Translocation of *Watermelon chlorotic stunt virus* (WmCSV) in its insect vector *Bemisia tabaci* (Genn.)

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Braunschweig, im Juli 2006

Inas Farouk Fahmy

This work is dedicated

To the memory of my late Mom who died too soon and,

in appreciation of my Dad's invaluable contributions to my education;

To my dear husband who stands with the most sincere efforts he could offer to finalize this work;

To my lovely daughter who suffered with me in her early years of life.

To my dear family

Abstract

Keywords: *Bemisia tabaci*, virus transmission, begomoviruses, *Watermelon chlorotic stunt virus*, translocation pathway, immunolocalization.

To elucidate the interactions between begomoviruses and their whitefly vectors leading to virus transmission, the acquisition and translocation of *Watermelon chlorotic stunt virus*, WmCSV, by *Bemisia tabaci* (Genn.) was studied. A whitefly transmissible WmCSV isolate and 4 different non-transmissible virus mutants carrying a single amino acid change in the capsid protein at position 133 were used in these investigations.

B. tabaci was fed on infected watermelon plants for an acquisition access period (AAP) of 48 h then transferred for 48 h to non host plants, to subsequently inoculate healthy watermelons. Transmission was taken as an evidence for a viable interaction between virions, the gut membrane and the primary and/or the accessory salivary glands. Fresh, dissected organs from viruliferous whiteflies feeding on wild type or mutant virus were examined by PCR to determine presence of virus. For the transmissible, wild-type virus a pathway already described for the translocation of *Tomato yellow leaf curl virus* (TYLCV) similar to luteovirus / aphid interaction, was found. Virus is ingested with plant sap through the stylet into the oesophagus, is concentrated in the filter chamber to subsequently passage through the gut wall into the haemocoel to reach the salivary glands. The virus is translocated in the salivary duct and is finally excreted with the saliva for plant inoculation.

Virus DNA was detected in the midgut, in the haemocoel and in salivary glands of *B. tabaci* for both the transmissible and non-transmissible mutants. In *Trialeurodes vaporariorum*, a non vector for geminiviruses, the WmCSV wild-type was not capable to cross the gut wall and hence was not detected in haemocoel or salivary glands. In these non vector whitefly insects, the gut wall represents the essential epithelial barrier to virus passage.

Geminiviruses are transmitted by *Bemisia tabaci* (Genn.) in a circulative, non propagative manner. To be translocated; the ingested virus has to cross two cellular

barriers; the gut epithelial cells, to be released into the haemocoel and, the salivary glands, to be re-injected for plant infection. To elucidate this putatively receptor-mediated process and to reveal the sites of endocytosis, insects fed on plants infected with WmCSV were subjected to immunolocalization studies in electron microscopy. To test for the best fixation as well as the best embedding resin resulting in maximum ultrastructural preservation with high and specific labelling, a number of fixation and embedding methods and resins were tested. Ultrathin sections of insect organs embedded in different resins such as Epon 812, LR White or Lowicryl, were subjected to immunolocalization experiments. Transmission Electron Microscopy (TEM) observations demonstrate that the specific labelling using WmCSV antiserum was concentrated in the microvilli lining the gut wall of the epithelial cells of the food canal (the descending midgut and the filter chamber) which would point to a putative virus storage site allowing further internalisation for delivery to the haemocoel.

Both transmissible and the non-transmissible virus mutants were capable of crossing the midgut (PCR studies), while different observations were made with glands - primary salivary glands (PSG) and accessory salivary glands (ASG). At these organs, virus mutants were detected, however, in all cases there was no virus transmission. Consequently, virus localization studies concentrated especially on these organs.

For these investigations, optimisation of methods preserving membrane structures of multilamellar/tubular vesicles or -bodies, which are suggested to play a significant role in virus transcytosis and transmission, was imperative for this study. Thus, different etching and antigen retrieval procedures were investigated on specimens that were fixed and embedded in Epon or Lowicryl resins respectively.

A highly specific labelling in the primary salivary glands PSG, especially in the electron lucent and in multilamellar vesicles was observed. This labelling intensity as well as specificity was not observed in examinations of the accessory salivary glands (ASG). Hence, for translocation of WmCSV in *B. tabaci* insects, the primary salivary glands represent the major epithelial barrier. These organs therefore play the most decisive role for a successful vector transmission of begomoviruses by *Bemisia tabaci*.

Zusammenfassung

Schlagwörter: *Bemisia tabaci*, Weiße Fliege, Virus Übertragung, Begomoviren, *Watermelon chlorotic stunt virus*, Translokationsweg, Immunolokalisierung

Die Beziehungen zwischen Begomoviren und Weiße Fliege Insektenvektoren, die zu Virusübertragung führen, sollen aufgeklärt und Virusakquisition und die Translokation der Viren in *Bemisia tabaci* (Genn.) untersucht werden. Ein Weiße Fliege übertragenes watermelon chlorotic stunt virus Isolat, WmCSVwt, und vier verschiedene Virusmutanten des WmCSV, die jeweils einen einzigen Aminosäureaustausch im Kapsidprotein in Position 133 aufweisen, wurden für diese Studien herangezogen.

B. tabaci Insekten wurden zur Virusaufnahme für eine Akquisitionszeit von 48 h auf infizierten Wassermelonen gehalten, danach für 48 h auf Nicht-Wirtspflanzen gebracht, um schließlich gesunde Wassermelonen zu inokulieren. Eine erfolgreiche Virusübertragung wurde als Zeichen einer effektiven Interaktion zwischen Virionen, Darmmembran und Speicheldrüsen (primary, PSG, und accessory salivary glands, ASG) bewertet. Frisch entnommene Organe von Weißen Fliegen, die auf WmCSV infizierten Wassermelonen gehalten wurden, wurden mittels PCR untersucht, um an/in den Organen vorhandenes Virus zu bestimmen. Für das übertragbare WmCSVwt wurde so ein bereits für das *Tomato yellow leaf curl virus* (TYLCV) beschriebener Übertragungsweg gefunden, der ähnlich der Luteovirus / Aphiden Interaktion verläuft. Virus wird mit dem Pflanzensaft durch das Stylet in die Speiseröhre aufgenommen, in den Filterkammern konzentriert, um schließlich durch die Darmwand in das Hämozöl zu dringen und an die Speicheldrüsen zu gelangen. Das Virus wird dann in die Speichelkanäle transloziert und schließlich mit dem Speichel ausgeschieden, um erneut Pflanzen zu inokulieren.

Sowohl das Weiße Fliege übertragbare WmCSVwt als auch die nicht-insektenübertragbaren Virusmutanten wurden im Mitteldarm, im Hämozöl und in den Speicheldrüsen von *B. tabaci* gefunden. In *Trialeurodes vaporariorum* konnte WmCSVwt nicht die Darmwand durchdringen und war deshalb weder im Hämozöl

noch in den Speicheldrüsen zu finden. Hier stellt die Darmwand die wesentliche epitheliale Begrenzung für die Viruspassage dar.

Geminiviren werden durch *Bemisia tabaci* (Genn.) in einem zirkulativen nicht-propagativen Modus übertragen. Um in den Insekten zu zirkulieren, muss das Virus zwei bedeutende zelluläre Barrieren, die epithelialen Zellen des Darms, um in das Hämozöl zu gelangen und die Speicheldrüsen, passieren. Dieser möglicherweise Rezeptor -vermittelte/gesteuerte Prozess sollte in Weiße Fliege Insekten untersucht werden, vor allem um die Orte der Endozytose aufzuzeigen. Hierfür wurden Weiße Fliege Insekten auf WmCSV infizierten Pflanzen gehalten und dann elektronenmikroskopischen Immunolokalisierungsstudien unterzogen. Zunächst wurde eine Vielzahl verschiedener Fixierungs- und Einbettungsverfahren und Medien geprüft, um jenes Verfahren zu finden das eine maximale Erhaltung der Ultrastrukturen mit bestmöglicher und spezifischer Markierung gewährleistet. Dünnschnitte von Insektenorganen, die in verschiedenen Kunstharzen wie Epon 812, LR White oder Lowicryl eingebettet waren, wurden für die Translokationsstudien verwendet. In den elektronenmikroskopischen Untersuchungen war eine spezifische Markierung des WmCSV mit Antiserum in den Microvilli zu finden, die die Darmwände der epithelialen Zellen des Verdauungskanals auskleiden (absteigender Mitteldarm, Filterkammer). Das könnte auf eine mögliche Sammelstelle der Viren, für die weitere Passsage in das Hämozöl hinweisen.

Sowohl WmCSVwt als auch die nicht insektenübertragbaren Virusmutaten konnten den Mitteldarm durchdringen, während an PSG und ASG unterschiedliche Beobachtungen gemacht wurden. Hier konnten zwar einige Virusmutanten mittels PCR nachgewiesen werden, eine Virusübertragung blieb jedoch in allen Fällen aus. Lokalisierungsstudien konzentrierten sich deshalb im Besonderen auf diese Organe. Auch für diese Studien mussten zunächst Verfahren gefunden werden, welche Antigenität und Strukturen der Zellmembranen und der multilamellaren und tubulären Vesikel, denen eine wesentliche Rolle bei der Transzytose und der Virusübertragung zukommen soll, erhalten.

Eine hoch spezifische Markierung mit WmCSV Antikörpern wurde in den multilamellaren Vesikeln der PSG gefunden, was für ASG nicht bestätigt werden konnte. Für die Translokation von WmCSV in *B. tabaci* Insekten stellen somit die

primären Speicheldrüsen (PSG) eine wesentliche epitheliale Barriere dar. Diese Organe spielen für eine erfolgreiche Vektorübertragung von Begomoviren durch *Bemisia tabaci* eine entscheidende Rolle.

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Abbreviations

aa	Amino acid(s)
AAP	Acquisition Access Period
AbMV	*Abutilon mosaic virus*
ACMV	African cassava mosaic virus
aP	alkaline Phosphatase
APS	Ammonium Persulphate
AR	Antigen Retrieval
ASG	Accessory Salivary Glands
B. tabaci	*Bemisia tabaci*
BBA	Biologische Bundesanstalt
BCTV	*Beet curly top virus*
BGMV	*Bean golden mosaic virus*
bp	base pairs
BSA	Bovine Serum Albumin
BSA-c	Acetylated Bovine Serum Albumin
CabLCV	Cabbage leaf curl virus
CBB	Coomassie Brilliant Blue
CLCuV	*Cotton leaf curl virus*
CLCrV	*Cotton leaf crumple virus*
CP	Coat Protein
CR	Common Region
d	Day(s)
dd H_2O	double distilled water
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic Acid
dsDNA	double stranded DNA
EACMV	*East African cassava mosaic virus*

EDG	Electron Dense Granules
EDTA	Ethylene diaminetetraacetic acid disodium salt dehydrate
EM	Electron microscopy
Fab	Fragment antigen binding
g	gram(s)
GA	Glutaraldehyde
GAR	Goat anti-Rabbit
GFP	Green Fluorescent Protein
HD	Head
HL	Haemolymph
HPF	High Pressure Freezing
h	Hours
IAP	Inoculation Access Period
ICMV	Indian cassava mosaic virus
IC-PCR	Immunocapture- PCR
IgG-aP	Immunoglobulin G conjugated Alkaline Phosphatase
IPS	Insect Physiological Saline
IR	Intergenic Region
ISEM	Immunosorbent electron Microscopy
ISH	*In-situ* Hybridization
kb	kilo bases
kDa	kilo Dalton
Mab	Monoclonal Antibody
MD	Midgut
MES	2-(N-morpholino) ethane sulfonic acid
mg	Milligramm
min	Minute(s)
ml	Milliliter

mM	millimolar
MMA	MS+MES+Acetosyringone
MP	Movement Protein
mPJ	Metaperiodate
MS	Murashige and Skoog Medium
MSV	Maize streak virus
mtCOI	Mitochondria Cytochrome Oxidase
Mw	Molecular weight
µl	microliter
µm	micrometer
N	Nucleus
NGS	Normal Goat Serum
NSP	Nuclear Shuttle Protein
nt	nucleotides
ORF	Open Reading Frame
OsO_4	Osmium tetroxide
PAGE	Polyacrylamide Gel Electrophoresis
PB	Phosphate buffer
PBS	Phosphate buffered Saline
PBS-T	Phosphate buffered Saline with Tween®20
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pI	Isoelectric Point
PLT	Progressive Lowering Temperature
PSG	Primary Salivary Glands
PVP	Polyvinyl pyrrolidon
RAPD	Random Amplification of Polymorphic DNA
RCR	Rolling Circle Replication

REn	Replication Enhancer Protein
Rep	Replication Associated Protein
RER	Rough Endoplasmic Reticulum
Rpm	Revolutions per min
RT	Room Temperature
RuBisCo	Ribulose-1,5-bisphosphate Carboxylase/oxygenase
SCB	Sodium citrate buffer
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
SG	Salivary Glands
SiGMV	Sida golden mosaic virus
SLCV	Squash leaf curl virus
ssDNA	single-stranded DNA
ST	Stylet
T.vap	*Trialeurodes vaporariorum*
TAE	Tris-acetate EDTA
TAS-ELISA	Triple Antibody Sandwich-Enzyme linked Immunosorbent Assay
TEM	Transmission Electron Microscopy
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGMV	*Tomato golden mosaic virus*
ToMoV	*Tomato mottle virus*
TPCTV	*Tomato pseudo-curly top virus*
TrAP	Transcriptional activator protein
TYDV	*Tobacco yellow dwarf virus*
TYLCV	*Tomato yellow leaf curl virus*
UAc	Uranyl acetate
V	Vesicles
WDV	*Wheat dwarf virus*

WFTG	Whitefly transmitted Geminiviruses
WmCSV	*Watermelon chlorotic stunt virus*
wt	Wild type virus
YEBKnRif	YEB,Kanamycin/Rifampicin

1 Introduction

1.1 Geminiviruses

The family *Geminiviridae* comprises a large number of plant viruses that produce significant losses in economically important crops of both monocotyledonous and dicotyledonous plants. Especially since the late 1980s, the horticultural-producing areas of the Mediterranean, Spain, Morocco, Italy, Central America, Venezuela and Brazil, and Southern USA, Arizona and Florida, the Caribbean and Mexico have been heavily attacked by whitefly-borne geminiviruses, with devastating economic consequences for the respective agro-industries. Geminivirus diseases pose a serious threat to agricultural production in tropical and sub tropical world.

Geminiviruses are small plant viruses appearing as twin (geminate) icosahedral particle structures in electron microscopy. The family *Geminiviridae* comprises four genera, *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus*. Their small genomes consist of covalently closed circular single-stranded (ss) DNA molecules. The taxonomy of the family is based on genome organization, taxon of insect vector and host association (Gafni *et al*., 2002).

Mastreviruses like *Wheat dwarf virus* (WDV) are transmitted by leafhoppers, have monopartite genomes and mostly infect monocotyledonous plant species while some members such as *Tobacco yellow dwarf virus* (TYDV) or *Bean yellow dwarf virus* (BeYDV) infecting dicotyledonous plants. Curtoviruses like *Beet curly top virus* (BCTV) are also transmitted by leafhoppers, infect dicotyledonous plants and have monopartite genomes with a genetic organization distinct from the mastreviruses. The sole member of the genus *Topocuvirus*, *Tomato pseudo-curly top virus* (TPCTV), is transmitted by treehoppers, and represents the least-well characterized genus of geminivirus (Gutierrez, 2002).

Begomoviruses with the type member *Bean golden mosaic virus* (BGMV) are transmitted by *Bemisia tabaci* whiteflies. Most members of the genus have bipartite circular single-stranded DNA A and DNA B genomic components (approximately 2.5-3.0 kb) and infect dicots. Some very important members of the genus *begomovirus*, *Tomato yellow leaf curl virus* (TYLCV) or *Cotton leaf curl virus* (CLCuV) have all genes resident on one DNA A like component of about 2.7 kb. Geminiviruses invade the nuclei of infected plants and since their genomes do not encode DNA or RNA polymerases, their replication relies entirely on the host cellular replication and transcription machinery for gene expression and genome amplification (Gutierrez, 1999). DNA replication occurs within the nuclei of infected cells by a rolling circle replication (RCR) employing circular dsDNA replicative form intermediates serving as templates for replication and transcription (Gutierrez, 2002).

1.2 Diseases caused by *Bemisia tabaci* transmitted Begomoviruses

Begomoviruses cause many diseases of crops and wild plants. Symptoms typically consist of leaf-curling, mosaic, vein yellowing or a more generalized leaf chlorosis often accompanied by stunted growth of infected plants. In the past two decades, Begomoviruses have emerged as serious and devastating pathogens and now present major constraints to the cultivation of significant food and fibre crops in various parts of the world. Some of the diseases are newly emerging hence providing evidence that these viruses are still evolving with increasing impact threatening sustainable agricultural production world wide and particularly in the tropics and sub-tropics. The recent discovery that these viruses can be accompanied by even smaller DNA molecules (ca. 1.3 kb), so called satellite DNA (DNA ß) leading to serious disease complexes (Saunders *et al*., 2002; Varma and Malathi, 2003; Bull *et al*., 2004; Stanley, 2004), contributes to the notion that begomoviruses are the most dangerous plant viruses to date.

Diseases caused by begomoviruses result in the world's economically most important crop losses. For example, mosaic diseases of cassava in sub-Saharan Africa cause annual yield losses exceeding $2 billion in value of this most significant

staple food for millions of poor people (Thresh *et al*., 1998). Tomato leaf curl and tomato yellow leaf curl diseases are devastating in tomato crops in more than 20 countries (Czosnek and Laterrot, 1997, Green and Kalloo, 1994). Cotton leaf curl disease, the monopartite begomovirus trans-replicating the satellite DNA ß complex, has spread enormously affecting more than 2 million acres in Pakistan (Ali *et al*., 1995) where cotton covers about 60% of the country's exports, with serious effects on yield and on the nation's economy. From 1992 to 1995 the accumulated losses in this crop in Pakistan were calculated exceeding $5 billion (Briddon and Markham, 2000). Solanaceous crops, tomato and pepper are most favoured hosts for a large number of begomoviruses. Until now more than 50 begomoviruses with 35 distinct species were described in natural infections of tomato, pepper and cucurbits in the New and Old World (Fauquet *et al*., 2003). Some of the virus species further occur with a large number of distinct virus strains (Jones, 2003) emphasizing the high diversity of these pathogens most evident in the tropical and subtropical tomato growing regions of the Americas.

In North Africa, besides TYLCV infections seriously impeding the cultivation of tomato and *Phaseolus* beans, there is yet another begomovirus, *Watermelon chlorotic stunt virus* (WmCSV) threatening the production of melons and related cucurbits. This serious disease in watermelon is characterised by vein yellowing, chlorotic mottling and a severe stunting of young leaves with drastic reduction of fruit yield. It was firstly reported from the former People Democratic Republic of Yemen (PDRY) where incidence exceeding 90% infected watermelon plants was common with WmCSV present wherever watermelon was grown (Jones *et al*., 1988). The infected plants were found to contain virus particles with a twinned morphology typical of geminiviruses and the virus was subsequently described as *Watermelon chlorotic stunt virus* (Bedford *et al*., 1994) a begomovirus transmitted by the whitefly *B. tabaci*. WmCSV has been found in central and eastern Sudan (Lecoq *et al*., 1994, Kheyr-Pour *et al*., 1997; Dafalla *et al*., 1998) and was reported in 1998 from watermelon fields in southern regions of Iran (Bananej *et al*., 1998).

1.3 Genome structure and replication of Begomoviruses

Bi-segmented, 'geminate' particles are characteristic for all viruses in the family *Geminiviridae* including the members of the genus Begomovirus. This structure is contained by the only structural protein of the virus; the coat protein (CP) encoded by the AV1 gene which is located on DNA A genomic component of the bipartite members of this genus (Fig. 1). It codes for a protein of ca 28-30 kDa of which approximately 110 CP molecules are required to form the geminate particle. The CP enforces the structural features of the virion hence geminivirus CP especially that of begomoviruses are highly conserved.

The circular ssDNA genomes of the geminiviruses contain coding regions in virion-sense and complementary-sense strands diverging from an intergenic region (IR). The circular dsDNA replication intermediates are the transcriptionally active templates. Transcription occurs bidirectionally and is dependent on the activity of two divergent promoters separated by a non-transcribed region where most (if not all) of the *cis*-acting signals regulating viral replication are also located.

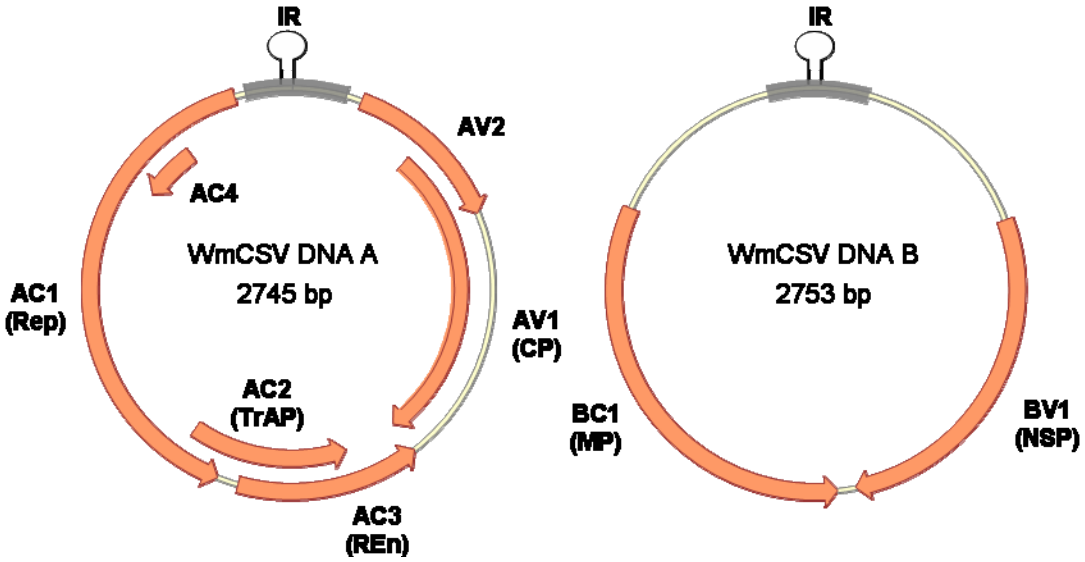

Figure 1: Genome organisation of a typical bipartite begomovirus, WmCSV. On WmCSV DNA A, CP is the viral coat protein in virion sense orientation, the replication initiation protein (Rep), the transcriptional activation protein (TrAP) and the replication enhancer protein (REn) is in complementary sense orientation. ORF AV2 and AC4 are present only in Old World begomoviruses. On WmCSV DNA B, NSP is the nuclear shuttle protein in virion sense orientation, while MP, the movement protein, is in virion complementary sense.

This IR forms a stem loop structure in which ssDNA is initiated by the replication-associated protein (Rep) cleaving an absolutely conserved TAATATT/AC nona-nucleotide motif in virion sense, forming a potential loop structure. In the bipartite begomoviruses, the IR of the DNA A and DNA B components are highly similar with identical reiterated motifs (iterons) distinct for a species, so that the DNA A Rep will only and specifically recognise the IR of its cognate DNA B for replication.

On DNA A in complementary C-sense, four proteins: Rep, TrAP, REn and AC4 are located while DNA B contains two ORFs coding for proteins directly involved in movement of viral DNA (NSP, nuclear shuttle protein; MP, movement protein).

As a general rule, the complementary, C-sense-encoded proteins are involved in DNA replication, regulation of transcription and interference with cellular processes needed for the replicative cycle. The Rep protein is the only viral protein absolutely required for viral DNA replication interacting with the viral REn. This protein enhances viral infection and symptom development, an effect that is likely a consequence of stimulation of viral DNA replication. TrAP is a transcriptional activator gene (Gutierrez, 2002) and it is the viral gene silencing suppressor gene for most begomoviruses counteracting the plants natural defence mechanisms (Gutierrez, 2002).

Recent studies with the begomovirus SLCV have elucidated the role of proteins encoded by DNA B in cell-to-cell movement of viral genomes. Thus, NSP is believed to associate with newly-formed viral ssDNA and translocate it outside the nucleus. The NSP and the MP interact mediating the transport of viral genomes to the cortical cytoplasm and the plasmodesmata, with the MP being an essential part of virus-infected cells (Gafni and Epel, 2002).

The CP enforces the structural features of the virion hence geminivirus CP genes especially that of begomoviruses, are highly conserved. CP has several functions and is the basis of serological methods for detecting and identifying individual begomoviruses (Harrison *et al.,* 2002). One of the most noteworthy characteristics of begomoviruses is the extent of serological relationships among different viral species. This was first discovered in work on *African cassava mosaic virus* (ACMV), *Bean golden mosaic virus* (BGMV), *Tomato golden mosaic virus* (TGMV) and

Squash leaf curl virus (SLCV), in which the reactions of polyclonal antibodies were tested by immunodiffusion, ELISA, and immunosorbent electron microscopy. The nature of these relationships has been explored in more detail by tests with monoclonal antibodies (MAbs), especially the panels of MAbs raised against particles of ACMV, *Indian cassava mosaic virus* (ICMV), or *Okra leaf curl virus* (Swanson, 1992; Swanson and Harrison, 1993; Thomas *et al.,* 1986; Swanson, 1992). The conclusion drawn from comparing the epitope profiles of about 50 distinct begomoviruses is that many of these profiles are specific for a given virus, which can thereby be distinguished from other begomoviruses (Harrison 1994; Harrison *et al.,* 1991). Further comparisons of epitope profiles have led to another, more interesting, conclusion: Begomoviruses from different hosts in the same geographical region tend to be more closely antigenically related to one another than to viruses causing indistinguishable diseases in other regions. For instance, the epitope profile of ICMV is more similar to that of another begomovirus from the Indian subcontinent, *Cotton leaf curl virus* from Pakistan (CLCuV-PK), than to that of either of two cassava geminiviruses from Africa, ACMV and *East African cassava mosaic virus* (EACMV). In turn, the epitope profile of CLCuV-PK differs greatly from that of *Cotton leaf crumple virus* (CLCrV, from the United States), which is much more closely related to TGMV (from Brazil) and BGMV (from Puerto Rico). The obvious relation of epitope profile to geographical source among a set of begomoviruses that have different host ranges, and the apparently limited influence of plant host species, leads to two further propositions (Hong, and Harrison 1995): (*a*) Begomovirus particle proteins have evolved differently in different geographical regions, almost without reference to host range, suggesting that region-specific selection pressures are operating; and (*b*) plant host range may be more flexible over a period of years, for begomoviruses than it is for RNA plant viruses. Begomoviruses occurring in a particular region may thus be able in time, to adapt to additional or alternative plant species.

Sequence comparisons of begomovirus genomes with emphasis on the CP has grouped these viruses according to their geographical origin: (1) new world, with subgroups including Central and South America and the Caribbean Islands (except the newly introduced Middle Eastern TYLCV); (2) western Mediterranean basin, (3)

Middle East; (4) Indian subcontinent; and (5) East and Southeast Asia and Australian (Rybicki, 1994; Padidam *et al.,* 1994).

As for the geographic diversification of begomoviruses, the insect vector *B. tabaci* can be separated into five major groups based on a partial mitochondria cytochrome oxidase (mtCOI) DNA sequences (Fröhlich *et al*., 1999) revealing almost identical phylogenetic patterns separating *B. tabaci* whiteflies according to geographical origin. This co-adaptation is likely to be the result of co-evolution processes between begomoviruses and their vectors (Harrison and Robinson, 1999; Harrison *et al.,* 1991; Maruthi *et al*., 2002).

1.4 Geminivirus factors for vector transmission

The geminivirus coat protein plays a key role in insect transmission determining vector specificity and transmission competence. The CPs of begomoviruses are antigenically related (Harrison *et al.*, 2002) while CPs of leafhopper-transmitted geminiviruses (mastreviruses) are antigenically unrelated and have different vector species, indicating a specific correlation between vector and antigenic affinity (Roberts *et al.*, 1984).

The geminivirus coat protein gene determines vector specificity. This was demonstrated by replacing the CP of *African cassava mosaic virus* (ACMV) with that of *Beet curly top virus* (BCTV), a leafhopper (*Ciculifer tenellus*) (Baker) transmitted curtovirus. The ACMV / BCTV CP chimera was transmitted by the leafhopper (Briddon *et al.*, 1990). More specific indications for CP sequences involved in virus transmission were presented by exchange of the CP of a non transmissible *Abutilon mosaic virus* (AbMV) isolate with that of *Sida golden mosaic virus* (SiGMV-Co), a closely related transmissible geminivirus. The resulting chimeric AbMV restored transmissibility by *B. tabaci* (Höfer *et al.*, 1997).

The CP amino acid composition determines whitefly transmission. This was first described by Noris *et al*. (1998) with amino acid substitutions in the CP of *Tomato yellow leaf curl virus* (TYLCV) abolishing whitefly transmissibility in a naturally occurring TYLCV strain from Sicily (SicRcv). Comparing CP sequences with the

closely related *Tomato yellow leaf curl Sardinia virus* (TYLSCV; Sar), aa exchanges were found in positions 129 (P in SicRcv, Q in Sar), 134 (H in SicRcv, Q in Sar) and 152 (E in SicRcv, D in Sar). When TYLCSV mutants were generated with amino acid substitutions in those positions it was found that whitefly transmission is determined by a glutamine (Q) at position 134 but also a Q at position 129.

Höhnle *et al.* (2001) used the closely related Central American begomoviruses *Abutilon mosaic virus* (AbMV) and *Sida golden mosaic virus* (SiGMV) isolated to study transmission by *B. tabaci*. Comparison of highly conserved C termini of transmissible and non transmissible begomovirus CP sequences showed 6 amino acids differing between the non transmitted AbMV and the transmission competent SiGMV at positions 124, 149, 174, 179, 193 and 249 . Mutagenesis of the AbMV coat protein then revealed that the exchange of two amino acids, Q124K and H149Q, restored whitefly-transmissibility. However when in addition, L at 174 was replaced with M, AbMV transmission efficiency was increased. Höhnle *et al.* (2001) concluded that not a concise motif, such as the amino acid triplet, aspartate-alanine-glycine (DAG), involved in aphid transmission of potyviruses, determines transmissibility of begomoviruses by *B. tabaci* but rather a coat protein domain from amino acid 123 to 149, acting as a structural context for transmission, with amino acids positions 149 to 174 contributing to transmission efficiency.

While investigating whitefly-transmission of WmCSV, Kheyr-Pour *et al.* (2000) found a single mutation in the CP at position 131 (D, aspartate) of an Sudanese isolate abolished whitefly transmissibility of this isolate maintained on infected plants through vegetative propagation by multiple grafting cycles. The engineered mutant virus WmCSV D131N was transmissible by *B. tabaci,* hence it was concluded that asparagine at position 131 conserved in all whitefly-transmitted geminiviruses restores transmissibility of WmCSV.

With the recent elucidation of the ACMV begomovirus capsid morphology, studied in detail by cryo-electron microscopy and image reconstruction (Böttcher *et al*., 2004), structural information became available that located the critical amino acids essential for begomovirus transmission from the studies by Noris *et al*. (1998), Kheyr-Pour *et al.* (2000) and Höhnle *et al.* (2001) to a protruding region on the CP

capsomer. This exposed position is suggestive for a structural role of this region in receptor recognition.

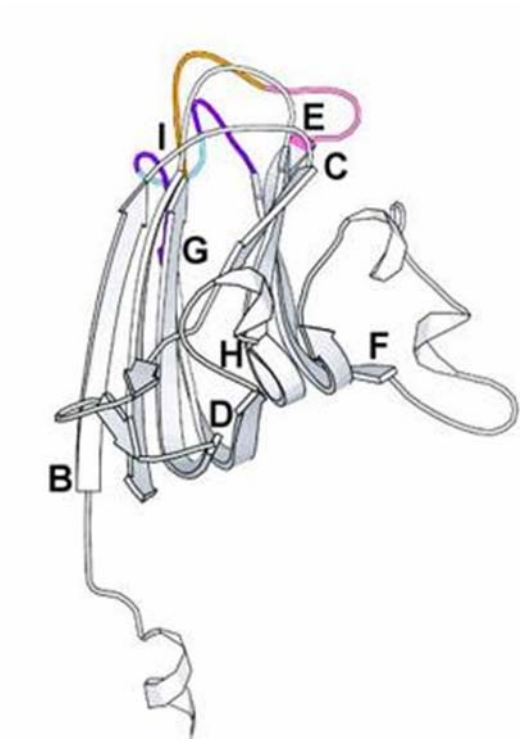

Figure 2: Structural model of a single ACMV CP capsid with a six aa motif essential for begomovirus transmission by *Bemisia tabaci* exposed in E (violet). Model from Böttcher *et al.*, 2004.

1.5 Whiteflies as virus vectors

Whiteflies are insects belonging to the family *Aleyrodidae*. They occur in warm climates where they are pests of herbaceous and woody plants. In temperate climates, they are usually pests of protected crops. About 1300 whitefly species in over 120 genera have been described (Annon., 2001a; Mound and Halsey, 1978) but relatively few are virus vectors. One-hundred and fourteen virus species are transmitted by limited genera of whiteflies. In the *genus Bemisia*, only *Bemisia tabaci* (Genn.) is a virus vector whereas in the *Trialeurodes genus, Trialeurodes vaporariorum, T. abutilonea* and *T. ricini* transmit viruses. *Bemisia tabaci* transmits 111 virus species while *T. vaporariorum* and *T. abutilonea* transmit three species each. *B. tabaci* and *T. vaporariorum* are present in the European-Mediterranean region, though the former is restricted in its distribution to the Southern parts of Europe up to the South

of France. Of the whitefly transmitted virus species, 90% are begomoviruses, 6% criniviruses and the remaining 4% are in the genera *Closterovirus, Ipomovirus* or *Carlavirus (*Jones 2003).

1.6 Characteristics of *Bemisia tabaci* virus transmission

The life span of *B. tabaci* depends on temperature but on average is 21 days. The adult whitefly must feed for only about 3.5 h (Acquisition Access Period, AAP) for acquisition of most begomoviruses with a latency period of at least 8 h and about 10 min of inoculation feeding (Inoculation Access Period, IAP) for virus transmission. The whiteflies then remain viruliferous for more than a week and up to 28 days which was found for TYLCV. Transovarial transmission was only reported by Ghanim *et al.,* (1998) for a laboratory whitefly culture transmitting TYLCV.

Parameters of acquisition, inoculation, retention, latent periods and number of viruliferous *B. tabaci* required for transmission of begomoviruses by adult *B. tabaci* have been studied extensively with *Tomato yellow leaf curl virus* (TYLCV). The minimum AAP was found to be between 5 min and 1 hour with TYLCV detectable in the head of the specimen already 5 - 10 min after access feeding, in the thorax after 10 min and the abdomen after 25 - 40 min (Ghanim *et al.*, 2001; Atzmon *et al*., 1998). TYLCV was translocated and reached the haemolymph after 90 min and the salivary glands 5.5 h later (Ghanim *et al.*, 2001). For WmCSV transmission by whiteflies a 1 hour AAP and 1 hour IAP was found the minimum time necessary (Marchelo *et al*., 1997).

A single whitefly can acquire TYLCV, transmit it to tomato plants (Cohen and Nitzany, 1966) and introduce virus infection. In contrast, attempts to transmit WmCSV from and to watermelon plants using one insect / plant were not successful (Marchelo *et al*., 1997). The rate of virus transmission increases with AAP and IAP and with the number of viruliferous insects. The frequency of WmCSV transmission increased from 12.5% after one hour AAP to 85.7% after 24 h AAP and from 11.1% after one hour IAP to 88.9% after 24 h IAP.

The rate of infection was 37.5% with 3 insects / plant and reached 100% with 15 insects / plant (Marchelo *et al*., 1997), with frequency of TYLCV transmission increasing from 10 - 20% after a 30 min AAP to 100% after 24 – 48 h AAP (Brown and Czosnek, 2002). The minimum latent period for begomoviruses that was studied with different TYLCV isolates was between 17 – 24 h (Caciagli *et al*., 1995; Cohen and Nitzany, 1966; Mehta *et al*., 1994).

B. tabaci transmission of begomovirus involves complex interactions between viral proteins and vector compounds (Gray and Banerjee, 1999), while the involvement of geminivirus capsid proteins in virus transmission has been clearly demonstrated only little is known about the whitefly factors determining begomovirus transmission.

B. tabaci transmits begomoviruses in a circulative manner. The viruses are ingested from the plant (phloem) into the alimentary canal, translocated across the midgut into the haemocoel, move to the accessory salivary glands for subsequent re-inoculation of plants. Circulative transmission of plant viruses involves active translocation in the vector across multiple cell membranes. Ultimately the virus must associate with the vector salivary system to be inoculated into a host (Ng and Perry, 2004). In circulative virus/vector interactions, transmission is determined by the virus to successfully passage the critical barrier tissues of the alimentary canal and the salivary gland (Reinbold *et al*., 2003; Rouze-Jouan, *et al*., 2001; Reinbold *et al*., 2001; Rosell *et al*., 1999; Gildow, 1993; Gildow and Gray, 1993; Ullman *et al*., 1992). This mechanism is regarded as a specific interaction between the virus coat protein and receptors in the vector.

During transmission, begomoviruses circulate in their vector, requiring virus recognition, penetration, and transport through whitefly tissues and organs. It is generally assumed that geminiviruses do not replicate in their whitefly vector. The type of virus transmission is a circulative-non propagative transmission (Cohen and Nitzany, 1966) and (Rubinstein and Czosnek, 1997) observed first for the luteoviruses *Barley yellow dwarf virus*, *Potato leaf roll virus* and *Carrot mottle virus* vectored by aphids. It is generally assumed that begomovirus transmission follows a strategy similar to the aphid transmitted luteoviruses (Cohen and Antignus, 1994).

Geminivirus transmission could be divided into four distinct processes (Hunter *et al.,* 1998):

a) virus ingestion from the host plant into the lumen of the whitefly alimentary canal,

b) acquisition of the virus through the gut,

c) retention in the tissues and haemocoel, and

d) transmission through the salivary gland and into the phloem tissue of a host plant.

1.7 Virus translocation in *B. tabaci*

The path taken by the virus after being ingested by the insect vector has been explored by Hunter *et al*., (1998) for two begomoviruses, *Tomato mottle virus* (ToMoV) and *Cabbage leaf curl virus* (CabLCV) which were visualized by indirect-fluorescent microscopy in dissected whiteflies. More recently, Rossel *et al*., (1999) conducted a more detailed study using Polymerase Chain Reaction (PCR) to trace *Squash leaf curl virus* (SLCV) in whole whitefly body extracts including saliva, haemolymph and honeydew.

