
3) ND = not determined.

Table 7.2. Concentration of CCMV in leaf tissue estimated by ELISA at different times after 3 days of differential temperature treatment.

	µg CCMV/g leaf				
t	1	2	3	1'	2'
0[1]	<<[2]	<<	<<	<<	<<
3	<<	<<	1	ND[4]	ND
6	1	2	2	ND	ND
12	7	4	9	20	22
24	45	59	79	398	>>[3]

1) Samples consisted of discs cut from the leaf at t=0 and incubated on nutrient solution (1, 2 and 3) and of the remaining leaflets from plants 1 and 2 left attached to the stem (resp. 1' and 2'). At each time, one disc or one leaflet was sampled.
2) << = virus not detectable.
3) >> = A_{405} exceeded 2.0.
4) ND = not determined.

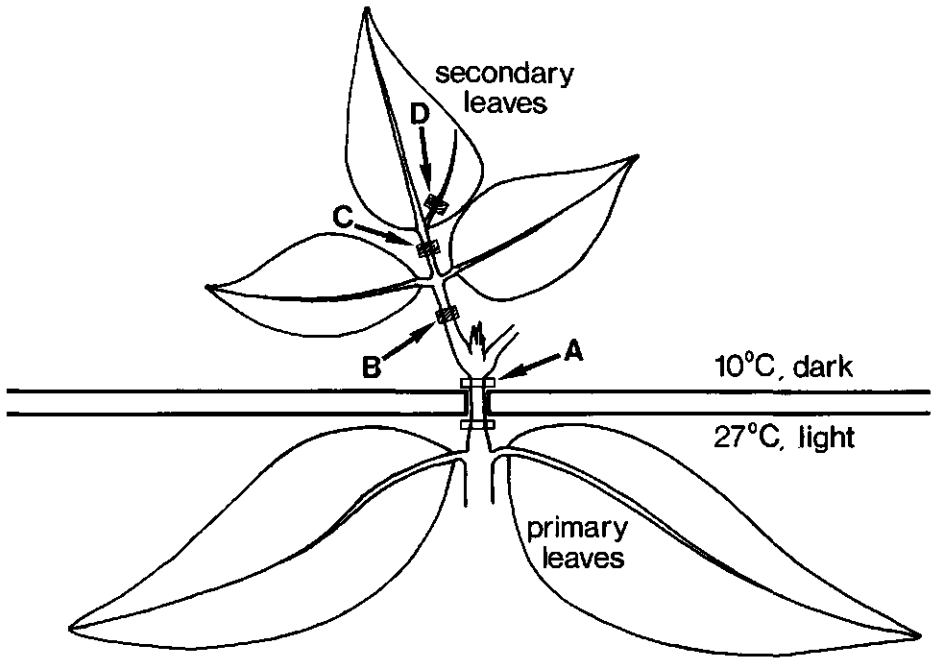


Fig. 7.1. Schematic drawing of a young cowpea plant during differential temperature treatment. The positions of samples for light microscopic study are indicated: A = stem; B = petiole; C = petiolule; D = leaf.

branching vein and neighbouring blade tissue.

At t=0, CCMV was detected in cross-sections of the stem (Fig. 7.2A), petiole (Fig. 7.2B) and petiolule (Fig. 7.3A), but not in the vein (Fig. 7.5A) or blade (Fig. 7.6A) tissue of the leaf at t=0 after 4 days of differential temperature treatment. In the stem (Fig. 7.2A), virus was detected in numerous cells of the phloem and some cells of the bundle sheath of all vascular bundles, but not in the pith, xylem, procambium and cortex cells. In the petiole (Fig. 7.2B) virus was detected in a limited number of phloem cells of all vascular bundles, whereas in the petiolule (Fig. 7.3A) only four cells with detectable virus were found in the phloem. These cells were identified as phloem parenchyma cells. Both in the petiole and petiolule virus was not detected at t=0 in the pith, xylem, procambium, bundle sheath and cortex. In time the number of cells with label increased rapidly. At t=6 large numbers of phloem cells and some bundle sheath cells were labelled in the petiole, petiolule (Fig. 7.3B) and vein. In the course of time label was observed in an

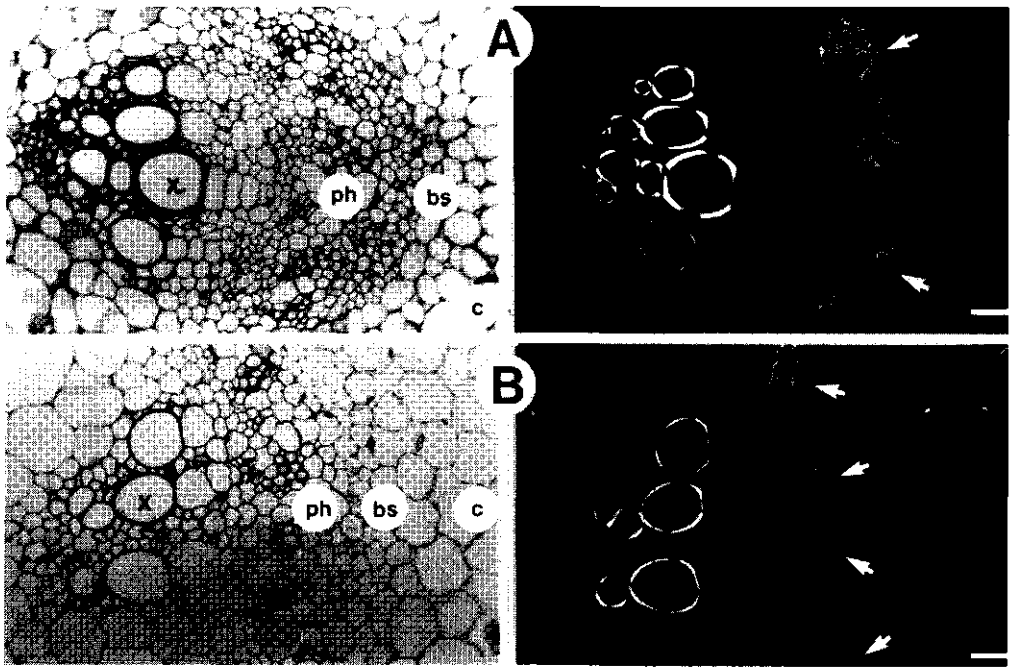


Fig. 7.2. Immunogold/silver staining of CCMV in cross-sections of the stem (A) and petiole (B) at t=0 after differential temperature treatment. Silver stain (arrows) is observed in the phloem (ph) and bundle sheath (bs) of the stem (A) and in the phloem of the petiole (B). Sections were stained with toluidine blue. The micrographs on the left are made with transillumination and those on the right with epipolarization illumination. c = cortex; x = xylem. Bar represents 10 μm.

increasing number of phloem cells, bundle sheath cells and neighbouring cortex cells and in some cells of the procambium. At t=24 h label was found in large areas of the cortex in both petiole and petiolule (Fig. 7.3C), but also in some undifferentiated tracheary elements of the primary xylem. In longitudinal sections of vascular tissue in the petiole and petiolule (Fig. 7.4) where sieve elements (sieve-tubes and companion cells) could be clearly identified, label was not found on these cells at any time after systemic inoculation. In these tissues label was only found in the phloem parenchyma. When counting labelled cells from the location of sieve elements in the phloem in radial direction towards the outer side of the petiolule, the maximum number of cells containing label was 7, 12 and 22 at t=6, 12 and 24 h, respectively.

