Molecular and Biochemical Characterization of Viral and Vector Components Required for Cucumber Necrosis Virus Transmission

By

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THE FACULTY OF GRADUATE STUDIES
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Abstract

Natural transmission of *Cucumber necrosis virus* (CNV) occurs via zoospores of the chytrid fungus *Olpidium bornovanus*. Transmission involves specific adsorption of virus particles onto the zoospore plasmalemma prior to infestation of cucumber roots by virus bound zoospores. In order to determine if specific regions of the CNV capsid are involved in transmission, several naturally occurring CNV transmission mutants were isolated and characterized. Analysis of the mutants showed that the CNV trimer cavity at the particle quasi three-fold axis plays an important role in transmission, and, moreover that the reduction in transmission is at least partially due to the reduced ability of mutants to bind to zoospores. *In vitro* virus/zoospore binding studies have shown that pre-treatment of zoospores with trypsin and sodium periodate each decrease CNV binding by approximately 80%, whereas no reduction in binding was found when zoospores were treated with phospholipase C. These studies suggest an important role for zoospore proteins and/or glycoprotein(s) in virus attachment. In virus overlay assays, CNV virions bound to specific-sized zoospore proteins, but CNV transmission mutants showed little or reduced binding. Several sugars were used to study their inhibitory potential on CNV binding to zoospores *in vitro*. It was found that a variety of mannose-containing sugars inhibited CNV binding to zoospores whereas several others did not. These studies suggest that the putative zoospore receptor may be a mannose-containing glycoprotein.

Many animal virus particles undergo conformational changes upon binding to their cellular receptors. CNV is an icosahedral virus and like many other isometric plant viruses, undergoes expansion in the presence of EDTA at an alkaline pH. In the case of
CNV, we have demonstrated that during expansion, the internally located coat protein RNA binding domain (R) and arm domains translocate to the particle exterior, becoming protease sensitive. Protease digestion experiments of zoospore-bound virus have revealed that CNV undergoes conformational change upon binding to zoospores and that the conformationally altered virion resembles the swollen conformation. In addition, we have found that a CNV mutant defective in vector transmission is unable to undergo this conformational change. This is the first time that conformational change in a plant virus particle has been shown to be essential for vector transmission.
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<tr>
<td>3'</td>
<td>three prime</td>
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<tr>
<td>a</td>
<td>arm</td>
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<td>A</td>
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<td>aphid transmission factor</td>
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<td>readthrough domain</td>
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<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>S</td>
<td>serine in the context of protein sequence; shell domain in the context of CNV CP</td>
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SBWMV  Soil borne wheat mosaic virus
SDS  sodium dodecyl sulphate
sec  seconds
Ser  serine
sg  subgenomic
SLRSV  Strawberry latent ringspot virus
SqNV  Squash necrosis virus
T  thymidine in the context of nucleotide sequence; threonine in the context of protein sequence; triangulation number in the context of icosahedral virus structure
TB  Tris borate
TAE  Tris acetate EDTA
TBE  Tris borate EDTA
TBRV  Tomato black ring virus
TBS  Tris buffered saline
TBSV  Tomato bushy stunt virus
TBSV-Ch  cherry strain of TBSV
Thr  threonine
TM  transmembrane
TMV  Tobacco mosaic virus
TNV  Tobacco necrosis virus
TPA  Tetragonolobus purpureas agglutinin
Tris  Tris-hydroxymethyl amino methane
Trp  tryptophan
TRV  Tobacco rattle virus
TSWV  Tomato spotted wilt virus
TuMV  Turnip mosaic virus
TVMV  Tobacco vein mottling virus
Tyr  tyrosine
U  uridine in the context of nucleotide sequence; units when referring to amount of enzyme
V  volts in the context of electrophoresis; valine in the context of protein sequence
Val  valine
VAP  viral attachment protein
VP  viral protein
W  tryptophan
WT  wild-type
Y  tyrosine
ZYMV  Zucchini yellow mosaic virus
Dedication