From different studies, it appears that a definite scenario of virus passage through the insect exists, starting with virus ingestion and passage into the oesophagus and foregut (Harris *et al*.,1995). As food enters the filter chamber, excess water is shunted to the ileum of the hindgut (Lindsay and Mashall, 1981), thus nutrients and virus become concentrated in the filter chamber. The virus may adsorb to specific sites on the alimentary membranes or at anterior region of the midgut with the exact mode of the virus entry into the cells still unknown. The virus then moves out of these cells into the haemolymph (Rossel *et al.*, 1997) and eventually invading the salivary glands (Briddon *et al.*, 1990; Ghanim *et al*., 2001) Once the virus reaches the salivary gland, it passes through the salivary gland membranes via small ductules to the salivary ducts, where it be salivated out through the salivary canal, thus being injecting into plant cells during insect feeding (Hunter *et al*., 1998).

Selective, or receptor-mediated, endocytosis is a ubiquitous mechanism for internalisation functionally important macromolecules in animal cells (Goldstein *et al*., 1985). The selectivity of receptor-mediated endocytosis is dictated by the presence of specific receptors on the plasma membrane of the cell, thus enabling the internalisation of only particular macromolecules.

For viruses that enter the host via ingestion, as with begomoviruses, the peritrophic membranes must be passaged in order to penetrate midgut cells. This process is not fully understood. Studying the interactions of transmissible and non-transmissible begomoviruses with vector and non vector insects may assist in identifying the viral and cellular determinants involved in transmission and also elucidate the history of begomovirus / whitefly co-evolution. Furthermore and from a more pragmatic point of view, effective virus control and disease management strategies can also consider interfering with whitefly transmission. This however requires a solid and profound knowledge of the route viruses take in their vectors and the interactions underlying virus translocation and transmission.

1.8 Objectives of the study

A detailed study of the interactions between begomoviruses and their vectors may permit the identification of viral and cellular determinants of insect transmission. This shall be reached by an investigation of the begomovirus translocation pathway in whitefly insects, to localise the virus in insect organs. By including transmissible and non transmissible viruses and virus vectors and non vector insects, the epithelial barriers for virus translocation shall be discovered. Microscopical studies, to reveal virus at sites on whitefly organs, filter chamber, gut membrane, primary salivary gland and/or accessory salivary gland that function as virus recognition/receptor sites further contributes to the detailed description of the interactions between begomovirus(es) and their whitefly vector.

2 Materials and Methods

2.1 Maintenance of whitefly cultures

B. tabaci (biotype B) whiteflies were reared in colonies on healthy cotton plants, (*Gossypium hirsutum L*.) and tomato plants (*Lycopersicon esculentum* Mill.) that are non host plants of WmCSV. Whiteflies were maintained in insect-proof cages in a growth chamber at 26°C with a 16 h photoperiod. Cultures of *T. vaporariorum* insects that are not transmitting begomoviruses were established on virus-free tobacco (*Nicotiana tabacum* L.) plants and maintained in cages at 22°C, for comparison and reference. Adult whiteflies were collected using a hand-held aspirator.

2.2 Transmission of WmCSV by *Bemisia tabaci* and *Trialeurodes vaporariorum* insects

Virus free whiteflies were collected from *B. tabaci* and *T. vap* colonies by aspiration. Insects were starved for 4 h before allowing them a 48 h AAP on watermelon plants infected with a whitefly transmitted WmCSVwt. Insects were discharged for 72 h by transferring them onto a non host, healthy tomato plants. 100 specimen, discharged insects, were allowed a 72 h IAP on 10-15 healthy watermelon plants for inoculation at the first true leaf stage. Plants were sprayed with Confidor® (Bayer CropScience, Leverkusen, Germany) or Applaud® (Syngenta Agro, Maintal, Germany) following the manufacturer's prescriptions. Plants were checked for characteristic virus symptoms every 2-3 days for a period of 6 weeks. Virus infections were confirmed by TAS-ELISA.

All WmCSV mutants used in this study were subjected to the similar experimental conditions to verify their transmissibility and to generate viruliferous whiteflies insects for translocation studies.

2.2.1 Blocking WmCSV transmission by *Bemisia tabaci*

To test for receptor recognition specificity, experiments were conducted to inhibit transmission of the whitefly transmissible WmCSVwt by saturating insects with acquisition of a non transmissible virus mutant WmCSVasp. 100 insects were fed on WmCSVasp infected watermelon plants for 5 days and following a 48 h discharge period, insects were transferred to WmCSVwt infected plants for further 5 days, discharged for 48 h and subsequently transferred to 20 healthy watermelon plants at the 2 leaf stage. After 3 days IAP, plants were sprayed with insecticides, observed for symptom development and tested by TAS-ELISA.

2.2.2 Feeding *Bemisia tabaci* on artificial diets for WmCSV transmission and increased uptake of virus particles

For ultrastructural investigations of insect organs, midgut, primary and accessory salivary glands respectively, the concentration of virus needed to be increased to facilitate localisation of virus particles. Hence purified WmCSV preparations (100 µg/ml) were added to filter sterilized 15% sucrose in 0.1 M phosphate buffer pH 7.2, as artificial diet. This virus solution was placed between 2 layers of stretched parafilm onto which insects were allowed to feed.

Insects from virus free cultures were collected using a hand-held aspirator, starved for 4-5 h, cooled on ice for 5 min to arrest movement and subsequently transferred to feed for 48-72h AAP on the virus-sucrose diet.

Following the AAP, insects were placed on watermelon plants for 24 h IAP and collected thereafter for virus localisation experiments, insect dissection and organ excision and fixation.

2.3 *Watermelon chlorotic stunt virus*, origin, virus mutants, plant inoculation and maintenance of virus infected plants

WmCSV is not mechanically transmitted and in nature relies on insect transmission by *B. tabaci* for plant infection and spread. In the laboratory virus infections were

established for the virus/insect interaction studies using infectious clones of WmCSV previously described by Kheyr-Pour *et al*., (2000). The original virus materials, cloned genomic components of WmCSV in *E. coli* bacteria, were kindly provided by Dr. Bruno Gronenborn, CNRS, Gif sur Yvette, France.

In a previous study by Kheyr-Pour *et al*., (2000), full length WmCSV genomic components DNA A and DNA B were cloned and sequence analysed. For plant infections, virus constructs comprising redundant genome sequences, that are dimeric DNA components position cloned in a head-to-tail orientation, were generated for WmCSV DNA A and DNA B genomic components. These were sub-cloned into the binary vector pBin19 and transformed into the *Agrobacterium tumefaciens* strain LBA 4404, to infect plants by agroinoculation (Kheyr-Pour *et al*., 1991) or by biolistic delivery of DNA constructs coated on gold particles using a Helios gene gun (BioRad).

An infectious virus clone, comprising DNA A and DNA B genomic components, represented the wild type virus, WmCSVwt with characteristic symptomatology and whitefly transmission features. The coat protein of this virus consisted of 258 amino acids with a characteristic asparagine aa conserved in all whitefly transmitted geminiviruses at position N131.This is part of a potential N-linked glycosylation site (NXS/T) and hence mutation were induced in this motif (Fig. 3 bold) to provide proof for this hypothesis.

```
  1  MAKRTGDILI STPVSKVRRK LNFDSPYMSR ALAPTVLVTS KRRQWANRPM
 51  YRKPRMYRMY RSPDVPKGCE GPCKVQSYEQ RDDVKHTGIV RCVSDVTRGS
101  GITHRVGKRF CVKSIYILGK IWMDENIKKQ NHTNQVMFFL VRDRRPYGSS
151  PMDFGQVFNM FDNEPSTATV KNDLRDRFQV MRKFHATVVG GPSGMKEQAL
201  VKRFYRVYNH VVYNHQETAK YENHTENAML LYMACTHASN PVYATLKIRI
251  YFYDSVTN
```

Figure 3: Coat protein amino acid sequence of a whitefly transmitted isolate of WmCSVwt.

Several WmCSV mutants created by site directed mutagenesis (Fig. 4) to alter the amino acids in aa position 133 retained infectivity to watermelon and subsequently were made available for this study to investigate on vector translocation and whitefly transmission.

Aa position						131	132	133			
Wild type	WmCSV wt	I	WMDENIK	KQ		**N**	H	T	N	QVMFFLVR	DRRPYGSS
Mutant D133	WmCSV asp	I	WMDENIK	KQ		N	H	D	N	QVMFFLVR	DRRPYGSS
Mutant S133	WmCSV ser	I	WMDENIK	KQ		N	H	S	N	QVMFFLVR	DRRPYGSS
Mutant A133	WmCSV ala	I	WMDENIK	KQ		N	H	A	N	QVMFFLVR	DRRPYGSS
Mutant V133	WmCSV val	I	WMDENIK	KQ		N	H	V	N	QVMFFLVR	DRRPYGSS

Figure 4: WmCSV DNA A mutants generated by site directed mutagenesis to exchange thereonine at position T133 with D133 asparagine, S133 serine, A133 alanine and V133 valin. Boxed area reflects the protruding 6 aa motif in the structural model of the ACMV CP capsid presented in Fig. 2

2.3.1 WmCSV host plants

The watermelon cultivar *Citrullus lanatus* cv. Sugar Baby (Petroseed, France) and *Nicotiana benthamiana* were used for virus propagation. Watermelon plantlings were agroinoculated as 3-4 weeks old plants, with the first true leaf just unfolding. *N. benthamiana* plants were used in the 5-8 leaf stadium. Infection was confirmed by characteristic virus symptom development becoming visible approximately 10-12 days post inoculation. For agroinoculation experiments the plants were maintained under greenhouse conditions with additional light provided for an 18 h photoperiod. Temperature was controlled in a closed circuit and conditioned chamber at 25 °C, to guarantee *vir*-gene induction.

2.3.2 Establishment of WmCSV infections by agroinoculation

The infection of host plants with cloned viruses using the plant bacterium *Agrobacterium tumefaciens* harbouring a binary plasmid carrying viral sequences was used for inoculation of plants with DNA viruses (Kheyr-Pour *et al*., 1991). It utilises the principle of *A. tumefaciens*, to transfer the T-DNA integrated viral full length clones, into plant cells. To establish viral infection, replication competent viral intermediates need to be generated, with the induction of agrobacterial *vir*-genes a significant pre-requisite for T-DNA transfer. This activation is initiated at temperatures below 28 °C, at low pH and stimulated by the phenolic compounds, acetosyringone.

2.3.3 Preparation of bacterial suspension cultures for agro-inoculation

All media, solutions and distilled water used were sterilized by autoclaving at 121°C, 1 bar for 30 min, or filter sterilized (pore size 0.2 µm).

YEB medium (per liter)

3-5 g	Beef meat extract
5 g	Peptone (0.5%)
5 g	Sucrose (0.5%)
1 g	Bacto yeast (0.1%)
15 g	Agar if plates were needed

pH adjusted to 7.2 with 1 N NaOH

YEB KnRif plates (per liter) pH 7.2

3-5 g	Beef meat extract
5 g	Peptone
5 g	Sucrose
1 g	Bacto yeast

15 g BiTek^{TM}-agar

1 ml Kanamycin (80 mg/ ml H_20)

1 ml Rifampicin (100mg/ml Methanol)

MS medium

4.6 g MS (Murashige & Skoog)

20 g Sucrose

pH was adjusted to 5.6 using 1N KOH

MMA

MS medium

1.95 g MES (2-(N-morpholino)ethane sulfonic acid)

pH was adjusted to 6.3 using 1N KOH

150-200 mM Acetosyringone (Sigma, Germany)

A glycerol stock of the Agrobacterium strain LBA 4404 carrying dimers of the respective DNA A and DNA B genomic components of wild type or mutant WmCSV were streaked onto YEB KnRif plates containing the antibiotics kanamycin and rifampicin and grown for 48 hrs and incubated at 28 °C for 48 h. A single colony was transferred to 3 ml YEB KnRif adjusted to 2 mM $MgSO_4$ and incubated overnight at 28 °C while shaking incubator. 300 µl of each bacterial suspension was then used to inoculate 50 ml YEB KnRif medium adjusted to 2 mM $MgSO_4$ and 20 µM acetosyringone and 1 M MES. After shaking at 28 °C for 48 h, cells were harvested by centrifugation in an Eppendorf centrifuge (Germany) at 4000 rpm for 10 min. Sedimented cells were resuspended in MMA and 200 mM acetosyringone, diluted to OD_{600nm} at 0.5-0.7 Bacterial cultures harbouring plasmids with DNA A and respective WmCSV DNA B genomic components were mixed to equal amounts and left for 2-3 h at room temperature to sediment debris prior to agroinoculation.

2.3.4 Agroinoculation of watermelon plants with cloned WmCSV genomic components

About 100 µl of bacterial suspension 0.5 x 10^{12} cells were injected into each plant at the base of the stem of the plant leaves. Plants were inoculated using a 1 ml disposable syringe with a 28 gauge insulin needle attached. Injection was done by inserting several wounds into stem and leaf petioles. For controls, some plants were left either uninoculated or were mock inoculated with water. Agroinfection experiments were carried out in the BBA Biocontainment facility with all plants maintained under strict containment conditions following safety precautions on handling and disposal of bacteria and plant materials as prescribed in guidelines on biosafety for the BBA facility.

To confirm presence of WmCSV DNA A in agrobacteria a polymerase chain reaction (PCR) was conducted as described in section (2.8). For template preparation bacteria were lysed using lysosyme and DNA was extracted using the high pure PCR template preparation kit, (Roche, Mannheim, Germany).

2.3.5 Inoculation of watermelon plants using the biolistic Helios gene gun

Biolistic inoculation to infect plants with cloned WmCSV constructs, was done with watermelon plants at the true leaf stage or with *N. benthamiana* at the 3-5 leaf stage. For each DNA delivery, two shots on the underside of leaves from the hand-held biolistic device were applied in a helium stream set at a pressure of 200-300 psi. Inoculation experiments were done under strict considerations of the biosafety guidelines governed by the German law (Gentechnik Gesetz, Gen TG).

For biolistic bombardment of DNA into plant tissues for virus infection, either plasmid preparations containing the respective DNA clone or, total DNA preparations from WmCSV infected plants containing virus ssDNA and replicative dsDNA forms, were used.

Plasmid preparations harbouring multimeric DNA constructs were mixed (0.5 µg of DNA A + 0.5 µg of DNA B) to reach approximately 1µg DNA per shot prior to coating onto gold microcarriers (section 2.3.5.1). When total DNA preparations from

virus infected plants were used to prepare inoculum coated on microcarriers, DNA preparations were prepared using the DNAzol reagent (Invitrogen, Karlsruhe, Germany)

2.3.5.1 Preparation of DNA-coated micro-carriers and gold-coated tubings for biolistic inoculation

For total DNA delivery, 2 µg/shot of DNA was used, while for biolistic inoculation of cloned virus constructs 0.5 µg/µl of plasmids carrying DNA A and 0.5 µg/µl of plasmids harbouring DNA B were mixed for precipitation onto the gold.

To coat extracted total DNA onto gold particles, 25 mg gold (BioRad, Hercules) with particle sizes of 1.6 micron was weighed into an eppendorf reaction tube, 100 µl of 0.05 M spermidine was added, vortexed and immediately sonicated for 5 seconds. DNA attachment to the gold particles was accomplished by precipitating the DNA from solution to the gold micro-carriers by dropwise addition of 100 µl 1 M $CaCl_2$ while vortexing. The mixture was then left to stand for 10 min at room temperature, then vortexed to resuspend the remaining gold, left to sediment and the gold pellet was washed extensively three times in 1 ml of fresh absolute ethanol to remove residual water. The final pellet was transferred into a Falcon tube and resuspended in 2.6 ml of 0.01 mg/ml ρolyvinyl pyrrolidone (PVP, MW 360 kD, BioRad, Hercules) in ethanol.

Gold-coatTM tubing (50ft, BioRad, Hercules) was washed with fresh absolute ethanol using a syringe to suck in the ethanol at one end of the rubber tube. This was then inserted into the "Tubing Prep Station" (Biorad, Hercules) and dried for 15 min under as stream of Nitrogen (N_2) gas connected to the "Tubing Prep Station". The DNA micro-carrier solution was vortexed and immediately applied into the gold-coat tubing using a syringe. This was then rotated immediately in the Tubing Prep Station to evenly coat the inner tube wall with a layer of DNA/micro-carriers. The remaining particles in solution were drawn out slowly with the syringe followed by immediate rotation of the Gold-coat tubing for a few seconds. The tubing was then dried with N_2 by opening the gas valve at one end of the tubing. So prepared tubing was then

cut into 0.5 inch length cartridges representing the projectiles that could be stored at 10 °C until use.

For biolistic inoculation, cartridges, where inserted into the Helios Gene Gun and shot to deliver DNA coated gold particles by helium discharge to generate wounds and entry sites into plant cells.

2.4 Purification of *Watermelon Chlorotic Stunt Virus* WmCSV Homogenization buffer

0.5 M Na-Phosphate buffer pH 6.0

2.5 mM EDTA

10 mM $Na_2 SO_3$

0.1% 2-mercaptoethanol

1% Triton-X 100

0.1% Driselase

Pellet buffer

0.5 M Na-Phosphate buffer pH 7.0

2.5 mM EDTA

Sucrose buffer

10% sucrose in pellet buffer

N. bentamiana plants that were infected by agroinoculation were harvested 21-30 days post inoculation after pronounced symptoms of WmCSV infection had developed. The purification procedure for WmCSV was adopted from the method described by (Luisoni *et al.,* 1995) for tomato yellow leaf curl virus. Homogenisation and high speed centrifugation for virus concentration was followed by a sugargradient centrifugation step replacing the buoyant density gradient**.**

Virus purification scheme

All steps of the purification were carried out at 4 °C.

1. Leaves were frozen in liquid nitrogen, crushed to a fine powder, and homogenized in a Warring blender after adding ice cold homogenisation buffer (1:3 w/v) containing Driselase for degradation of cell walls.
2. The homogenate was stirred overnight at 4 °C, squeezed through 3 layers of cheesecloth and then emulsified by stirring in 15% ice cold chloroform for 10 min.
3. Phase separation for removal of the chloroform and plant debris was achieved by centrifugation at 10.400g for 15 min at 4 °C in a Sorvall^{R} RC - 5B Refrigerated Superspeed Centrifuge (Du Pont, GmbH, Bad Homburg, Germany) using a GSA rotor.
4. The aqueous phase was removed, transferred to Ti 45 tubes and layered over a 15 ml sucrose cushion. High speed centrifugation in a Optima^{TM} LE-80K Ultracentrifuge (Beckman, Palo Alto, California, USA) was then conducted in a Beckman Ti 45 rotor at 125.000g for 4 h at 4 °C.
5. After high speed centrifugation, the supernatant was discarded and pellets were resuspended in pellet buffer and left overnight at 4°C to allow complete dissolving.
6. A further clarification step at 10.000g for 15 min at 4 °C followed and virus preparation where then loaded onto linear 10-40% sucrose gradients and directly centrifuged in a Beckman SW 41 rotor at 151.000 g for 3 h.

7. Following the centrifugation, the sucrose gradients were fractionated manually by use of a peristaltic pump and collecting 20 drops aliquots.

A 10 µl sample of each fraction was then checked in SDS page for virus coat protein and presence of other protein impurities.

Virus containing fractions were combined and sucrose was removed by high speed centrifugation in a Beckman Ti 70 rotor at 225.000 g for 2 h at 4 °C.

Purified virus preparations were re-checked either by polyacrylamide gel electrophoresis PAGE or by ISEM to confirm virus purity, identity and the approximate concentration needed for artificial feeding experiments.

2.4.1 Separation of proteins by SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

To determine purity of the WmCSV preparation and molecular weight of the coat protein samples from partially purified virus and purified virus preparations were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 12% discontinous polyacrylamide gels. For molecular weight determination, a molecular weight marker (BioRAD, Munich, Germany; Protein standard, phosphorylase b 97,400 kDa; bovine serum albumin 66,200 kDa; ovalbumin 45,000 kDa; carbonic anhydrase 31,000 kDa; soybean trypsin inhibitor 21,500 kDa; lysozyme 14,400 kDa) was separated in parallel to the virus samples.

Solutions for SDS-PAGE

30% Polyacrylamide stock solution

Resolving gel buffer (4x): 1.5 M Tris-HCl, pH 8.8

Stacking gel buffer (4x): 1 M Tris-HCl, pH6.8

10% (w/v) sodium dodecylsulphate (SDS)

10% (w/v) ammonium persulfate (APS)

TEMED: N,N,N`,N`-tetramethylethylenediamine

2x sample buffer, pH6.8, (Laemmli, 1970)

10% SDS	12.5 ml
Stacking gel buffer	20.0 ml
Glycerol	10.0 ml
1% bromophenol blue	2.5 ml
Mercaptoethanol	5.0 ml

Electrophoresis buffer, pH 8.3

25 mM	Tris base,
192 mM	Glycine
0.1% (w/v)	SDS

Coomassie staining solution

Coomassie brilliant blue R 250	0.25%
Methanol	50%
Glacial acetic acid	10%

Destaining solution (per liter)

Methanol	25%
Glacial acetic acid	10%

Table 1: Solutions for preparing 12% resolving gels and 5% stacking gels for SDS-PAGE.

Solution	*20 ml Resolving gel (12%)*	*5ml Stacking gel 5%*
Distilled water	*6.6 ml*	*3.4 ml*
30% acrylamide	*8.0 ml*	*0.85 ml*
Resolving gel buffer (4x)	*5.0 ml*	-
Stacking gel buffer (4x)	-	*0.625 ml*
10% SDS	*200 µl*	*50 µl*
10% APS	*200 µl*	*50 µl*
TEMED	*8 µl*	*5 µl*

Using a dual gel caster (Mighty Small TM SE 245, Amersham Pharmacia Biotech, Freiburg, Germany), solutions for the resolving gel were mixed carefully (without introducing air bubbles), poured between glass plates and overlaid with 1ml isopropanol. When polymerization was complete (approx. in 30 min.) the overlay was poured off and the gel well drained using Whatman 3MM paper. A comb used to form gel slots was inserted and solutions for the stacking gel were mixed and poured on top of the resolving gel. The stacking gel was allowed to polymerize for 20 min. The comb was removed and the gel was carefully washed with distilled water to remove unpolymerized gel solution. The gel plates were subsequently detached from the caster and fixed to the gel apparatus to form upper and lower buffer chambers, which were filled with cold electrophoresis buffer.

2.4.1.1 Sample preparation, electrophoresis and protein staining

Semi-purified and purified virus preparations were mixed (1:1 w/v) with 2x Laemmli sample loading buffer and proteins were boiled for 6 min for denaturation, placed on ice for immediate loading on the gels. 20 µl of each sample to be analysed was

loaded on gels (10cm long x 8 cm wide x 2mm thick) with the respective protein molecular weight marker loaded on lane 1.

Gel electrophoresis was conducted at 100 volts in a vertical gel electrophoresis apparatus Mighty Small II, Amersham Pharmacia Biotech) for about 1.5 h or until the bromophenyl blue dye had migrated to the bottom of the gel.

After electrophoresis, the gel was carefully removed from the glass plates and stained by soaking in the staining solution for 20 min with gentle agitation. Excess stain was then removed by immersing the gel for 1 h in destaining solution changing for fresh solution every 15-20 min.

2.5 Detection of WmCSV by Enzyme linked immunosorbent assay (ELISA)Sample preparation

Virus infection in host plants of WmCSVwt and the different WmCSV mutants was tested using Triple Sandwich Antibody ELISA (TAS-ELISA) essentially as described by Thomas *et al.* (1986). Microtitre plates (96 wells) were coated with a polyclonal WmCSV IgG (DSMZ AS-0830) while monoclonal antibodies raised against TYLCV (general begomovirus Mab, DSMZ AS-0546/1) were used as detecting antibodies.

For ELISA, the youngest plant leaves were collected and ground 1:10 (w/v) in sample extraction buffer.

Sample extraction buffer

Tris pH 8.4 (per liter):

6.05 g Tris

7.56 g Na_2SO_3

add up to 1 liter with distilled water.

ELISA Buffers

Phosphate buffered saline (PBS); 10x PBS pH 7.4 (per liter):

80 g NaCl

2 g NaN_3

2 g KCl

14.4 g $Na_2HPO_4.2H_2O$

2 g KH_2PO_4

dissolve in 800 ml distilled water, adjust pH, make up to 1 liter.

Coating buffer, pH 9.6 (per liter):

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.20 g NaN_3

dissolve in 900 ml distilled water, adjust pH, make up to 1 liter.

Washing buffer-PBST (per liter):

100 ml 10x PBS, pH 7.4

500 µl Tween 20

make up to 1 liter with distilled water.

Conjugate buffer, pH 7.4 (per liter):

100 ml 10x PBS

500 µl Tween 20

20 g PVP 10,000 (M_r)

0.2 % egg albumin

make up to 1 liter with distilled water.

Substrate buffer, pH 9.8 (per liter):

97 ml Diethanolamine

0.2 g NaN_3

dissolve in 600 ml distilled water,

adjust pH with HCl, make up to 1 liter.

The alkaline phosphatase IgG conjugate was detected using the substrate, *p*-nitrophenyl phosphate (Sigma, N9389) at a concentration of 1 mg/ml in 1 M diethanolamine buffer pH 9.8.

TAS-ELISA

For all ELISA tests, Microtitre plates (Greiner Microlon medium binding) were used and generally volumes for each reactant were kept at 100 µl/well.

Between incubations 3 intensive washing steps were carried out by repeated soaking of the plates in PBS-T washing buffer, tapping dry the wells and plate after the final third PBS-T removal. After step 2, application of the blocking solution, the plate was not washed and only the blocking solution was removed.

Microtitre plates (96 wells) were coated with WmCSV IgG (DSMZ AS-830) diluted 1:1000 (v/v) in coating buffer and incubated for 3 h at 37 °C.

After washing in PBS-T, blocking was done by adding 2% skimmed milk in PBST and incubating for 30 min at 37 °C.

Sap extracts were prepared by grinding plant materials in extraction buffer (1:20 w/v) adding 100 µl extract to the microtitre well and incubating it overnight at 4 °C. Negative and positive controls, which were extracts from healthy plants and from plants infected with known begomoviruses were used as negative and positive controls, respectively.

After thorough washing in PBS-T, Mabs raised against TYLCV (general begomovirus Mab, DSMZ AS 546/1) were used as detecting antibodies at dilutions in conjugate buffer of 1:1000 (v/v). Mabs were incubated for 3 h at 37 °C.

After washing the plates, an alkaline phosphatase labelled (aP), rabbit-anti-mouse IgG-aP, (DAKO A/S, Denmark) diluted 1:1000 (v/v) in conjugate buffer was added (100 µl/well) and the plates incubated for 45 min at 37 °C.

The substrate, p-nitrophenyl phosphate diluted 1 mg/ml in substrate buffer was added and incubated for 1 h and 2h at 37 °C to monitor substrate conversion.

Quantitative measurements of the p-nitrophenyl substrate conversion, resulting in a yellow colour was made by determining absorbance at 405 nm (A_{405}) in a Titertek Multiscan® MCC/340 model spectrophotometer (Labsystems Co., Finland). The mean absorbance readings of non-infected controls were determined and twice the values were used as the positive/negative thresholds.

2.6 Dissection of whiteflies and excision of insect organs

After 48 h AAP, insects were collected from their respective host plants by aspiration and placed on ice for 5 min to arrest movement. Specimen were placed on a glass slide in a drop of insect physiological saline (IPS) and dissected under a stereo microscope (Zeiss, Stemi 2000C) essentially as described by Bandla *et al*., (1998) for thrips. Isolated organs were washed thoroughly with dd H_2O and adhered to an eyelash for transfer to fresh 10 µl sterilized dd H_2O in an eppendorf tube.

Insect physiological saline, pH 7.5

4.5 g	NaCl	
0.1 g	KCl	
0.1 g	$CaCl_2$	
0.1 g	$MgCl_2$	
0.1 g	$NaHCO_3$	
2.0 g	Glucose	adjust volume to 1 liter, autoclave.

Removal of midgut

To dissect midgut, the abdomen was separated from the thorax at their junction. Then its content was expelled into IPS by pushing gently the abdominal part. Midguts were isolated, gently cleaned from other tissues and flushed several times with water (Ghanim *et al*., 2000). Midguts were also isolated using a fine insect needle (Ehlert & Partner, Germany) placed with its fine tip into the last third of the abdomen to pull the midgut out of the abdomen essentially free of other organ sections.

Isolation of salivary glands

To isolate salivary glands, the pro-thorax with the head was separated from the meso-thorax and abdomen. The glands were teased away from the head and after pushing it the salivary gland appeared aside. Salivary glands were left for 2 min in Toluidine blue until they adsorbed the dye for better visualisation during dissection. The pair of primary salivary glands and accessory salivary glands was flushed several times with sterile dd H_2O and collected using surface sterilized eyelashes for transfer.

Dissection of stylet and head

Stylets were pulled out from the head section, flushed several time with sterilized dd H_2O and collected in PCR tubes. Head segments were separated from the whole body, washed as described above and collected in a PCR tube.

Collection of haemolymph fluid

Adult whiteflies were collected by aspiration from respective host plants, immediately cooled on ice for 5 min to arrest movement. Female whiteflies were fixed by their dorsal side in a fresh mineral oil droplet. A haemolymph droplet was forced out from an immersed individual by removing a hind wing using a fine tip (0.15 µm) insect needle from. Haemolymph droplet was collected using a sterile

glass 1-5 µl capillary pipette and immediately transferred to a tube containing 100 µl of sterile TE buffer (pH 8.0) and 10 mM phenylmethyl sulfonyl fluoride (Sigma chemical Co., St. Louis) to prevent coagulation (Rosell *et al,* 1999).

2.7 Isolation, quantification and electrophoretic analysis of DNA

2.7.1 Extraction of DNA from whole whitefly insects

Total genomic DNA of whiteflies was isolated using the high pure PCR template preparation kit (Roche, Mannheim, Germany). Extractions were carried out essentially following the manufacturer's instructions with modifications according to Abdullahi (2001).

2-3 whitefly individuals were transferred into a sterile 1.5 ml eppendorf tube and homogenized with a sterile micro pestle in 15 µl tissue lysis buffer. After addition of another 35 µl lysis buffer and 10µl proteinase K (20 mg/ml), the whitefly homogenates were gently mixed and incubated for 1 h at 55 °C.

After addition of 50 µl of binding buffer, mixing and further incubation for 10 min at 72 °C, 25 µl isopropanol was added prior to transfer of the homogenate to a filter tube for centrifugation at 8000 rpm for 1 min. The flow through was discarded, 250µl wash buffer was added to the upper reservoir of the filter tube and centrifugation was repeated. After another round of washing, the filter tube containing DNA bound to the matrix was inserted in a clean 1.5 ml sterile eppendorf tube and purified DNA was released by addition of 50 µl pre-warmed (70 °C) elution buffer and centrifugation for 1 min at 8000 rpm. Aliquots of the DNA preparations were analysed by agarose gel electrophoresis to assess the integrity and the quantity of insect genomic DNA.

2.7.2 Extraction of DNA from dissected insect organs and haemolymph fluids

Isolated insect organs from viruliferous and non viruliferous whiteflies presented sufficiently accessible template DNA for PCR analysis to amplify WmCSV

sequences. Excessive washing with double distilled sterilized water (dd H_2O) prior to submersion in PCR reaction buffer and boiling released sufficient viral DNA for PCR. All other DNA extraction methods used for whitefly DNA preparations (Abdullahi, 2001), resulted in poor or irreproducible PCR due to loss of template DNA during extraction.

Haemolymph fluids collected from 10 insects were adjusted to 3 % sodium dodecyl sulphate (SDS) and 1mg/ml proteinase K, then incubated at 55 °C for 1 h, boiled at 95 °C for 5 min and extracted with phenol/chloroform (1:1 v/v). Samples were mixed by vortexing, and phase separation was reached by centrifugation at maximum speed >12 000 rpm for 5 min. The aqueous phase was collected and nucleic acid was precipitated adding 2.5 vol ethanol and 0.3 M sodium acetate and overnight incubation at -20 °C. Total nucleic acids were pelleted by centrifugation, washed with 70% ethanol, dried and dissolved in 20 µl sterilized dd H_2O preheated to 70 °C (Rosell *et al*, 1999).

2.7.3 Extraction of total DNA from virus-infected plants

Total DNA was extracted from plant tissues using either a plant DNA minipreparation method (Dellaporta *et al*., 1983) for PCR analysis or by using the DNAzol reagent (Invitrogen, Germany) for DNA extraction when total DNA from WmCSV infected plant tissues was prepared as inoculum for gold particle coating and subsequent biolistic delivery to infect plants using the Helios gene gun.

2.7.4 Extraction of plant DNA by a plant minipreparation method (Dellaporta et al., 1983)

Fresh leaf tissue (100 – 150 mg) harvested in a polythene sample bag was shortly placed in liquid nitrogen and then crushed in 1 ml extraction buffer (100 mM Tris HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% 2-Mercaptoethanol) and 10 µl of 10 µg/µl RNAse A, using a wallpaper roller.

An aliquot of 500 µl was transferred into a 1.5 ml eppendorf tube, 33 µl of 20% SDS was added and incubated for 10 min at 65 °C. After addition of 160 µl of 5M potassium acetate, the mixture was thoroughly vortexed and then centrifuged for 10 min at 13.000 rpm. About 400-450 µl of the clarified supernatant was carefully transferred to a new tube, 0.5 vol isopropanol was added, the mixture was vortexed and centrifuged for 10 min at 4 °C to precipitate nucleic acids. Isopropanol was carefully decanted; DNA pellets were washed with 500 µl of 70% ethanol and centrifuged.

The final DNA pellet obtained was air dried at 37 °C and resuspended in 100 µl of dd H_2O. An aliquot (5 µl) of each sample was subjected to agarose electrophoresis to check for DNA quantity and integrity of the preparation.

2.7.5 Extraction of total DNA from diseased plants using the DNAzol reagent

Plant DNA preparations extracted by the Dellaporta DNA minipreparation protocol were not of sufficiently clean for an even coating of gold particles Hence total DNA preparation were made from diseased plant leaves using the DNAzol reagent (Invitrogen, Karlsruhe, Germany).

Portions of one or two fresh diseased leaves, approximately 0.1 g in weight were ground with a pestle and mortar in liquid nitrogen. 300 µl freshly prepared DNAzol reagent (20 µl 2-mercapthoethanol and 100 mg PVP MW 10000 (SERVA, Heidelberg, Germany) added to 20 ml DNAzol) was added and incubated at 25 °C for 5 min while shaking. Then 300 µl chloroform was added, mixed vigorously and incubated at 25 °C for another 5 min. Phase separation was reached by centrifugation for 10 min at 10.000 rpm, the aqueous phase was carefully removed and transferred to a fresh tube. DNA was precipitated subsequently by mixing 150 µl absolute ethanol to the sample.

Samples were mixed by vortexing and kept for 10 min at room temperature to settle the DNA precipitate. The supernatant was carefully decanted and 300 µl of a DNA-ethanol wash solution was added (DNAzol mixed with 0.75 volume of 100% ethanol). Samples were kept for 5 min, centrifuged for 10 min at 10. 000 rpm and

DNA pellets were subsequently washed with 300 µl of 70% ethanol and centrifuged for 10 min at 10.000 rpm; Residual ethanol was removed by air drying and DNA was dissolved in 70 µl Tris/EDTA buffer (10/1 mM, pH 8.0) adjusted with 2 µl of RNAse A (10 µg/µl).

Quantification of DNA

Total DNA was quantified taking spectrophotometric absorbance readings at wavelengths (λ) of $A_{260\ nm}$ and A_{280nm} permitting an estimation of nucleic acid content and purity (Sambrook *et al*., 1989).

Sample absorbance $A_{260nm} = 1$ corresponds to 50 µg/ml dsDNA or 40 µg/ml/ssDNA or RNA. The $A_{260nm}/A_{280\ nm}$ ratio provides an estimate of the purity with pure preparations of DNA having an $A_{260\ nm}/A_{280\ nm}$ coefficient between 1.8 and 2.0.

Agarose gel electrophoresis

For analysis of total DNA preparations, PCR amplicons or plasmid DNA, standard agarose gels (1 or 2 % w/v) prepared in 1x TAE electrophoresis buffer (0.04 M Tris acetate, pH 8.0; 1 mM EDTA) were used.

Agarose powder was added to TAE buffer and microwaved for 2 min to dissolve the powder. To the cooling solution, 0.005 % ethidium bromide was added and the solution subsequently poured into a tray in which a comb was inserted to form sample slots. The agarose gel was allowed to solidify for approximately 30 min before the comb was removed and the gel immersed in the electrophoresis tank containing TAE buffer.

To 3-10 µl of DNA sample, 3 µl of sample buffer were added and the total volume (6-13 µl) loaded into a slot in the gel. The gel was run at 120 volts and maximum current for 1-1.5 h before being viewed under UV light and photographed. λ *Pst*I digested phage DNA (Fermentas NBI, Germany) was used as molecular size markers.

The amount of DNA was estimated in agarose gels after electrophoresis. The fluorescence emitted by ethidium bromide intercalating into the dsDNA during electrophoresis was compared with the fluorescence emitted by the dsDNA fragments of the DNA size marker.

2.7.6 Southern blot hybridization analysis for PCR amplicon verification

Preparation of labelled DNA probes

To generate a labelled DNA probe by PCR, a WmCSV DNA A full length clone in the plasmid pBluescript SK- was used as template. Labelling was done by PCR incorporating the hapten digoxigenin dUTP (DIG-dUTP) into DNA during DNA synthesis. Prior to PCR, an aliquot of the a DNA minipreparation was linearised outside the amplification region, to prevent formation of circular DNA products from circular templates. For labelling, the PCR DIG Probe Synthesis Kit (Roche, Germany) was used following the manufacturer's recommendations.

Reaction mix

PCR buffer	5 µl
PCR DIG labelling mix	5 µl
WmCSVA 1286c	1 µl
WmCSVA 839s	1 µl
Taq polymerase	0.5 µl
Template DNA (plasmid, 50 ng)	1µl
H_2O adjusted to	50 µl

The reaction mix was prepared and subjected to PCR. DNA amplification was done in 30 cycles following an initial denaturation step at 95°C for 5 min. 30 cycles of 95°C for 30 sec, 50 °C for 40 sec, 72 °C for 40 sec were conducted and PCR was terminated by a final extension at 72 °C for additional 10 min. The amplification

reaction, essentially comprising a DIG-dUTP labelled 429 bp WmCSV DNA A fragment, was kept at – 20°C prior to use.

DNA transfer

Southern blot: For hybridization analysis, DNA separated by agarose electrophoresis was transferred to nylon membranes in a Southern blot procedure.

Membranes: Boehringer (Nylon membranes, positively charged)

Transfer buffer pH 7.0

20 x SSC

3 M NaCl

0.3 M tri-Na-Citrate ($C_6H_5Na_3O_72H_2O$)

Denaturation buffer

1.5 M NaCl

0.5 M NaOH

Neutralization buffer

0.5 M Tris HCl, pH 7.5l

1.5 M NaCl

0.01 M Na_2EDTA

PCR reactions were separated by agarose gel electrophoresis and photographed for documentation. For transfer to nylon membranes, DNA in gel was denatured by submersion of the agarose gels in denaturation buffer for 20 min while shaking, then changing into neutralisation buffer for 15 min followed by equilibration in transfer buffer prior to vacuum blotting. DNA was blotted onto positively charged nylon membranes for 90 min, applying 25 psi vacuum. Slots were marked with a pencil before removing the gel from the vacuum apparatus: Upon completion, the

membrane was quickly checked under UV light and then was washed for 5 min in transfer buffer to remove residual agarose on the gel surface. The membrane was placed on dry filter paper to remove excess moisture and DNA was fixed by UV cross linking at a wavelength of 254 nm and 1200 joules. Membranes were then dried and stored in the dark at room temperature or, directly used for hybridization.