In the leaf virus was first localized at t=3 in the phloem of the major veins (Fig. 7.5B) and minor veins (Fig. 7.6B). With time the number of phloem

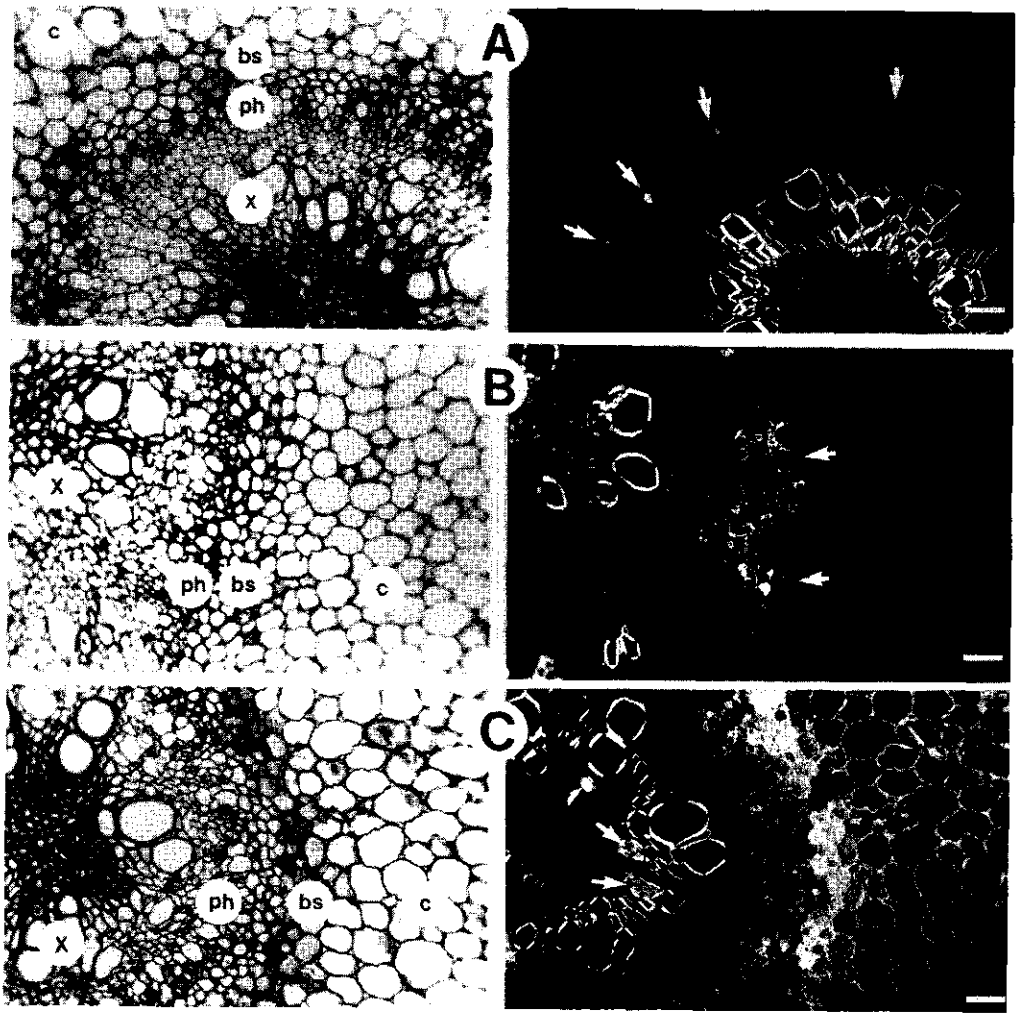


Fig. 7.3. Immunogold/silver staining of CCMV in cross-sections of the petiolule at t=0, 6 and 24 h after differential temperature staining (A, B and C, respectively). Silver stain is observed in four phloem parenchyma cells at t=0 (A) and in an increasing number of cells in the phloem, bundle sheath and cortex at 6 and 24 h. For further explanation see Fig. 7.2. Bar represents 10 µm.

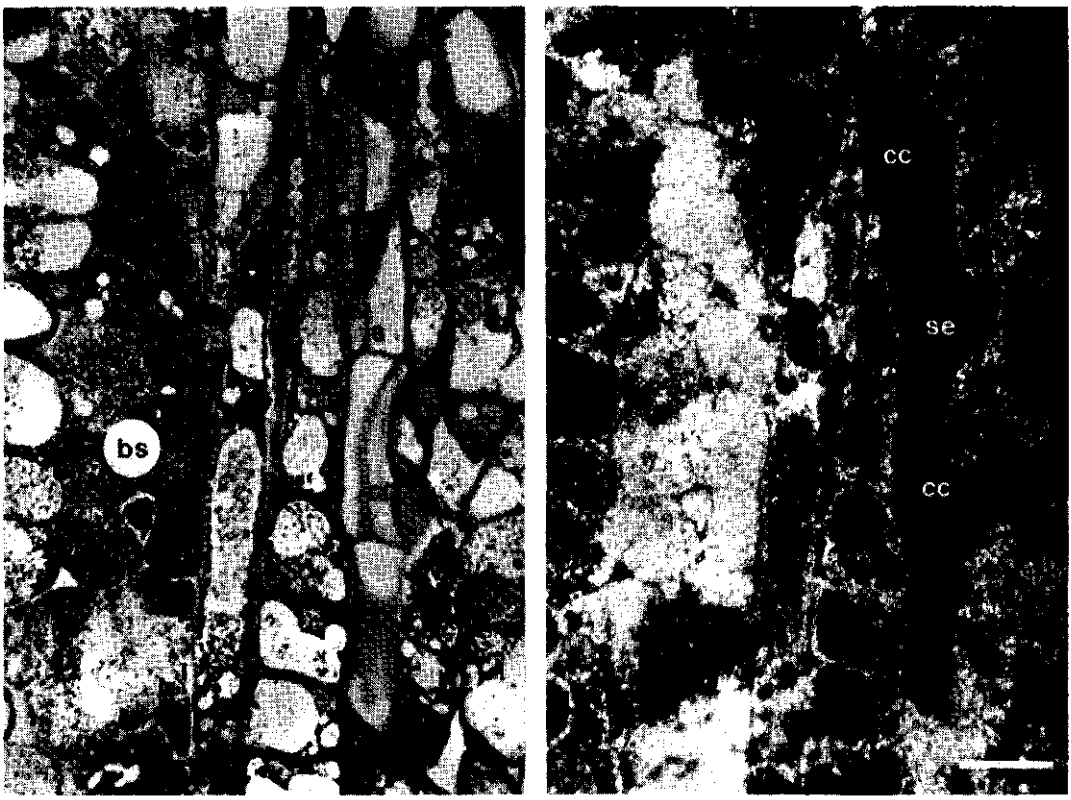


Fig. 7.4. Longitudinal section showing details of the phloem of the petiolule at 24 h after differential temperature treatment. Silver stain was observed in phloem parenchyma cells, but not in sieve elements (se) and companion cells (cc). Left: brightfield illumination. Right: epipolarization illumination. Bar represents 10 μm.

cells with label increased and label was also found in bundle sheath and collenchyma cells of the vein (Fig. 7.5C) and bundle sheath, mesophyll and epidermal cells of the blade (Fig. 7.6C). In the blade labelled mesophyll cells (spongy parenchyma and palisade parenchyma) were always associated with (minor) veins. The maximum number of mesophyll cells with label counted in parallel direction to the leaf surface from the vascular tissue in the blade was 5, 9 and 16 at t=6, 12 and 24h, respectively.

Similar results were found in secondary leaves which were systemically inoculated during 3 days. The only difference was that in this experiment no virus could be detected in the petiolule at t=0 h and the maximum number of

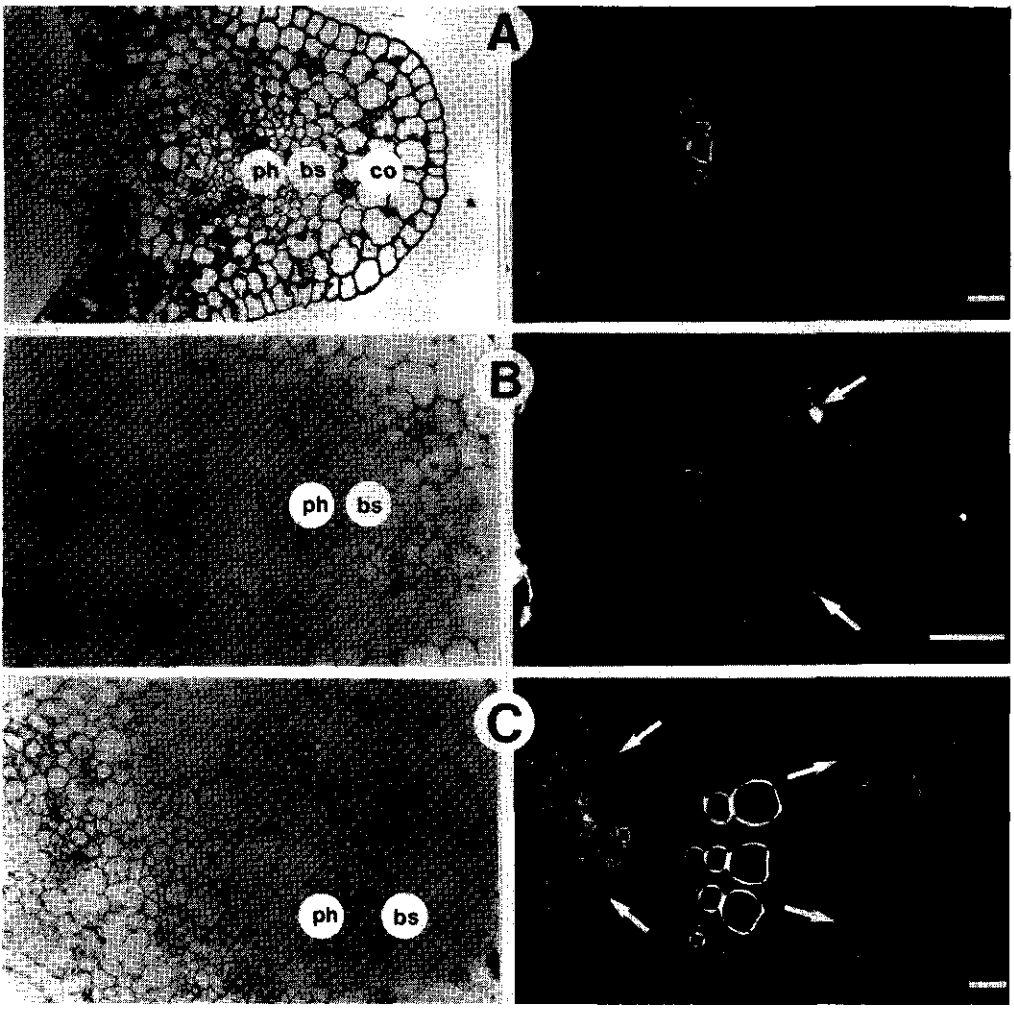


Fig. 7.5. Immunogold/silver staining of CCMV in cross-sections of the first branching vein at t= 0, 3 and 24 h after differential temperature treatment (A, B and C, respectively). Silver stain is absent in the vein at t=0 (A), and first observed in the phloem at t=3 (B) and on numerous phloem, bundle sheath and collenchyma (co) cells at t=24 (C). For further explanation see Fig. 7.2. Bar represents 10 μm.