DNA hybridisation

Hybridisation solution

50% (v/v)	formamide
7%	SDS
2%	ROCHE blocking reagent
5 x	SSC
0.1 (w/v)	N-lauroylsarcosine,
50 mM	Sodium hydrogen phosphate pH 7.2

In a prehybridisation step, membranes were first prewetted in 2 x SSC and carefully placed in a hybridisation tube to which 10-25 ml of pre-hybridisation solution were added. Prehybridisation was done in a hybridisation oven (Techne, UK) for 90 to 120 min at 42 °C by slowly rotating the tubes. During this step, membranes were blocked to inhibit unspecific DNA binding and cross-hybridisation to non target molecules leading to false interpretations.

Hybridization was initiated following this membrane equilibration by changing the hybridization solution and adding 10 ml fresh liquid. For DNA annealing and to form specific labelled DNA/DNA hybrids, the DNA probe was boiled for 10 min at 95 °C to separate the double DNA strands and added directly to the hybridization solution. Hybridization was done overnight at 42 °C by gently rotating the tube to keep membranes immersed in solution at all times.

The post hybridization washing removed unbound and excess DNA probe and DNA probe mismatching with non homologous DNA at low stringency. First, the

hybridization solution was removed and stored at -20 °C for subsequent use. The membrane was then washed with 2 x SSC, 0.1% SDS for 10 min at room temperature, followed by a 2 times washing cycle at high stringency in 0.1 x SSC, 0.1% SDS for 15 min at 68 °C . After the post hybridization washing, the membranes were either stored dry or subjected directly to the immunological detection of digoxigenin labelled DNA.

Immunological detection of DNA hybrids

For hybridization analysis, a protocol following the procedure and recommendations of ROCHE, for the detection of digoxigenin in labelled DNA hybrids was adopted.

Maleic acid buffer

0.1 M Maleic acid pH 7.5

0.15 M NaCl

Washing buffer

0.1 M Maleic acid pH 7.5

0.15 M NaCl

0.3% Tween

Blockung stock

Blocking reagent (Roche), 10% (w/v) in Maleic acid buffer

Blocking buffer

1:10 (v/v) dilution of blocking stock in Maleic acid buffer

Substrate buffer

0.1 M Tris pH 9.5

0.1 M NaCl

Membranes were equilibrated for 5 min in washing buffer and unspecific antibody binding sites were blocked by incubation in blocking buffer for 30 min at RT. Subsequently, the membrane was incubated with an anti-digoxigenin antibody fab fragment conjugated to alkaline phosphatase (Roche, Cat. No. 1 093 274), diluted 1:10000 (v/v) in blocking buffer, for 45 min. Excess antibody was removed by washing the membranes 2 times in washing buffer for 15 min followed by equilibration for 3 min in substrate buffer prior to adding the chemiluminescent substrate CSPD (Roche, CSPD ready-to-use) for the detection of DIG labelled DNA.

This was done in a moist cover and under exclusion of light. Excess moisture was first removed and membranes were placed on plastic sheets. Then 20 drops of CSPD were applied, membranes were covered with a second folia and CSPD was evenly spread over the membranes by wiping the surface of the sheets with a tissue paper, to also squeeze out excess liquid. The damp membrane sandwich was placed between Whatman filter papers in an X-ray cassette to exclude light, incubated for 5 min at RT and 10 min at 37 °C. The membrane was then exposed to X-ray film (Agfa Curix MR 800) for 15–25 min placed in the cassette. After exposure, which was sequentially repeated with new X-ray films to obtain the best signal, the X-ray film was developed following standard procedures for black/white film development.

2.8 Detection of WmCSV by Polymerase Chain Reaction, PCR

A PCR reaction was conducted using DNA A primers located in the coat protein and REn genes. The combination

WmCSVA 1286c (downstream, complementary)

5'- GCGATCGTTTCCAAGTTATGCGAA -3'

and WmCSVA 839s (upstream, sense)

5'- CGGCCTCAGACTGGTCGTTTCTTAA -3'

amplified a 429 bp DNA A fragment indicating for the presence of WmCSV sequences in whole insects and in dissected whitefly organs.

Dissected whitefly organs were subjected to PCR in a total volume of 50 µl. The reaction consisted of 2.5 µl $MgCl_2$ (50 mM), 1µl of each primer (50 pmol), 5 µl of Taq DNA polymerase buffer, 1µl dNTPs (25 mM) and 0.5 µl Taq DNA polymerase (Invitrogen, Karlsruhe, Germany) was added to the mix. DNA amplification was reached using the following temperature cycles: an initial denaturation step of 3 min at 95 °C followed by 35 cycles of 1 min denaturation at 95 °C, 1.5 min primer annealing at 56 °C and 2 min strand extension at 72 °C. PCR amplification was terminated with a final extension step for 7 min at 72 °C. Samples were removed and kept at – 20 °C or analysed directly in agarose gel electrophoresis. Here fore, 10 µl of the amplified reaction mix were analysed by electrophoresis in 2% agarose gels.

DNA from whole viruliferous insect extracted using the high pure PCR template preparation kit (Roche, Mannheim, Germany) were included as a positive control, DNA from non-viruliferous insect served as negative PCR control.

2.9 Detection of WmCSV mutants using immunocapture PCR (IC-PCR)

Begomoviruses do not require coat protein for plant infection and transport through the plant. To verify that WmCSV mutants are assembled into virions an immunocapture PCR (IC-PCR) was conducted using the polyclonal antibody raised against watermelon chlorotic stunt virus (DSMZ AS-0800) as the trapping antibody.

IC buffers

Coating buffer, pH 9.6

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.2 g NaN_3

adjust to 1 liter

TBST, pH 8.0

10 mM Tris-HCl

150 mM NaCl

0.05% Tween-20

Extraction buffer I

2.4 g Tris

8 g NaCl

0.5 ml Tween 20

0.2 g KCl

0.2 g NaN_3

adjust to 1 liter, pH 9.0

Extraction buffer II

2.4 g Tris

20 g PVP

8 g NaCl

0.5 ml Tween 20

0.2 g KCl

0.2 g NaN_3

make up to1 liter, pH 9.0

Immunocapture PCR (IC-PCR)

For sample preparation, infected leaves were homogenized (1:20 w/v) in extraction buffer I. Alternatively, small pieces (0.1 g) of infected leaves or 1-5 whitefly specimen were homogenised in 100-200 µl extraction buffer II.

200 µl of WmCSV IgG (diluted 1:1000 (v/v) in coating buffer) were added to each to 0.2 ml PCR tube and incubated at 37 °C for 3-4 h.

WmCSV antibody solutions were removed from the tubes which were subsequently washed 3 times with TBST.

100-200 µl of the virus homogenates were added to the PCR tubes and incubated for 18 h at 4 °C.

Samples were discarded and the tubes were thoroughly washed 3x with TBST.

PCR tubes were dried; and10 µl dd H_2O was added and boiled at 100°C for 5 min.

A 50 µl PCR reaction was set up and conducted directly in those capture tubes as stated in section 2.8.

2.10 Electron Microscopy

2.10.1 Preparation of specimen grids and carrier films

For electron microscopy, nickel grids (75 Mesh per inch) or copper grids (400 mesh pro inch), (Plano, Wetzlar, Germany), (Stockem, 1970) were used throughout the experiments. A support film of 0.5 % pioloform dissolved in chloroform was used to coat the grids which subsequently were treated with carbon vapour to stabilize the specimen sections caught on the respected grid. Sections were suspended into water and adsorbed onto the carbon-coated pioloform films on the grids.

Since carbon surfaces become contaminated upon storage and thus hydrophobic, it is recommended to glow discharge the grids surface prior to use. This was done under vacuum in a vacuum evaporator (Balzers, WF006) applying 5 x 10^4 mbar at 2400 Volt. To render the coated grids hydrophilic just before use, grids were lightly treated again with carbon vapour. For *in-situ* hybridization carbon treated grids were heated for 10 min at 100 °C to stabilize the pioloform layer against mechanical damage during long hybridisation procedures and, to render these grids more hydrophilic.

2.10.2 Adsorption preparations

To confirm presence of virus particles, adsorption preparations were done with sap of infected plants, or with purified virus preparations in virus buffer. A leaf disc was excised from a WmCSV infected watermelon plant and crushed with a sterile glass pestle in 50 µl of phosphate buffer (100 mM KH_2PO_4/Na_2HPO_4 pH 7.0). Two sets of carbon coated cupper grids (400 mesh pro inch) were floated on a 10µl drop of infected and healthy plant homogenate to adsorb to virus in virus preparations for 15 min or in plant homogenates for 3 h. Grids were subsequently washed with 40 drops dd H_2O, then without being dried, contrasted using 5 drops of 1% aqueous uranyl acetate and subsequently dried using filter paper prior to electron microscopical examination (Milne, 1984). Virus particles observed in EM were counted in a surface area of (10.3 μm^2) where 1-10 of virus particles per screen surface area was sufficient estimation of virus concentrations used for insect feeding experiments and to ascertain virus presence.

2.10.3 Immunosorbent Electron Microscopy (ISEM)

The technique, developed by Derrik (1973), involved trapping of virus particles from suspension by virus specific antibodies coated on the grid prior to virus adorption. Virus particles become selectively attached to the grid while host plant material is easily removed by subsequent washing steps (Dijkstra and de Jager, 1998). This method is highly sensitive by increasing the number of virus particles trapped on EM grids.

ISEM was done as described by (Milne and Lesemann 1984). Freshly prepared copper grids were incubated for 5 min on a 10 µl drop of WmCSV antiserum (DSMZ AS-0803) diluted 1:1000 (v\v) in sample buffer (100 mM KH_2PO_4/Na_2HPO_4 pH 7.0, 2% PVP, 0.2% sodium sulfite). After washing with 20 drops of phosphate buffer (100 mM KH_2PO_4/Na_2HPO_4 pH 7.0), the grids were floated onto infected plant leaf extracts for 3 h at room temperature homogenized in buffer 3 in order to facilitate virus particle attachment. After washing with 40 drops of distilled water, grids were negatively stained with 5 drops of 1% aqueous uranyl acetate.

All EM preparations were viewed in a Zeiss TEM (906) transmission electron microscope.

2.10.4 ISEM and Decoration tests

In this experiment, ISEM captured virus particles were incubated with 10µl of polyclonal IgG in two dilutions 1:5 (v/v) and 1:50 (v/v) for at least 15 min, subsequently washed with dd H_2O and contrasted using 1% aqueous uranyl acetate. This experiment provides an indicating for the antibody affinity to the respective virus, and is used for specific identification of a virus in a given sample (Milne, 1984; Milne & Luisoni, 1977). The antibody decoration is displaying a more or less dense halo on the virus particle depending one the labelling density.

2.10.5 Immunogold- labelling of purified virus particles

Gold immunolabelling experiments were applied to investigate the effect of aldehyde fixation on virus antigenicity (to evaluate eventual denaturation of virus epitopes) and to determine the stability and intensity of gold particle binding to virus particles. Captured virus particles were fixed in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde in PBS, pH 7.4, for 10 min. Decoration with polyclonal antibody was done as described in section 2.10.3, subsequently followed by gold immunolabelling of decorated virus particles. This was done by using a goat anti rabbit fab`2 fragment conjugated to 10 nm gold (British Biocell Internaional) in a dilution of 1:50 (v/v) for 30 min.

2.11 Localization of WmCSV in insect organs

To achieve the maximum retention of antibody binding, different immunolocalization strategies were adopted.

In the pre-embedding immunolocalization, the specimen was fixed through a light fixation treatment prior to immunolocalization or first subjected to immunolocalization and then fixed. After a dehydration step following immunolocalization and fixing, embedding was done in LR white medium at 60 °C.

In post-embedding immunolocalization, specimen were embedded either using LR white as embedding medium followed by polymerization at 60 °C or using Lowicryl as an embedding medium with polymerization at -30°C using the Progressive Lowering Temperature technique (PLT).

For pre- and post-embedding methods organs were isolated in IPS to maintain tissue integrity replacing IPS directly with fixation solution depending on the method described below.

2.11.1 Pre-embedding Immunolocalization

Three methods were adopted to improve antigen antibody binding and reaction.

Method I: This method was essentially followed as described by Driss-Ecole *et al*. (2000)

1. Dissected insect organs were fixed in a mix of 0.5% of glutaraldehyde and 4% paraformaldehyde for 2-3 h at 4 °C;

2. subsequently treated with 0.5% Triton X 100 for 5-15 min, to enhance permeability;

3. free aldehyde groups from earlier fixation steps were inactivated by treatment with sodium borohydride for 15 min (Yi *et al*., 2001) or with 50 mM glycine in PB (Momayezi *et al*., 2000) for 10 min;

4. organs were subjected to blocking for 30 min in 5% BSA, 5% goat normal serum in PBS-Tween (Yi *et al*., 2001) at room temperature;

5. organs were soaked overnight at 4 °C in dilutions of primary antibody (WmCSV IgG, DSMZ AS-0803) at 1:100, 1:500, 1:1000 (v/v) in incubation buffer (1% BSA, 1% NGS in PBS-T);

6. organs were subjected to goat anti rabbit fab`2 fragment conjugated to 10 nm gold (British Biocell International) diluted 1:100 (v/v) in incubation buffer at room temperature for 4 h or with affinity purified goat anti rabbit IgG conjugated to 0.8 nm gold (Bio-Trend, Köln, Germany);

7. organs were then subjected to several washing steps for 15 min each in PBS pH 7.4, then post-fixed in 1.25 % glutaraldehyde and subjected to silver enhancement (SEM-R Gent) using the Amersham silver enhancement kit for 10 min (Yi *et al*., 2001);

8. After silver staining treatment, organs were subjected to osmication, in 0.5 % of osmium tetroxide for 30 min, then washed several times with water and stained en block in the dark for 10 min using 0.5 % aqueous uranyl acetate;

9. Fixed tissues were dehydrated through a series of ethanol concentrations starting from 25%, 50%, 75%,100% each for 15 min prior to infiltration with a 50/50 mix of LR white Ethanol for 30 min followed by an infiltration of 100% L white at room temperature for 30 min;

10. Infiltration with 100% LR white at 4 °C overnight followed by a further LR white infiltration for one h at room temperature finalised the embedding process.

11. LR white embedded organs were transferred into polymerization capsules containing LR white which were completely filled and then kept for 24-48 h at 60 °C prior to sectioning into 60-90 nm sections and staining with 2% aqueous uranyl acetate .

Controls

Organs of non-viruliferous insect were treated as described and used as a negative control. Organs from viruliferous insects were treated similarly but omitting the primary antibody treatment to determine unspecific antibody binding.

Method II: In this method labelling was applied first and then followed by fixation, and post-fixation as as described by J.Boyes and J.P. Bolam (2003).

1. To enhance penetration with reagents, organs were treated with 0.5% Triton X-100 as described by Driss-Ecole *et al*. (2000):

2. organs were subjected to blocking for 2 h using 10 % normal goat serum in PBS;

3. organs were incubated overnight in primary antibody (WmCSV IgG, DSMZ AS-803) at dilution 1:100 and 1:500 (v/v) at 4 °C in 2% NGS;

4. organs were incubated in goat anti rabbit fab´2 fragment conjugated to 10 nm gold (British Biocell International) diluted 1:100 (v/v) in incubation buffer at room temperature for 4 h or with affinity purified goat anti rabbit IgG conjugated to 0.8 nm gold (Bio-Trend, Köln, Germany);

5. organs were fixed in 1% glutaraldehyde for 10 min at room temperature, followed by several washing steps, subsequently post-fixed in 1% osmium tetroxide in PBS for 10 min at room temperature;

6. organs were washed three times in PBS pH 7.4, dehydrated in ethanol series and soaked in 1% uranyl acetate dissolved in 70 % ethanol during dehydration at room temperature;

7. organs were embedded and left for polymerisation at 60 °C for 24-48h and then sectioned and contrasted using lead citrate for 3-4 min at room temperature.

Controls were used as described for Method I.

Method III: Pre-embedding immunolocalization, as described by (Thomas Kurth, 2003).

1. Organs were pre-fixed in 4% fresh paraformaldehyde in PBS pH 7.4 overnight at 4 °C, post-fixed in 20% dimethylsulfoxide (DMSO) in methanol overnight at 4 °C;

2. organs were rehydrated in a series of decreasing methanol/water 100%, 90%, 70% and in methanol/PBS 50%, 30% for 15 min each step;

3. organs were blocked in 20% NGS for 2 h at room temperature and incubated in 20% NGS/PBS containing primary antibody (WmCSV IgG, DSMZ AS-0803) at dilution 1:100 with incubation time varying from overnight to 2 or3 days at 4 °C ;

4. organs were washed several times for 30 min, 4x 1 h, or 1x 2 h and subsequently incubated for 3 days at 4 °C in affinity purified goat anti rabbit IgG conjugated to 0.8 nm gold (Bio-Trend, Köln, Germany); in 20% NGS/PBS at 1:100 (v/v) dilution.

5. samples were washed in PBS then post fixed in 2% glutaraldehyde for 2 h, washed 4x 10 min in PBS and in distilled water 3x 10 min;

6. following a silver enhancement for 1-2 h, organs were washed several times in water and stained en bloc for 2 h in 1% aqueous uranyl acetate on ice, dehydrated in ethanol series from 30%,50%,70%,80%,90%,100% for 15 min each step;

7. organs were infiltrated in 50/50 resin/ethanol (v/v), then 75/25 resin/ethanol, 1h resin (used), 1 h resin (new), 2 h resin (new), resin (new) overnight and finally embedded in Epon (Serva) plastic resin.

2.11.1.1 Silver enhancement

The developer and the enhancer were allowed to reach room temperature.

After the immunogold incubation, grids were washed and post-fixed following the descriptions of the R-Gent SE-EM (Aurion, The Netherlands). Prior to silver enhancement, excessive washing steps with distilled water were done to remove residual buffer that may impede enhancement.

Once temperature equilibrium was established, 20 drops of the enhancer solution were pipetted to a 1.5 ml eppendorf vial to which 1 drop of developer solution was added and the mixture was mixed well by vortexing.

Post-embedding application

Enhancement solution was dropped on a sheet of parafilm and grids were floated on top of it for an enhancement time typically between 20 and 30 min.

Pre-embedding application

Silver enhancement was done either before or after osmium tetroxide fixation. When silver treatment was applied before osmium fixation, a longer incubation time was required due to potential removal of silver by OsO_4. When enhancement wass complete the specimen were washed extensively with distilled water (at least 3x 5 min) and subsequently contrasted for 15 min with uranyl acetate.

In order to preserve the silver signal grids were stored in a dry environment.

2.11.2 Post-embedding immunolocalization

To achieve maximum ultrastructural preservation with minimum effects on antigenicity preservation, different concentrations and combinations of glutaraldehyde and paraformaldehyde (Karnovsky, 1965) were tested to optimize fixation conditions of dissected insect organs for immuno-labelling experiments (Table 2). This was done essentially following the procedure of Ramandeep *et al*. (2002) and applied only on the dissected midgut.

Table 2: Combinations of fixative used to evaluate effect on preservation of antigenicity (Ramandeep *et al*., 2002).

No.	*Fixative combinations (preliminary fixation)*	*Post-fixation*
1	*0.5 % Glutaraldehyde* *4 % Paraformaldehyde*	*No*
2	*4 % Paraformaldehyde*	*No*
3	*2.5 % Glutaraldehyde*	*No*
4	*0.2 % Glutaraldehyde* *4 % Paraformaldehyde*	*0.5% Osmium tetroxide*

Fixation

Insect organs were isolated on a drop of IPS and covered with 2% Nobel agar to facilitate handling. For all ultrastructural investigations, organs were fixed on 2.5% glutaraldehyde for 4-6 h to overnight, followed by post-fixation in 1% osmium tetroxide.

Epon-embedded organs were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for at least 2 h to overnight, followed by post-fixation in 0.5% osmium tetroxide for 2 h (Reinbold *et al*., 2001) and subsequently contrasted in 2 % aqueous uranyl acetate. The fixation solutions were infiltrated *in vacuo* using a water pump for at least 15 min.

2.11.2.1 Embedding of insect organs using Epon- 812

Solution I 62 ml DDSA + 38 ml Epon 812 = 111 g

Solution II 56 ml Epon 812 + 44 ml MNA = 119 g

A polymerization mix was prepared by mixing solution I and II that contained the resin Epon and DDSA or MNA hardeners 1:1 (v/v). To achieve complete polymerization an accelerator (DMP) was added using 0.15-0.2 ml DMP-30 per 10 g mixed solution.

Prior to resin polymerization, organs for embedding needed to be dehydrated in a series of acetone:

Osmium fixed organs were washed 4 times with dd H_2O for 45 min

1. contrasted by incubation in dark overnight in 1-2 % aqueous uranyl acetate.
2. dehydrated two times each for 30 min in 50% acetone.
3. dehydrated two times each for 30 min in 70% acetone.
4. dehydrated 4 times each for 30 min in 100% acetone.
5. infiltrated for 1 h in 100% acetone/ Epon 1:1 at 40°C.

6. infiltrated 1 h in Epon at 40°C.

7. Organs were infiltrated 1 h in freshly prepared Epon at 40 °C.

Dissected organs were place in silicon-block forms for embedding in Epon resin at 60 °C for 48 h. One day before embedding, these blocks were filled to 1/3 volume with Epon and left overnight at 40 °C for partial polymerisation to facilitate positioning of dissected obtaining in the blocks.

Preparation of ultra-thin sections

Ultra-thin sections (60-100) nm of embedded insect organs were obtained using a diamond knife (DuPont Instrument) in an ultramicrotome (Ultratome III, LKB). Sections were subsequently collected on carbon coated pioloform nickel grids (75 mesh per inch) as stated section 2.10.1.

2.11.2.2 Embedding of insect organs using LR White

Fixation Buffer

0.5 % glutaraldehyde

4 % paraformaldehyde

Dissolved in 0.1 M phosphate buffer pH 7.2

Viruliferous and non-viruliferous whiteflies were dissected under a dissection microscope in insect physiological saline (IPS) to extract midgut and primary and accessory salivary glands as described in section 2.6.

1. IPS was immediately replaced with fixation solution to preserve the ultrastructure of the tissues. Dissected organs were kept in a light shield container for at least 2 h;

2. preliminary fixed organs were washed two times with 1x PBS for 20 min;

3. to inactivate residual aldeyhyde groups which may cause gold conjugate coagulation and non-specific labelling; tissues were submerged with agitation on 0.1% sodium borohydride in 0.1 M PB for 15 min followed by a 4x wash with PB for 10 min (Yi *et al*., 2001);

4. to improve reagent penetration, sections where 0.05 % Triton X-100 in PB followed by 4x washing in PB for 10 min;

5. tissues were secondary fixed with 1% osmium tetroxide for not less than one h while tissues used for immunolocalization studies and for *in situ* hybridization analysis were not subjected osmium tetroxide fixation;

6. osmium fixed organs were washed two times with 1x PBS for 20 min and subsequently dehydrated by incubation for 15 min in a series of increasing ethanol concentrations from 25%, 50%,75% to 100%;

7. dehydrated organs were incubated with LR white in 100% ethanol (50%:50% v/v) for 30 min and then infiltrated overnight at 4 °C in 100% LR white;

8. the dissected organs were transferred to fresh LR white and infiltrated for 1 h at room temperature, then transferred into polymerization capsules and covered with LR White avoiding air bubbles.

9. Polymerization in capsules was for 24-48 h at 60 °C.

Embedding of insect organs using Lowicryl K4M

The polar (hydrophilic; K4M) embedding medium Lowicryl can be photo-polymerized in long wavelength (366nm) ultraviolet light. This was used for better structural preservation and to improve antigenicity and significantly lower background signal development. Lowicryl K4M can be used at room temperature or at low temperatures. This section mainly addressed the technique of low temperature embedding, as a solution to the most common problems encountered in high temperature embedding.

Dehydration was done using the Progressive Lowering of Temperature (PLT) Technique as follows.

1. Dissected organs from viruliferous and non-viruliferous insects were fixed in 1% paraformaldehyde and 0.125% glutaraldehyde for 1 h on ice to preserve ultrastructural features,

2. fixed organs were washed 2x for 20 min at 4 °C with 1x PBS containing 10 mM glycine to inactivate residual aldehyde groups which may cause gold conjugate coagulation;

3. dehydration was done with ethanol following the PLT method (progressive lowering of temperature);

    a. organs were dehydrated in 10%, 30% ethanol, 30 min for each step on ice;

    b. further dehydrated in 50% ethanol for 30 min at –20°C;

    c. and subsequently dehydrated to completion in 70, 90 and 100 % ethanol steps for 30 min at –30 °C with 2x exchange of 100% ethanol;

4. Infiltration with Lowicryl K4M resin was by overnight incubation in K4M/100% ethanol (1:1 v/v) exchanging with K4M/100% ethanol (2:1 v/v) for 8 h and incubation with 100% Lowicryl K4M resin over 2 days with several changes for fresh resin;

5. polymerization was done by placing samples into gelatine capsules filled with resin and incubating under UV-light (366 nm) for 1 day at –30°C followed by a 2 days incubation period at room temperature;

6. Ultrathin-sections were prepared as described in section 2.11.1 and subjected to immunolabelling experiments as described in section 2.10.4 without etching treatments and with etching treatment as described in section 2.11.3.

2.11.3 Etching of Epon and Lowicryl embedded sections for antigen retreival

For post-embedding immuno-electron microscopy ultra-thin sections (120nm) of Epon embedded organs fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde, were mounted on pioloform carbon coated Nickel grids. Etching treatment was carried out using 10 µl drops of saturated sodium metaperiodate (mPJ) solution (Pierce, Germany).

Sections were incubated in a moist chamber for 1 h at room temperature with 15 % mPJ. Antigen retrieval was performed by heat incubation of sections in different solutions as described by (Röcken and Rössner, 1999) and by (Saito *et al*., 2003).

Immuno-labelling of embedded sections treated for antigen retrieval was essentially applied as described in section 2.11.4 below. Sections were first washed three times with dd H_2O. Antigen retrieved sections were blocked for 30 min in 5% BSA and 5% NGS in PBS (Yi *et al*., 2001), then floated overnight on a 10µl drop of WmCSV IgG, subsequently washed 3 times with PBS-T and incubated for 3 h with anti rabbit fab´2 fragment conjugated to 10 nm gold (British Biocell International, Plano, Magdeburg, Germany) diluted 1:100 (v/v) in PBS. Grids were incubated on a 10 µl drop of 1% GA for 10 min washed 3 times with PBST followed by a brief washing with dd H_2O prior to contrasting with 2% uranyl acetate for 20-30 min.

2.11.4 Immunogold labelling of ultra-thin sections

To achieve best results and low background signals, besides the use of antibodies of high quality especial emphasis was on the optimization of the blocking procedure with reagents and solutions for blocking advised by several authors. Blocking solutions that were used are compiled in Table 3.

Table 3: Reagents and solutions used to reveal optimum blocking in immunolocalization experiments

No	Blocking reagent	Use and preparation	Incubation
1	Nil	PBS	PBS
2	1% Skim milk in 1x PBST buffer (Tween- 20, 0.05%) (Ramandeep *et al.,* 2002)	3% w/v dissolved with stirring in PBS-T for 2-3 h, supernatant used after allowing the solution to stand for 1 h.	0.3% skim milk in PBST buffer
3	5% BSA in PBS-Tween	Dissolved overnight with occasional stirring	1% BSA in PBS-Tween
4	BSA-c Aurion (Ramandeep *et al.,* 2002)	2% and 0.2 % were diluted from 10% w/v stock solution	1% BSA in PBS-Tween
5	10% Normal Goat Serum Ramandeep *et al.,* 2002) (J.Boyes and J.P. Bolam, 2003).	Dissolved 10% v/v in PBS-T	2% Normal goat serum in PBS,(J.Boyes and J.P. Bolam, 2003).
6	20% Normal Goat Serum Ramandeep *et al.,* 2002) (J.Boyes and J.P. Bolam, 2003).	Dissolved 10% v/v in PBS-T	2% Normal goat serum in PBS,(J.Boyes and J.P. Bolam, 2003).
7	5% BSA in PBS-Tween, 5% Normal goat serum (Hong *et al.,* 2001)	Dissolved overnight with occasional stirring	1% BSA in PBS-Tween,1% Normal goat serum
8	5% BSA in PBS-Tween, 5% Normal goat serum (Yi *et al.,* 2001)	Dissolved overnight with occasional stirring	1% BSA in PBS-Tween,1% Normal goat serum, 0.2 BSA-c (Momayezi *et al.,* 2000; Yi *et al.*, 2001) (Aurion newsletter 3)

For immunolocalization experiment, blocking and incubation buffers as stated in Table 3, (line 8) proved best and were used throughout the immunolocalization experiments.

2.12 Immunolocalization of WmCSV in ultra-thin sections

Blocking buffer

5% BSA

5% Normal goat serum

in PBST pH 7.4

Incubation buffer

1% BSA

1% Normal goat serum

0.2% BSA-C (Aurion)

in PBST pH 7.4

1. To prevent non specific binding of immunoreagents, sections were incubated on a 10 µl drop of blocking solution for 30 min;

2. grids were washed 3x with PBST and then incubated overnight on a 10 µl drop of WmCSV IgG, DSMZ AS-803). For optimization, antibody dilutions from 1:50 to 1:2000 (v/v) in PBS were tried. For acrylic resins, a 1:50 (v/v) dilution of WmCSV IgG and a 1:25 (v/v) dilution of WmCSV IgG for plastic resin (Epon 812) resulted in low background to noise signal;

3. after antibody incubation, grids were washed 3x with PBST and subsequently incubated on a drop of 10 µl organs were incubated in goat anti rabbit fab`2 fragment conjugated to 10 nm gold (British Biocell International Plano, Germany) or, using goat anti-rabbit 0.8 nm conjugated gold fab`2 fragment (Aurion, The Netherlands) followed by silver enhancement (R-Gent SE-EM,

Aurion, The Netherlands) in the case of pre-embedding immunolocalization. The working dilutions for secondary antibodies were 1:200 (v/v) for acrylic resins while 1:100 (v/v) dilutions were used for the plastic resin (Epon 812) that was treated by etching for antigen retrieval.

4. grids were washed 3x with PBST followed by 3x washing in dd H_2O prior to incubation for 15-30 min in 1-2% uranyl acetate and ending the procedure with 3x washing in dd H_2O.

2.13 Quantifying antibody reactions in immunogold labelled thin sections

To estimate the densities of antibody labelling and as a quantitative comparison, gold particles were counted manually on several loop fields approximately (0.237 μm^2), at a standard final magnification of 12930 x. Particle counts per μm^2 section were determined for different subcellular compartments of the midgut and the primary and the accessory salivary glands. Gold particle counts of the nucleus were taken as an estimate of background labelling. The mean values were calculated for the number of gold particle found in each figure set. Since considerable differences were found in sections and repetitions, the standard deviation was determined to support the evaluation and interpretation of the labelling data.

2.14 In-situ hybridization to localise WmCSV in thin sections of insect organs

This protocol was adopted from Wachtler *et al.* (1996) and used with minor modifications. For the *in situ* hybridization experiments, Sigma hybridization slides were used requiring 100 µl hybridization or probe solutions. The digoxigenin labelled DNA probe was generated as described in section 2.7.6 with a final concentration of labelled probe used in the experiments of approximately 2 ng /µl.

Pre-hybridization and Hybridization solution

Hybridization mixture

50 % formamide in 2x SSC

2 ng/µl labelled probe

100 ng/µl salmon sperm DNA

100 ng/µl yeast tRNA

10 % dextran sulphate

Pre-hybridization solution

50 % formamide in 2x SSC

200 ng/µl denatured salmon sperm DNA

200 ng/µl yeast tRNA

0.1 % Ficoll 400

0.1 % BSA

0.1 % PVP

***In- situ* Hybridization protocol**

1. Wash grids for 10 min in Tris $CaCl_2$.
2. Incubate the grids in proteinase K at 37 °C for 10 min, stop reaction with 2x 10 min wash in PBS $MgCl_2$.and apply RNAse A for 1 h;
3. Rinse the sections rapidly at room temperature on three drops of dd H_2O and wash extensively with f distilled water;
4. fix the grids by incubation in 4% paraformaldehyde followed by washing for 2x 10 min in PBS;
5. wash 3x for 5 min in distilled water and incubate grids for 1 h in pre-hybridization solution at 45 °C;

6. denature the probe at 100°C for 10 min and immediately quench on ice,
7. soak away the pre-hybridization mixture using filter paper, immediately afterwards distribute the hybridization mixture over the grids;
8. Place the cover slips on the grid and make sure that no air bubbles are left under the glass;
9. Put the loaded slides for 10 min in an incubator adjusted to 80 °C followed by overnight incubation at 45 °C.

Post-hybridization treatments

Wash the grids 2x 15 min in 2 x SSC at 45 °C followed by 1 x SSC 10 min, then 0.5 x SSC for 10 min, then in 0.1 x SSC for 10 min at 50 °C.

Immunological detection of DNA – DNA hybrids

The DIG labelled DNA-DNA hybrids were detected using labelled antibodies against digoxigenin incorporated in the DNA probe during DNA synthesis. Sections were first treated with a blocking reagent to reduce the non-specific labelling, then in for immunological reaction in buffers containing BSA. After several washes, the dig antibody was detected using a gold conjugated monoclonal antibody fab`2 fragment (British Biocell International, Plano, Germany).

3 Results

3.1 *Watermelon chlorotic stunt virus,* WmCSV, propagation, purification and whitefly *B. tabaci* transmission

3.1.1 WmCSV infections of test plants

The agro-inoculation technique described in chapter 2.3.4 proved efficient for delivery of cloned WmCSV comprising WmCSV DNA A wt and mutant clones mixed with DNA B genomic components.

More then 95% infections by agro-inoculation were reached with *N. benthamiana* and watermelon, *Citrullus lanatus,* for all DNA A and DNA B combinations with symptoms of the mutant viruses resembling that of WmCSVwt and with somewhat milder symptoms of plant infections with WmCSVser.

Upon virus inoculation, first symptoms in watermelon became visible after 10-14 days developing into definite WmCSV disease symptoms after approximately 20 days. In watermelon, chlorotic mottle symptoms were first signs of infection followed by severe leaf deformation, leaf curling and severe stunting characterising chronical infections. WmCSV infected *C. lanatus* plants were used as source plants for whitefly transmission studies.

Figure 5: Typical symptoms of *Watermelon chlorotic stunt virus* disease on field grown watermelon with characteristic chlorotic mottling and mosaic with stunting and leaf distortion in the chronic phases of the disease.

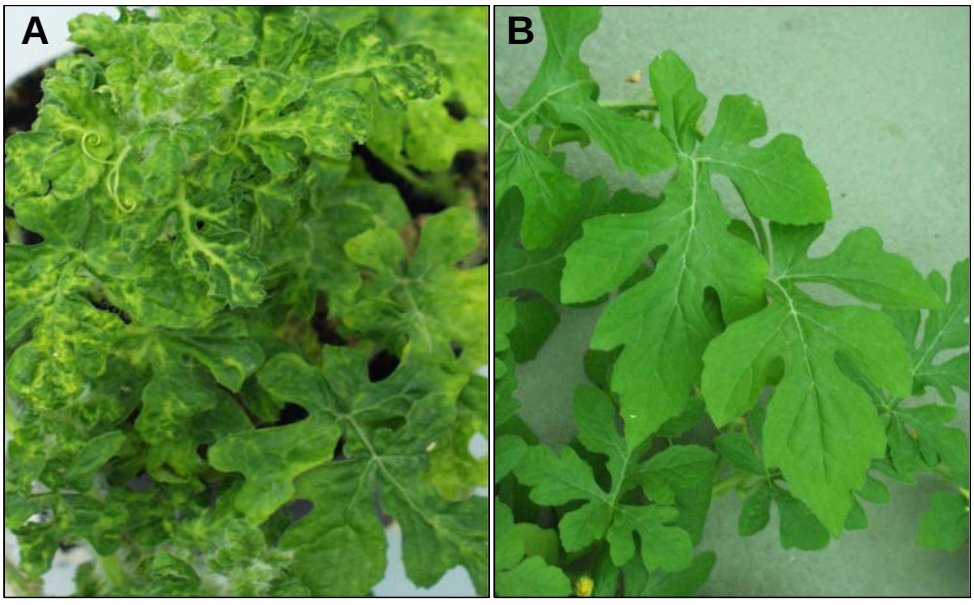

Figure 6: A, Chlorotic mottling and mosaic symptoms of WmCSVwt infected watermelon, 3-4 weeks post-inoculation by biolistic delivery of infectious virus clones. B, Healthy watermelon plants.

First symptoms in *N. benthamiana* for WmCSV appeared later than in watermelon and consisted of leaf blistering and deformation accompanied by mild and relatively inconspicuous mosaic symptoms. Later, symptoms of WmCSV infections developed into a a diffuse mild chlorosis with ystemic curling and leaf deformation. Plants infected with WmCSVwt were found showing a rapid symptom development with severe leaf curling and stunting than the plants infected with WmCSV mutant clones.

Figure 7: N. benthamiana infected with WmCSVwt by agro-inoculation 3-4 weeks post-inoculation at an infection stage ready for harvesting for virus purification.

In general, plants infected by agro-inoculation developed symptoms faster with severe stages of infections reached earlier than plants infected by biolistic inoculations using the Helios gene gun. This was most likely because of the delivery of a higher number of infectious units with agrobacteria harbouring the DNA A and DNA B infectious genomic components.

However, to unequivocally exclude any effect of whitefly uptake or eventual transmission of residual or contaminating agrobacteria, biolistic delivery of WmCSV was pursued in cases where whitefly transmission or WmCSV localisation in the insect was of crucial significance.

3.1.2 Preparation of purified WmCSV

For virus purification symptomatic leaf and stem tissues of *N. benthamiana* plants were used 3-4 weeks after agro-inoculation with WmCSV.

For WmCSV purification, the procedure described for *Tomato yellow leaf curl virus* purification (Luisoni *et al.*, 1995) and modified using a sucrose gradient centrifugation instead of a buoyant density centrifugation in caesium salts, resulted in relatively pure virus particle preparations.

After centrifugation through a 10-40% sugar gradient, the main contaminant of virus preparations, Ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO, accounting for 30–50% of total soluble protein in chloroplasts was largely removed from the virus preparations. The pooled sucrose fractions concentrated by high speed centrifugation was essentially free of contaminating plant proteins and a pure virus particle preparation was obtained (Fig. 8 lane 4). This was characterised by a single polypeptide of approximately 32 kDa in CBB stained SDS-PAGE gels of WmCSV particle preparations representing the mass of the WmCSV coat protein. Virus thus prepared was regarded as purified virus and used for subsequent transmission experiments by artificial feeding and to optimize further immunological fixing and staining processes.