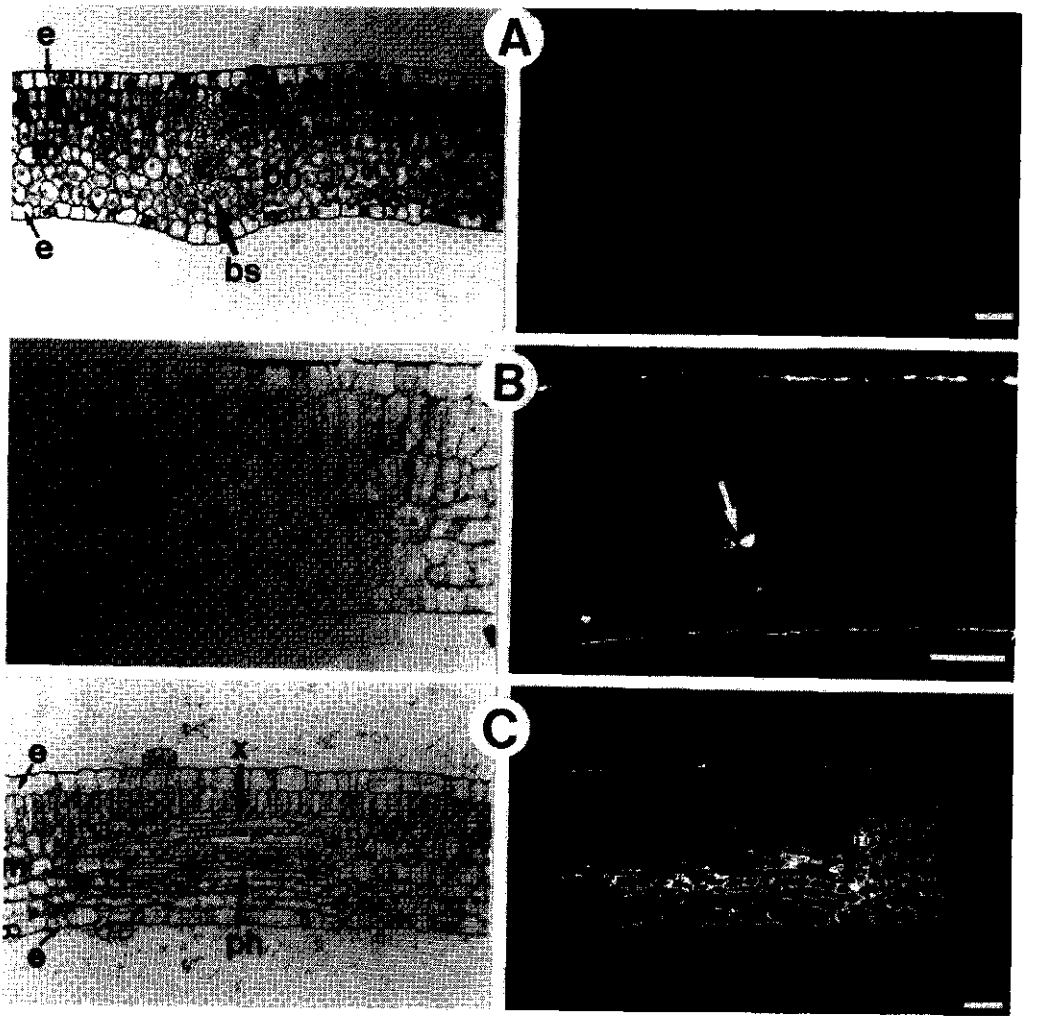


Fig. 7.6. IGSS of CCMV in cross-sections of leaf blade at t= 0, 3 and 24 h after differential temperature treatment (A, B and C, respectively). Silver stain is absent at t=0 (A), and first observed at t=3 (B) in the phloem of a minor vein and in phloem, bundle sheath, mesophyl (m) and epidermal (e) cells at t=24. For further explanation see Fig. 7.2. Bar represents 10 μm.

cells with label counted from the phloem in radial direction towards the outer side of the petiolule was 4, 7 and 15 at t=6, 12 and 24 h respectively. In the leaf 4, 6 and 14 cells were counted at t=6, 12 and 24 h, respectively. Furthermore, in both experiments no differences were observed between secondary leaves which were left attached to the stem or leaves separated from the inoculum source (primary leaves) at t=0.

DISCUSSION

Differential temperature treatment of cowpea plants, inoculated at the primary leaves with CCMV, resulted in rapid increase of virus concentration in leaves. After an initial lag period of about 6 h CCMV was detected with ELISA and virus concentration increased to as much as 1 mg virus per gram of leaf material within 24 h after transfer of the plants. Virus synthesis occurred in leaves left attached to the plant (not separated from the primary leaves which served as inoculum source) as well as in leaves detached from the plant or in discs taken from the leaves at the moment of transferring the plants to a higher temperature (t=0). The systemic infection, therefore, can not be attributed to a fast invasion of the trifoliate leaf by virus from the primary leaves after shifting the plants to a higher temperature, but infectious virus must have been present in the leaf at t=0. These results agree with similar findings of Dawson and Schlegel (1973; 1976). The authors showed high rates of virus multiplication in systemically inoculated leaves. Furthermore, they showed a synchrony in CCMV replication by using inhibitors effective at different periods of the replication cycle (Dawson and Schlegel, 1976).

With IGSS we located CCMV, either particles or coat protein molecules, in different parts of systemically inoculated cowpea plants. In sections of the stem, petiole and in one experiment also in the petiolule. Virus was localized exclusively in the phloem of the petiolule at t=0. In the next 24 h CCMV was localized in increasing numbers of phloem cells, but also in cells of neighbouring tissues. In both petiole and petiolule the sequence of tissues becoming progressively labelled with time was: phloem, bundle sheath and finally cortex. Only at t=24 h virus was also detected in the primary xylem and cambium of the petiolule. Similar patterns were observed for the vein and leaf blade, where virus was always detected in cells next to the phloem. These results suggest that CCMV is transported through the phloem of vascular bundles. However, within the phloem, virus was always detected in parenchyma cells, but never in

the differentiated sieve elements including companion cells.

Evidence presented for other viruses indicate that long-distance transport through the phloem along with the flow of metabolites is the most common route of virus spread (Matthews, 1981; De Zoeten, 1981). Absence of detectable virus in sieve elements and companion cells does not exclude the participation of these cell types in virus spread. The amount of virus transported may well be below a detectable level or the form in which infective virus is transported may be another than encapsidated viral RNA (Atabekov and Dorokhov, 1984). Companion cells apparently do not sustain multiplication of CCMV but probably only serve in transport to surrounding parenchymous cells. Capoor (1949) already showed that virus can pass infectible tissue without causing infection and De Zoeten (1981) suggested that long-distance transport does not seem to be dependent on multiplication of virus in conducting tissue. For several other viruses, however, the presence of virus particles in sieve elements and companion cells has been reported, e.g. potato leafroll virus (Shepardson et al., 1980; Barker and Harrison, 1982; Barker, 1987) and tobacco ringspot virus (Halk and McGuire, 1973). Transport of virus through tracheary elements of the xylem may provide an alternative route for some viruses as was shown by Schneider and Worley (1959a; 1959b) for southern bean mosaic virus (SBMV). However, the presence of CCMV in a few xylem cells at later stages of systemic infection (24 h) may be explained by virus multiplication in young undifferentiated tracheary elements, rather than virus transported through these elements.

The increasing number of cells in the petiole, petiolule, vein and leaf blade tissue becoming labelled at different periods after systemic inoculation, indicate little or no synchrony in the infection of cells in these tissues. In previous work on the infection of cowpea protoplasts with CCMV we have reported that newly synthesized virus could be detected by immunogold labelling at 6-9 h after inoculation (Chapter 4). As individual silver-coated gold particles can be observed in a microscope with epipolarization illumination (De Brabander et al., 1985; Chapter 6) this early stage of infection can also be recognized in light microscopic preparations. Dawson and Schlegel (1973; 1976) reported that virus accumulated in systemically inoculated leaves and an initial step of CCMV replication already took place before the plants were shifted to a higher temperature. For the latter reason newly synthesized virus may even be detected earlier in individual cells of systemically inoculated plant tissue as compared to protoplasts subjected to inoculation in vitro. With IGSS all cells infected at the time of temperature shift are likely to be identified as early as 6 h

after this shift. Additional cells with label appearing at later stages and at a further distance from the phloem of petiole, petiolule, vein and leaf blade have attracted infection by cell-to-cell transport of an infective entity after shifting the plants to a higher temperature. A maximum cell-to-cell spread of 1 cell in every 1-2 h was recorded. These results agree with similar findings of Dorokhov et al. (1981), that during differential temperature treatment the 'infective entity' spreads mainly into the vascular system of the leaf but not in the surrounding mesophyll tissue.

CCMV replication is relatively synchronous upon systemic inoculation when looking at plant tissue as a whole. However, at the cellular level in the same tissue there is hardly any synchrony in infection of these cells. The manipulation of systemic infection by differential temperature treatment nevertheless may supply a useful model system for the in situ study of plant virus replication and transport. In systemically inoculated tissue virus replication can be switched on at will and nearly all events in virus infection can be studied in relatively small and defined areas of plant tissue within a short period up to 48 h and with relatively simple means.

CHAPTER 8

SUMMARY AND CONCLUSIONS

This thesis describes the application of an immunocytochemical technique, immunogold labelling, new in the light and electron microscopic study of the plant viral infection. In Chapter 1 the present state of knowledge of the plant viral infection process, as revealed by in situ studies of infected cells, is briefly reviewed. Until now, light and electron microscopic studies have merely described morphological changes in cells and tissue as a result of viral infection, but have failed to provide information on the functional role of these structures in the viral infection process and their association with viral components. A common cytopathological feature of many different plant viruses seems to be the induction of membranous vesicles or membranous bodies, which have been implicated in viral replication. However, only in a few cases some evidence was obtained with regard to the intracellular location of viral replication and the association of replication and membranes. Available cytochemical techniques have apparently failed to provide a tool for the identification of virus particles and virus-encoded proteins within cellular structures. The impact of a suitable detection techniques to elucidate the molecular processes of viral replication and transport in situ is obvious, as it would link findings obtained by in vitro experiments to the events observed in the cell.

Immunogold labelling seems to provide such a tool for the tracing of antigens in light and electron microscopic preparations of biological specimens. Gold particles are excellent markers for electron microscopy, because of their high electron density which makes them appear as black dots in EM preparations. Furthermore, by a simple silver staining following gold labelling, viral antigens can be dete cted in semi-thin sections with the light microscope. The application of immunogold labelling for the light and electron microscopic localization of antigens is described in Chapters 2, 3, 4, 5, 6 and 7.

In Chapter 2 the preparation of homodisperse suspensions of colloidal gold particles is described. By adsorption of protein A to the surface of the gold particles, a marker (protein A-gold, pAg) is obtained which can be used for labelling antigen-antibody complexes. The specificity of the technique was demonstrated by gold labelling of antibodies bound to plant viruses in mixed

suspensions of two viruses. Each virus was labelled using its homologous antiserum and pAg, and no significant cross-reaction with the other virus occurred. Simultaneous identification of two different viruses (CCMV and SBMV) with similar morphological appearance was achieved by double labelling with pAg-complexes containing gold particles of 7 and 16 nm, respectively. Immunogold labelling of viral antigens in suspension has been applied to distinguish between different serologically related viruses like strains of TMV (Pares and Whitecross, 1982), and the potyvirus sugarcane mosaic virus and maize dwarf mosaic virus (Alexander and Toler, 1986; 1985). A clear advantage of the immunogold labelling over conventional decoration of antigens is that the discrete gold particles allow quantification of the results.

The immunogold labelling of viral antigen in ultrathin sections of infected protoplasts is described in Chapter 3. Best results were obtained when the protoplasts were only mildly fixed with aldehydes, dehydrated and finally embedded in Lowicryl K4M at -30°C. The antigenicity of viral coat protein was well preserved. A disadvantage of the method is the limited preservation of cell structures, especially membranes due to extraction of lipids. Weibull et al. (1983) reported that approximately 50% of the lipid content of cells may be extracted, despite the low temperatures used in the Lowicryl K4M embedding procedure. Ashford et al. (1986) questioned the low temperature character of Lowicryl embedding, and found that during polymerization of the resin, temperature rises due to the exothermic nature of the reaction. With plant tissue (not protoplasts), low temperature dehydration and infiltration of the embedding resin must be prolonged, to allow sufficient penetration of the chemicals through the thick walls surrounding the plant cells, and this may result in even more extraction than reported by Weibull and colleagues. Rapid dehydration in ethanol and infiltration of plant tissue with a polar resin like LR White at ambient temperatures, therefore, seems to be a good alternative (Newman et al., 1983; Causton, 1984; Newman and Jasani, 1984).

Light microscopic localization of viral antigen in semi-thin sections of LR White embedded plant tissue is described in Chapter 6. CCMV was successfully localized in petiolules of systemically inoculated cowpea plants by immunogold labelling and subsequent silver staining (immunogold/silver staining: IGSS). The silver stain could be observed in the light microscope by brightfield, darkfield and phase-contrast illumination. Most sensitive detection, however, was obtained with epi-illumination using polarized light (epipolarization microscopy). Combining epipolarization illumination with brightfield illumination allowed the simultaneous observation of silver stain and cell morphology.

Immunogold labelling and IGSS in combination with appropriate fixation and embedding of biological specimens, appear to be efficient and simple techniques for the in situ identification and localization of antigens, with many advantages over other immunochemical and cytochemical techniques, like ferritin-labelling, peroxidase-anti-peroxidase, immunofluorescence and autoradiography, which have only incidentally been used in plant virus research. Recently, Patterson and Verduin (1987) have reviewed the literature on the use of immunogold labelling in animal and plant virology, showing numerous fields of applications and discussing progress made in virus research. With respect to the technique the authors rightly concluded that immunogold labelling is a flexible technique with little limitation for the improvement of existing assays and the development of new ones.

Using immunogold labelling to identify and localize virus particles and coat protein, CCMV-infection in cowpea protoplasts was studied as function of the infection time. Observations with regard to virus entry into protoplasts are reported in Chapter 3. Upon inoculation aggregates of virus particles were observed attached to the plasmamembrane, or sometimes penetrating the plasmamembrane at places where the membrane appeared to be damaged. Virus was also found inside vesicles formed by invagination of the plasmamembrane. These vesicles with inoculum-virus particles were stable over long periods of time. Large vesicles (vacuoles) containing viral antigen were also detected at 24 h post-inoculation in protoplasts which were not infected by CCMV.

The mechanism by which plant viruses enter their host cells is still disputed (Shaw, 1986). Passage of the plasmalemma by endocytosis was suggested by Takebe (1975), and through pores or lesions by Burgess et al. (1973) and Watts et al. (1981). Our observations do not favour endocytosis to be the mechanism of virus entry leading to infection of the protoplasts as virus containing vesicles are stable. Recently, Roenhorst et al. (1988) presented data supporting a mechanism of virus entry by initial physical association of virus particles with the protoplast membrane and subsequent invasion of virus particles through membrane lesions. Such a mechanism may be also applicable to the cytoplasmic extrusions observed by Laidlaw (1987) after puncturing plant epidermal cells. The author suggested that virus particles may adsorb to the plasmalemma covering the extrusions, which are then withdrawn into the cell. Invasion of whole particles through membrane lesions may then be followed by a uncoating and initial translation (cotranslational disassembly) at the cytoplasmic ribosomes as suggested by Wilson (1985).