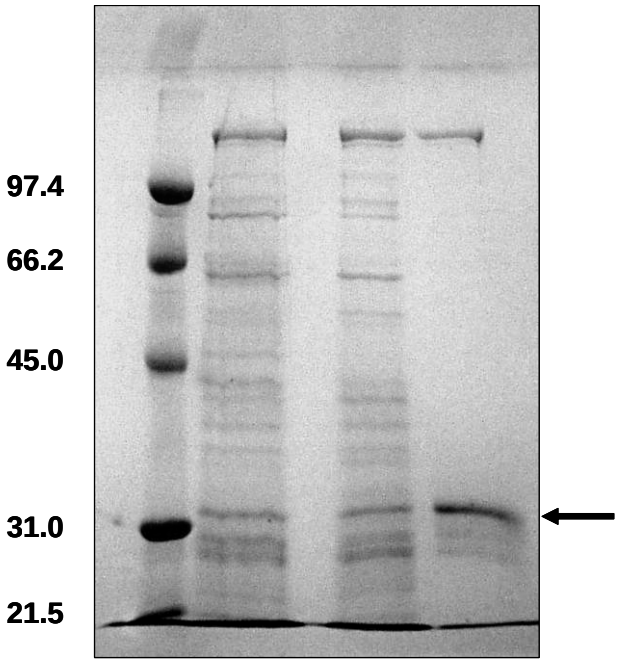

Figure 8: WmCSV preparations to produce purified virus. M, Molecular weight marker (Protein standard in kDa, BioRad); 2,3 semi purified WmCSV preparation after high speed centrifugation; 4, Final WmCSV particle preparation. Arrow pointing at CP size of purified WmCSV

3.1.3 WmCSV transmission by whitefly insects

To study WmCSV transmission by *B. tabaci* and *T. vaporariorum* insects were allowed a 48 AAP on WmCSV infected watermelons that were either inoculated by agro-inoculation or by biolistic-particle delivery. After feeding, on infected plants insects were allowed 48-72 h on a non-host plant *Lycopersicon esculentum* Mill. for discharge. After this, insects were transferred to inoculate healthy watermelon plants allowing a 48-72 h IAP.

B. tabaci insects feeding on WmCSVwt infected plants reached almost 100% infection of watermelon plants (8/8), while attempts to transmit WmCSVwt by *T. vap* as expected, were not successful (0/15).

Transmission studies conducted with the virus DNA A mutant clones, WmCSV asp, WmCSV ser, WmCSV ala, WmCSV val (Fig. 3) revealed that virus infections were not transmissible hence it was found that all infectious WmCSV mutant viruses resulting from agroinfection or from biolistic inoculations, were lacking the whitefly transmissibility (Table 4).

Whitefly inoculated plants developed no symptoms and by further TAS-ELISA analysis none of the experimented plants tested positive.

Table 4: *B. tabaci* transmission of WmCSV infectious virus clones to watermelon plants. Inoculation was done allowing 100 insects a 48 h AAP, 48 h discharge and 72 h AAP. TAS-ELISA was done to prove WmCSV infections.

	WmCSVasp D 133	WmCSVser S 133	WmCSVval V 133	WmCSVala A 133
Mean OD value	0.030	0.029	0.025	0.033
Controls +/-	0.87/0.40	0.88/0.31	1.1/0.04	1.1/0.37
ELISA result	neg	neg	neg	neg
No. of positives/ No. of plants tested	0/12	0/10	0/8	0/9

3.1.3.1 Blocking virus transmission in *B. tabaci*

To investigate the possibility of transmission of the transmissible WmCSV wt by saturation of putative receptor sites with a non transmissible WmCSV mutant, 100 insects were allowed feeding for 5 days on WmCSV asp prior to acquisition of WmCSVwt from infected plants.

This experiment has shown that only 8 out of 20 test plants became infected and transmission therefore was reduced to 40% of the total number of tested plants. This provided a first indication that virus transmission sites in the vector can be blocked by non transmissible virus, but that the putative receptors are not completely saturated. A further transmission experiment feeding high concentration of purified virus might provide clarification of this particular aspect. However since purification of mutant WmCSV did not result in particle preparations with sufficient concentration and quality, proof of this significant observation is still pending.

3.1.3.2 Feeding *B. tabaci* on artificial diets to increase virus concentrations in insects

To increase virus uptake by whiteflies for use in immunolocalization experiments, insects were fed on purified virus preparations adjusted to 15% sucrose. Several virus concentrations in the diet were tested ranging 50-800 µg/ml. Insect populations were unaffected by virus concentrations up to approximately 200 µg/ml, while virus concentrations of about 500 µg/ml resulted in a death rate of 75% of a population of 500-1000 insects that were fed for 72 h on diets of purified virus preparations. A virus concentration of approximately 800 µg/ml in the insect diet was fatal and eliminated the insect population. When midguts of these insects, after a 24 h AAP, were isolated, the organs appeared totally fragile with severe damage found on the tissues. This has also been reported for *Squash leaf curl virus* (SLCV) associated with cytopathological abnormalities in vector tissues and with detrimental effects on vector biology and reproduction.

These observations have provided arguments suggesting for begomovirus replication in *B. tabaci*, however despite few reports based on TYLCV observations (Rubinstein and Czosnek, 1997) replication intermediates of begomoviruses in their respective

vector insects have not been detected. In a recent publication, Xiomara *et al*. (2005) by applying real time PCR, revealed that TYLCV actively produces *de novo* viral transcripts while Tomato mottle virus (ToMoV) does not, hence reflecting differences in transcript profiles but still not providing evidence for begomovirus replication in *B. tabaci*.

3.1.4 Detection of WmCSV by Polymerase Chain Reaction, PCR

PCR was the most versatile method for virus detection in watermelon plants, whole insects and dissected insect organs; since amplification of viral DNA sequences during PCR and reliable detection of begomovirus sequences was largely independent from initial virus DNA concentrations. Total DNA extracted from plants using a DNA minipreparation method as described by Dellaporta *et al.* (1983) provided good quality DNA preparations with sufficient template for reliable and robust PCR amplification of WmCSV sequences. An approximately 1:10 to 1:100 (v/v) dilution of each DNA schown in Fig. 9A (ca. 100 ng total plant DNA) was subjected to PCR using the WmCSVA c/s primers designed (chapter 2.8) and resulting in well defined PCR and specific amplification of WmCSV sequences.

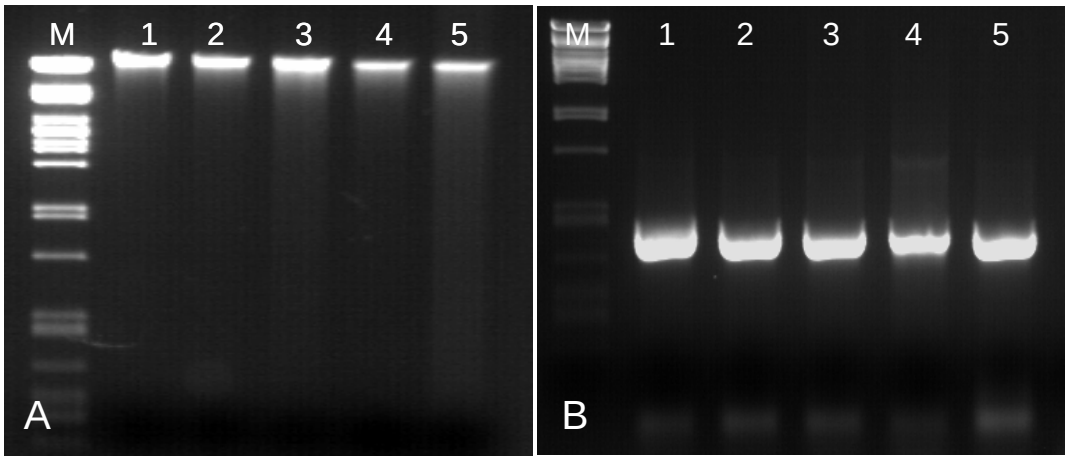

Figure 9: A) DNA extracts from watermelon plants infected with WmCSVwt, lane 1; and WmCSV mutant clones laned 2 – 5; B) PCR performed with total DNA extracts permitting robust WmCSV DNA detection.

3.1.5 Localisation of WmCSVwt in *B. tabaci* and *T. vaporariorum* insects

Organs of the whitefly insects were dissected as described in chapter 2.6. For virus detection using PCR, insects were arrested for 5 min on ice, organs were isolated, flushed with sterile water and immediately subjected to PCR which was conducted with isolated organs of 3 specimen. Isolated primary salivary glands were subjected as paired organs to PCR and except extensive washing, template DNA was exposed by boiling only. The major elements of the stylets as described by Czosnek *et al*. (2002) including labium (outside the head), bundle (inside the head) and the cibarial pump protractor muscles were excised and subjected to PCR. The digestive tract including the midgut (descending and ascending) with hindgut removed was also subjected to PCR for virus detection.

Only female whiteflies were used in the experiments due to easier handling of larger size animals and efficiency of female insects in virus transmission reported (Cohen & Nitzany, 1966; Muniyappa *et al*., 2000).

Viral DNA was detected in all organs isolated from *B. tabaci* feeding on WmCSVwt infected plants (Fig. 10 A) from stylet to primary salivary glands, while in the non-vector *T. vaporariorum* virus was detected in the stylet reaching the midgut only (Fig. 10 B). These results are consistent with evidence reported from similar studies on TYLCV translocation (Czosnek *et al*., 2002).

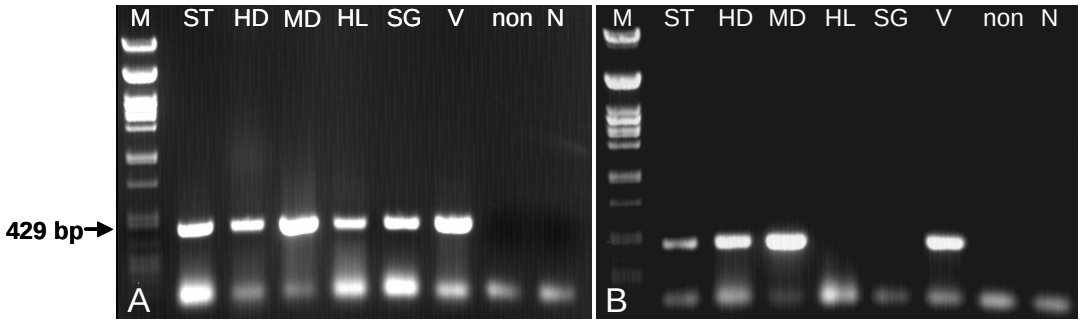

Figure 10: PCR for detection of WmCSV in organs isolated from A, *B. tabaci* and B, *T. vaporariorum*. ST, stylet; HD, head; MD, midgut; HL, haemolymph: SG, salivary glands; non, non-viruliferous whitefly; V, viruliferous insect; N, negative control. 429bp is the expected size of the WmCSV PCR amplicon.

The identity of the amplified viral DNA fragments detected by PCR were confirmed by Southern blot hybridization using a digoxigenin labelled WmCSV DNA A probe as described in chapter 2.7.6.

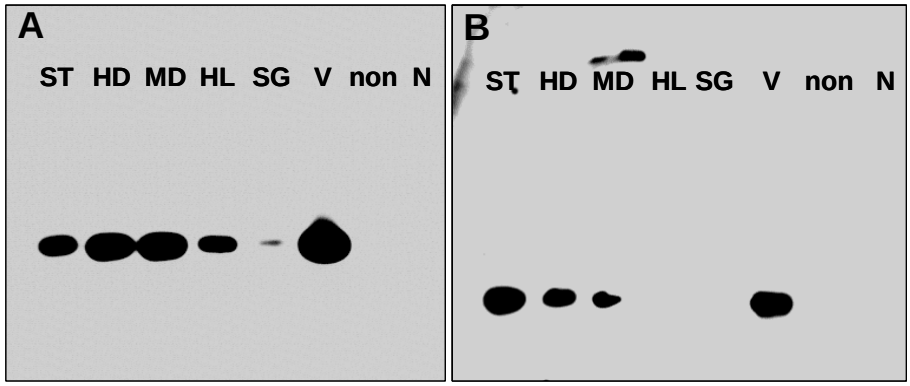

Figure 11: Southern blot hybridization analysis verifying results of the PCR analysis in Figure 10. A, dissected organs of *B. tabaci* and B, organs of *T. vaporariorum.*

These experiments provided evidence that the midgut provides an efficient barrier preventing passage of the begomovirus WmCSV into the haemolymph of the non-vector species *Trialeurodes vaporariorum*.

3.1.6 Localisation of WmCSV mutant virus clones in the vector *B. tabaci*

In a similar experiment with dissected organs *B. tabaci* insects fed on watermelon plants infected with WmCSV coat protein mutants, virus DNA was found in all organs, except for the WmCSVser mutant which was found in the haemolymph but apparently not capable of attaching to the salivary glands hence it was not detected in this isolated organ. The failed detection of WmCSVser (Fig. 12 D) was probably due to low concentration or instability of this mutant virus in this preparation. However, WmCSVser detection was subsequently reached in these organs in later experiments.

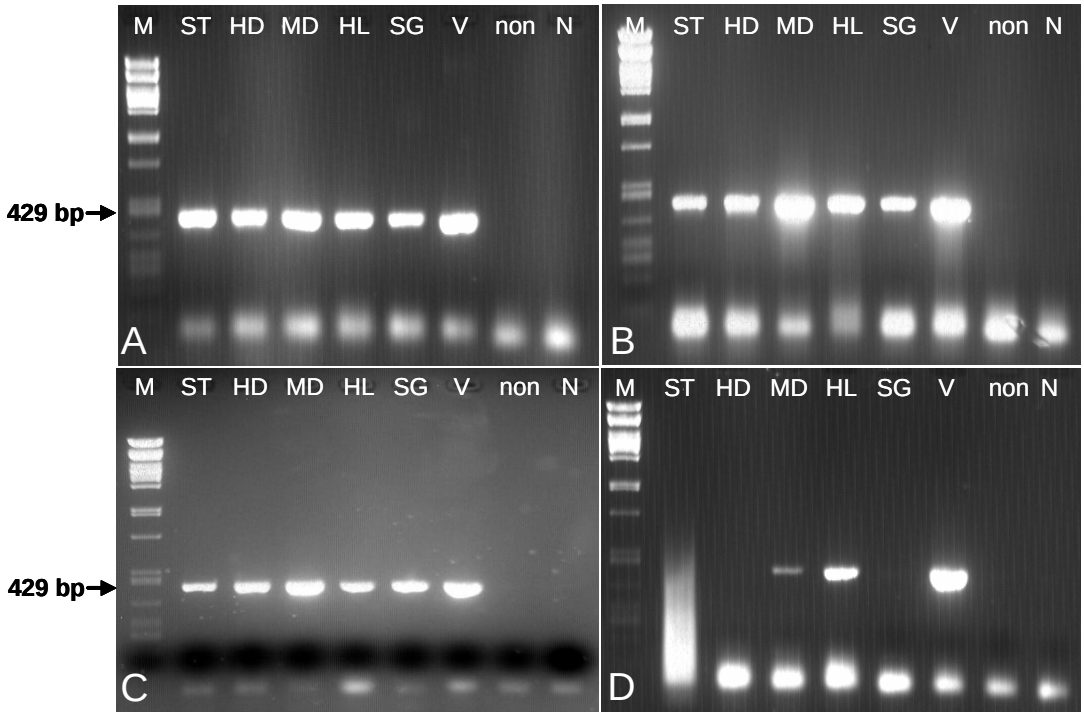

Figure 12: Translocation of WmCSV mutant virus clones in *B. tabaci*. A, WmCSVala; B, WmCSVasp; C, WmCSVval; D, WmCSVser. ST, stylet; HD, head; MD, midgut; HL, haemolymph: SG, salivary glands; non, non-viruliferous whitefly; V, viruliferous insect; N, negative control. 429 bp is the expected size of the WmCSV PCR amplicon.

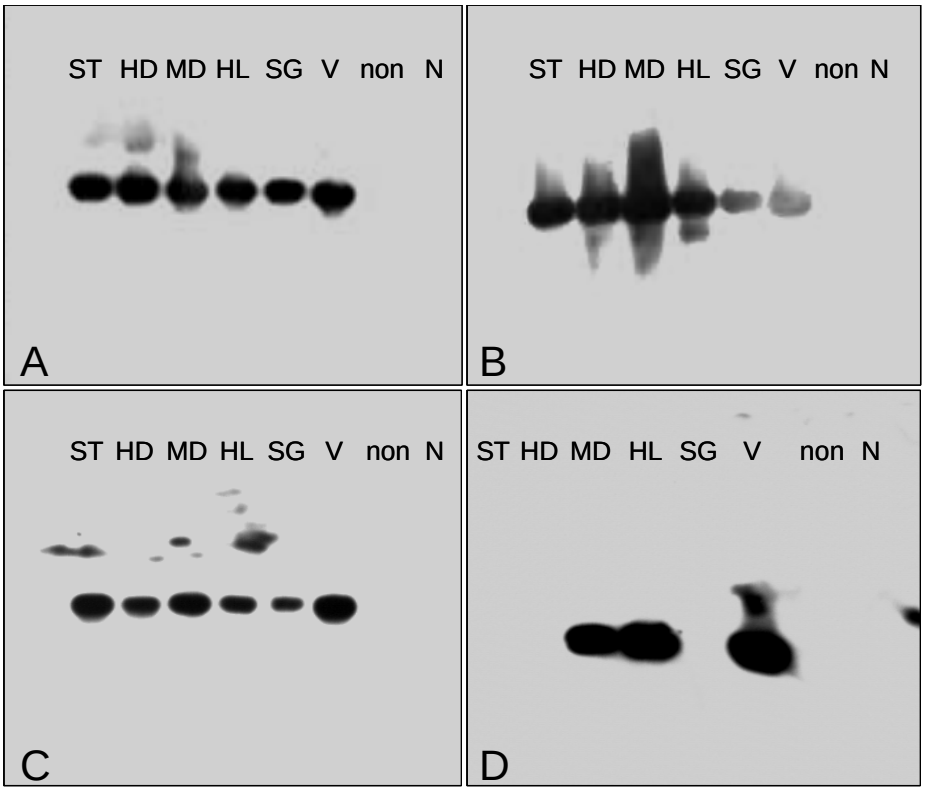

Figure 13: Southern blot analysis confirming results obtained for PCR analysis of dissected *B. tabaci* organs. A, WmCSVala; B, WmCSVasp; C, WmCSVval; D, WmCSVser. ST, stylet; HD, head; MD, midgut; HL, haemolymph: SG, salivary glands; non, non-viruliferous whitefly; V, viruliferous insect; N, negative control.

Feeding *B. tabaci* with coat protein WmCSV mutant viruses to trace the pathway of these viruses in their begomovirus vectors, it was found that the midgut does not present a barrier to virus translocation. All mutants were detected in the haemolymph and either adhering to salivary glands or, within the salivary system. This is in contrast to non vector insects, where the midgut apparently is the barrier to virus passage.

In the interesting case of the WmCSVser mutant, it can be speculated that coat protein is not capable of attaching to the salivary glands or that the virus does not assemble into a coat protein structure, which is not required for begomovirus movement in the plant hence systemic infection. To proof the latter, WmCSVser was subjected to immunocapture PCR analysis.

3.1.7 Immunocapture PCR analysis to verify WmCSVser virion assembly

Immunocapture PCR was conducted to investigate the possibility that WmCSVser did not form a coat protein hence providing argument for its failure to bind to salivary glands. As stated above, the virus was detected in haemolymph and at low titers in midgut (Fig 12 D) albeit at low concentrations. Virus purification attempts to provide virion preparations for electron microscopy also failed hence immunocapture PCR should at least reveal information about assembly of this mutant virus into virions. As shown in Fig. 14, WmCSVser was detected by PCR following an immunocapture of virion capsid protein using a WmCSV specific antibody for trapping of virus particles. Hence, it was confirmed that indeed, WmCSVser forms virus particles.

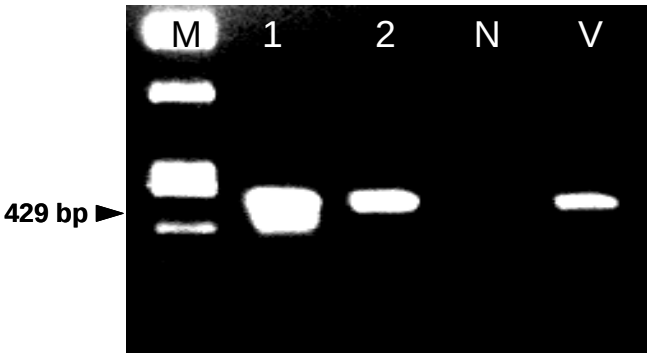

Figure 14: PCR analysis using IC-PCR confirming the assembly of WmCSVser into virions. Lane 1, WmCSVwt, lane 2, WmCSVser; N, negative control; V, WmCSVwt in viruliferous insect used as a positive control.

3.1.8 Tracing WmCSV in organs of whitefly insects

Virus transmission studies and experiments for virus translocation done with whole specimen and with isolated organs of begomovirus vectors, *B. tabaci* and non-vector insects, *T. vaporariorum*, are summarised in Table 5.

Table 5: Virus detection by PCR in isolated organs of whitefly insects fed on watermelon plants infected with WmCSVwt or WmCSV mutant viruses.

	Bemisia tabaci			*Trialeroudes vaporariorum*		
	MD	HL	SG	MD	HL	SG
WmCSVwt Transmitted virus	+	+	+	+	-	-
WmCSVasp Non transmissible virus	+	+	+	---	---	---
WmCSVser Non transmissible virus	+	+	-	---	---	---
WmCSVala Non transmissible virus	+	+	+	---	---	---
WmCSVval Non transmissible virus	+	+	+	---	---	---

3.1.9 Electron microscopical studies with purified WmCSV particle preparations

3.1.9.1 WmCSV particle structure

Upon electron microscopical examination of crude sap extracts from virus infected watermelon plants and from purified virus preparations, geminate, icosahedral particles typical for begomoviruses were observed (Fig. 15). When sap preparations of infected plants were subjected to electron microscopy, virions however were only found by ISEM, with crude sap of virus-infected plants incubated overnight on grids coated with WmCSV polyclonal antiserum (Fig. 16). ISEM conducted on WmCSVser mutant infected plants did not trap any particles hence virus identification was inconclusive.

Purified virus preparations were routinely subjected to EM examination prior to artificial feeding experiments.

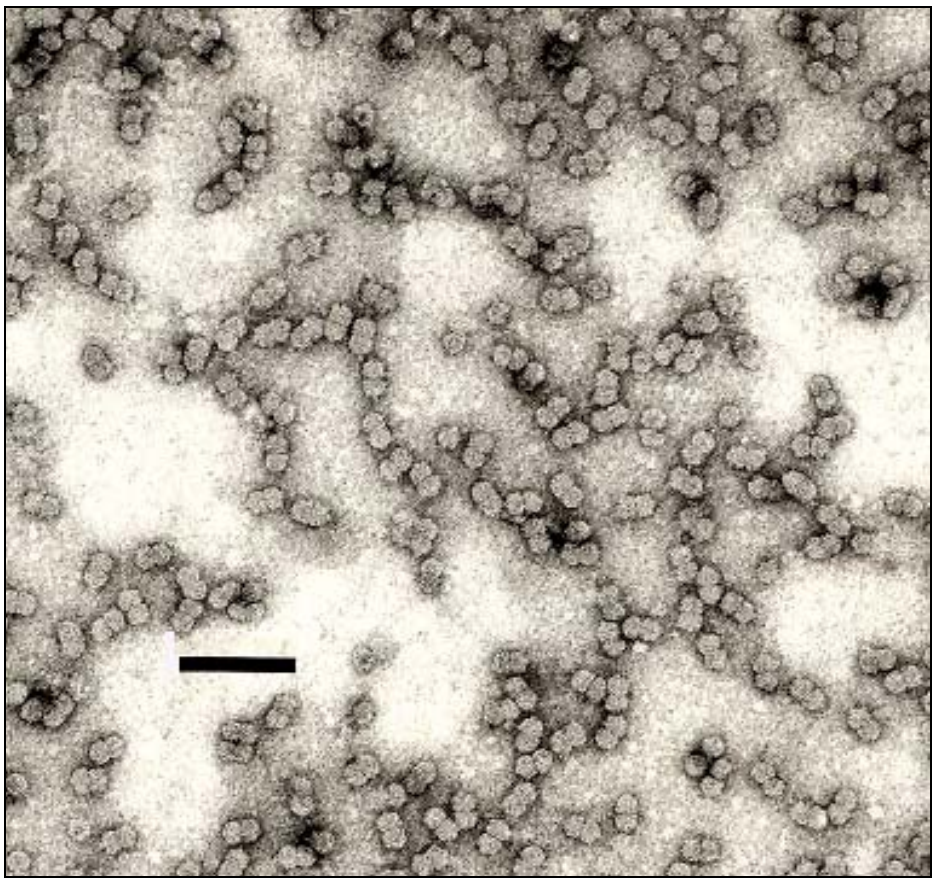

Figure 15: Adsorption preparation of WmCSV particle preparations diluted 1:10 (v/v) in phosphate buffer. Scale bar represents 100 nm.

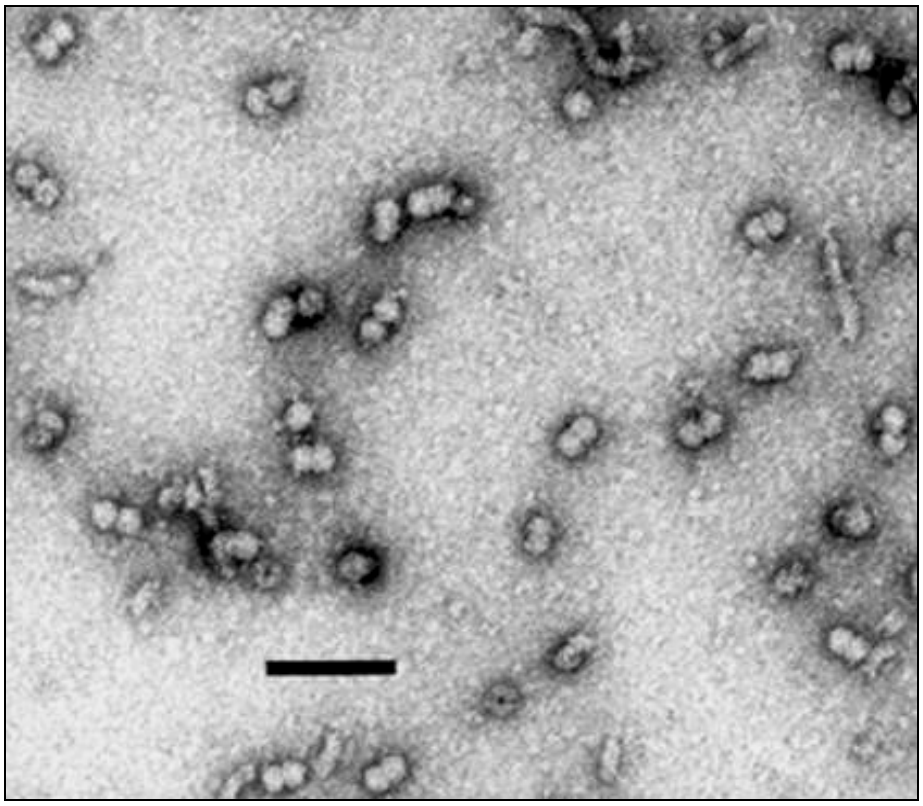

Figure 16: Electron micrograph showing a negatively stained preparation (1% UAc) of WmCSV particles from crude sap of infected watermelon plants trapped by ISEM with WmCSV IgG diluted at 1:1000 (v/v) in phosphate buffer. Scale bar represents 100 nm.

3.1.9.2 Decoration of purified virus particle preparations with polyclonal antibodies

Virus particles captured by ISEM were incubated on 10 µl of preadsorbed IgG 1:5 (v/v) and 1:50 (v/v) in phosphate buffer for at least 15 min, subsequently washed with dd H_2O and contrasted using 1% aqueous UAc. This experiment was conducted to investigate antibody binding affinity to WmCSV and to assess quality of the antigen-antibody interaction.

The assessment of the foggy clouds from antibody decoration of respective virus particles, hence to determine labelling density, is an important precondition for all labelling reactions and especially for *in situ* experiments.

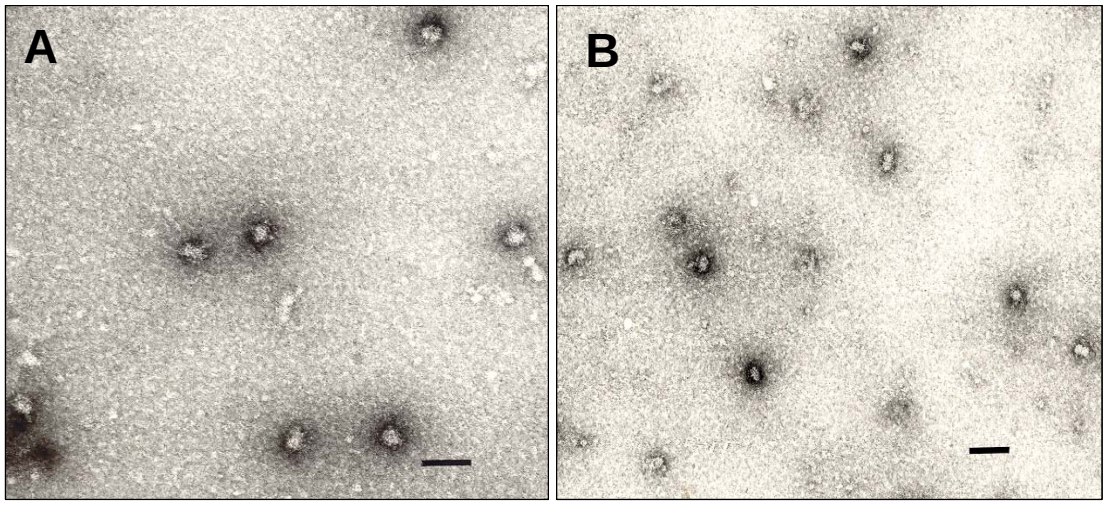

Figure 17: Decoration of purified virus particles trapped with pre-adsorberd WmCSV IgG at 1:1000 (v/v) and fixed in 0.5% GA, 4% PFA for 15 min prior to decoration with WmCSV IgG at dilutions A, 1:5 (v/v); B, 1:50 (v/v). Scale bar represents 100 nm.

In general, using the WmCSV antibody decoration of virus particles with antibody dilutions 1:5 (v/v) resulted in very high and dense labelling with fixed or unfixed virus particles indicating for only minor adverse effects of fixation on decoration and staining intensity. Still, unfixed virus particles subjected to decoration revealed higher antibody binding at 1:5 (v/v) dilutions (Fig. 18).

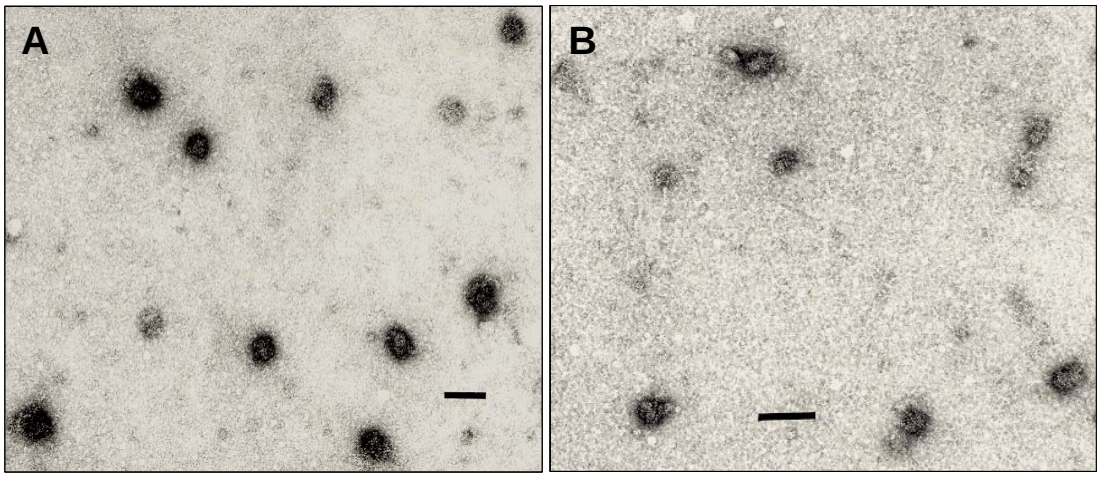

Figure 18: Decoration of purified virus particles trapped with pre-adsorbed WmCSV IgG at 1:1000 (v/v) and decoration with WmCSV IgG at dilutions A, 1:5 (v/v); B, 1:50 (v/v). Scale bar represents 100 nm.

3.1.10 Immunogold-labelling of purified WmCSV particle preparations

Decoration tests with WmCSV antibodies followed by detection of antibody reactions with gold labelled anti-antibody conjugates was pursued to assess affinity of antibody binding and the intensity of gold labelling that can be reached per virus particle. Using antibody dilutions and with fixed particles subjected to decoration, WmCSV antibody dilutions of 1:5 (v/v) and 1:50 (v/v) reached similar results with 2-10 gold particles adhering to one virus particle (Fig. 19, A,B). In contrast omitting fixation of virus particles (Fig. 19, D, E); only 2 gold particles were counted per virus structure at dilution 1:5 (v/v). Thus fixation of virus sample not only increased the density of gold labelling, probably by stabilising virus particles but also decreased unspecific background.

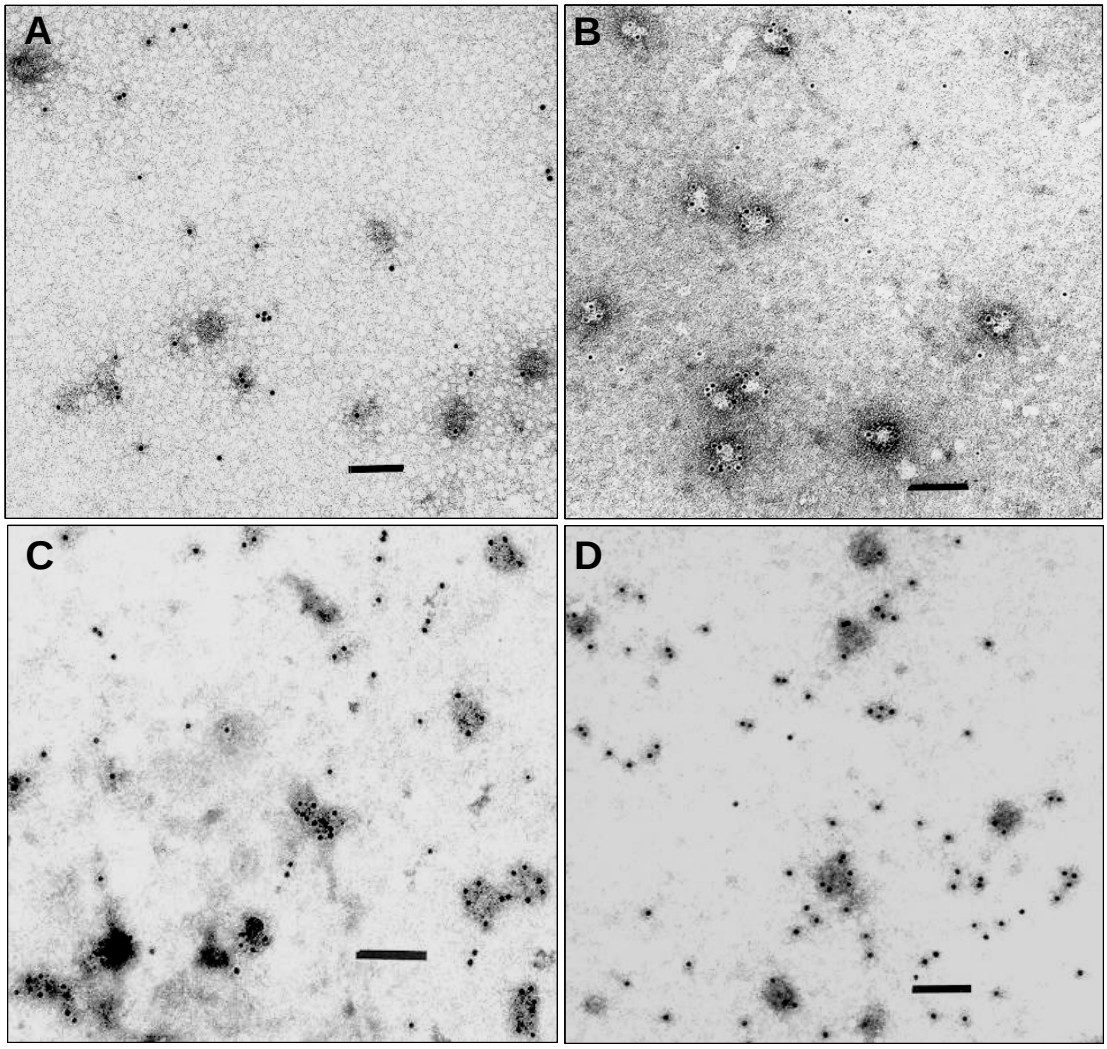

Figure 19: Immunogold labelling of purified virus particles trapped with pre-adsorberd WmCSV IgG at 1:1000 (v/v), fixed in 0.5% GA, 4% PFA for 15 min, A&B, or subjected unfixed, B&D; to decoration with WmCSV IgG at dilutions A&C, 1:5 (v/v); B&D, 1:50 (v/v). Gold labelling was applied using fab`^{2} fragment goat anti- rabbit conjugated 10 nm gold antibody at a dilution of 1:50 (v/v). Scale bar represents 100 nm.

Optimization of embedding and immunogold labelling protocols

Once tissues are excised from its context, autolysis is initiated soon after cell death by intracellular enzymes causing proteolysis and breakdown of cellular components. Autolysis is independent of any bacterial action, retarded by cold, greatly accelerated at temperatures of about 30 °C and almost inhibited at 50 °C.

Autolysis is more severe in tissues rich in enzymes and autolysed tissue presents a `washed-out' appearance with swelling of cytoplasm, eventually converted into a granular, homogeneous mass not amenable to staining for microscopy. Nuclei of autolytic cells may show signs of necrosis, condensation (pyknosis), fragmentation (karyorhexis) and lysis (karyolysis). Diffusion of intracellular substances might occur and desquamation of epithelium separating cells from membranes. All this occurs within a very short time hence for cytological studies suitable fixation of the tissue to be examined is a precondition.

Fixation is to preserve cells and tissue constituents in a close to life-like state and to still support all further preparative procedures without change. Fixation arrests autolysis and stabilises the cellular and tissue constituents, preserving cellular constituents and proteins for subsequent stages of tissue processing. Fixation therefore is the first step in a sequence of events that culminate in the final examination of a tissue section.