Ultrastructure of RNA-inoculated protoplasts was studied in sections of

aldehyde- and osmium-fixed protoplasts (Chapter 4). Cytological alterations attributed to virus infection consisted of dilation of the endoplasmic reticulum (ER) and the formation of vesicles early in infection. Distended ER and vesicles seemed to form a kind of membranous area in the cytoplasm. In protoplasts fixed and embedded in Lowicryl K4M newly synthesized virus particles or coat protein were first localized in restricted areas of the cytoplasm at 6-9 h post-inoculation. The rough appearance of the cytoplasm in these areas suggested the presence of membranous structures like observed in osmium-fixed protoplasts. However, due to poor membrane preservation in Lowicryl embedded material this could not be proven. Within one protoplast several of these labelled areas were identified. At later stages of infection viral antigen was located throughout the cytoplasm, but also in the nucleus and in particular the nucleolus. No viral antigen was detected in or specifically associated with chloroplasts, mitochondria, microbodies and vacuoles. The specificity of gold labelling was demonstrated by quantification of the labelling density on sections of infected and non-infected protoplasts. These results indicate that CCMV coat protein synthesis and virus assembly take place in the cytoplasm of plant cells, but the involvement of cellular structures, in particular membranes, remains to be established. Protein synthesis and virus assembly may occur in certain restricted sites (compartments) in the cytoplasm possibly formed by the membranous bodies. Compartmentalization of the cytoplasm, creating different environments in the cell, may explain the occurrence of both disassembly and assembly in the same cell, and furthermore account for the phenomenon of specific assembly of viral RNA and homologous coat protein in cells infected with two related viruses like CCMV and BMV (Sakai et al., 1983; Zaitlin and Hull, 1987). Whether RNA-replication also occurs in the same location as coat protein synthesis and virus assembly could be established by localization of non-structural virus encoded proteins involved in viral replication. However, antisera against these products of the CCMV-genome were not available. The function of CCMV coat protein or virus in the nucleus and especially the nucleolus is not known. Coat protein may have an affinity for ribosomal proteins and/or fulfill some functional role in the viral replication. Kim 1977 described the occurrence of filamentous inclusions (FI) in the nucleus often associated with the nucleolus. These FI were not found in the nuclei of cowpea protoplasts (this study) or tobacco protoplasts (Burgess et al., 1974), but may be formed later in the infection by excess coat protein. Bancroft et al. (1969) showed the ability of CCMV-coat protein to form narrow tubules under specific conditions. The FI described by Kim (1977) may represent

this type of coat protein aggregation, although the chemical composition of the FI is not yet known.

In Chapter 5 preliminary observations are reported on the localization of sites of CPMV replication in cowpea protoplasts, by in situ detection of coat proteins and non-structural proteins involved in viral replication and proteolytic processing. With regard to virus entry and subsequent locations of inoculum virus inside vesicles, similar phenomena were observed as in infection with CCMV. Infection of CPMV generates large inclusion bodies in the cytoplasm, consisting of membranous vesicles with fibrillary material and adjoining amorphous electron-dense material which have been observed as early as 12 h post-inoculation. Virus particles and/or coat protein were first detected 24 h after inoculation throughout the entire cytoplasm and in between the membranous vesicles and electron dense material. The 24K, 170K and their precursor proteins were exclusively localized in the electron dense material and not in association with the membranous vesicles or any other location in the cell. These results show that the electron-dense material consists at least in part of CPMV-encoded non-structural proteins and may represent a site for accumulation of non-functional proteins. The membranous vesicles have been implicated in viral RNA synthesis (Goldbach and Van Kammen, 1985). The failure to detect non-structural proteins in association with these membranes may be explained by either a low concentration of these proteins at the site of replication or by extraction of these proteins during the fixation and embedding procedure, despite the low temperature.

With IGSS the distribution of CCMV in cowpea plants was monitored at different times after systemic inoculation according to Dawson and Sehlegel (1976) (Chapters 6 and 7). No virus was detected at the time of temperature shift (t=0) in petiolule and leaves of plants subjected to 3 days of differential temperature treatment. Virus was first localized in phloem parenchyma cells of petiolule and veins at t=3 h and from there it spread to neighbouring tissues. Twenty four hours after systemic inoculation virus was located in the phloem, bundle sheath, cortex, but also in the cambium and some xylem cells. These results show that CCMV is transported from the inoculated primary leaves to the secondary leaves through the phloem, apparently following the route of metabolites. This finding is in agreement and further supports the generally accepted concept of plant virus long-distance transport through phloem tissue (Matthews, 1982; Atabekov and Dorokhov, 1984). The failure to detect CCMV in differentiated sieve elements may indicate that the form in which the infectious entity is transported is another than virus particles (Atabekov and

Dorokhov, 1984), or that the amount of virus transported through the sieve elements is below detectable levels. The true character of the synchrony of infection of leaf mesophyll cells obtained by differential temperature treatment is disputed. Infection of mesophyll cells may have been accomplished after shifting the plants to higher temperature by fast transport of infectious particles from the vascular tissue, as was also suggested by Dorokhov et al. (1981).

For the first time a suitable method for localization of antigens is available, which can be routinely applied for both light and electron microscopic study of the plant viral infection process. The application of the gold labelling technique in the localization of viral structural and non-structural proteins has been demonstrated, using CCMV- and CPMV-infections of plant cells as model system.

With regard to the technique, future work must be done on the improvement of the preservation of cellular structures, especially membranes, as these appear only poorly in Lowicryl embedded plant tissue even with dehydration, infiltration and polymerization at low temperatures. Alternatives, may be found in cryofixation and cryosectioning or freeze-substitution techniques.

With regard to the study of the plant viral infection process, the localization of virus-encoded proteins involved in replication and transport, but also the localization of plant viral nucleic acids by in situ hybridization, will contribute to the understanding of the mechanisms underlying these events. New biochemical techniques like the production of infectious transcripts from cloned viral cDNA (Ahlquist et al. 1984) enabling genetic manipulation of the viral genome, and integration of plant viral genes into the plant genome (Gardner et al., 1984; Abel et al., 1986) will supply future model systems for the study of virus-host interactions.

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SAMENVATTING

In het infectieproces van een plantecel met een virus kunnen verschillende stappen worden onderscheiden: het binnendringen van het virus in de cel, de ontmanteling van het virus-nucleïnezuur, d.w.z. het virusgenoom, de translatie en transcriptie van dit virusgenoom, de assemblage van nieuwe virusdeeltjes en het transport naar andere cellen. Over de mechanismen volgens welke de verschillende stappen in het infectieproces verlopen is nog weinig bekend. Een infectie wordt gestuurd door een samenspel van factoren die bepaald worden door zowel het virus als de gastheercel. Virus kan zich namelijk uitsluitend vermenigvuldigen in een levende cel.

Biochemische processen die een rol spelen bij de replicatie van het virale genoom, met name translatie en transcriptie, kunnen voor een deel bestudeerd worden door nabootsing van deze processen in vitro en door biochemische analyse van geïnfecteerde cellen. Dissociatie en associatie van virusdeeltjes kunnen eveneens in vitro worden bestudeerd als nabootsing van de ontmanteling en de assemblage van virusdeeltjes in de cel. Aanvullende informatie over de plaats waar de processen van ontmanteling, replicatie en assemblage in de cel plaatsvinden en de functie van celstructuren in deze processen, kan worden verkregen door licht- en elektronenmicroscopisch onderzoek van geïnfecteerde cellen. Conventioneel licht- en elektronenmicroscopisch onderzoek heeft geleid tot de beschrijving van een groot aantal structurele veranderingen in de cel als gevolg van infectie met verschillende virussen. Een gemeenschappelijk kenmerk van veel virussen is de inductie van membraanstructuren die vermoedelijk betrokken zijn bij de replicatie, maar ook bij de assemblage van virusdeeltjes. In situ lokalisatie van virus en virale eiwitten die een functie hebben bij de replicatie of het transport, zou meer inzicht geven in de betrokkenheid en functie van dergelijke (door virus geïnduceerde) celstructuren in het infectieproces.