However, fixation already constitutes a major artefact since in contrast to the fluid or semi-fluid state of living cells, fixation produces coagulation of proteins and cellular constituents, a step however necessary to prevent loss or diffusion during tissue processing. Fixation methodologies should be adopted taking in consideration achieving the minimum antigen denaturation, on the other hand attaining the maximum fine structure preservation especially the membrane system of the cell which facilitates the correlation of the localization signal with the well defined cellular structure. To achieve this goal, different embedding methods in different embedding resins as well as different immunogold labelling techniques were adopted.

3.1.11 Fixation and embedding

Several combinations of fixative mixtures as described by Karnovsky (1965) were used for fixing of dissected organs and to achieve maximum preservation of antigenicity while maintaining ultrastructural features. For ultrastructural studies embedding in 2.5% glutaraldehyde followed by post-fixation in 0.5% OsO_4 and contrasting *en bloc* in 1% UAc was applied as described by Riedel (Ph.D thesis, 1997). This resulted in a high level of cell structure preservation, especially the membranes in the cytoplasm and with an excellent preservation of the vesicular structures in embedded organs of whiteflies. Due to its reaction with unsaturated fats, OsO_4 fixation provides good fixation of membranes & lipids and the fine protruding, finger like microvilli structures were fully resolved after fixation and embedding in Epon 812. When LR White was used for embedding, even high concentrations of glutaraldehyde resulted in loss of the membrane structures.

A summarised in Table 6, best preservation of antigencity was achieved by specimen fixation in 4% paraformaldehyde (PFA). However, this was also accompanied with a great loss of fine structure of cells, in particular the cellular membranes. Labelling density was reduced from a mean of 56.4 gold particles/5µm^2 using 4% PFA to 20.8 gold particles/5µm^2, when 2.5% glutaraldehyde (GA) was used as a fixative. To compromise antigencity preservation and maintenance of ultrastructure of immuno-stained specimens, a mix of 0.5% GA and 4% PFA was used in for organ studies prior to LR White embedding.

To assess the effect of OsO_4 on antigen preservation, an immuno-staining experiment was conducted with dissected organs embedded in LR White and organs post-fixed with 0.5% OsO_4. Here, it was observed that this treatment dramatically reduced the immuno-staining intensity compared with GA and PFA. Consequently OsO_4 fixation was never used in for immuno-staining experiments conducted with organs embedded in acrylic resins.

To eliminate high temperature effects reducing antigenicity Lowicryl K4M was used for organ embedding permitting tissue processing under very low temperatures from -20 up to -35 hence reducing cellular damage during processing. Generally, embedding in Lowicryl K4M resin resulted in better preservation of ultrastructures with higher contrast compared with LR White acrylic resins.

Table 6: Effects of fixatives on immunogold staining of WmCSV antigen in embedded midgut (MD) tissue sections . Viruliferous insects fed on artificial diets containing purified WmCSV (50 µg/ml) were fixed immediately after AAP in freshly prepared fixative solutions. Embedding was in LR White. MD (midgut), N (nucleus) Out of microvilli is area adjeacent to the microvilli.Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

No.	**Fixative combinations (preliminary fixation)**		**Post fixation**	**Viruliferous insects Mean ± SE**			**Non viruliferous insects Mean ± SE**		
				Microvilli	Out of the microvilli	N	Microvilli	Out of the microvilli	N
1	0.5 %	Glutaraldhyde	No	31.8 ± 8.2	5.1± 2.1		13± 2.5	3.7± 1.0	
	4 %	Paraformadhyde							
2	4 %	Paraformadhyde	No	56.4 ±4.5	9.35 ±1.12	8.9 ±1.6	13.3 ±1.5	4.9 ±1.7	6.3 ±1.4
3	2.5 %	Glutaraldhyde	No	20.8 ±3.1	3.1 ± 0.6	3.1 ±1.1	13.6 ±0.17	1.9 ± 0.98	1.75 ±0.49
4	0.2 %	Glutaraldhyde	0.5 % OsO_4	12.5 ±1.2	5.5 ±0.5	10.6 ±3.9	7.7 ±0.8	5.4 ±0.1	7.4 ±2.8
	4 %	Paraformadhyde							

3.1.12 Optimization of tissue blocking

One of the major problems that confront all immunolocalization studies is the elimination of background and increasing the signal/ background noise ratio, thus permitting unequivocal interpretation of results. To achieve best and most reproducible signal, several blocking solutions were tested. The use of skimmed milk powder as a blocking reagent (Ramandeep *et al*., 2001) resulted in complete saturation of epitopes with no signals obtained, while use of BSA in blocking buffer resulted in a very high background (Table 7).

Table 7: Comparison of different blocking solutions to reduce background in dissected midgut tissues, embedded in LR White, fixed in 0.5% GA + 4% PFA. Blocking buffers, 1% non- fat dry milk in phosphate buffer pH 7.4; 1% BSA in PBST. Nil (control, phosphate buffer; Out (outer regions surrounding microvilli); D (midgut) N (nucleus). Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	Nil **Mean ± SE**	**1% non fat dry milk** **Mean ± SE**	**1% BSA / PBS-T** **Mean ± SE**
Viruliferous insects	N 100.9±11.45	N ~1-2 particles	N 95.6±8.2
	Microvilli 101.5 ±8.5	Microvilli 1-2 particles	Microvilli 82.8±14.4
	Out 30.6±1.7	Out 0	Out 14.7 ±2.6
Non viruliferous insects	N 53.3±15.1	N ~1-2 particles	N 67.6±2
	Microvilli 101.3±2.8	Microvilli ~1-2 particles	Microvilli 74.3±2.7
	Out 28.2±2.3	Out 0	Out 16.4 ±4.8

Increasing the BSA concentration to 3% and up to 5% resulted in a very high background when comparing embedded tissue from viruliferous and non viruliferous insects. However, a mix of 5% BSA and 5% NGS (normal goat serum) as described by Hong *et al*. (2001) improved background considerably while a further addition of BSA-c as described by Momayezi *et al*., (2000) and Yi *et al*. (2001) resulting in a incubation buffer composed of 1% BSA in PBST, 1% NGS and 0.2% BSA-c reached the most reproducible results with low background, however also reducing the signal intensity. This solution was subsequently used in all experiments for blocking and as incubation buffer, respectively.

Table 8: Comparison of blocking solutions for background reduction in dissected midgut tissues, embedded in LR White, fixed in 0.5% GA + 4% PFA. Blocking buffers, 3% BSA in PBS-T, 5% NGS + 5% BSA, 5% NGS + 5%BSA (*incubation buffer composed of 1% BSA, 1%NGS, 0.2% BSA-c). Out (outer regions surrounding microvilli); N (nucleus). Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	3% BSA /PBS-T Mean ± SE	**5% NGS+ 5% BSA Mean ± SE**	***5% NGS+ 5%BSA Mean ± SE**
Viruliferous insects	N 82.5 ±8.9	N: 98.3 ±7.4	N: ±**
	Microvilli 66.4 ±15.2	Microvilli 75.7 ±3.6	Microvilli 31.8 ±8.2
	Out 16.6 ±2.3	Out 30.4 ±4.3	Out 5.1 ±2.1
Non viruliferous insects	N:62.4 ±7.3	N: 52.1 ±0.7	N: ±**
	Microvilli 78 ±3.4	Microvilli 64.15 ±9.6	Microvilli 13 ±2.5
	Out 19.5 ±0.3	Out 30.15 ±4.5	Out 3.7 ±1.0

Using 2 %BSA-c (Ramandeep *et al*., 2002) or increasing NGS to 10% Normal Goat Serum as described by Boyes and Bolam, 2003 failed to give reliable results and reducing the overall signal obtained.

As shown in Fig 20 and 21, unspecific immunogold labelling of embedded and sectioned whitefly organs presented a major problem and the main impediment of image data interpretation and straightforward analysis.

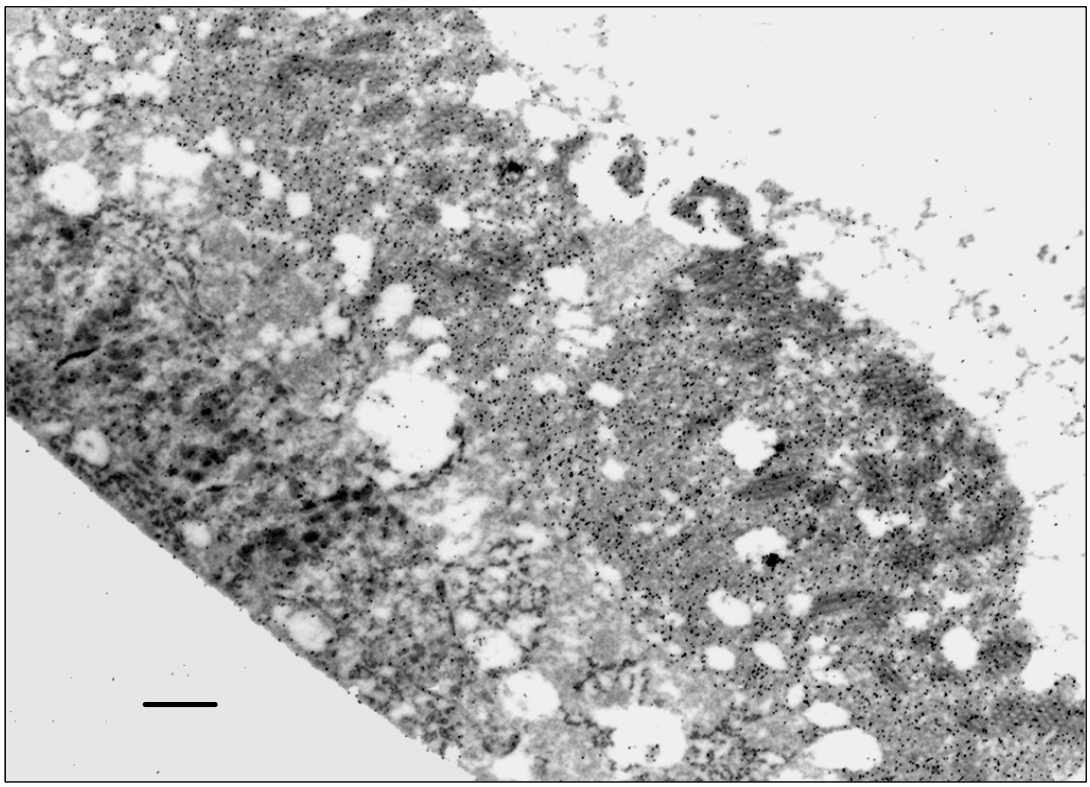

Figure 20: Thin section of midgut isolated from *B. tabaci* reared on WmCSVasp infected watermelon. Scattered gold particles on section surface indicated high non-specific binding. Fixation in 0.2% GA + 4% PFA prior to embedding in LR White resulted in poor preservation of ultrastructural features. Scale bar represents 1µm.

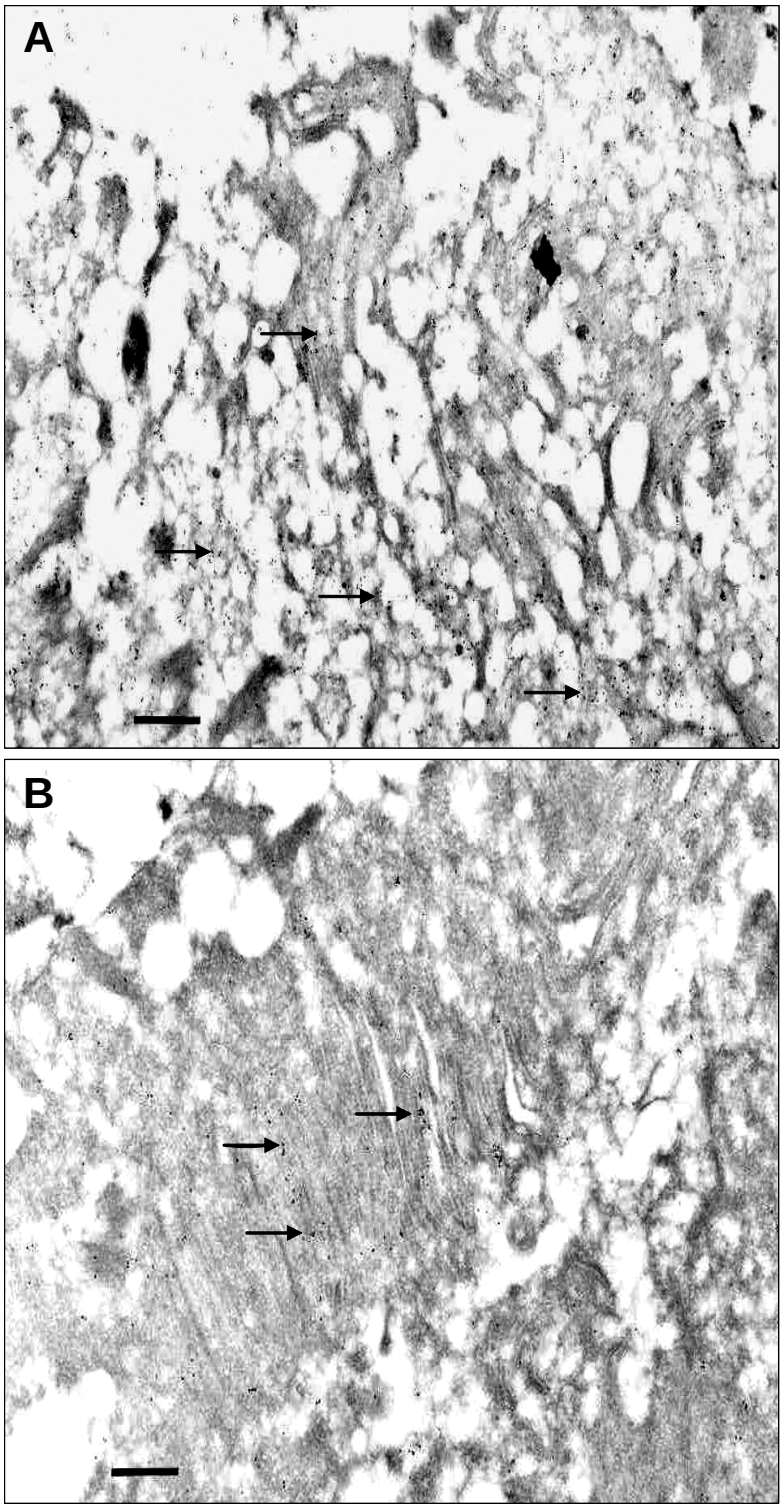

Figure 21: Immunolocalization of WmCSV by immunogold labelling of LR White embedded organ sections. Organs were fixed in 2.5% GA for 2 h then subjected to Glycine and Na BHO4 treatments for background reduction. A, high unspecific labelling with gold particles scattered over the surface (arrows) of the section; B) Specific gold labelling concentrated in the microvilli of the anterior part of the midgut (arrows). Scale bar represents 1µm.

3.1.13 Ultrastructural studies of *B. tabaci* organs

3.1.13.1 Primary salivary glands

Ultrastructural studies with whitefly organs are prerequisites for interpretation of immunolocalisation experiments.

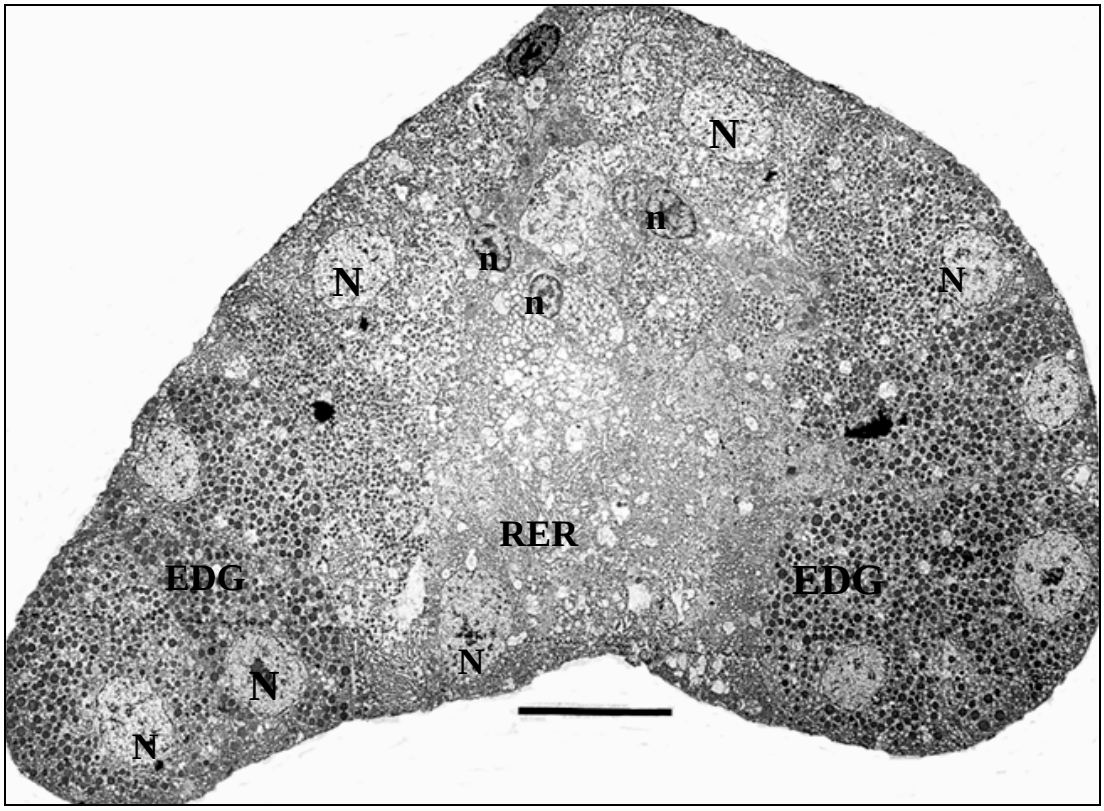

Figure 22: Thin section of a primary salivary gland of *B. tabaci.* The dissected organ was fixed in 2.5% GA, post fixed in 0.5% OsO4 and embedded in Epon 812. N, nucleus; n, nucleoli; EDG, electron dense granules; RER, rough endoplasmic reticulum. Scale bar represents 5µm.

Osmication as a post-fixation treatment provides a good contrast fxing membrane bound lipids and revealing cell boundaries. Fig. 22 shows a thin section through a primary salivary gland fixed in OsO_4 and embedded in Epon 812 plastic resin.

The gland is composed of 13 large cells with large nucleus and small nucleoli, containing condensed and organized whorls of rough endoplasmic reticulum, and large numbers of electron dense granules (Fig 22 EDG) differing in size and of yet

unknown function. Upon higher magnification (Fig. 23) the gland tissue is filled with electron luscent vesicles (Fig. 23 arrows) or similar structures, surrounded by membrane bi-layers. These are probably responsible for transport of proteins or, for virus transport through cells barriers and from cell to cell. However pending a definite proof, this remains speculative.

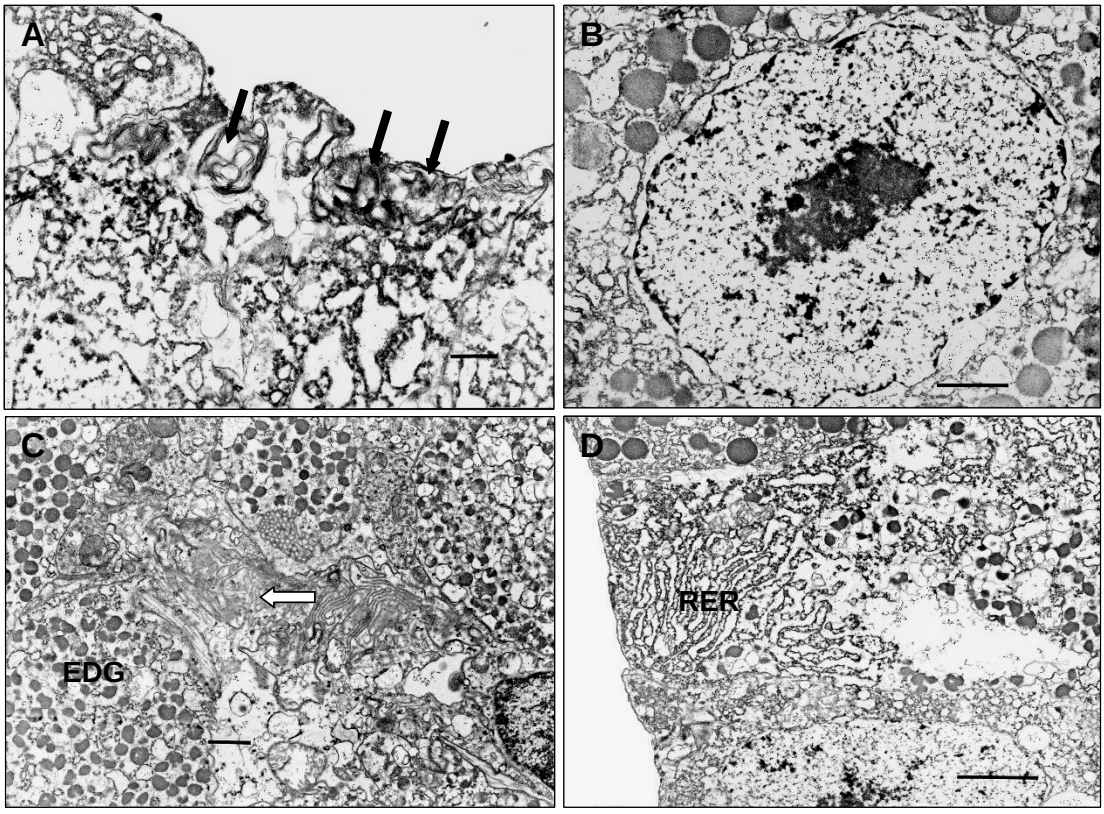

Figure 23: Electron micrograph of a thin sectioned primary salivary gland from *B. tabaci* showing segments of figure 22, at higher magnification. EDG, electron dense granules, RER, rough endoplasmic reticulum; Arrows, membranous structures (vesicles); open arrow, undefined structure. Scale bar represents 1μm.

3.1.13.2 Accessory salivary glands

For ultrastructural investigations of the pear shaped accessory salivary glands (Fig. 25), the excised organs were fixed as described for PSG.

The accessory salivary gland is composed of 4 large cells (Fig. 24 and 25), each cell characterized by a large nucleus, and a huge number of vesicles. However, electron dense granules as observed with primary salivary glands were not found. The four cells are composed of one layer of epithelial cells characterized by an extensive lining of microvilli assembled to meet in the salivary duct (Fig 24 A, B) Fig 25 shows a section of a whole accessory salivary gland with pear-like shape, while the PSG is kidney shaped (Fig. 22). In this section from the ASG enlarged in Fig. 25, microvilli are not found however this segment contains a large number of bi-layered vesicles ands is rich in mitochondria.

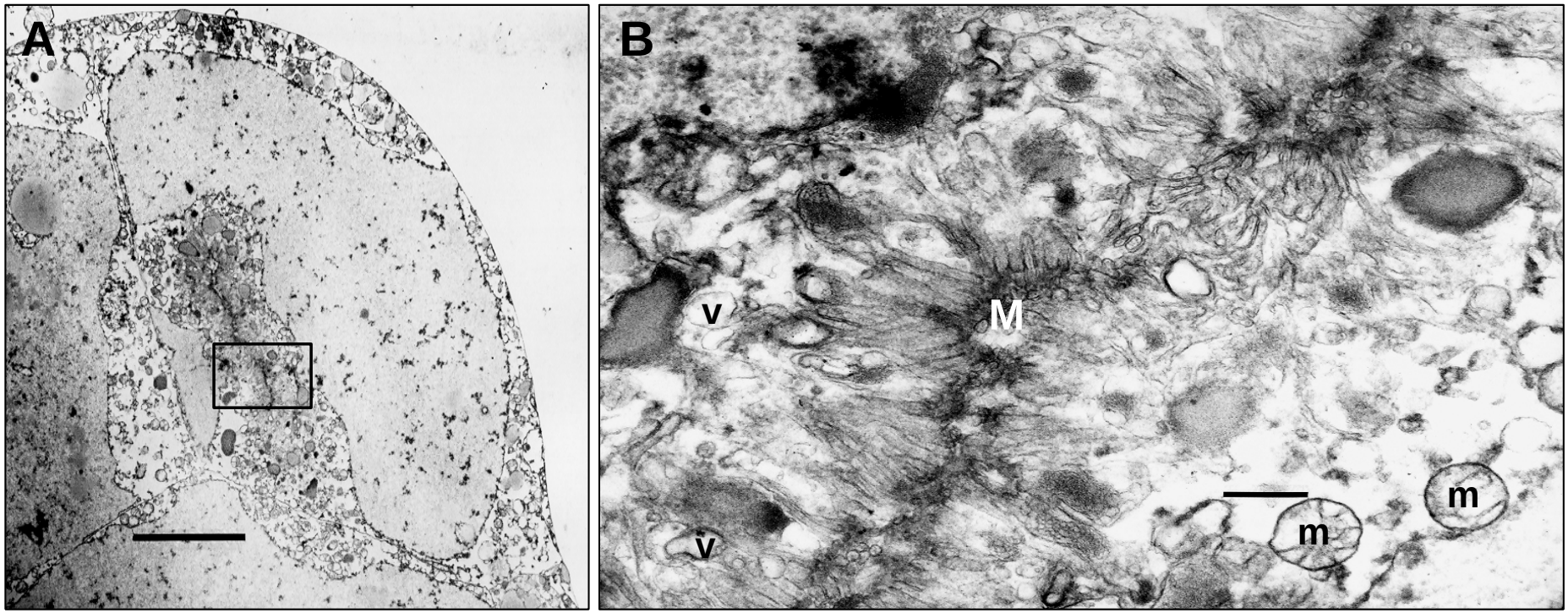

Figure 24: A, Ultrastructure of Epon embedded ASG. Organs were immediately fixed after excision in 2.5% GA for 2h, followed by post-fixation in 0.5% OsO_4 and staining in 2% aquous UAc; B, Enlargement of boxed section of A, Microvilli (M) in the centre of the gland and the four cells composing ASG, (m) mitochondria, (v), vesicles. Scale bar in A represents 5µm; scale bar in B is 1 µm.

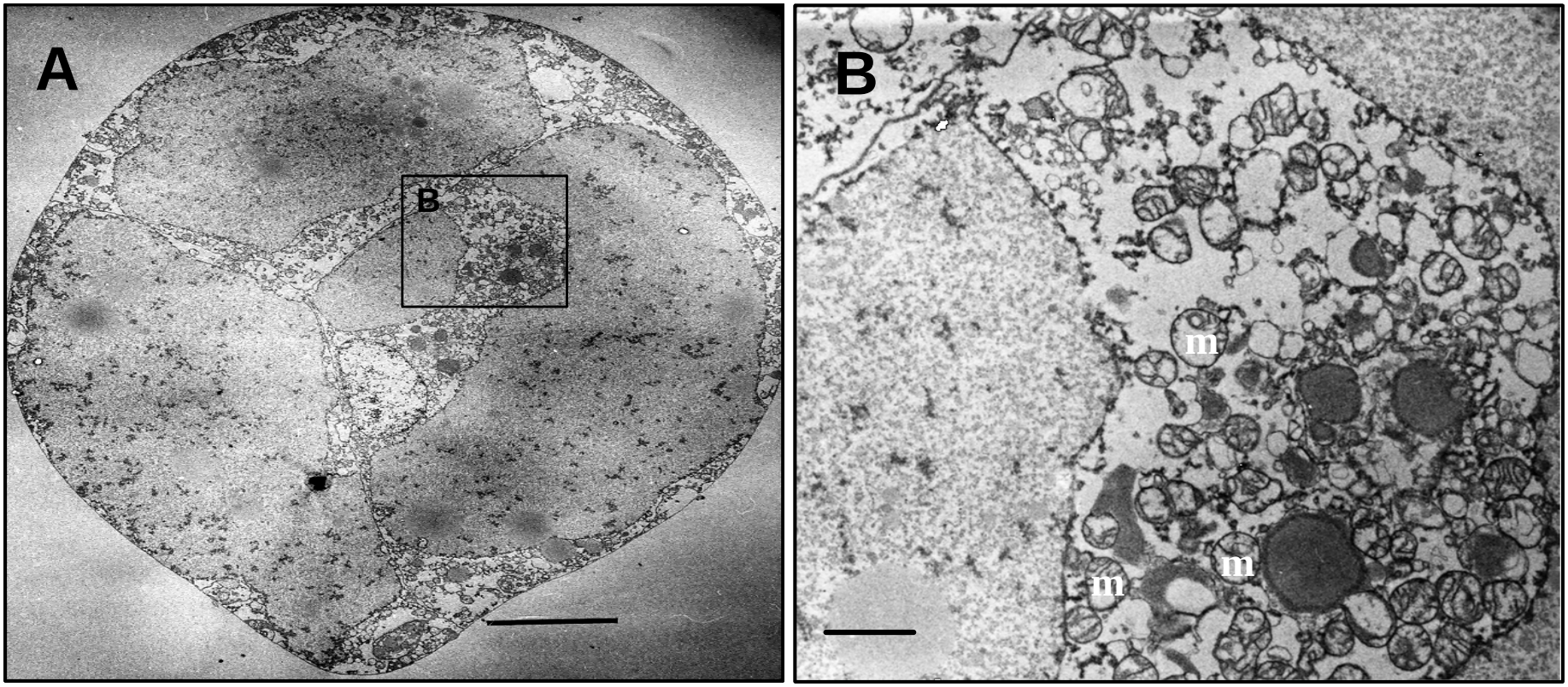

Figure 25: A, Ultrastructure studies of Epon embedded ASG. Organs were immediately fixed after excision in 2.5% GA for 2h, followed by post-fixation in 0.5% OsO_4 and staining in 2% aquous UAc; B, Enlargement of boxed section of A, in an area of the organ where no microvilli are found instead a large number of coated vesicles and mitochondria (m) can be observed. Scale bar represents 1µm.

3.1.14 Immunolocalization of WmCSVwt in dissected organs of *B. tabaci*

3.1.14.1 WmCSVwt immunoloalisation studies in Epon 812 embedded organs

Immunocytochemical localization of antigens at the ultrastructural level is a powerful technique to demonstrate relationships between cell structure and function. However, the central problem in imunocytochemistry is to retain antigenicity without sacrificing cell morphology. A major aspect of this problem is to preserve membrane ultrastructure adequately in the absence of OsO_4 fixation, a treatment known to destroy many antigens. In general, immunological detection is influenced by tissue fixation and the embedding procedure. However, the effect of tissue fixation and embedding is often the limiting factor, particularly when specimens are embedded for electron microscopical examination. Preservation of membranous structures such as multilamellar vesicles, and/or tubular vesicles or multilamellar bodies, which are suggested playing a significant role in virus transcytosis (Reinbold *et al*., 2001), is a key to investigations of the virus translocation mechanisms. Thus, investigations on the effects of etching and antigen retrieval (AR) procedures on post-embedding immuno-labelling of our target antigen were preconditions for all immunolocalization studies.

In our experiment, etching was achieved best using saturated sodium metaperjodate, mPJ (Table 9). Treatment with mPJ significantly reduced the contrast of the sections, thus in later experiments, thicker sections about (120-150 nm) were used and section contrast was enhanced. Four different antigen retrieval solutions were evaluated for best signal obtained upon each treatment with dd H_2O, SCB pH 6.0, EDTA pH 8.0 or Tris buffer pH 10.0, respectively. After AR using SCB or EDTA as described by (Röcken and Roessner, 1999), a number of holes with variable sizes were observed in the sections, although integrity and contrast of the ultrathin section appeared to be maintained.

Table 9: Effect of antigen retrieval treatments on post-embedding immunolabelling of WmCSV in embedded primary salivary gland sections. undef. s., undefined structure; multi. ves., multilamellar vesicles; EDG, electron dense granules; RER, rough endoplasmatic reticulum; N, nucleus; * comparable structures were not found for comparison Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	No treatment **Mean ± SE**	**Etching only with mPJ** **Mean ± SE**	**Etching & heating in H_2O** **Mean ± SE**
Viruliferous insects	N 10.6 ± 0.7	N 23.5± 0.5	N 54.8± 4.6
	EDG 2.45± 0.5	EDG 17.9±2.8	EDG 19.3 ±5.0
	vesicles 15.2 ± 0.2	vesicles 45.1± 0.5	versicles 62.6± 3.4
	undef. s. 7.8± 0.5	undef. s. 49.8 ± 0.34	undef.s. 78.8±9.7
	multi.ves. 8.9±1.0	multi. ves. 41.7± 1.46	multi.ves. 52.1 ± 3.6
	RER 8.4±0.4	RER 28.9 ± 1.4	RER 52.8±4.7
Non viruliferous insects	N 10.1±0.4	N 34.8± 2.47	N 47.9± 4.6
	EDG 3.6 ± 0.2	EDG 20.8 ± 1.2	EDG 16.9 ±1.67
	vesicles *	vesicles 37.9± 1.8	vesicles 49.5±2.7
	undef. s. 8.2 ±0.1	undef. s. 30.1± 0.8	undef. s. 51.5±2.9
	multi.ves. 8.1±0.95	multi.ves.. *	multi.ves. 42.1±3.1
	RER 12.2 ± 1.0	RER 32.4± 1.2	RER 40.8±4.8

Different antibodies were used in immunolocalization studies after etching and antigen retrieval treatments. The monoclonal antibody against TYLCV, DSMZ AS-0546/2 (1C1), and the ACMV polyclonal IgG DSMZ AS-0241/2 showed very good

reactions with WmCSV in ELISA and in Western-blot analysis. TYLCV 0546/2 was used to evaluate the effectivity of antigen retrieval. Sections were subjected to saturated sodium metaperjodate (15%) for 1 h and subsequently subjected to antigen retrieval in dd H_2O for 30 min at 91 °C. Unfortunately, there was no positive effect on the retrieval of the target epitope and only very few scattered gold particles were observed on PSG tissue sections. With the polyclonal antibody ACMV 0241/2 applied to etched sections subjected to H_2O antigen retrieval, a very weak signal was obtained and only few gold particles were observed. Using mPJ without further antigen retrieval treatment had moderate effects on labelling signal intensity, but there was no significant difference observed between PSG sections from viruliferous and non-viruliferous insects. As summerized in table (9) the mean value of gold particles scored in the undefined structure without etching treatment was 7.8 and after etching with mPJ it has been raised to 49.8 then to 78.8 upon antigen retrieval after heating in H_2O.

However when etching was combined with H_2O antigen retrieval the signal intensity was increased and most intense in multilamellar vesicles. Unspecific signals were found on the electron dense granules and nuclei; hence these two organelles may not play a significant role in virus translocation. This was in contrast to the report by Ghanim *et al.* (2001) observing TYLCV immunoreactions at high levels in the nucleus of PSG.

To reduce non-specific antibody binding, several blocking solutions were used, with non fat dry milk (0.1, 0.5, 3%) in PBS-T resulting in a total blocking of all antigen-antibody reactions. In parallel experiments with 2 % and 0.5 % BSA-C (Aurion-Germany) The only blocking method which gave better results was the combination of 5% NGS with 5% BSA in PBS-T however, this blocking was also accompanied with a relatively higher non-specific binding of gold particles on the nucleus and EDG which may not be significant sites for virus translocation. For ultrastructural examinations it was found that etching followed by antigen retrieval using H_2O resulted in satisfactory levels of antigen retrieval and good immunological signals (Fig. 27), while etching treatment alone resulted only in very weak immunological signals (Fig. 28).

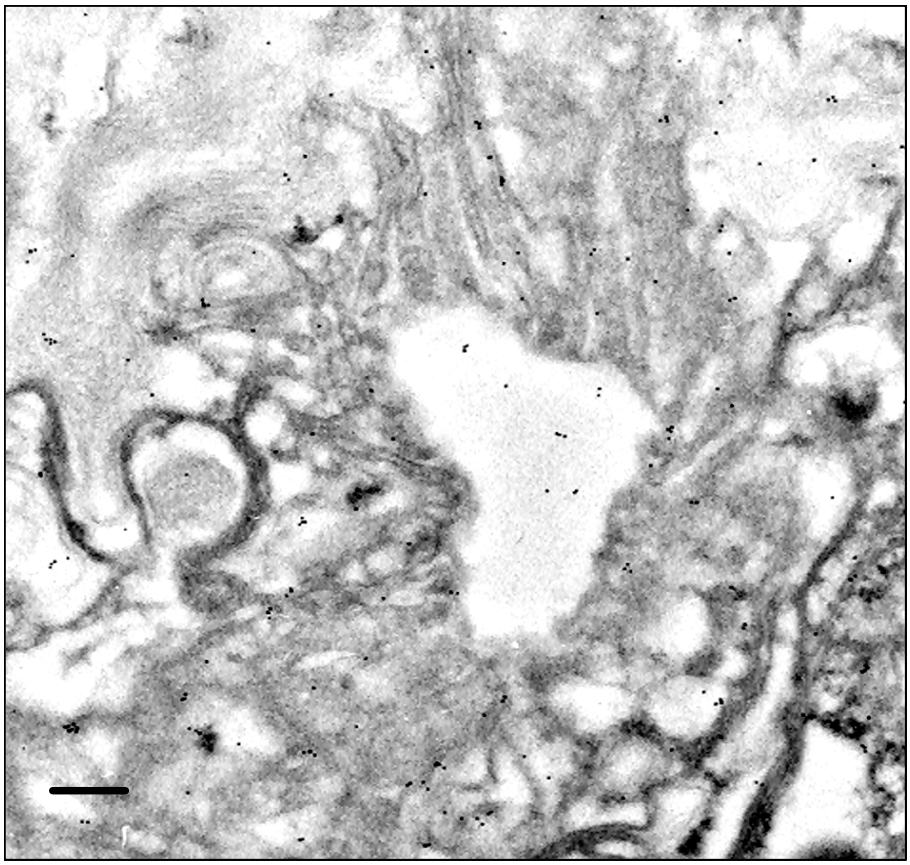

Figure 26: Immunolocalization of WmCSV in Epon embedded organs sections subjected to mPJ etching and antigen retrieval using dd H_2O. Polyclonal WmCSV AS-803 was used as a primary antibody. Signal was observed in vesicle aggregates. Scale bar represents 1µm.

These methods were carried out to determine the most effective antigen retrieval treatment. Generally, it can be concluded that etching treatment with mPJ followed by antigen retrieval has resulted in a great improvement of antigen binding, although non-specific binding to insect proteins or proteinaceous structures also was increased. In these experiments antigen retrieval of Epon embedded PSG using Tris-buffer at pH 10.0 for 24h at 65 °C as described by Saito *et al*. (2003), resulted in a very poor antigen retrieval while this treatment improved signal intensities with Lowicryl embedded PSG sections.

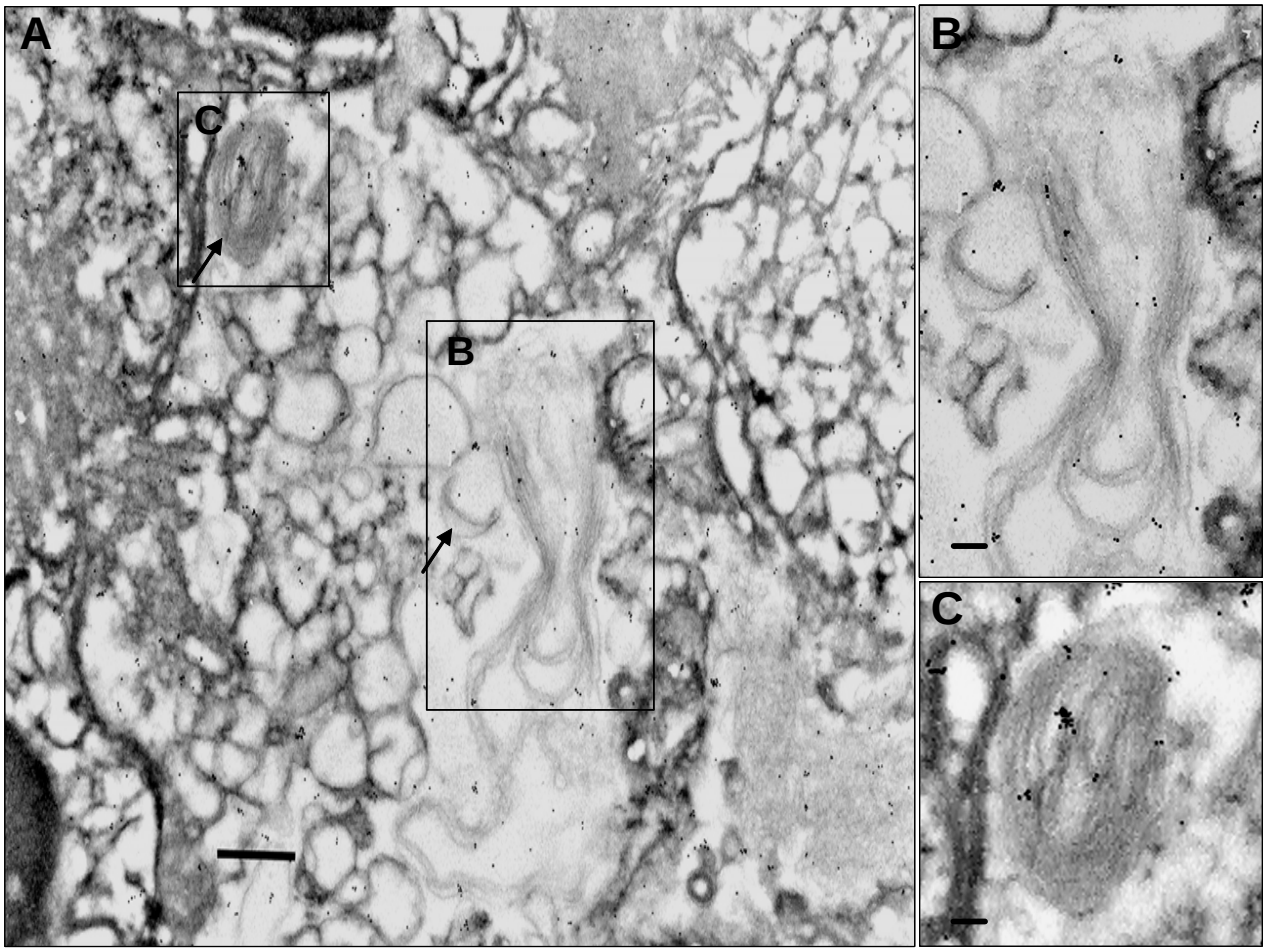

Figure 27: Immunolocalization of Epon embedded PSG sections subjected to antigen retrieval after mPJ etching. Polyclonal WmCSV was used in dilution of 1:25 (v/v) as primary antibody. Immunological signals were observed in vesicle aggregates and in multilamellar vesicles indicated by arrows. B,C is an enlargement of boxes in A. Scale bar represents 1 µm.

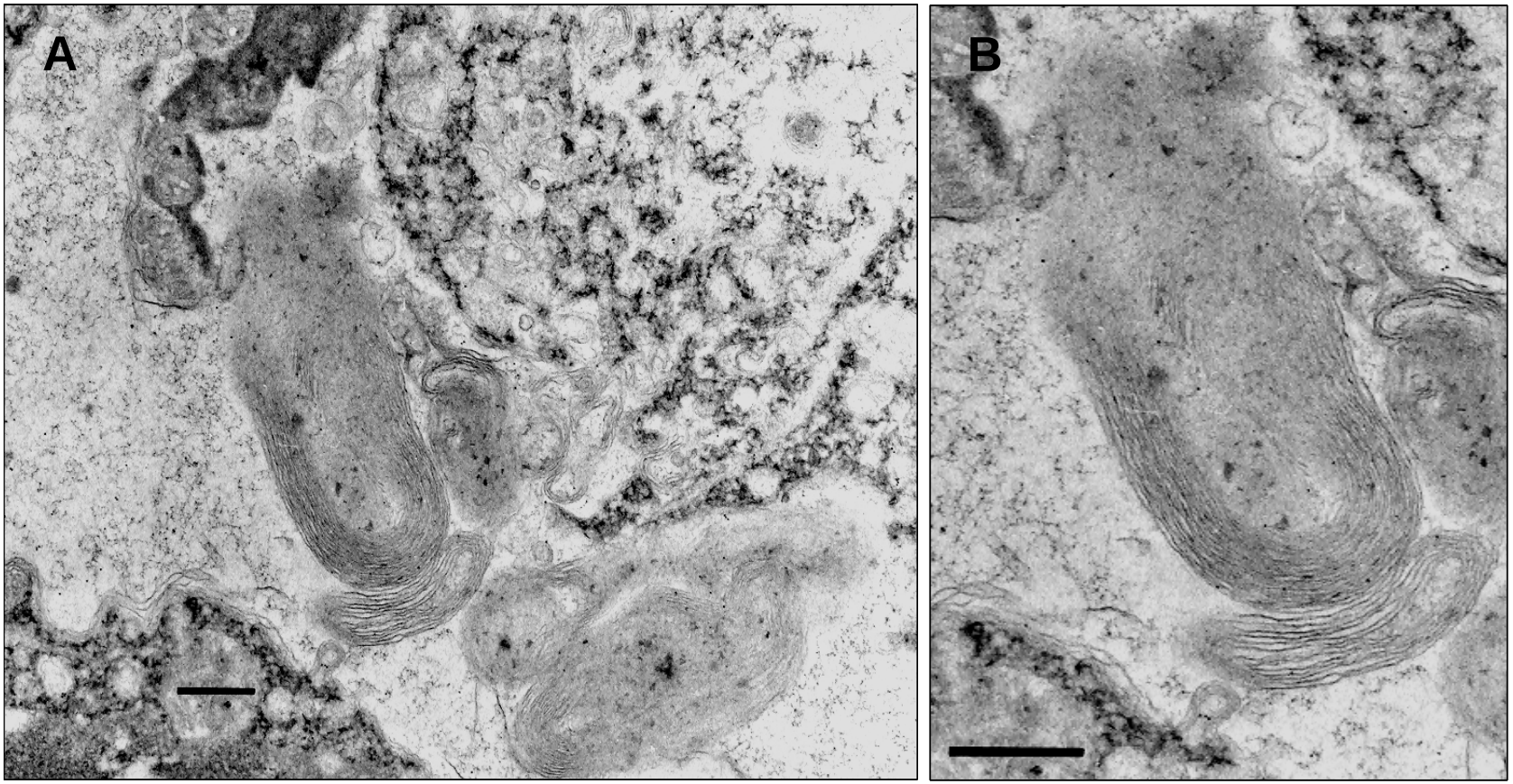

Figure 28: Immunolocalization of PSG sections embedded in Epon subjected to etching treatment only, without further antigen retrieval. Only very weak signal were observed in multilamellar vesicles. B is enlargement of a multilamellar vesicle observed in A. Scale bar represents 1µm.

3.1.14.2 Optimisation of blocking conditions for immunological experiments on sectioned insect organs

Several blocking buffer combinations were tried with etched and AR treated sections. Here it has been found that neither blocking using non fat dry (skim) milk (0.1%, 0.5%) nor the use of 2%BSA-c resulted in a satisfactory background reduction since the overall signal was almost quenched. The most acceptable signal was found using 5% NGS + 5% BSA as a blocking buffer. Immunological signals, evident by agglomeration of GAR-gold particles on the section was found to be concentrated in vesicles with a mean value of about 62.6 gold particles per 5µm^2 compared with only 49.5 particles found in non-viruliferous insects. In structures with a so far not clear function (unidentified structure) a mean value of 78.8 gold particles per 5µm^2 with viruliferous insects compared with a mean value of 51.5 reached with the non-viruliferous insect sections provided a good indication for specific agglomeration of virus in these structures. Immunogold labelling of WmCSVwt in embedded midgut tissuesImmunolocalization experiments preformed with dissected organs embedded in LR White at 60 °C and primarily fixed in 0.5% GA+4% PFA revealed striking and often ambiguous results for each organ subjected to experiments. For the midgut sections, the signal obtained was found to be concentrated in the microvilli adjacent to the lumen of the insect gut. In addition the signal was found to be intense in the filter chamber of the insect (Fig 29) while no signal was observed in the lumen of the midgut. Using LR White as embedding medium, the ultrastructure of the organs embedded was not resolve to high resolution as it was later found with organs embedded in Lowicryl acrylic resin.

3.1.15 Immunogold labelling of WmCSV in embedded midgut tissues

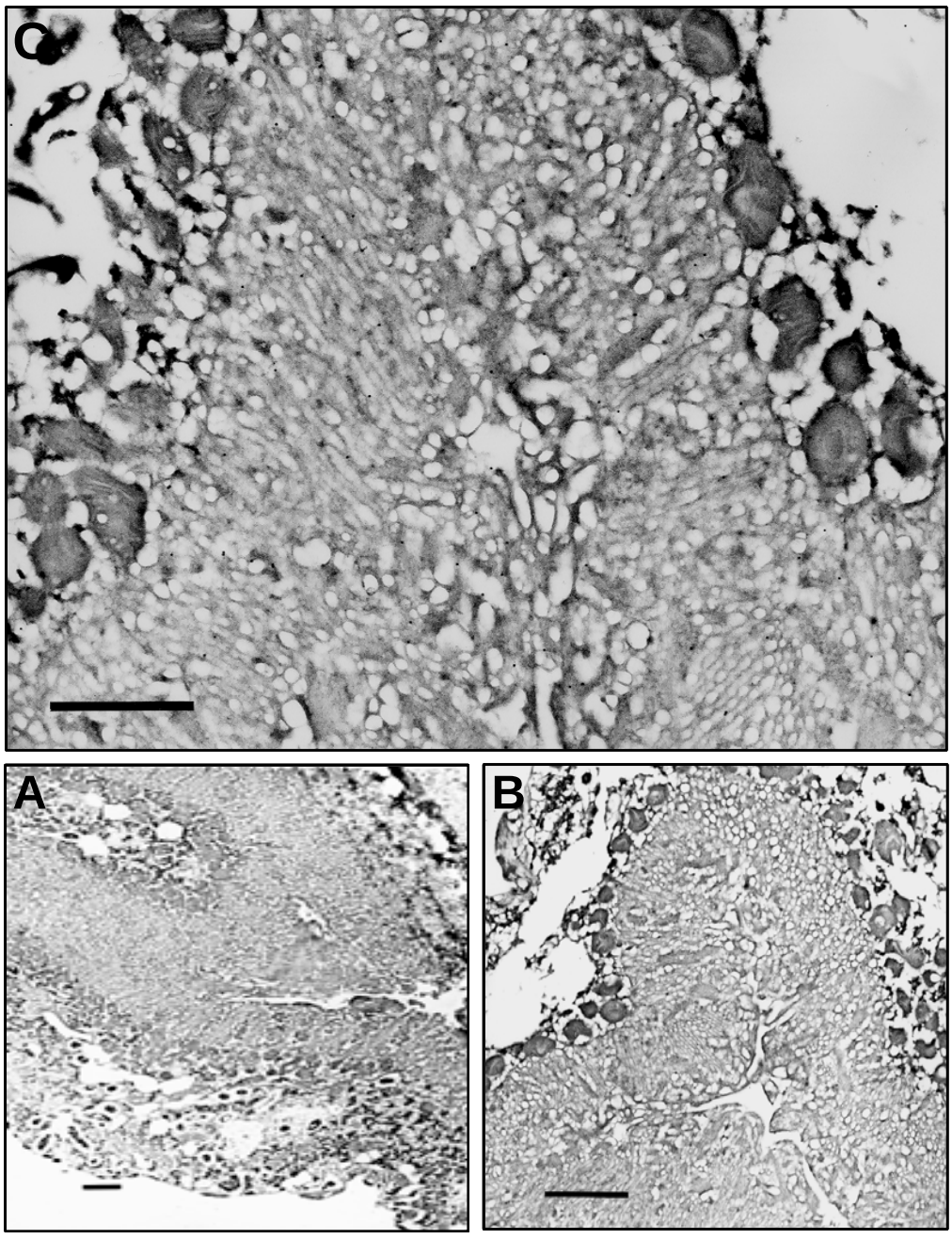

Figure 29: Immunogold labelling and localisation of WmCSV in embedded midgut tissues. To preserve antigenicity OsO4 treatment was not done hence the low contrast particularly with membranes. Immunogold granules are concentrated in the microvilli with few gold particles found out of the microvilli especially the mitochondria and the nucleus. Scale bar in A,C represents 1µm; in B scale is 2µm.

The observed signal recorded from assessment and gold granule counting of 20 sections taken from 2 midgut blocks revealed a mean value of 31.8 gold particles found in a 5 μm^2 area in viruliferous insect compared with 13 gold particles per 5μm^2 for non-viruliferous whiteflies (Table 10).

Table 10: Gold particle distribution on subcellular compartments of the proximal part of the ascending midgut counted on a 5 μm^2 area (at a magnification of 12930x). Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recording.

	Subcellular location	**Distribution of gold particles**	
Viruliferous insect		Mean	± SE
	Microvilli	31.8	± 8.2
	Out of the microvilli	5.1	± 2.1
Non viruliferous insect	Microvilli	13	± 2.5
	Out of the microvilli	3.7	± 1.0

3.1.16 Immunolocalization of WmCSV in embedded primary salivary glands

Primary salivary glands embedded in LR White subjected to immunolocalization studies revealed signals concentrated in structures which are in general not well defined and hitherto unknown. (Table 30). Especially when post-fixation treatment was omitted, these structures remained quite diffuse and not well contrasted. However, these structures revealed the most intensive signal regardless of the method used for fixation or type of the embedding .

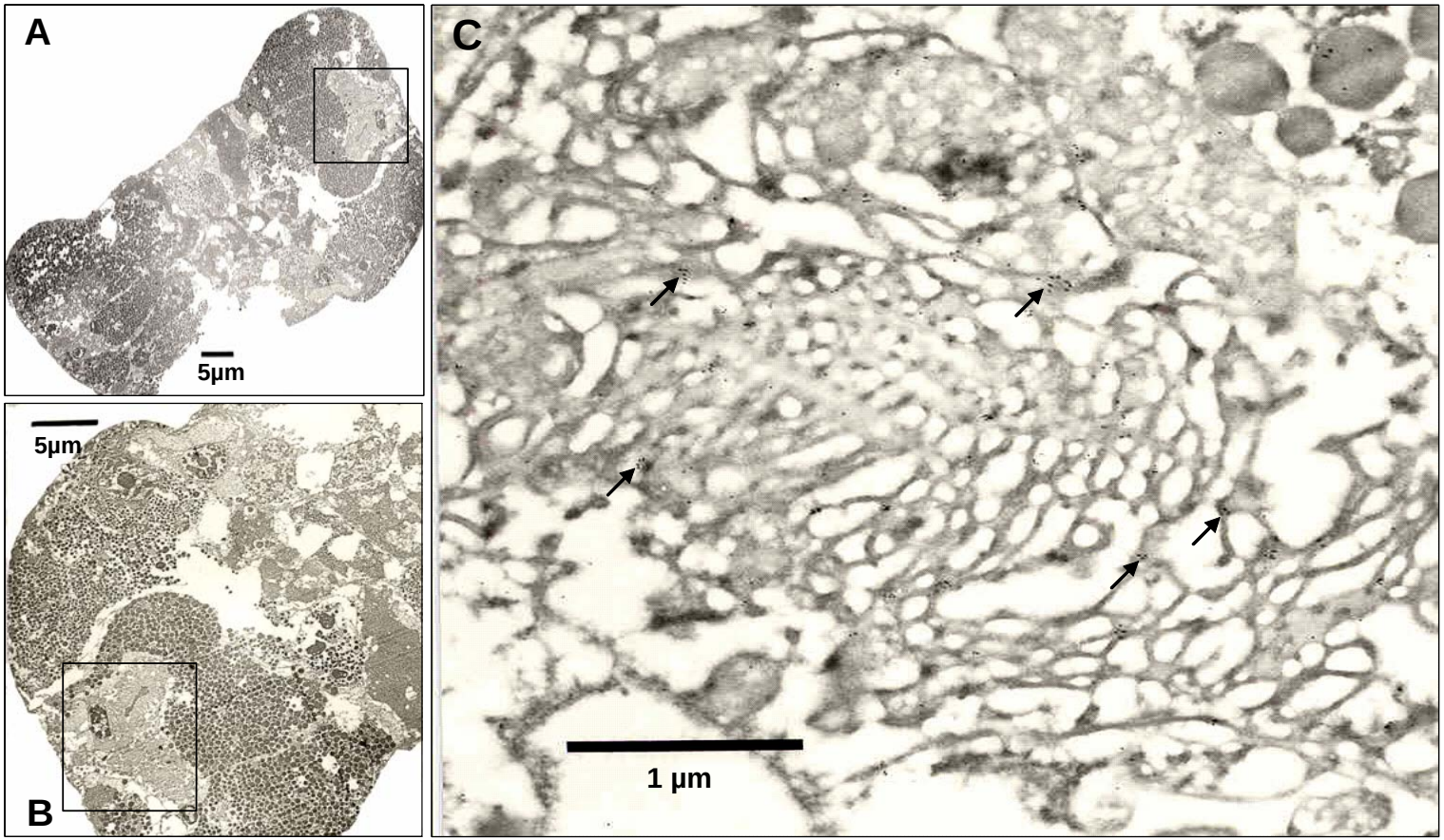

Figure 30: Immunogold localisation of WmCSV in primary salivary gland sections isolated from *B. tabaci* fed on purified virus preparations for 72 h. WmCSV polyclonal antibody was used in 1:25 (v/v) dilution; the secondary antibody, a goat anti rabbit fab`2 fragment conjugated to 10 nm gold (British Biocell Internaional) was used in a dilution of 1:50 (v/v). Scale bar A, B = 5µm, in C, scale bar is 1µm. Arrows point at gold aggregations.

The signal observed in this structure using LR White reached a mean value of 23.5 gold particles per a 5 μm^2 area compared with 9 gold particles per 5μm^2 for the control section (Table 11), taking into consideration that these experiment were done on dissected organs from insects feed on infected plants and not on artificial diets from purified virus preparations.

Table 11: Distribution of gold particles on subcellular compartments of primary salivary glands counted on a 5 μm^2 area (at a magnification of 12930 x). Figures representing mean values of 10 sections obtained from two glands. Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	Subcellular location	**Distribution of gold particles**	
Viruliferous insects		Mean	± SE
	Nucleus	10.9	± 3.1
	Undefined structure (rich of multilammelar vesicles)	23.5	± 10.6
	Electron dense granules	8.2	± 1.8
	Vesicle aggregates	18.8	± 9.8
Non viruliferous insects	Nucleus	3.9	±1.0
	Undefined structure (rich of multilammelar vesicles)	9.1	±0.9
	Electron dense granules	4.6	±1.0
	Vesicle aggregates	4.1	± 0.5

It has further been observed that a high level of gold particles was found in the nuclei of viruliferous insects with about 10.9 gold granules per area observed compared with 3.9 gold particles for the control section. The signal observed in the vesicle aggregates which was rich of multilamellar and electron lucent vesicles, with

putative role in protein transport was significantly higher in viruliferous insects (18.8 particles) than with non viruliferous insect (4.1 gold particles per 5µm^2)

For a more detailed investigation on these undefined structures, salivary gland sections embedded in Epon plastic resin after fixation for ultrastructure preservation and osmication were used for reference and interpretation of the results (Fig, 31). Observations in EM revealed a good preservation of the fine structures in these organ sections.

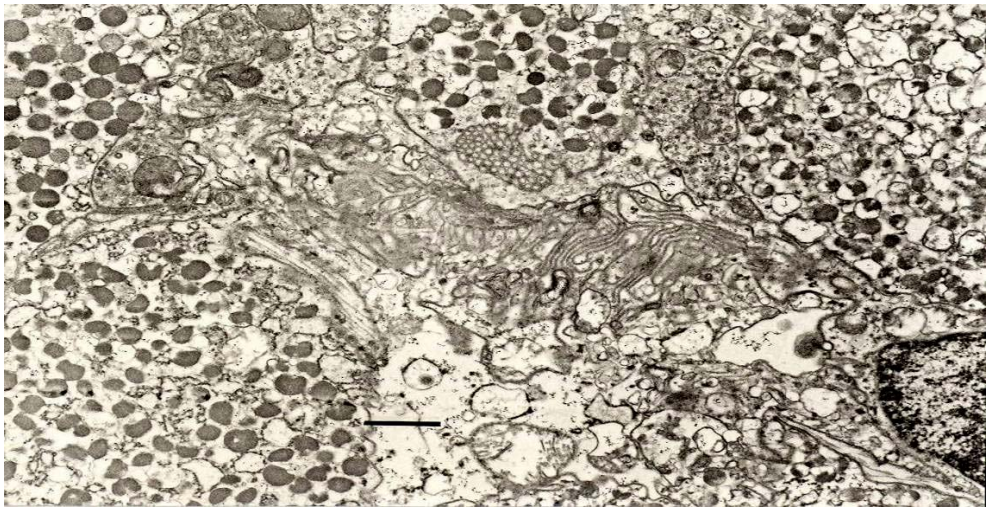

Figure 31: Section through a primary salivary gland embedded in Epon 812 to resolve cytology for ultrastructural investigations. Scale bar represents 1µm.

3.1.17 Immunolocalisation of WmCSV in embedded accessory salivary glands

The immunological experiments performed with accessory salivary glands overall resulted in weak labelling reactions providing an indication that accessory salivary glands may not play a role in virus translocation. The overall signal obtained was weak and only slightly higher in intracellular vesicle aggregates than in microvilli (Fig 32) of the viruliferous insects compared with the control sections of the ASG (Table 12).

Table 12: Distribution of gold particles on subcellular compartments of accessory salivary glands counted on a 5 μm^2 area (at a magnification of 12930 x). Figures represent mean values from 10 sections obtained from two glands of viruliferous and non-viruliferous insects respectively. Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	Subcellular location	**Distribution of gold particles**	
Viruliferous insects		Mean	SE
	Microvilli	7.1	± 2.9
	Intracellular vesicle aggregates	12.1	± 3.2
Non viruliferous insects	Microvilli	2.8	± 1.3
	Intracellular vesicle aggregates	2.7	± 0.7

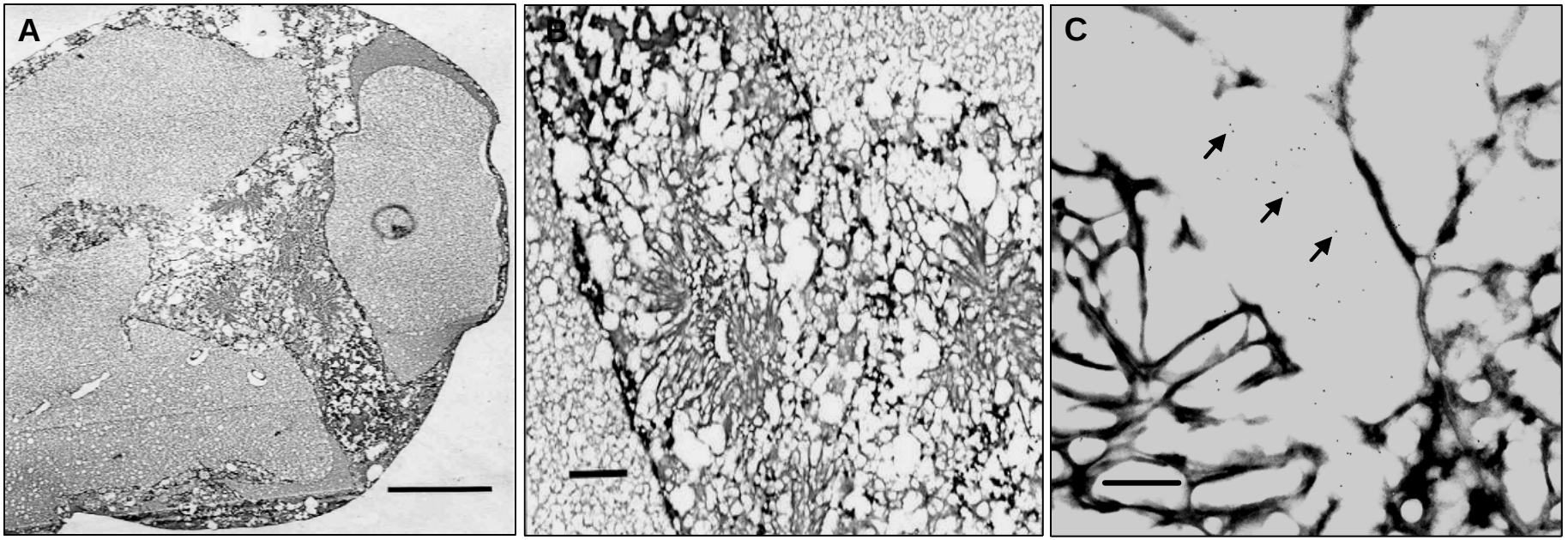

Figure 32: Immunolocalization study of acessory salivary gland sections embedded in LR White. WmCSV antibody was used at 1:25 (v/v). The signal observed with scattered gold granules in C, was too weak to be considered a specific binding (arrows). Scale bar in A represents 5μm; in B and C represents 1μm.

3.1.18 Immunolocalization studies with insect organ sections embedded in Lowicryl K4M

3.1.18.1 Immunolocalization of WmCSV in primary salivary glands embedded in Lowicryl

Lowicryl resins are highly cross linked acrylate and methacrylate-based embedding media which have been designed for use over a wide range of embedding conditions. These resins have been formulated to provide low viscosity at low temperatures -35 to -80°C. K4M resins are polymerized by long wavelength (360nm). Since the initiation of the polymerization is largely independent of temperature, blocks can be polymerized at the same temperatures which are used for infiltration. The hydrophilic properties of K4M provide two distinct advantages. During dehydration and infiltration the specimens may be kept in partially hydrate state, since K4M can be polymerized with up to 5% residual water. Secondly, K4M is particularly useful for immunolabelling of sections using specific antisera. In the case of this study use of K4M resulted in a better ultrastructural preservation (Roth *et al*., 1981) than with LR White, improved preservation of antigenicity (Bendayan and shore 1982; Roth *et al*., 1982) and showed a significantly lower background labelling.

Embedding in Lowicryl was done using the Progressive Lowering of Temperature (PLT) Technique: This procedure involves stepwise reductions in temperature as the concentration of dehydration agent is increased. The temperature is selected at each step above the freezing point of the concentration used in the preceding step. For the freezing points of various dehydrating agents, most polar and nonpolar dehydrating agents may be used with both resins. Due to its hydrophobic nature, K4M resin is freely miscible with methanol and ethanol.

Lowicryl as an embedding medium gave better immunostaining results especially after antigen retrieval using Tris-buffer pH 10.0 for 24 h at 65°C (Fig. 35). Using Tris- buffer as an antigen retrieval solution was mainly used as described by (Saito *et al*., 2003) for retrieving monoclonal antibody specific epitopes. This treatment combined with immunolabelling using the monocloanl antibody TYLCV 0546/2 has however failed to achieve such goal and only a very faint signal was observed. In contrast, a highly specific immunostaining was obtained when antigen retrieval was

applied in immunolocalization experiments using cross absorbed WmCSV polyclonal antibody instead of the Mab.

Labelling obtained upon immunolocalization of retrieved sections was specifically observed in the undefined vesicular structures and in vesicle aggregates directly attached to this structure. In Fig 33 presenting an overview of the primary salivary glands, this study for the first time shows that these structures are probably microvilli and represented 3 times in this organ. Two identical structures are located at both sides of the gland in the middle of electron dense granules rich cells, while the third bigger in size structure is located in the centre of the gland.

Lowicryl embedding has resulted in an improved resolution of ultrastructural features with low background staining and higher labelling intensity of primary salivary gland sections (Fig 34, 35). Counting of gold particle revealed specific signal within these structures while negligible numbers of gold particles were occasionally observed in control sections. The signal recorded in antigen retrieved sections was found again at highest level in the undefined structure, rich in multilammellar vesicles with a mean value of 133.7 gold particles per examined field in viruliferous insect compared with 43.6 gold particles per $5\mu m^2$ in control sections.

In vesicle aggregates, the mean value of the scored signal was about 43.5 in viruliferous insect compared with 17.3 gold particles per $5\mu m^2$ in control sections (Table 13).

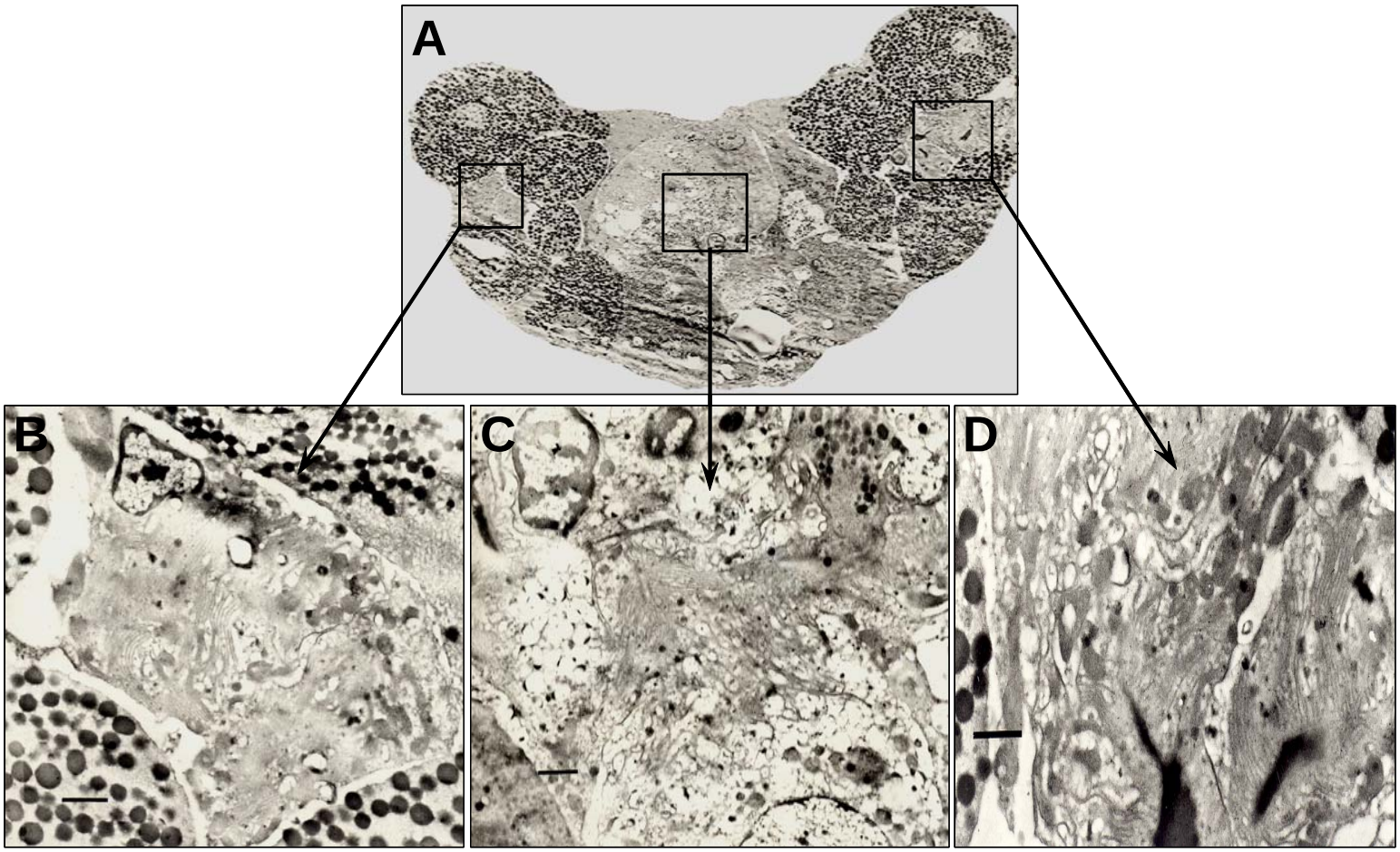

Figure 33: Primary salivary gland section embedded in Lowicryl K4M. A, undefined structure is represented 3 times in the organ; B and C are enlargements of respective sections in A. Scale bar in B,C and D represents 1µm.

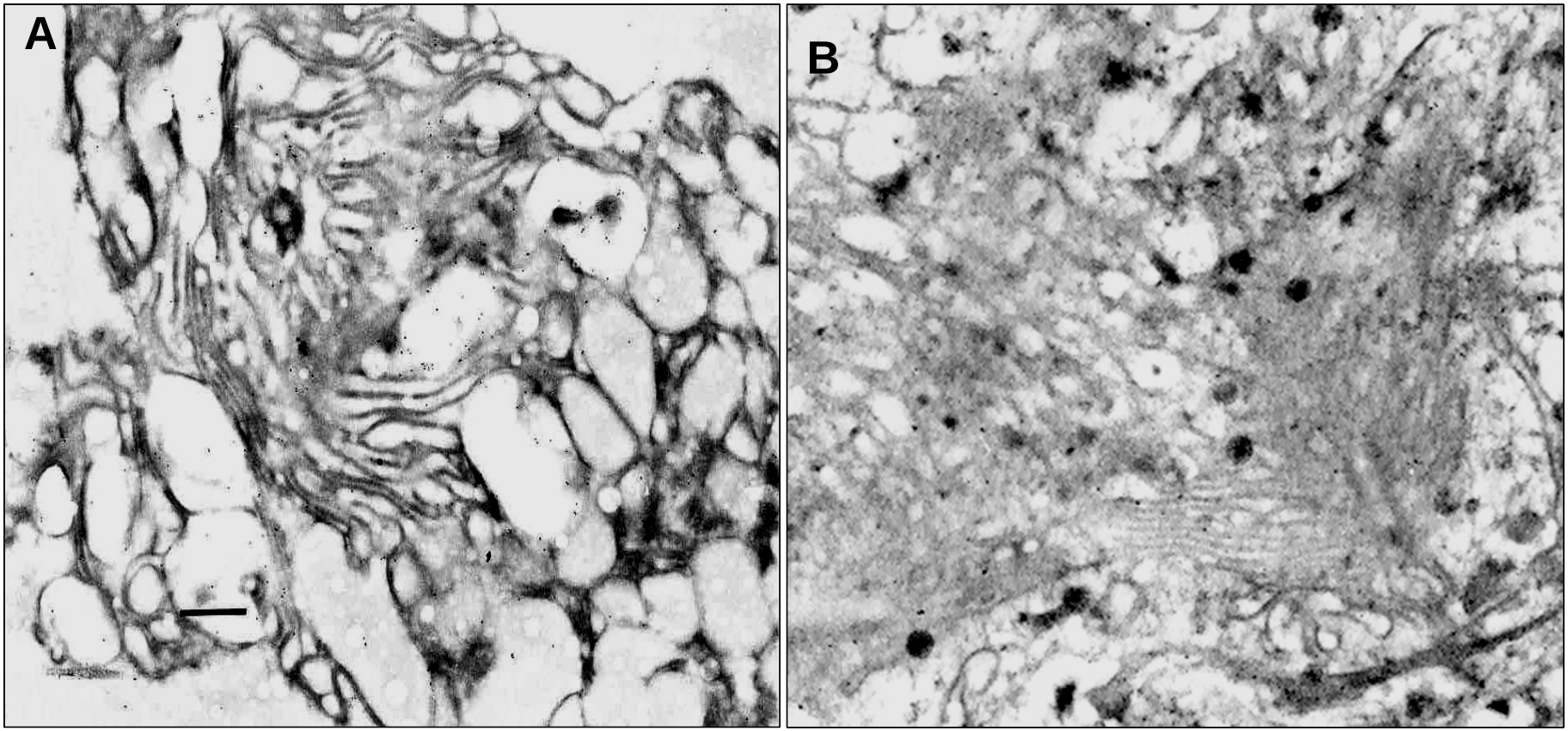

Figure 34: Effects of antigen retrieval treatment for immunolocalization of WmCSV in Lowicryl embedded primary salivary gland sections. Sections were fixed in 0.125 GA + 1%PFA, WmCSV adsorbed IgG diluted 1:25 (v/v) was used as primary antibody. A, represents immunolocalisation with antigen retrieved sections of PSG from viruliferous insects. B, shows immunolocalization experiment with PSG from viruliferous insects not treated for antigen retrieval. Scale bar represents 1µm.