Dit proefschrift beschrijft de toepassing van een nieuwe immunocytochemische techniek, de immunogoudlabeling, ten behoeve van de bestudering van het infectieproces met behulp van licht- en elektronenmicroscopie. Als model voor het onderzoek is gekozen voor de infectie van cowpea (Vigna unguiculata) met het "cowpea chlorotic mottle virus" (CCMV). Het infectieproces van CCMV is bestudeerd door lokalisatie van CCMV-manteleiwit in synchroon geïnfecteerde cowpea-protoplasten en cowpea-planten.

De probleemstelling van het onderzoek is weergegeven in de inleiding van dit proefschrift. In hoofdstuk 1 is een literatuuroverzicht gegeven met betrek-

king tot de cytopathologie van enkelstrengs RNA-virussen en de (immuno)cytochemische technieken voor licht- en elektronenmicroscopische detectie van virussen en eiwitten.

In hoofdstuk 2 is de bereiding van homodisperse kolloïdale goudsuspensies beschreven. De diameter van de gouddeeltjes kan daarbij, binnen bepaalde grenzen, naar believen worden gevarieerd. Door adsorptie van proteïne A aan het oppervlak van de gouddeeltjes verkrijgt men een merkteken ter identificatie van antigeen-antilichaamcomplexen. Het produkt van de binding van proteïne A aan gouddeeltjes (proteïne A-goud) wordt met de afkorting pAg aangeduid. Daar het proteïne A affiniteit heeft voor het constante deel (F_c) van gammaglobulinen (de antilichamen) worden de gouddeeltjes aanwezig in het pAg aan de gewenste complexen gebonden. In de elektronenmicroscoop zijn de goudbolletjes als zwarte deeltjes zichtbaar. De specificiteit van de immunogoudlabeling werd aangetoond door het labelen van afzonderlijke virussen in een gemengde suspensie van twee virussen. Afhankelijk van het gebruikte antserum werd een van beide virussen gelabeld door het pAg. Gelijktijdige labeling van beide virussen met pAg-deeltjes van verschillende diameter was ook mogelijk. Zo konden twee verschillende virussen met dezelfde morfologie in een preparaat van elkaar onderscheiden en geïdentificeerd worden.

De detectie van virus in ultradunne coupes van geïnfecteerde cowpea-protoplasten is beschreven in hoofdstuk 3. Een milde fixatie van het biologisch materiaal met aldehyden gevolgd door ontwatering in ethanol en inbedding in het polaire Lowicryl K4M bleek de beste resultaten te geven met betrekking tot de detectie van virale antigenen door middel van immunogoudlabeling op ultradunne coupes. Het behoud van de ultrastructuur van met name celmembranen was echter slecht. Bestudering van de ultrastructuur werd daarom uitgevoerd aan materiaal dat was gefixeerd met aldehyden en osmiumtetroxyde.

Na inoculatie van cowpea-protoplasten met CCMV werden aggregaten van virusdeeltjes waargenomen op verschillende lokaties bij de plasmamembraan. De aggregaten van virusdeeltjes bleken geadsorbeerd te zijn aan het oppervlak van de plasmamembraan, of deze membraan te penetreren. Losse virusdeeltjes of aggregaten daarvan werden ook waargenomem in blaasjes gevormd door instulping van de plasmamembraan. Dergelijk blaasjes met virus, die na vorming migreerden in het cytoplasma, bleken stabiel gedurende een lange periode na inoculatie en werden zelfs waargenomen in protoplasten die niet door het virus waren geïnfecteerd. Deze waarnemingen geven aan dat endocytose van virus niet hoeft te leiden tot infectie van de cel. Het is waarschijnlijk dat infectie van de cel wordt bewerkstelligd door virus dat, na inoculatie, direkt in het cytoplasma is

terecht gekomen via beschadigingen (lesies) in de plasmamembraan. Het model van "co-translational disassembly", volgens hetwelk de ontmanteling van het virusgenoom en de translatie ervan aan de cytoplasmatische ribosomen gelijktijdig plaatshebben, ondersteunt het voornoemde mechanisme van binnendringing.

In cowpea-protoplasten werd replicatie van het virus voor het eerst aangetoond op 6 uur na inoculatie met CCMV-RNA (hoofdstuk 4) door lokalisatie van nieuw gesynthetiseerd manteleiwit in het cytoplasma. Vroeg in de infectie (6 tot 12 uur na inoculatie) werd manteleiwit voornamelijk gevonden in beperkte delen van het cytoplasma, maar op latere tijdstippen kon het virus worden aangetoond in het gehele cytoplasma. In protoplasten die met aldehyden en osmiumtetroxyde waren gefixeerd werden veranderingen in het endoplasmatisch reticulum (ER) waargenomen als gevolg van infectie met CCMV. Het ER was gezwollen en kleine blaasjes werden gevormd, waarschijnlijk door afsplitsing van het ER. Blaasjes en ER leken een soort van membraanachtige massa te vormen in het cytoplasma. Door het slechte behoud van membranen in protoplasten die waren ingebed in Lowicryl K4M kon niet met zekerheid worden vastgesteld of de lokaties waar het manteleiwit werd gevonden, dezelfde waren als de plaatsen in het cytoplasma waar membraanstructuren werden waargenomen. Eiwitsynthese en assemblage van nieuwe virusdeeltjes zouden mogelijk plaats kunnen vinden in dergelijke, door membranen afgezonderde gebieden in het cytoplasma (compartimenten), waar lokaal de condities heersen die nodig zijn om deze processen gaande te houden. Door middel van kwantificering van de goudlabeling waarbij het aantal gouddeeltjes per oppervlakte eenheid (um^2) werd bepaald, kon worden aangetoond dat organellen zoals chloroplasten, mitochondriën, microbodies en vacuolen geen detecteerbare hoeveelheden viraal manteleiwit bevatten. Tevens werd aangetoond dat op latere tijdstippen in de infectie manteleiwit in de kern en met name in het kernlichaampje aanwezig was. Mogelijk heeft het manteleiwit van CCMV een affiniteit voor ribosomale eiwitten, waaruit de specifieke ophoping in het kernlichaampje kan worden verklaard.