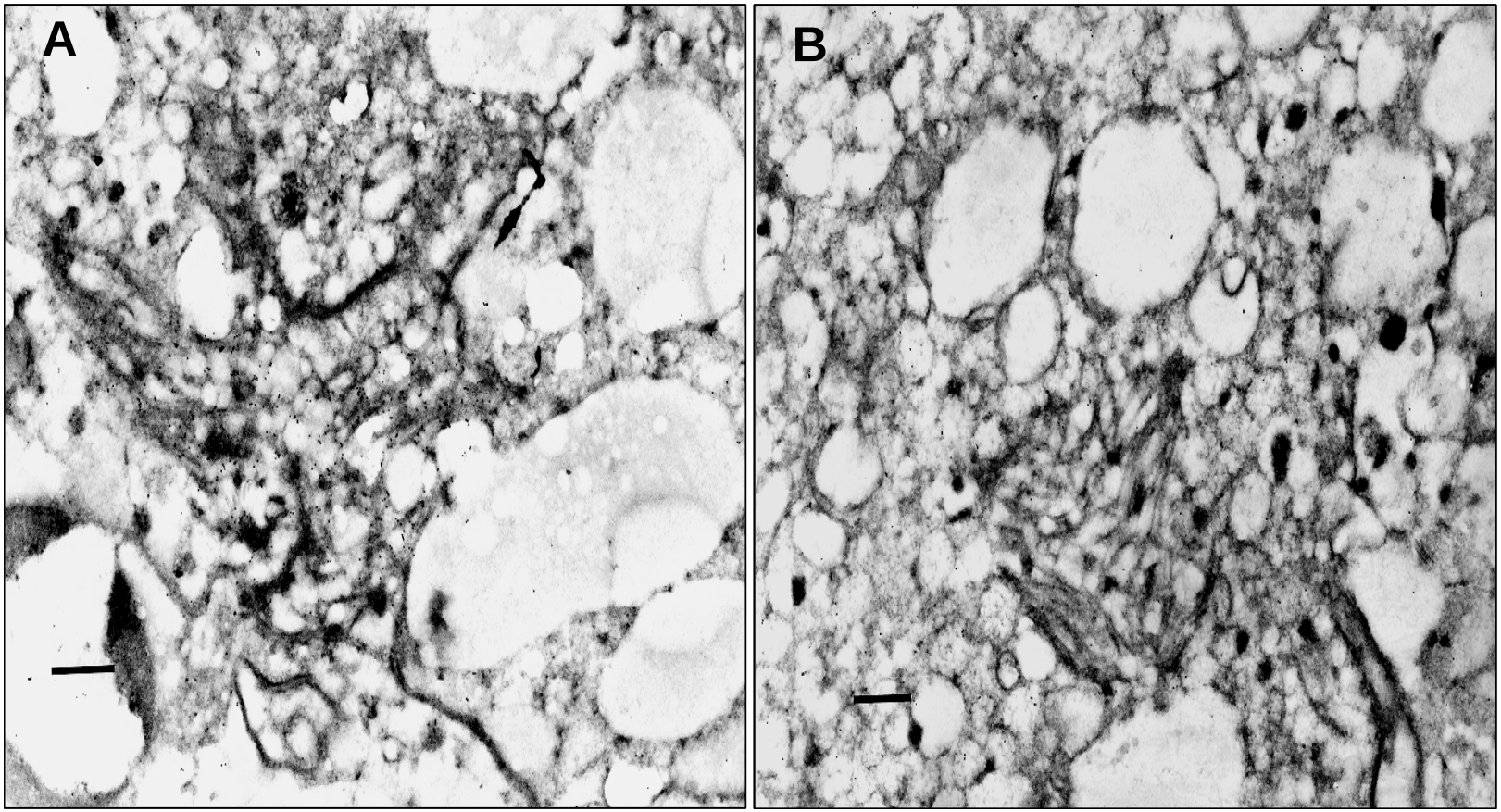

Figure 35: Immunolocalization of WmCSV in Lowicryl embedded primary salivary gland sections. Organs were fixed in 0.125 GA+1%PFA, incubated with WmCSV adsorbed IgG diluted 1:25 (v/v) and GAR conjugated gold. Sections were subjected to antigen retrieval. A), PSG from viruliferous insects. B, PSG from non viruliferous insects. Scale bar =1µm.

Table 13: Distribution of gold particles on subcellular compartments of Lowicryl-embedded primary salivary glands. Immunolabelling was applied on antigen retrieved sections. Gold particles were counted on a 5 μm^2 area (at a magnification of 12930x). Figures represent mean values from 5 sections of viruliferous and non viruliferous whiteflies. RE Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings. R, rough endoplasmatic reticulum

	Subcellular location	**No antigen retrieval Mean/ SE**	**Antigen retrieval Mean/ SE**
Viruliferous insects	Nucleus	11.4 ±1.97	26.7 ±7.7
	Undefined structures rich of multilammellar vesicles)	24.9 ±2.3	133.7 ±38.9
	RER	8.8 ±2.2	10.4 ±2.9
	Electron dense granules	7.1 ±1.2	5.27 ±1.3
	Vesicle aggregates	53.3 ±8.5	41.5 ±2.5
Non viruliferous insects	Nucleus	9.4 ±1.4	8.6 ±4.5
	Undefined structures (rich of multilammelar vesicles)	15.9 ±6.1	43.6 ±22.6
	RER	9.6 ±2.1	6.5 ±5.3
	Electron dense granules	7.6 ±1.6	5.8 ±2.3
	Vesicle aggregates	23.4 ±2.0	17.3 ±3.3

Immunolocalization of WmCSV in Lowicryl embedded accessory salivary gland organs

Immunolocalization experiments to localise WmCSVwt in Lowicryl embedded ASG tissue resulted in a very weak signal in the microvilli of viruliferous insects (fig 36). When compared with non-viruliferous insect up to 50% more gold particles could be observed, however the estimation of the standard error also revealed ambiguity of the results obtained. Examination of sections embedded in LR White and also the Epon embedded ASG resulted in no significant differences in all ASG immunolocalization experiments.

Table 14: Distribution of gold particles on subcellular compartments of Lowicryl embedded accessory salivary glands. Immunolabelling was applied on antigen retrieved sections; gold particles were counted on a 5 μm^2 area (at a magnification of 12930 x). Figures show mean values from 5 sections of viruliferous and non viruliferous whiteflies. Counts of gold particles were calculated as mean values (Mean) with standard error (± SE) determined as an indicator of the significance of the differences obtained in the recordings.

	Subcellular location	**Distribution of gold particles Mean value/SE**	
Viruliferous insects	Nucleus	9.6	±1.9
	Microvilli	49	±21.2
	Intracellular vesicle aggregates	8.3	±1.2
Non viruliferous insecst	Nucleus	6.3	±3.6
	Microvilli	24.7	±5.5
	Intracellular vesicle aggregates	5.6	±0.5

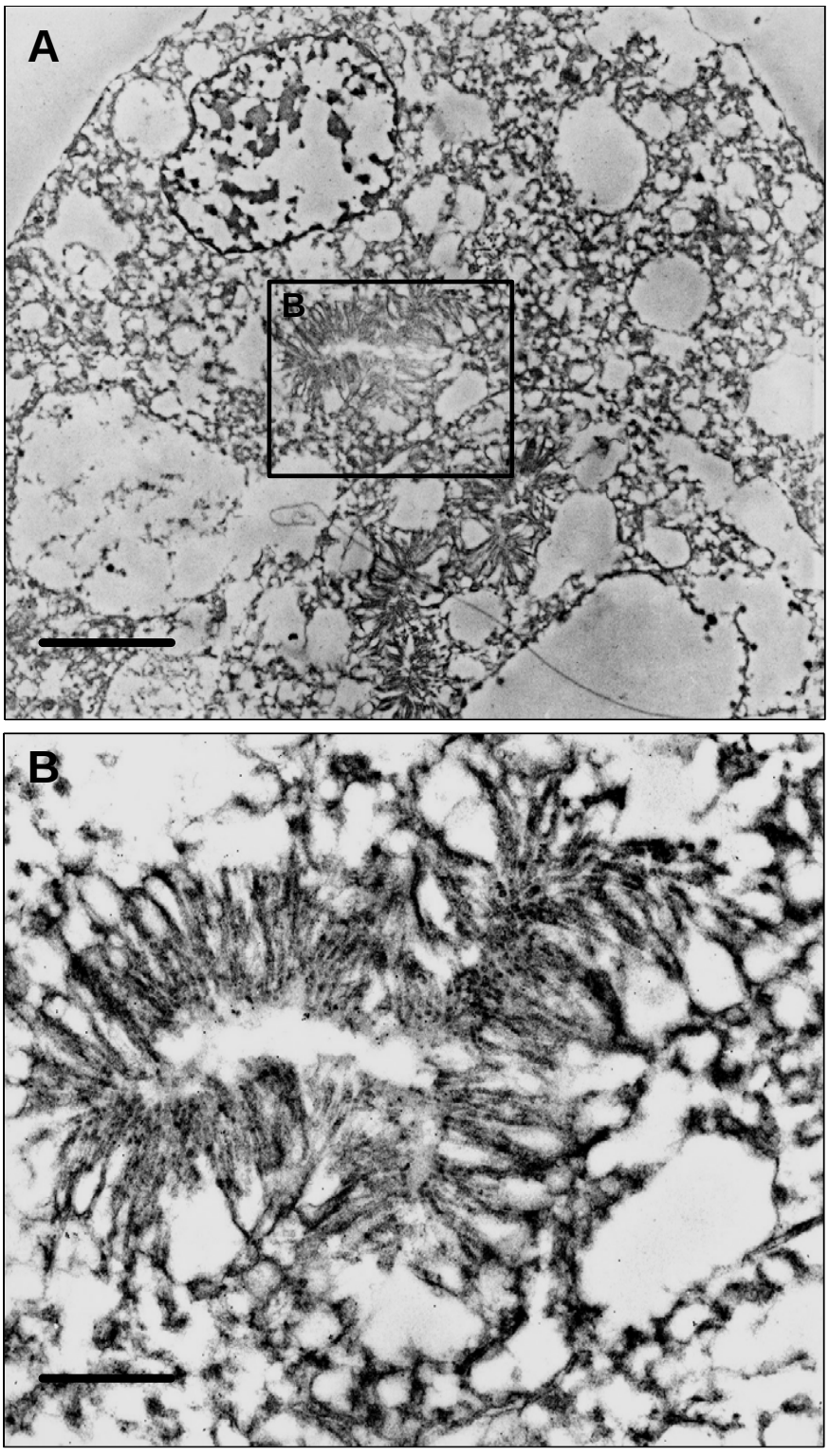

Figure 36: Immunolocalization of WmCSV in Lowicryl embedded acessory salivary gland sections (ASG) treated by antigen retrieval. WmCSV IgG 1:25 (v/v) was used as primary antibody. A section with microvilli, boxed, is enlarged in B. Scale bar represents 1μm.

3.1.19 In-situ hybridization

In situ hybridization is a multiple procedure, including:

(1) preparation of the biological material,

(2) nucleic acid probe selection and labelling,

(3) hybridization with labelled probe,

(4) cytochemical probe detection,

(5) microscopy and imaging analysis.

Thís first trial for an *in situ* hybridization experiment to reveal the ultrastructure of tissue sections obtained from LR White embedded organs, it was found that generally all fine structures are lost hence resolving fine details in dissected organ sections of insects might not be possible. There was a major background staining obtained upon hybridization and especially this was considered the major problem in virus localization by *in situ* hybridization with non radioactive nucleic acid probes. When *in-situ* hybridization was attempted on etched Epon embedded sections only very weak signals were found but with results for salivary gland tissues obtained, essentially verifying the immunolocalization studies

Weak, however, specific signals were obtained for localisation of WmCSV by *in situ* hybridisation in multilamellar vesicles of primary salivary glands excised from viruliferous *B. tabaci* insects (fig 37).

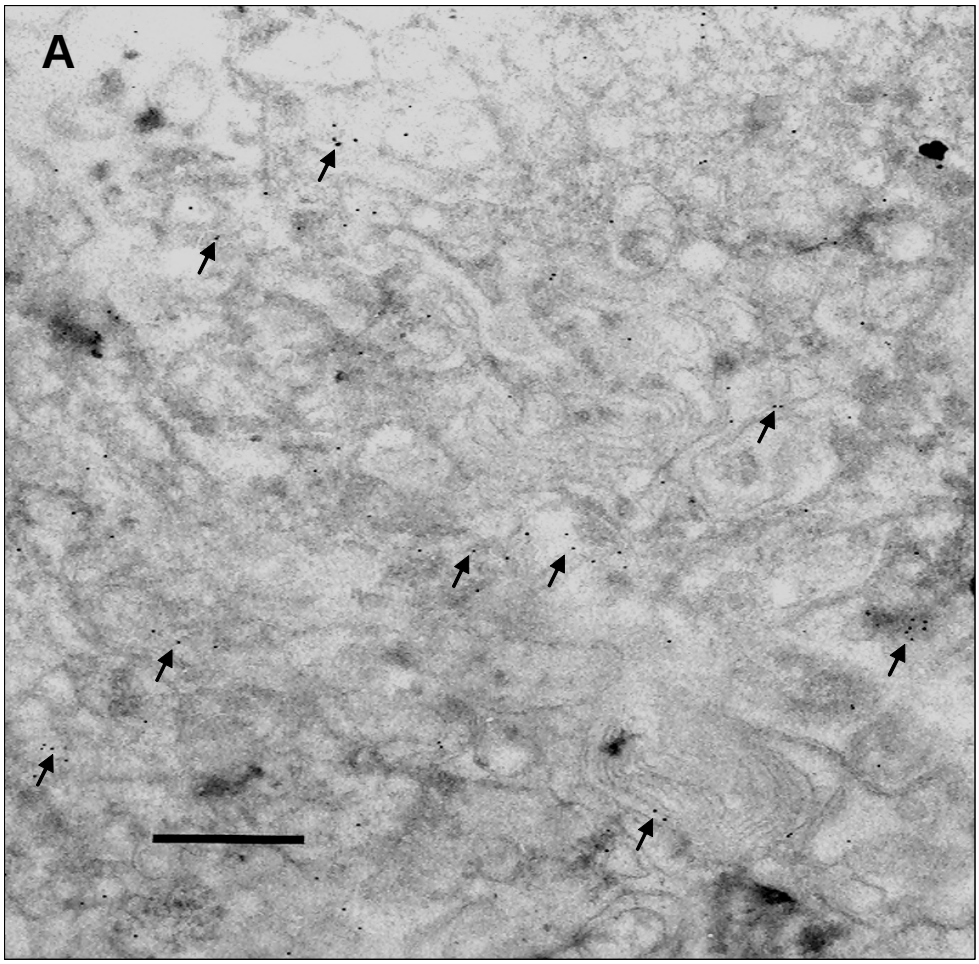

Figure 37: *In situ* hybridization analysis of Epon embedded primary salivary glands sections. Arrows pointing to gold agglomerates. Scale bar represents 1µm.

In Summary,

The results of the immunolocalisation study to detect WmCSV in excised organs of *B. tabaci* insects and to elucidate the translocation pathway in the vector indicate:

- The ASG apparently does not play a role in the circulative pathway of begomoviruses
- The primary salivary gland, PSG, presents the organ most decisive for begomovirus transmission by their *B. tabaci* whitefly vectors.

4 Discussion

4.1 Translocation of WmCSV in *Bemisia tabaci* vector and *Trialeurodes vaporariorum* non-vector species

The application of PCR for amplification and detection of geminiviruses is proven as a sensitive assay to trace geminiviruses in plants and in insects (Deng *et al.*, 1994; Ghanim *et al*., 1998; Navot *et al*., 1992; Rojas *et al*., 1993; Wyatt *et al*., 1996). The PCR primers designed in this study resulted in highly specific amplification of WmCSV DNA A sequences that, coupled with DNA hybridization of PCR products using a digoxigenin labelled DNA probe allowed for an extremely high resolution of begomovirus detection required for tracing virus DNA in single whiteflies and in minute volumes of whitefly isolated organs from virulent insects. However, virus detection in insects indicates for virus ingestion and does not provide a prove for transmission, as demonstrated by Rosell *et al*. (1999). The *Bemisi tabaci* transmitted begomovirus WmCSV can be detected in the non-vector whitefly *T. vaporariorum*, although transmission assays consistently remain negative. These results are consistent with the results of this study, where *T. vaporariorum* insects served as controls for detection and localization studies. Similarly the begomoviruses *Squash leaf curl virus* (SLCV) reported by Polsten *et al.*, (1990) and *Tomato yellow leaf curl virus* (TYLCV) as experimented by Antignus *et al*. (1993) are only transmitted by *B. tabaci* insects.

In this study acquisition access time for virus transmission was not under investigation. However, it has been demonstrated (Ghanim *et al*., 2001), that the latent period of a TYLCV virus from Israel was much shorter than reported by Cohen and Nitzany in 1966 more than 35 years ago. As it has been reported by Cohen and Nitzany (1966), and Cohen *et al*., (1983) that increasing AAP results in a higher PCR detection and consequently also increased the transmission rate. It has been observed by Rosell *et al*. (1999) that the transmission frequency of SLCV decreased between the 12 and the 24-h AAP and increased again after the 72-h AAP. These observations led to the definition of rather precise AAP ranging between 48-72 h AAP.

The purpose of this study was to obtain information about the passage of begomoviruses through the vector and to identify organs participating or rather governing vector transmission. To date, information about the translocation pathway of begomoviruses is scarce. Despite this, much is known about viral factors involved in transmission with coat proteins of begomoviruses as the structures defined. The specificity of geminivirus transmission results from an intimate relationship between surface structures of the CP and putative insect receptors. There is no evidence for any role of other virus-encoded proteins in insect transmission, as for transmission factors of caulimoviruses and potyviruses (Pirone and Blanc, 1996) in aphids or the read-through domain of luteoviruses (Brault *et al*., 1995). Still, receptors in insects remain speculative as the translocation events, as active virus/vector interaction in the vector, are rather dubious.

Using a non- transmissible isolate of *Abutilon mosais virus*, AbMV-Is, with whitefly transmissibility most probably lost as a result of constant vegetative propagation, Morin *et al*., (2000), could not trace the virus neither in haemolymph nor in the salivary glands of the vector species, suggesting that the virus is blocked at the level of the gut epithelia.

The involvement of the epithelial cells of the gut wall in the regulation of virus acquisition by the insect has been reported for different viruses and insects. Ultrasturctural analyses have shown that the midgut barrier is the cause of the inability of adult western flower thrips *Frankliniella occidentalis* to acquire *Tomato spotted wilt virus* (Ulmann *et al*., 1992). In aphids, recognition of luteoviruses however, occurs at the level of hindgut membrane (Gildow 1987, 1993).

The observation by Rosell *et al*. (1999) that SLCV DNA was detected in saliva and salivary products and in honeydew produced by *B. tabaci* insects feeding for 48h on virus infected plants and with viral DNA only found in honeydew excreted by *T. vaporariorum* as a control suggested that whitefly transmitted begomoviruses are unable to cross the gut membrane barrier of non vector insects. This was also observed in this detailed study providing proof that indeed the midgut presents a major impediment to begomovirus translocation in *T. vaporariorum* insects. This is however is not the case for *Bemisia tabaci*, the vector insects for begomoviruses.

4.2 Translocation of transmissible and non transmissible WmCSV begomoviruses in the whitefly vectors

In experiments with wild type WmCSV and several coat protein mutants generated in a transmission sensitive region of the CP, it was found that all mutant viruses were infectious to watermelon but not transmissible by *B. tabaci* insects. Since CP is the structural feature required for transmission but not for virus infectivity and movement i*n planta* it was first required to provide proof for virus assembly into virions, which was done by immunocapture PCR. This method was used because begomoviruses and geminiviruses in general are elusive in electron microscopy and hence require special treatment (ISEM) or enrichment to semi- purified preparations. Using IC-PCR omitted cumbersome virus purification and proved that the WmCSV mutants are assembled into virions. It did however not prove the stability of these particles in the insect.

All WmCSV mutants assembled in virions were not transmitted by *B. tabaci* but were capable of crossing the gut wall of the vector. All non transmissible virus mutants were found within the salivary glands, except for WmCSVser which; although found in haemolymph, most probably failed to attach to the salivary gland.

The result gained from experiments using wild type transmissible WmCSV supports the circulative transmission of geminiviruses with virus detection in all organs thus with features similar to other well studied circulative plant virus interactions, especially found with luteoviruses.

The results gained from our experiments suggest that the midgut epithelia in the vector species may act as a recognition site for virus transmission, and that two levels of barriers exist; the first is the midgut epithelial barrier suggested to play a recognition/barrier for vector and non-vector species. The second is the salivary gland barrier which plays the most significant role for virus transmission. This can also be deduced from the model for circulative persistent non-propagative transmission generated from experiments with *Potato leafroll virus* PLRV (Day, M.F., 1955) and *Beet western yellows virus*, BWYV (Duffus, 1960) verifying that luteoviruses entering the aphid vector haemocoel as part of the transmission process. However, Rochow and Pang (1961) discovered that some aphid vectors can acquire

luteoviruses and translocate it to the haemocel but were unable to transmit virus while non-vector insects did not transmit purified luteoviruses even when injected into the haemocel. Paliwal and Sinha (1970) demonstrated an association of luteoviruses with aphid salivary glands and hypothesized that BYDV vector specificity was not due to recognition and passage through the aphid gut but rather determined primarily by interactions of the virus proteins with membranes of the aphid salivary gland.

Hence, the results of this study, to trace the virus translocation pathway with dissected insect organs carrying WmCSV mutants, revealed a general rule, that transmission specificity is associated with inoculation of the virus (i.e., movement of the virus through the salivary system) rather than acquisition (uptake of the virus by the gut epithelial cells and delivery into the haemocoel.

4.3 Begomoviruses interfering with vector transmission

Begomviral DNA accumulates with increasing AAP on infected plants up to a peak at approximately 12 h for TYLCV, 24 h for TYLCSV (Zeidan and Czosnek, 1991; Caciagli and Bosco, 1997), and 48 h for SLCV (Polston *et al*., 1990). At its peak approximately 600 million (about 1ng viral DNA) viral genomes can accumulate in these insects. From a single feeding event this finite amount can be acquired reaching a steady state between ingestion and discharge after 12-48 h AAP. Feeding studies with insects first fed on TYLCV and subsequently on TYLCSV for 48 h each, while TYLCV concentration in the insect after first uptake remained, TYLCSV still accumulated in the insect during the secondary feeding. This showed that virus uptake is independed and additional virus does not chase virus already present in the insect. However experiments done by Cohen *et al*., (1989) showed that whiteflies that were fed for 48 h on SLCV-infected squash and then transferred to *Melon leaf curl virus* (MLCuV) infected watermelon for 24 h exhibited a 35-90% reduction in transmission of MLCuV. This is a clear result of interference of transmission.

In this study conducted with a first acquisition feeding with the non transmissible WmCSVasp followd by feeding with the transmissible WmCSVwt virus transmission dropped considerably to 40 % which points to blocking the virus

transmission and the putative receptor-mediated endo/exocytosis. This might be by saturation of insect receptors so that further virions fail to attach to these sites.

4.4 Immunolocalization studies to trace virus in insect organs and define sensitive translocation points

Cytological studies with begomoviruses and their vector insects are presenting a problem in itself. This is because of the nature of the whitefly insects which can not be studied as a whole sectioned organisms as with aphids due to the high complexity of structures, the enormous amounts of symbiontic organisms also present in the insects, the almost completely missing cytological data and the limited number of experts competent in this field. In addition, geminiviruses are very difficult to visualise by any method and even at high concentrations, morphological studies with these viruses are difficult. Despite the fact that good antisera for virus detection by ELISA are available reaching highly specific detection in these formats, the presentation of geminivirus epitopes for immunolocalisation studies is entirely different and presents a further technical aspect to consider.

Consequently this study concentrated merely to find and optimize methods that can be used for virus immunolocalization experiments and dealing with low binding-specificities, problems of virus localization despite low signal intensities of immunogold complexes etc.. There are several reasons for such difficulties among which, unspecific reactions of antiserum used and low intensity of a specific signal, are the most prominent. For example, Epon embeddings of insect tissues were characterized by a very good contrast of the membranes but represented a very high level of denaturation so that antigenicity was almost lost compared to LR White embedded materials where antigenicity was better preserved but with loss of contrast to resolve fine structure of the organ sections.

There is no universal fixative for all types of antigens, so the choice of fixative depends on type of epitope and tissue under study, hence complex optimization procedures are required to compare and then find the best suited process. As all needed to be done with dissected organs excised from insects and then sectioned, this process proved to be most complicated and cumbersome. The best level of antigencity preservation was achieved by specimen fixation using 4%

paraformaldehyde in phosphate buffer pH 7.4. This may be due to the ability of formaldehyde to cross-links amino groups of proteins, in a reversible manner.

While glutaraldhyde reacts with many nucleophiles in the cell (most commonly amines), this dialdehyde introduces extensive protein cross-link networks throughout the cytoplasm in a very short time which are mostly irreversible and significantly reducing antigencity (Hayat, 2000a). This was reflected by the very low immunolocalization obtained upon using 2.5% GA. In that case reviving and liberating cross-linked and compacted proteins via antigen retrieval treatments of denatured proteins upon primary fixation with GA and secondary fixation with osmium tetroxide significantly improved the overall signal.

The fixation conditions using only paraformaldehyde were characterized by a great loss of fine cellular ultrastructure in particular when the preservation of cellular membrane structures was concerned. Hence, in a study done by Hayat (2000a) superior structural preservation was reached with glutarladehyde and showing that fixation with formaldehyde is unacceptable for routine electron microscopical examinations demanding superior structural preservation. For most immunocytochemical studies a mixture of formaldehyde and glutaraldehyde is recommended and this was also found in the studies presented here.

Another common problem, background staining, is one of the most common problems in immunohistochemistry. A major cause for this is protein hydrophobicity which can occur between different protein molecules. Fixation with aldehydes render proteins more hydrophobic and as a result of cross-linking between reactive amino acids, the cross-links are both intramolecular and intermolecular (Hayat, 2000a). It should be noted that the greater the proximity of the pH of the antibody diluent and the isoelectric point (pI) of the antibody, the stronger the hydrophobic interaction. In all experiments reducing the background, blocking did not eliminate background neither by increasing BSA concentration nor by the addition of detergents.

To eliminate the different sources of background staining a number of treatments were tested and applied sequentially on fixed tissue sections. To reduce antibody cross reactions in the immunolocalization studies; the antibody was cross-absorbed with healthy insect tissues sap, which then was affinity purified. To avoid non-specific staining caused by Fc receptor glycoproteins present on the cell membrane

$F(ab)^2$ fragments were used instead of whole IgG molecule as reported by Boenisch (2001). To avoid antigen diffusion, isolated organs were immediately transferred to a fixation solution after insect dissection or the insect physiological saline was immediately replaced by fixation solution after dissecting the organ. It has been also observed that the overall recorded signal has been dropped to some extent during the combination of different background eliminating factors, which is to be expected in this case.

4.5 Immunolocalization of WmCSV in embedded insect organs

Three pre-embedding immunolocalization procedures described by Driss-Ecole *et al*. (2000), Boyes and Bolam (2003), and Kurth (2003) have been tested for the WmCSV localization studies, with special emphasis to elimination of factors that lead to antigen denaturation. These factors would be ascribed to the primary chemical fixation or to further tissue processing steps which might be the cause of weak antigen-antibody interaction. In the method adopted by Driss-Ecole *et al*., (2000), there was no signal obtained even after using the 0.8 nm gold particles instead of the recommended 10nm particles, to improve tissue permeability. In the second method described by Boyes and Bolam (2003), chemical fixation was not applied before primary and secondary antibody incubation however, neither this method nor light fixation with 4% paraformaldhyde (Kurth, 2003) and none of the methods in general proved suitable for our immunolocalization experiments which switched the focus on the post-embedding immunostaining techniques.

In these studies the most intensive signal was obtained in the filter chamber of the midgut and in the region surrounding the filter chamber. Immunolocalization studies have been suggested that *B. tabaci* filter chamber and the anterior portion of the midgut are possible sites for geminvirus transport from the gut lumen to the haemoceol (Hunter *et al*., 1998), while for aphids it has been reported that the hindgut is significant for virus uptake and transmission.

Aldehyde fixatives, such as formalin and glutaraldhyde, are cross-linkers that are preferred for tissue fixation because they are superior for preservation of tissue structure for both light and electron microscopy (Prento and Lyon 1997). They are

commonly used and guarantee standardization and reproducibility in morphological studies. Cross-linking joins two molecules by covalent bonds and antigen retrieval may loosen or break cross linkages caused by aldehyde fixation. In addition, AR may also unmask antigen by extracting diffusible proteins. It may precipitate and stabilize proteins and rehydrate sections to improve the diffusion of the antibody into the tissue section (Shi, 1997). Specifically, low contrast often resulted after prolonged incubation of thin sections with immunoreagents or after even short treatments with reagents that contained detergents which were often essential to minimize background staining. This problem can be largely overcome by including osmium tetroxide as a post-fixative to provide additional membrane contrast. Cell membrane, bio-membranous structures and vesicles in the cell are composed of lipid bilayer, which could be successfully fixed and preserved through a heavy metal precipitation like osmium tetroxide, however with the detrimental effect of removing virus antigenicity.

These probable mechanisms demonstrate that the effect of AR depends not only on the mode of fixation, but also on the antigen under investigation. There is no evidence thus far for a 'one and only' AR method, and it has been recommended that a test of different AR procedures should be applied to develop an optimal AR method for a given antigen or antibody (Boon and Kok 1994; Shi, *et al*., 1997). In this study such a number of etching and AR procedures to improve the post embedding immunohistochemical localization of virus were tried. However, while an optimum antigen retrieval on Epon embedded sections was found with well preserved ultrastructures, the signal intensity upon immunolocalization of viruliferous insect organs was reduced to levels similar to the ones obtained with non-viruliferous insects. Antigen retrieval of Epoxy resin may have released the protein partly from the epoxy network at the section surface leading to unsatisfactory observations and switching to other reisns for better resolution and improved immunolabelling.

The immunogold technique in conjunction with LR White embedding was used to localize WmCSV in *B. tabaci* tissues. Using LR White as a an embedding resin has several advantages which can be exploited for localization of antigens in sections of fixed and embedded tissue under the electron microscope because of its low viscosity and high infiltration rate.

It has been concluded from our study that the morphological preservation of Lowicryl embedded tissues was superior to that of LR White-embedded tissues, but significant differences in antigenicity were not observed. However, after employing antigen retrieval treatments a remarkable improvement in gold signal was found. Glutaraldehyde, with its greater power to cross-link proteins, undoubtedly stabilizes tissue structures to a greater degree than formaldehyd, but in turn, tissue thus fixed demonstrates reduced antigenicity, especially in the case of tissue fixing with high glutaraldehyde concentrations.

The results obtained show that LR White is advantageous over Epon for the study of nuclear antigens requiring delicate aldehyde fixation. The virus was localized on the microvillar surfaces of the apical plasma membrane in midgut tissues which points to a putative storage site for further delivering to the haemoceol. The signal also was observed in tubular like structure and occasionally in lucent vesicles in intercellular spaces between adjacent cells in PSG gland tissues which may imply a collective site for virus transfer to the salivary duct. But in the case of LR White embedding the borders of the vesicles were not clearly presented as it has been observed in Epon or in Lowicryl embedded tissues.

Several parameters affect the antigen preservation other than the fixative itself. While the fixation of specimens for standard histology is generally carried out at room temperature for convenience, for electron microscopy and some histochemical procedures, the temperature for fixation is usually 0-4 °C. At this lower temperature autolysis is slowed down as is the diffusion of various cellular components, allowing a more life-like appearance of the tissues. Therefore in order to minimize molecular thermal vibration, which can have adverse effects on specimens weakly fixed with paraformaldehyde, the samples can be dehydrated partially or totally at low temperature. Carlemalm *et al*., 1982 introduced the PLT technique (progressive lowering temperature) that combines increasing organic solvent concentration with decreasing temperature, after which infiltration and polymerization are carried out. The results obtained with Lowicryl clearly reflect the advantages of this approach resulting in good structural preservation of cellular contents and ultrastructure. Furthermore, the PLT method employs low temperature reducing protein denaturation and maintaining a degree of hydration, which may be important in preserving protein structural conformation.

It was therefore necessary to use Lowicryl as an acrylic resin which allows post-embedding immunostaining. In this experiment all temperature dependent problems at all steps were avoided. However in preliminary experiments with Lowicryl, there was no significant improvement in the intensity of the labelling signal observed. It was therefore concluded that the temperature itself was not the direct cause of loss of antigenicty loss and not the key factor affecting antigen binding. The fixing method used played affected antigenicity and epitope display by altering antigen binding sites due to linking. Immunolocalization experiments provided significantly higher level of signal enhancement upon antigen retrieval treatments especially when Tris-buffer pH 10 for 24 h at 65 °C as described by (Saito *et al*., 2003) was used.

Finally it can be stated that immunolocalization experiments with Lowicryl embedded tissues resulted in good resolution and antibody antigen binding. However it also needs to be made clear that immunolocalization studies to localize geminiviruses in whitefly vectors are confronted with technological limitiations where the use of antibodies in cytological studies is not indicated, due to complicated insect structures, unique epitope display of begomoviruses and extremely low target concentrations at locations not easily amenable to antibody antigen reactions.

Thus pilot experiments to study WmCSV localization by *in situ* hybridization (ISH) were followed. This has proven to be an important tool in molecular cell biology, genetics, and pathology for the detection and localization of specific nucleic acid sequences (DNA and RNA) within morphologically preserved chromosomes, cells and tissues.

The principle behind *in-situ* hybridization is the specific annealing of a labelled nucleic acid probe to its complementary sequences in fixed tissue or cells, followed by visualization of the hybridization signal with radioactive or fluorescent signals, or enzyme histochemistry. A critical aspect of the procedure is that the target nucleic acid be preserved *in situ* and be accessible for hybridization to the probe. Unlike the hybridization of nucleic acids in solution to target sequences on membrane filters, the target in this case is cross-linked and embedded in a complex matrix that hinders access of the probe and decreases stability of the hybrids.

The sensitivity of colloidal gold detection is usually lower than for fluorescence or enzyme cytochemical detection of hybridized probes *in situ*, which may be a problem for localizing low copy or unique nucleic acid sequences. Nevertheless, these ultrastructural nucleic acid detection procedures have contributed to a large extent to the current insight concerning the subcellular localization of mRNA transcripts, the functional sub-compartmentalization of the cell nucleus, and the analysis of intracellular viral life cycles (Bassell *et al*. 1994; Morey 1995; Dirks 1996; Puvion-Dutilleul and Puvion 1996).

A specific signal has been obtained in localization studies via *in-situ* hybridization of viruliferous insect of PSG tissues using a WmCSV DNA A specific probe. It has been observed that using Epon embedded organs provided a good preservation of the ultrasturcture as well as provding reliable signals that was found in the multilamellar vesicles. In a similar study by Brown and Czosnek, (2002) *in-situ* hybridization analysis was used for TYLCV detection in insects and this virus was also found in cells of the primary salivary glands of *B. tabaci*.

4.6 Virus vector interaction

4.6.1 Virus acquisition: Virus-Gut interactions

To initiate the circulative transmission process, geminiviruses must be transported from the gut lumen into the haemocoel of potential whitefly vectors. Thus, the whitefly gut is the first tissue barrier to transmission and the first site where begomoviruses recognition must occur. The different whitefly species studied, *B. tabaci* and *T. vaporariorum* have an almost identical gut morphology and cellular anatomy (Ghanim *et al*., 2001).

The alimentary canal begins where the stylet food canal empties into the praecibarium and cibarium. The cibarium and salivary syringe are found beneath the subesophageal ganglion. The cibarium empties into the pharynx, which traverses the circumesophageal passage, the tentorial bar and ultimately joins the external esophagus (that portion of the esophagus external to the filter chamber). The external esophagus is extremely slender and traverses the length of the thorax across the

dorsal side of the subesophageal and thoracic-abdominal ganglionic masses. It is formed by thin epithelial cells with a well-defined basal membrane and a heavy cuticular intima lining surrounding the ligqen. The external oesophagus has substantial musculature and the cuticular intima lining is generally folded accordion-like when these muscles are relaxed. The oesophagus enters the filter chamber anterior to the connecting chamber, at which point it is denoted the internal esophagus. The filter chamber are formed by a sheath of thin epithelial cells with a thin basal membrane and what appears to be a cuticular intima lining. The internal surface of these cells faces the continuous lumen of the filter chamber. A layer of epithelial cells on the opposing side of the continuous lumen appears to originate from the ascending midgut. These cells have extensive microvilli.

From this study it can be confirmed that the cellular site for geminivirus ingression through its whitefly vector is the filter chamber and the anterior part of the midgut, a result consistent with what earlier reports by Hunter *et al*. (1998) in immunolocalization studies by immunoflouresence of *Tomato mottle virus* (ToMoV) and *Cabbage leaf curl virus* (CaLCV).

In the experimental evidence presented here, a WmCVS signal was found localized in the microvilli lining the lumen of the anterior part of the midgut and the filter chamber. This was not reported in a similar study by Czosnek *et al*. (2002) where no signal was detected in the lumen of the insect. It has been shown that there are no particles freely in the lumen of the insect or in contact with the gut apical plasmalemma even upon feeding of insects with purified virus preparations at high concentration. The quantitative analysis in this study rather points to a specific interaction between virus and the anterior part of the gut.

Ingestion of plant viruses infecting the phloem cells is not specific, and many viruses not transmitted by a respective insect species may be ingested into the gut and exit the insect with e.g. honeydew excrements as has been reported for aphids by Gildow, (1993) or for *T. vaporariorum* by Rosell *et al*., (1999).

4.6.2 Virus entry via midgut cells

Viruses that enter the host via ingestion must pass through the peritrophic membrane in order to penetrate midgut cells. This process was not fully understood before

Derksen and Granados (1988) described an enhancing factor in a granulosis virus that disrupts the peritrophic membrane following ingestion by its lepidopteran host. Selective, or receptor-mediated, endocytosis is a ubiquitous mechanism for internalizing functionally important macromolecules in animal cells (Goldstein *et al*., 1985). The selectivity of receptor-mediated endocytosis is dictated by the presence of specific receptors on the plasma membrane of the cell, thus enabling the internalization of only particular macromolecules. An evidence of receptor mediated endocytosis/exocytosis as a mechanism for virus translocation is under investigation and has to be further investigated using different other embedding and localization methods in order to visualize this virus-receptor membranes interaction at the molecular level.

4.6.3 Virus-haemoceol interactions

The virus is released into the haemocoel where it presumably survives in the haemolymph, outside of any cells. Little is known about the specifics of geminivirus movement and survival in the haeolymph, but it may involve vector associated factors (Van den Heuvel, *et al*., 1999). Virus survives in the hamolymph for several weeks and appears to be passively transported to the salivary glands where it can continue its circulative transmission pathway. It has been observed through the PCR experiments that all non-transmissible virus clones were detected in the hamolymph and in the salivary glands; which means that passage through the haemolymph is without any harmful effects on the virus. In fact, the virus remains infectious for several weeks in the whitefly, presumably, much of this time in the potentially hostile haemolymph environment. Probably this protecting effect would be due to binding of the virus with the endosymbiotic bacteria housed in the haemolymph of the insect, that produce a GroEL protein homologue stabilising the virus in their insect vector (Morin *et al*., 1999).

4.7 Virus transmission: Virus-salivary gland interactions

Detection of the WmCSVwt in the salivary gland by immunolocalization provides evidence that the primary salivary glands are the likely most significant recognition sites for a successful begomovirus transmission. Through the different methods used in immunolocalization experiments conducted this type of complicated interaction at this organ not yet fully understood has at least pinpointed the site of entry to the organ and paved the way towards more detailed investigations focussed on the primary salivary gland barriers. The signal observed in the primary salivary glands of the viruliferous insects was mainly concentrated in tubular like structures in three different locations of the primary salivary glands two of them located at both sides of the gland and the third one in the centre of the gland which may point to what would be called a collection tubular structure with microvilli acting as a sieve or a storage site for the virus to sequester it to its final destination, the salivary duct. The virus is then transported via an end/exocytotic mechanism which is the only mechanism likely for receptor mediated transmission. However for more detailed studies, the type of resolution required and the small size and shape of the virus might present limitations which require novel methodical approaches.

4.8 Conclusion

The failure to detect WmCSV in haemolymph and in the salivary glands of the non-vector species and the detection of all not whitefly transmitted WmCSV mutant clones in the haemolymph and in the salivary glands provides evidence that geminivirus recognition with vector species occurs at the midgut and particularly at the filter chamber membrane, suggesting that virus recognition regulating the membrane attachment is a precursor of virus acquisition. This might explain why whiteflies transmit certain viruses in a circulative non-propagative manner.

All virus clones passaged the midgut of *B. tabaci* suggesting that there are two distinct CP recognition events, at the midgut of *B. tabaci* and at the primary salivary gland. Current evidence indicates that the primary salivary gland is acting as the major site determining the high level of vector specificity observed for WmCSV transmission. The immunolocalization experiments with dissected organs of the vector species *B. tabaci*, have further revealed that the accessory salivary glands doesn't play any role in virus transmission. However, despite all observations made, there is no evidence yet to the biochemical interactions involved in specific recognition of virus and any component involved is as yet only subject to speculations.

The results of this study presented here are merely to further our understanding of the complex virus insect relationships leading to virus transmission, to point to critical insect organs involved and to raise more questions directing future research efforts to elucidate the mechanisms of begomovirus transmission in the *Bemisia tabaci* insect vector.

References

Aaziz, R., Dinant, S., Epel, B. L. 2001. Plasmodesmata and plant cytoskeleton. *Trends in plant Sciences* **6**, 326-330.

Abdullahi, I. 2001. Diversity of whitefly (*Bemisia tabaci*) vector of African Cassava Mosaic Geminivirus. Ph.D. thesis, University of Ibadan, Ibadan, Nigeria.

Anon, 2001a. Crop protection Compendium, Global Module, 3rd edn. *CAB International CD-Rom Database.*

Antignus, Y., Perlsman, M., Ben-Yoseph, R., Cohen, S. 1993. The interaction of *Tomato yellow leaf curl virus* with its whitefly vector, *Bemisia tabaci. Phytoparasitica* **21**, 174-175.

Atzmon, G., **van Hoss, H. And Czosnek, H. 1998.** PCR- amplification of *Tomato yellow leaf curl virus* (TYLCV) from squashes of plants and insect vectors: application of the study of TYLCV acquisition and transmission. *European Journal of Plant Pathology* **104**, 189-194.

Azzam, O., Frazer, J., de la Rosa, D., Beaver, J.S., Ahlquist, P., and Maxwell, D.P. 1994. Whitefly transmission and efficient ssDNA accumulation of Bean golden mosaic geminivirus require functional coat protein. *Virology* **204,** 289–296.

Bananej, K., Ahoonmanesh, A., Kheyr-Pour, A., 2002. Host range of an Iranian Isolate of *Watermelon chlorotic stunt virus* as determined by whitefly-mediated inoculation and agroinfection, and its geographical distribution. *Journal of phytopathology* **150**, 423-430.

Bananej, K., Kheyr-Pour, A. and Ahoonmanesh, A. 1998. Identification of Watermelon chlorotic stunt Geminivirus in Iran. 194. In Proc 13th Iranian Plant Protection Congress.

Bandla, M.D., Campbell, L.R., Ulman, D.E., and Sherwood, J.L. 1998. Interaction between *Tomato spotted wilt virus* Tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for (TSWV). *Phytopathology* **88**, 98-104.

Bassell, G.J., Powers, C.M., Taneja, K.L., Singer, R.H. 1994. Single mRNAs visualized by ultrastructural in situ hybridization are principally localized at actin filament intersections in fibroblasts. *Journal of Cell Biology* **126**, 863–876.

Baumann, P., Moran, N.A., and Baumann, L. 2000. Bacteriocyte-associated endosymbionts of insects. *In* M. Dworkin (ed.), The prokaryotes. [Online.] Springer, New York, N.Y. http://link.springer.de/link/service/books/10125.

Bedford, I.D., Briddon, R.W., Brown, J.K., Rosell, R.C., and Markham, P.G. 1994b. Geminivirus transmission and biological characterisation of *Bemisia tabaci* (Gennadius) biotypes from different geographic regions. *Ann. Appl. Biol.* **125**, 311-325.

Bedford, I.D., Briddon, R.W., Jones, P., Alkaff, N., Markharm, P.G. 1994a. Differentiation of three whitefly-transmitted geminiviurses from the Republic of Yemen. *Eur.J. Plant Pathol.* **100,** 243-257.

Bendayan, M., and Shore, G.G. 1982. Immunocytochemical localization of mitochondrial proteins in rat hepatocyte. *J. Histochem. Cytochem*. **30**, 139-147.

Boenisch, T. 2001. Formalin-fixed and heat-retrieved tissue antigens: A comparison of their immunoreactivity in experimental antibody diluents. *Appl. Immunohistochem. Mol.Morphol.* **9**, 176-179.

Boon, M.E. and Kok, L.P. 1994. Microwaves for immunohistochemistry. *Micron* **25**, 151-170.

Boyes, J. and Bolam J.P. 2003. The subcellular localization of $GABA_B$ receptor subunits in the rat substantia nigra. *European Journal of neuroscience* **18**, 3279-3293.

Brault, V., **van den Heuvel, J.F.J.M., Verbeek, M., Ziegler-Graff, V., Reutenauer, A, A., Herrbach, E., Garaud, J.C. Guilley, H., Richards, K., and Jonard, G. 1995.** Aphid transmission of beet western yellows luteovirus requires the minor capsid read through protein *P74EMBOJ*. **14**, 650-659.

Briddon, R.W., Pinner, M. S., Stanley, J. and Markham, P. G. 1990. Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177**, 85-94.

Briddon, R.W. and Markham, P.G. 2000. *Cotton leaf curl virus* disease. *Virus Research* **71,** 151-159.

Brorson, S.-H., Andersen, T., Haug, S., Kristiansen, I., Risstubben, A., Tchou, H., and Ulstein, J. 1999. Antigen retrieval on epoxy sections based on tissue infiltration with a moderately increased amount of accelerator to detect immune complex deposits in glomerular tissue. *Hitstol. Histopathol*. **14**,151-155.

Brown, J.K. 2001. Molecular markers for the identification and global tracking of whitefly vector-begomovirus complexes. *Virus Research* **71**, 223-260.

Brown, J.K. and Bird, J. 1992. Whitefly-transmitted geminiviruses and associated disorders in the Americas and the carribbean Basin. *Plant Disease* **76**, 220-225.

Brown, J.K. and Czosnek, H. 2002. Whitefly transmission of plant viruses. *Advances in Botanical Research* **36,** 65-100.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms, p.332–338. John Wiley and Sons Interscience, New York, N.Y.

Byrne D. N., Bellows T. S. Jr. 1991. Whitefly biology. *Annual Review of Entomology* **36**, 431-457.

Caciagli, P., Bosco, D., Al-Bitar L. 1995. Relationships of the sardinian isolate of *Tomato yellow leaf curl* geminivirus with its whitefly vector *Bemisia tabaci* Gen. *European Journal of Plant Pathology* **101**, 163-170.

Carlemalm, E., Garavito, R.M., Villiger, W. 1982. Resin development for electron microscopy and an analysis of embedding at low temperature. *J Microsc* **126,** 123-143.

Cattoretti, G., Becker, M.H.G., Key, G., Duchrow, M., Schlter, C., Galle, J., Gerdes J. 1992. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J. Pathol.* **168**, 357–363.

Cohen, S., and Nitzany, F.E. 1966. Transmission and host range of the *Tomato yellow leaf curl virus*. *Phytopathology* **56**, 1127-1131.

Cohen, S., and Y. Antignus. 1994. *Tomato yellow leaf curl virus*, a whitefly-borne geminivirus of tomatoes. *Adv. Dis. Vector Res*. **10**, 259-288.

Cohen, S., Duffus, J.E., and Liu, H.Y. 1989. Acquisition, interference, and retention of *Cucurbit leaf curl viruses* in whiteflies. *Phytopathology* **79**, 109-113.

Cohen, S., Duffus, J.E., Larsen, R.C., Liu, H. Y., and Flock, R.A. 1983. Purificaion, serology, and vector relationships of *Sqush leaf curl virus*, a whitefly-transmitted geminivirus. *Phytopathology* **73**, 1669-1673.

Costa, H. S.,Toscano, N., C., and Henneberry, T., J. 1996. Mycetocyte inclusion in the oocytes of *Bemisia argentifolii* (Homoptra: Aleyrodidae). *J. Econ. Entomol.* **89,** 694–699.

Costa, H.S., Henneberry, T.J., and Toscano, N.C. 1997. Effects of antibacterial materials on *Bemisia argentifolii* (Homoptera: Aleyrodidae) oviposition, growth, survival, and sex ratio. *Ann. Entomol. Soc*. Am. **90,** 333–339.

Costa, H.S., Westcot, D.M., Ullman, D.E., and Johnson, M.W. 1993. Ultrastructure of the endosymbionts of the whitefly, *Bemisia tabaci* and *Trialeurodes vaporarorum. Protoplasma* **176,**106–115.

Costa, H.S., Westcot, D.M., Ullman, D.E., Rosell, R., Brown, J.K., and Johnson, M. W. 1995. Morphological variation in *Bemisia* endosymbionts. *Protoplasma* **189,**194–202.

Czosnek, H., Ghanim, M., Ghanim, M. 2002. The circulative pathway of begomoviruses in the whitefly vector *Bemisia tabaci* - insights from studies with *Tomato yellow leaf curl virus*. *Ann. Appl. Biol*. **140**, 215-231.

Czosnek, H. and Laterrot, H. 1997. A worldwide survey of *Tomato yellow leaf curl viruses*. *Arch Virol* **142**, 1391–1406.

Czosnek, H., Ghanim, M. and Ghanim, M. 2002. The circulative pathway of begomoviruses in the whitefly vector *Bemisia tabaci* insights from studies with *Tomato yellow leaf curl virus*. *Annals of Applied Biology* **140,** 215-231.

Dafalla, G.A., Gronenborn, B., Kheyr-Pour, A., Lecoq, F.L. 1998. *Watermelon chlorotic stunt virus*: A new emerging epidemic in export melons in Sudan. In: C.M. Faquet, D. H. L. Bishop, M. H. van Regenmortel, B. Gronenborn (eds), Second International Workshop Geminiviurses, San Juan, Puerto Rico. P. 37. *The American Phytopathology Society (APS), San Jose, Puerto Rico.*

Day, M.F. 1955. The mechanisms of the transmission of potato leafroll virus by aphids. *Aust. J. Biol. Sci.* **8**, 498–513.

Derksen, A.C.G., and Grandos, R. R.1988. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology, 167, 242-50.

Duffus, J.E. 1960. Radish yellows, a disease of radish, sugar beet and other crops. *Phytopathology* **50,** 389–94

Dellaporta, S.L., Wood, J., and Hicks, H.B., 1983. A plant DNA mini prepartion: Version II. *Plant Mol. Bio. Rep.* **14**, 19-21.

Deng, D., McGarth, P.F., Robinson, D.J., and Harrison, B.D. 1994. Detection and differentiation of whitefly-transmitted Geminiviruses in plants and vector insects by polymerase chain reaction with degenerate primers. *Ann.Appl.Biol*. **125**, 327-336.

Derksen, A. C. G. and Granados, R. R. 1988. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology* **167,** 242-250.

Derrick, K.S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* **56**,652-653.

Dijkstra, J., and de Jager, C. P. 1998. Practical Plant virology, Protocols and Excercises. Springer Lab Manual, Berlin, Germany.

Dirks, R.W. 1996. RNA molecules lighting up under the microscope. *Histochem Cell Biol* **106,**151–166.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera. Annu. Rev. Entomol.* **43,**17–37.

Driss-Ecole,D., Vassy, J., Rembur, J., Guivarc`h, A. Prouteau, M., Dewitte, W., Perbal, G. 2000. Immunolocalization of actin in root statocytes of *lens culinaris* L. *Journal of Experimental Botany* **51**, 521-528.

Duffus, J.E. 1960. *Phytopathology* **50,** 389.

Fauquet, C.M., Bisaro, D.M., Briddon, R.W., Brown, J.K., Harrison, B.D., Rybicki, E.P., Stenger, D.C., Stanley, J. 2003. Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, and an updated list of begomovirus species. *Arch. Virol*. **148,** 405–421.

Fröhlich, D.R., Torres-Jerez, I.I., Bedford, I.D., Markham, P.G., and Brown, JK.1999. A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. *Mol Ecol.***10**, 1683-9.

Gafni, Y., Epel, B. L., 2002. The role of host and viral proteins in inter-cellular trafficking of geminiviruses. *Physiological and Molecular Plant Pathology* **60**, 231-241.

Garret, A., Kerlan, C., Thomas, D. 1996. Ultrastructural study of acquisition and retention of potato leafroll luteovirus in the alimentary canal of its aphid vector, *Myzus persicae* Sulz. *Arch. Virol.* **141,** 1279–92.

Ghanim, M., Morin, S., Zeidan, M. and Czosnek, H. 1998. Evidence for transovarial transmission of *Tomato yellow leaf curl virus* by its vector the whitefly *Bemisia tabaci*. *Virology* **240**, 295-303.

Ghanim, M., Morin, S. and Czosnek, H. 2001. Rate of *Tomato yellow leaf curl virus* translocation in the circulative transmission pathway of its vector, the whitefly *Bemisia tabaci*. *Phytopathology* **91,** 188–196.

Ghanim, M., Rosell R. C., Campbell L.R., Czosnek H., and Brown J.k., 2001. Digestive, Salivary, and Reproductive organs of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) B Type. *J Morphol* **248**, 22-40.

Gibbs, M. 1999. Chaperonin camouflage. *Nature* **399**, 415.

Gildow, F.E. 1987. Virus membrane interactions involved in cirulative transmission of luteouviruses by aphids. *Curr.Topics Vector Res*. **4**, 93-120.

Gildow, F.E. 1993. Evidence for receptor-mediated endocytosis regulating luteouvirus acquisition by aphids. *Phytopathology* **83**, 270-277.

Gildow, F.E., Damsteegt, V.D., Stone, A.L., Smith, O.P., Gray, S.M. 2000. Virus-vector cell interactions regulating transmission specificity of soybean dwarf luteoviruses. *Phytopathol. Z.* **148**, 333–42.

Gildow, F.E. and Gray, S.M. 1993. The aphid salivary gland basal lamina as a selective barrier associated with vector-specific transmission of barley yellow dwarf luteoviruses. *Phytopathology* **83**, 1293-1302.

Gill, R.J. 1990. The morphology of whiteflies. In Gerling D (ed) Whiteflies, their Bionomics, Pest Status and Management (pp13-46) Intercept, Andover.

Green, S. K. and Kalloo, G. 1994. Leaf curl and yellowing viruses of pepper and tomato: an overview. *Technical Bulletin* No. **21**, Asian Vegetable Research and Development Centre, Taiwan, p. 51.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russel D.W., and Schneider, W.J. 1985. Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1**, 1-39.

Gray, S.M., and Banerjee, N. 1999. Mechanisms of arthropod transmission of plant and animal viruses. *Micobiol.Mol.Biol.Rev.***63,** 128-148.

Gray, S.M., and Gildow, F.E. 2003. Luteovirus-aphid interactions. *Annu. Rev. Phytopathol.* **41**, 539-66.

Gullan, P.J., and Martin, J.H. 2002. Sternorrhyncha (jumping plant-lice, whiteflies, aphids and scale insects), p. 1079–1089. *In* V. H. Resh and R. T. Carde´ (ed.), Encyclopedia of insects. Academic Press/Elsevier Science, New York, N.Y.

Gutierrez, C. 2002. Strategies for geminivirus DNA replication and cell cycle interference. *Physiological and Molecular Plant Pathology* **60**, 219-230.

Harris, K. F., Pesic-Van Esbroeck, Z. and Duffus, J. E. 1995. Anatomy of a virus vector. In ‘‘*Bemisia* 1995: Taxonomy, Biology, Damage, Control and Management’’ (D. Gerling and R. Mayer, Eds.), pp. 289–318.Intercept, Buckinghamshire, UK.

Harris, K.F., PesicVan Esbroeck, Z., and Duffus, J.E. 1996. Morphology of the sweet potato whitefly, *Bemisia tabaci* (Homoptera, Aleyrodidae) relative to virus transmission. *Zoomorphology* **116,** 143-156.

Harrison, B.D. 1985. Advances in geminivirus research. *Annual Review of Phytopathology* **23**, 55-82.

Harrison, B.D., Muniyappa, V., Swanson, M.M., Roberts, I.M. and Robinson, D.J. 1991. Recognition and differentiation of seven whitefly-transmitted geminiviruses from India, and their relationships to *African cassava mosaic* and *Thailand mung bean yellow mosaic viruses*. *Annals of Applied Biology* **118.** 299-308.

Harrison, B.D. and Robinson, D.J. 1999. Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (Begomoviruses). *Annual Review of Phytopathology* **37.** 369-398.

Harrison, B.D., Swanson, M.M., Fargette, D. 2002. Begomovirus coat protein: serology, variation and functions. *Physiological and Molecular Plant Pathology* **60**, 257-271.

Hayat, M. A. 2000a. *Principles and Techniques of Electron Microscopy*: Biological Applications, 4th ed. Cambridge University Press, Cambridge and New York.

Hayat, M. A. 2002. *Microscopy, Immunohistochemistry, and antigen retrieval methods*: for light and electron microscopy. Kluwer academic/Plenum Publishers New York.

Hoddle, M.S. The Biology and Management of Silverleaf Whitefly,*Bemisia argentifolii* Bellows and Perring (Homoptera: Aleyrodidae) on Greenhouse Grown Ornamentals Department of Entomology, University of California, Riverside, CA 92521 USA. WWW.biocontrol.ucr.edu

Höfer, P., Bedford I.D., Markham, P.G., Jeske, H., and Frischmuth, T. 1997. Coat protein gene replacement results in whitefly transmission of an insect non-transmissible geminivirus isolate. *Virology* **236**, 288-295.

Höhnle, M., Hofer, P., Bedford, I.D., Briddon, R.W., Markham, P.G. and Frischmuth, T. 2001. Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible *Abutilon mosaic virus* isolate. *Virology* **290.** 164-171.

Hong, Y. G. and Harrison, B. D. 1995. Nucleotide sequences from tomato leaf curl viruses from different countries : evidence for three geographically separate branches in evolution of the coat protein of whitefly-transmitted geminiviruses. *Journal of General Virology* **76**. 2043-2049.

Hong, Y., Leunissen, J.L.M., Shi, G.-M., Gutekunst, C.-A. and Hersch, S.M. 2001. A Novel Procedure for Pre-embedding Double Immunogold–Silver Labeling at the Ultrastructural Level . *Journal of Histochemistry and Cytochemistry* **49**, 279-284.

Hunter, **W.B., Hieberst**, **E., Webb, S.E., Tsai**, J**.H., Polston**, **J.E. 1998.** Location of Geminiviruses in the Whitefly *Bemisia tabaci* (Homoptera:Alerodidae). *Plant Dis.* **82**, 1147-1151.

Ishikawa, H. 1984. Characterization of protein species synthesised *in vivo* and *in vitro* by an aphid endosymbiont . *Insect Bionchem*. **14**, 417-425.

Jirikowski,G.F., Ramalho-Ortigao, J.F., Kesse, K.W., Bloom, F.E. 1990. *In-situ* hybridization of semi-thin Epon sections with BrdU labelled oligonucleotide probes. *Histochemistry* **94**, 187-90.

Jones, D.R., 2003. Plant viruses transmitted by whiteflies. *Eur.J. Plant Pathol.* **109**, 195-219.

Jones, P., Satar, M.H., Alkaff, N. 1987. Viruses of tropical crops, p.76 Rothamsted experimental station, Harpenden, Herts, UK.

Jones, P., Satar, N.W., Alkaff, N. 1988. The incidence of virus disease in watermelon and sweetmelon crops in the Peoples Democractic Republic of Yemen and its impact of cropping policy. *Aspects Appl. Biol*. **17,** 203-207.

Karnovsky, M.J. 1965. A formaldhyd-glutaraldhyd fixative of high osmolarity for use in electron microscopy. *J.Cell Biol*. **27**, 137 A.

Kheyr-Pour, A., Bananej, K., Dafalla, G.A., Caciagli, P., Noris, E., Ahoonmanesh, A., Lecoq, H., and Gronenborn, B. 2000. *Watermelon chlorotic stunt virus* from the Sudan and Iran: Sequence comparisons and identification of a whitefly-transmission determinant. *Phytopathology* **90**, 629-635.

Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G.P., Crespi, S. and Gronenborn, B. 1991. *Tomato yellow leaf curl virus* from Sardinia is a whitefly-transmitted monopartite geminivirus. *Nucleic Acids Res.* **19,** 6763-6769.

Kurth, T. 2003. Immunocytochemsitry of the amphibian embryo from overview to ultrastructure. *Int.J.Dev.Biol*.**47**, 373-383.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lazarowitz, S.G., Pinder, A.J., Damsteegt, V.D., Rogers, S.G. 1989. *Maize streak virus* genes essential for systemic spread and symptom development. *The EMBO Journal* **8**, 1023-1032.

Lecoq, H., Dafalla, G.A., Mohamed, Y.F., Pitrat, M., Omara, S.E., and Ali, H.A. 1994. A survey on cucurbit viruses in Kordofan, east and central regions of Sudan Khartoum. *Univ. J. of Agric. Sci*. **1**, 47-71.

Lindsay, K.L., and Mashal, T.A. 1981. The osmoregulatory role of the filter chamber in relation to phloem feeding in *Eurymela distincta* (Cicadelloidea, Homoptera). *Physiol. Entomol.* **6**, 413-414.

Liu, S., Bedford, I.D., Briddon, R.W., Markham P.G. 1997. Efficient whitefly transmission of African cassava mosaic geminivirus requires sequences from both genomic components. *Journal of General Virology* **78**, 1791-1794.

Luisoni *et al.*, E., Miline, R.G. and Vechiati, M.1995. Purification of *Tomato yellow leaf curl* Geminivirus. *Microbiologica* **18**, (3) 253-260.

Mansoor, S., Bedford, I.D., Pinner, M.S., Stanley, J., Markham, P.G. 1993. A whitefly transmitted geminivirus associated with cotton leaf curl disease in Pakistan. *Virology* **259**, 190-199.

Markham, P.G., Bedford, I.D., Liu, S., and Pinner, M.S. 1994. The transmission of geminiviruses by *Bemisia tabaci. Pestic. Sci.* **42**, 123-128.

Maruthi, M.N., Colvin, J., Seal, S., Gibson, G. and Cooper, J. 2002. Co-adaption between cassava mosaic geminiviruses and their local vector poulations. *Virus Research* **86,** 71-85.

Matsuno, A., Ohsugi, Y., Utsunomiya, H., **Takekoshi, S**., **Munakata, S., Nagao, K., Osamura, R.Y., Tamura, A., Nagashima, T. 1998.** An improved ultrastructural double-staining method for rat growth hormone and its mRNA using LR White resin: a technical note. *Histochem J*. **30**, 105-109.

Matthews, R.E.F. 1991. *Plant Virology*. *Fourth edition*. New York, Academic Press.

Metha, P. Wyman, J.A., Nakhla, M.K., Maxwell, D.P. 1994. Transmission of tomato yellow leaf curl geminivirus by *Bemisia tabaci* (Homoptera:Aleyrodidae).*Journal of Economical Entomology* **87**, 1291-1297.

Milne, R.G. 1984. Electron microscopy for identification of plant viruses *in virto* preparations. *Methods Virol.* **7**, 87-120.

Milne, R.G., Lesemann, D-E. 1984. Imunosorbent electron microscopy in plant virus studies. *Methods in Virology* VIII, 85-101.

Milne, R.G., Luisoni, E. 1977. Rapid immune electron microscopy of virus preparations. *Methods Virol*. **6**, 265-281.

Momayezi, M., Kissmehl, R., and Platter, H. 2000. Quantitative immunogold localization of protein phosphatase 2B (Calcineurin) in *Paramecium* cells. *J Histochem Cytochem* **48**, 1269-1281.

Moran, N.A., and Baumann, P. 2000. Bacterial endosymbionts in animals. *Curr. Opin. Microbiol.* **3,** 270–275. Proc. R. Soc. Lond. B 253:167–171.

Moran, N.A., and Telang, A. 1998. Bacteriocyte-associated symbionts of insects: a variety of insect groups harbour ancient prokaryotic endosymbionts. *BioScience* **48,** 295–304.

Moran, N.A., Plague, G.R., Sandström, J.P., and Wilcox, J.L. 2003. A genomic perspective on nutrient provisioning by bacterial symbionts of insects. *Proc. Natl. Acad. Sci.* USA **100**,14543–14548.

Morey, A.L. 1995. Non-isotopic *in-situ* hybridization at the ultrastructural level. *J Pathol* **176**, 113–121

Morin, S., Ghanim, M., Sobol, I., Czosnek, H. 2000. The GroEL protein of the whitefly *Bemisia tabaci* interacts with the coat protein of transmissible and non-transmissible begomoviruses in the yeast two-hybrid system.*Virology*. **276**, 404-16.

Morin, S., Ghanim, M., Zeidan, M., Czosnek, Verbeek, M., and van den Heuvel, J.F.J.M. 1999. A GroEl homolouge from endosymbiotic bacteria of the whitefly *Bemisia tabaci* is implicated in the circulative transmission of *Tomato yellow leaf curl virus*. *Virology* **256**, 75-84.

Mound, L. A. and Halsey, S.H. 1978. Whitefly of the world, a Systemic Catalouge of the Aleyrodidae (Homoptera) with Host Plant and Natural Enemy Data. *British Museum (Natural History), London and John Wiley and Sons, Chichester, UK.*

Muniyapaa, V., Venkatesh, H. M., Ramappa, H. K., Kulkarni, R. S., Zeidan, M., Tarba, C-Y., Ghanim, M., Cosnek, H. 2000. *Tomato leaf curl virus* from Bangalore (ToLCV-Ban4): sequence comparison with Indian ToLCV isolates, detection in plants and insects, and vector relationships. *Archives of Virology* **145**, 1583-1598.

Navot, N., Zeidan, M., Pichersky, E., Zamir, D., and Czosnek, H. 1992. Use of polymerase chain reaction to amplify *Tomato yellow leaf curl virus* DNA from infected plants and viruliferous whiteflies. *Phytopathology* **82**, 1199-1202.

Ng, J.C.K. and Perry, K.L. 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5,** 505-511.

Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B., and Accotto, G.P. 1998. Amino acids in the capsid protein of *Tomato yellow leaf curl virus* that are crucial for systemic infection, particle formation, and insect transmission. *J. Virol.* **72**, 10050-10057.

Padidam, M., Beachy, R.N., Fauquet, C.M. 1995. Classification and identification of geminiviruses using sequence comparisons. *J Gen Virol*. **76**, 249-63.

Paliwal, Y.C., Sinha, R.C. 1970. Mechanism of persistence and distribution of *Barley yellow dwarf virus* in an aphid vector. *Virology* **42,** 668–680.

Pirone, T.P., and Blanc, S. 1996. Helper-dependent vector transmission of plant viruses. *Annu.Rev.Phytopathol*. **34,** 227-247.

Polston, J.E., Al-Musa, A., Perring, T.M., Dodds, J.A. 1990. Association of the nucleic acid of Squash leaf curl geminivirus with the whitefly *Bemisia tabaci*. *Pytopathology* **80**, 850-856.

Prento, P. and Lyon, H. 1997. Commercial formalin substitutes for histopathology. *Biotech. Histochem.* **72,** 273–282.

Puvion-Dutilleul, F. and Puvion, E. 1996. Non-isotopic electron microscope in situ hybridization for studying the functional sub-compartmentalization of the nucleus. *Histochem Cell Biol* **106,** 59–78.

Ramandeep, K., Kanak L. Dikshit, and Manoj, R. 2002. Optimization of immunogold labeling TEM: An ELISA-based method for evaluation of blocking agents for quantitative detection of antigen. *J. Histochem. Cytochem.* **50**, 863–873.

Reinbold, C., Gildow, F.E., Herrbach, E., Ziegler-Graff, V., Goncalves, M.C. 2001. Studies on the role of the minor capsid protein in transport of *Beet western yellows virus* through *Myzus persicae. J. Gen. Virol.* **82**,1995–2007.

Reinbold, C., Herrbach, E. and Brault, V. 2003. Posterior midgut and hindgut are both sites of acquisition of Curcurbit aphid-borne yellows virus in *Myzus Persicae* and *Aphis gossypii*. *Journal of General Virology* **84,** 3473-3484.

Riedel, D. 1997. Immunoelectronmikrokopische Untersuchungen zur Lokalisierung des Akkumulations-und Funktionsortes von Genprodukten des *Plum pox virus* and anderer Potyviren. Dissertation , Fachbereich Biologie der Universität Hamburg, Germany.

Roberts, I.M., Robinson, D.J. and Harrison, B.D. 1984. Serological relationships and genome homologies among geminiviruses. *Journal of General Virology* **65,** 1723-1730.

Rochow, W. F., and Pang, E. W. A. 1961. Aphids can acquire strains of *Barley yellow dwarf virus* they do not transmit. *Virology* **15,** 382-384.

Röcken, C., and Rössner, A., 1999. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. *J. Histochem. Cytochem.* **47**, 1385-1394.

Rojas, M.R., Gilberston, R.L., Russell, D.R., and Maxwell, D.P. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Dis*.**77**, 340-347.

Rosell, R.C., Torres-Jerez, I., and Brown, J.K., 1999. Tracing the geminivirus whitefly transmission pathway by polymerase chain reaction in whitefly extracts, saliva, hemolymph and honeydew. *Phytopahtology* **89**, 239-246.

Rossel, R.C., Torres-Jerez, I., and Brown, J.K., 1997. Temporal monitoring of geminiviurs DNA in whitelfy hemolymph by polymerase chain reaction (PCR). (Abstr.) *Phytopathology* **87**, 583.

Roth, J. 1982b. The protein A-gold (PAG) technique. Qualitative and quantitative approach for antigen localization on thin sections. In: Techniques in Immunocytochemistry, Vol. I Academic Press, London pp104-137.

Roth, J., Berger, E.G. 1982a. Immunocytochemical localization of galatosyltransferease in HeLa cells: co-distribution with thiamine pyrophoshatease in trans-golgi cisternae. *J. Cell Biol*. **93**, 223-229.

Roth, J., Bendayan, M., Carlemalm, E., Villinger, W. and Garavito, R.M. 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedding pancreatic tissue. *J. Histochem. Cytochem*. **29**, 663-671.

Rochow, W.F., Pang, E. 1961. Aphids can acquire strains of *Barley yellow dwarf virus* they do not transmit. *Virology* **15,** 382–84.

Rochow, W.F. 1969. Biological properties of four isolates of *Barley yellow dwarf virus*. *Phytopathology* **59**,1580–89.

Rochow, W.F., Foxe, M.J., Muller, I. 1975. A mechanism of vector-specificity for circulative aphid-transmitted viruses. *Ann. NY Acad. Sci.* 266:293–301.

Rouze-Jouan, J., Terradot, L., Pasquer, F., Tanguy, S. and Ducray-Bourdin, D.G. 2001. The passage of *Potato leafroll virus* through *Myzus persicae* gut membrane regulates transmission efficiency. *Journal of General Virology* **82,** 17-23.

Rubinstein, G., and Czosnek, H. 1997. Long-term association of *Tomato yellow leaf curl virus* (TYLCV) with its whitefly vector *Bemisia tabaci*: Effect on the insect transmission capacity, longevity and fecundity. *J. Gen. Virol.* **78**, 2683-2689.

Ruizeveld de Winter, J.A., Trapman, J., Vermey, M., Mulder, E., Zegers N.D., van der Kwast, T.H. 1991. Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* **39**, 927–936.

Rybicki, E.P. 1994. A phylogenetic and evolutionary justification for three genera of Geminiviridae. *ArchVirol.***139**, 49-77.

Saito, N., Konishi, K., Takeda, H., Kato, M., Sugiyama, T., and Asaka, M. 2003. Antigen retrieval trail for post-embedding immunoelectron microscopy by heating with several unmaksing solutions. *J Histochem Cytochem* **51**, 989-994.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning – A laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, USA.

Saunders, K., Bedford, I.D. and Stanley, J. 2002. Adaptation from whitefly to leafhopper transmission of an autonomously replicating nanovirus-like DNA component associated with ageratum yellow vein disease. *Journal of General Virology* **83**, 907-913.

Shi, S. R., Cote, R.J., Taylor, C.R. 1997. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Chytochem* **45**, 327-343.

Shi, S.R., Key, M.E., Kalra, K.L. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem. Cytochem.* **39**, 741–748.

Stanley, J. 1995. Analysis of *African cassava mosaic virus* recombinants suggests strand nicking occurs with the conserved nonanucleotide motif during the initiation of rolling cycle DNA replication. *Virology* **206,** 707-712.

Stockem, W. 1970. Die Eignung von Pioloform-F für die Herstellung elektronenmikroskopischer Trägerfilme. *Mikroskopie* **26**, 185-189.

Suurmeijer, A.J.H. and Boon, M.E. 1993. Optimizing keratin and vimentin retrieval in formalin-fixed, paraffin-embedded tissue with the use of heat and metal salts. *Appl. Immunohistochem.* **1**, 143–148.

Swanson, M. M. and Harrison, B. D. 1994. Properties, relationships and distribution of cassava mosaic geminiviruses. *Tropical Science* **34**, 15-25.

Szklarzewicz, T., and Moskal, A. 2001. Ultrastructure, distribution, and transmission of endosymbionts in the whitefly *Aleurochiton aceris* Modeer (Insecta, Hemiptera, Aleyrodinea). *Protoplasma* **218,** 45–53.

Taylor, C.R., Shi, S.R., Cote, R.J. 1996. Antigen retrieval for immunohistochemistry. Status and need for greater standardization. *Appl. Immunohistochem*. **4**, 144–166.

Thao, M. L., Moran, N.A., Abbot, P., Brennan, E.B., Burckhardt, D.H., and Baumann P. 2000. Cospeciation of psyllids and their prokaryotic endosymbionts. *Appl. Environ. Microbiol.* **66,** 2898–2905.

Thomas, J.E., Massalski, P.R. and Harrison, B.D. 1986. Production of monoclonal antibodies to *African cassava mosaic virus* and differences in their reactivity with other transmitted geminiviruses. *J.Gen.Virol.* **67,** 2739-2749.

Thresh, J.M., Otim-Nape, G.W., Thankappan, M. and Muniyappa, V. 1998. The mosaic diseases of cassava in Africa and India caused whitefly-borne geminiviruses. *Review of Plant Pathology* **77,** 935-945.

Ullman, D.E., Cho, J.J., Mau, R.F.L., Westcost, D.M., and Custer, D.M. 1992. A midgut barrier to *Tomato spotted wilt virus* acquisition by adult western flower thrips. *Phytopathology* **82**, 1333-1342.

Van den Heuvel, J.F. J.M., Verbeek, M., and van der Wilk, F. 1994. Endosymbiotic bacteria associated with circulative transmission of *Potato leaf roll virus* by *Myzus persica*. *J. Gen. Virol*.**75**, 2559-2565.

Van den Heuvel, J., Hogenhout, S.A., van der Wilk, F. 1999. Recognition and receptors in virus transmission by arthropods. *Trends Microbiol.* 7, 71–76.

Varma, A. and Malathi, V.G. 2003. Emerging geminivirus problems: A serious threat to crop production. *Annals of Applied Biology* **142,** 145-164.

Von Arnim, A., Stanley, J. 1992. Determinants of *Tomato golden mosaic virus* symptom development located on DNA B. *Virology* 186:286-293.

Von Dohlen, C.D., Kohler, S., Alsop, S.T., and McManus, W.R. 2001. Mealybug proteobacterial endosymbionts contain proteobacterial symbionts. *Nature* (London) **412,** 433–436.

Wachtler, F., Schöfer, C., and Weipoltshammer, K. 1996. *In-situ* Hybridization. laboratory companion, M.Clark (Ed.) © Chapman &Hall, chapter 6.ISBN 3-8261-0038-7.

Wang, J.Y., Chay, C., Gildow, F.E., Gray, S.M. 1995. Readthrough protein associated with virions of barley yellow dwarf luteovirus and its potential

role in regulating the efficiency of aphid transmission.*Virology* **206**, 954–62.

Wu, Z.C., Hu, J.S., Polston, J.E., Ullman, D.E., and Hiebert, E. 1996. Complete nucleotide sequence of a nonvector-transmissible strain of Abutilon mosaic geminivirus in Hawaii. *Phytopathology* **86**, 608-613.

Wyatt, S.D., and Brown, J.K. 1996. Detection of subroupIII geminivirus isolated in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopahtology* **86**, 1288-1293.

Xiomara H. S., McKenzie, C. L., Wayne B. H., Charles, A. P. and Robert G. S., Jr. 2005. Differential transcriptional activity of plant pathogenic begomoviruses in their whitefly vector (*Bemisia tabaci*, Gennadius: Hemiptera Aleyrodidae) *Journal of General Virology*, **86**, 1525–1532.

Yi, H., Leunissen J. L.M., Shi, G.M., Gutekunst C.A., and Hersch, S.M. 2001. A novel procedure for pre-embedding double immunogold-silver labelling at the ultrastructural level. *J Histochem Cytochem* **49**, 279–283.

Zeidan, M., Czosnek, H. 1991. Acquisition of *Tomato yellow leaf curl virus* by the whitefly *Bemisia tabaci*. *J. Gen. Virol*. **72**, 2607–2614.

Zhang, P. and Guissem, W. 2003. Efficient replication of cloned *African cassava mosaic virus* in cassava leaf discs. *Virus Research* **92**, 47-54.

Publications

During the course of the experiments for this thesis, several aspects of the research work were presented at national and international conferences.

Fahmy, I., Winter, S. 2005. *In-situ* localization of the Geminivirus *Watermelon chlorotic stunt virus*, WmCSV, in its insect vector *Bemisia tabaci*. Microscopy conference, 6. Dreiländertagung 28.08-2.09.2005, Davos, Switzerland. Poster presentation.

Fahmy, I.F, Lesemann, D-E., and Winter, S. 2005. Translocation and immunolocalization of *Watermelon chlorotic stunt virus*, WmCSV, in its vector *Bemisia tabaci* (Genn.).Fourth Joint Meeting of Dutch and German Plant Virologists, 10-11.03.2005, WIIC, Wageningen. Presentation.

Fahmy, I., Abdullahi, I., Winter, S. 2004. Translocation of *Watermelon Chlorotic Stunt Begomovirus* in its Whitefly Vector *Bemisia tabaci*. Arbeitskreis Viruskrankheiten der Pflanze, 29-30.03.2004, Tagung in Braunschweig. Poster presentation.

Fahmy, I. F., Gadelseed, A., Abdullahi, I., Winter, S. 2004. Untersuchungen zur Translokation von Geminiviren im Vektor Weiße Fliege, *Bemisia tabaci*.p.78, ISSN 0067-5849. Deutsche Pflanzenschutztagung, in Hamburg, 20-23.09.2004. Presentation.

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