Aanwijzingen met betrekking tot de lokatie van de replicatie van het virusgenoom, kunnen worden verkregen door lokalisatie van niet-structurele virale eiwitten die betrokken zijn bij de replicatie. In hoofdstuk 5 is de lokalisatie van cowpeamozaïekvirus (CPMV) en enkele niet-structurele eiwitten van dit virus beschreven. Na infectie van een cowpea-protoplast met CPMV worden cytopathische structuren gevormd die bestaan uit amorf elektronendicht materiaal en grote aantallen blaasjes. Niet-structurele eiwitten van CPMV, met name het 24K eiwit dat betrokken is bij de proteolytische verwerking van polypeptiden en het 170K eiwit dat een functie heeft bij de replicatie van RNA, evenals het VPg werden

uitsluitend gelokaliseerd in de amorfe elektronendichte structuren van het cytopathisch complex. Viraal manteleiwit werd zowel in het cytoplasma als tussen de blaasjes en elektronendichte materiaal gelokaliseerd. Deze resultaten geven aan dat het elektronendichte materiaal tenminste voor een deel bestaat uit niet-structurele eiwitten van CPMV en waarschijnlijk wordt gevormd door opslag van niet (meer) functionele viruseiwitten. De betrokkenheid van de blaasjes in het proces van replicatie is niet aangetoond.

In hoofdstuk 6 is de immunogoud-zilverkleuring (IGSS) beschreven voor de lichtmicroscopische lokalisatie van virale antigenen. Op semidunne coupes van plantemateriaal dat was gefixeerd met aldehyden en ingebed in LR White, werden na immunogoudlabeling de pAg-deeltjes bedekt met zilver. Dit kan worden bewerkstelligd, aangezien de gouddeeltjes de reductie van zilverionen tot metallisch zilver in een ontwikkelaar katalyseren. Het zilverprecipitaat was waarneembaar als zwarting in een lichtmicroscoop met doorvallend licht en lichtte 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.

Met behulp van IGSS in combinatie met epipolarisatiemicroscopie werd CCMV gelokaliseerd in cowpea-planten als functie van de infectieduur. De verplaatsing en vermenigvuldiging van het virus werden gevolgd in planteweefsel dat systemisch was geïnoculeerd volgens de differentiële-temperatuur-inoculatietechniek (hoofdstuk 7). Hierbij werden primaire bladeren van cowpea-planten met een nog niet volledig gestrekt secundair blad geïoculeerd met CCMV. De planten werden vervolgens zodanig weggezet in een klimaatkast, dat de primaire bladeren zich in een compartiment met continu licht en 27°C en de secundaire bladeren zich in een compartiment zonder licht en 10°C bevonden. Na 3 of 4 dagen werden de planten overgebracht naar een verlichte klimaatkast van 27°C. Tijdens de toestand van differentiële temperatuur zal virus zich vermenigvuldigen in de primaire bladeren en vervolgens worden getransproteerd naar de secundaire bladeren, maar bij de lage temperatuur vindt in deze secundaire bladeren, die zich tevens in het donker bevinden, geen of weinig replicatie van het virus plaats. Komen deze bladeren vervolgens in het licht bij hogere temperatuur dan wordt een min of meer synchronische systemische infectie bewerkstelligd.

Op het moment dat de planten van de toestand van differentiële temperatuur naar de klimaatkast werden overgeplaatst (t=0 van systemische inoculatie) werd

geen virus gedetecteerd in het bladsteeltje of in het blad van de secundaire bladeren. Virus werd voor het eerst gelokaliseerd in floëemparenchymcellen in het bladsteeltje op t=3 uur en vandaaruit verspreidde het virus zich naar naburige cellen, zoals kon worden waargenomen op latere tijdstippen. Vierentwintig uur na systemische inoculatie werd virus gelokaliseerd in het floëem, de zetmeetschede en de schors, maar ook in het cambium en de xyleemcellen van het bladsteeltje. Deze resultaten wijzen erop dat het virus via het floëem wordt getransporteerd vanuit de geïnoculeerde bladeren naar de secundaire bladeren, analoog aan het transport van assimilaten. Virus kon niet worden gedetecteerd in gedifferentieerde zeefvaten en hun begeleidende cellen. Mogelijk wordt het virus in een andere vorm dan complete virusdeeltjes getransporteerd en zijn de begeleidende cellen niet in staat om het virus te vermenigvuldigen. Een andere verklaring kan zijn dat het virus in hoeveelheden in de zeefvaten voorkomen, die beneden de detectiegrens liggen.

Uit het verspreidingspatroon van CCMV binnen 24 uur na systemische inoculatie kan worden geconcludeerd dat de schijnbare synchronisatie van infectie, waargenomen door regristratie van de virusvermenigvuldiging, waarschijnlijk berust op een snel transport van het infectieuse deeltje vanuit het vaatweefsel naar naburige cellen, vanaf het moment dat de planten bij hogere temperatuur zijn weggezet.

Immunogoudlabeling blijkt een zeer geschikte methode voor de detectie van plantevirussen met behulp van zowel de lichtmicroscoop als de elektronenmicroscoop. Een milde fixatie van het biologisch materiaal en ontwatering en inbedding bij lage temperatuur, waarbij nieuw ontwikkelde polaire inbedmedia worden gebruikt, leidt tot het behoud van de antigene eigenschappen van eiwitten in het preparaat. Echter in veel gevallen gaat behoud van deze antigene eigenschappen ten koste van het behoud van de ultrastructuur. Alternatieve methoden zoals fysische fixatie door bevriezing van het materiaal en daaropvolgend het snijden van vriescoupes of vervanging van het bevroren water door een inbedmedium (vriessubstitutie) kunnen leiden tot verbetering van zowel het behoud van de ultrastructuur als van de antigene eigenschappen.

In de bestudering van het infectieproces van plantevirussen zal de lokalisatie van niet-structurele eiwitten en virale nucleïnezuren, die betrokken zijn bij de replicatie of bij het transport van virus door de plant, aanwijzingen kunnen geven omtrent de mechanismen volgens welke deze processen verlopen. Antisera tegen dergelijke functionele eiwitten en methoden voor <u>in situ</u> hybridisatie zullen, dankzij moleculair biologisch onderzoek, in de toekomst beschikbaar zijn. Nieuwe biochemische technieken zoals de productie van infec-

tieuse transcripten van gekloneerd cDNA, waardoor genetische manipulatie van het virale genoom mogelijk wordt, en integratie van genen van plantevirussen in het genoom van planten zullen in de toekomst de modelsystemen leveren voor de bestudering van het infectieproces van plantevirussen.

CURRICULUM VITAE

Johannes Willem Maria van Lent werd op 31 januari 1955 geboren te Beneden Leeuwen. Na in 1973 het eindexamen Atheneum B aan het Maasland College te Oss behaald te hebben werd in hetzelfde jaar begonnen met een studie aan de Landbouwhogeschool. Het doctoraalexamen werd behaald met lof, in september 1980, met als hoofdvakken Virologie en Dierkunde.

Tussen september 1981 en september 1983 was de promovendus als assistent deskundige voor het Directoraat Generaal voor de Internationale Samenwerking (DGIS) gestationeerd bij het "International Institute of Tropical Agriculture" (IITA) te Ibadan, Nigeria.

Van februari 1984 tot en met februari 1987 was de promovendus werkzaam als wetenschappelijk assistent bij de Vakgroep Virologie van de Landbouwhogeschool te Wageningen in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Het onderzoek dat daar door de promovendus werd verricht heeft geleid tot dit proefschrift.

Sinds 1 augustus 1987 is de promovendus verbonden aan de Vakgroep Virologie van de Landbouwuniversiteit te Wageningen als universitair docent.