To my parents for their love and support.
Acknowledgements

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1 CHAPTER ONE
LITERATURE REVIEW

1.1 Introduction

Viruses, being obligate parasites, must depend on susceptible hosts for their survival in nature. Animal viruses spread in nature by utilizing the host cell surface molecules as their receptors. Plant viruses on the other hand, are unable to penetrate plant cells due to the presence of the plant cuticle and cell wall (83). Thus successful spread of the majority of the plant viruses is achieved through a vector that induces wounds in the plant host and also carries virus from one susceptible host to another. Most virus-host interactions are quite specific in nature. In animal viruses, the specificity lies in the ability of the virus to recognize a particular receptor on the host cell surface which primes the virus for subsequent steps in the infection process (14, 79, 140, 210). In plant viruses, however, the specificity is determined at least in part by the ability of the virus to recognize a component or components of the vector. The ability of a plant virus to recognize a specific vector is probably controlled via one or more receptors in or on the vector (163).

Plant viruses are responsible for severe economic losses in agriculture worldwide (83). Although there is considerable information available on the biology of virus-vector relationships, very little is known about virus-vector interactions at the molecular level. Understanding the molecular mechanisms that govern the virus-vector recognition is an important part of the strategy to control plant virus diseases.
Cucumber necrosis virus (CNV) is a small icosahedral virus belonging to the family Tombusviridae, in the genus Tombusvirus. Vector transmission of CNV is facilitated by zoospores of the chytrid fungus Olpidium bornovanus (46). It is believed that CNV particles are adsorbed to the surface of the motile zoospores following independent release of virus and zoospores from roots of infected plants. Bound virus enters root cells upon zoospore encystment (34, 46). Previous work from our lab has shown that the CNV coat protein is important for fungus transmission (121). A single amino acid (aa) substitution in the shell domain (Section 1.5.4 and Figure 2.3) of the coat protein (CP) resulted in lowered transmission efficiency of CNV by O. bornovanus. In vitro binding assays demonstrated that this mutant bound zoospores less efficiently than wild type (WT) CNV indicating the role of specific regions on the CP shell domain in fungus transmission as well as zoospore attachment (153). In addition, it was shown that binding of CNV to zoospores is saturable suggesting that a specific number of recognition molecules exist on the zoospore surface for CNV attachment. Further in vitro binding studies demonstrated that binding between viruses and their respective vector zoospores was specific and reflective of their association in nature (154).

Chytrid zoospores are known to contain a prominent cell coat. Cytochemical studies demonstrated that the cell coat is made predominantly of carbohydrates, especially glucosyl and/or mannosyl residues (141). Lectin binding studies from our lab suggested that glucose and/or mannose and fucose sugars exist on the surface of O. bornovanus zoospores (157).

Plant virus-vector interactions are highly specific in nature (66, 67, 68, 139, 163). The specificity of the transmission process suggests the presence of specific recognition
molecules (receptors) on the vector surface for viruses (39, 163, 197). Plant virus-vector interactions may in some ways resemble animal virus/host interactions. Several animal viruses undergo extensive conformational changes as a part of their host cell entry process to expose the otherwise hidden fusion peptide (enveloped viruses) or membrane penetration proteins (nonenveloped viruses). These conformational changes are induced either by binding to the receptor or in endosomes at acidic pH (41, 48, 51, 140).

Prior to the work described in this thesis there were no reports describing: 1) specific regions of a plant virus capsid involved in vector recognition or attachment; 2) the nature and biochemical characteristics of putative vector receptors for a plant virus; and 3) conformational changes induced in a plant virus capsid upon vector attachment or the importance of conformational rearrangements in vector transmission.

This literature review is organized into five sections and provides background knowledge for the main areas of research covered in this thesis. The first section describes general aspects of plant virus transmission by arthropods, nematodes and fungi. The second section deals with primary and secondary cellular receptors for animal viruses with a special emphasis on the role of conformational changes in animal virus entry. In the third section, biological and molecular aspects of CNV are described. In the fourth and fifth sections, a brief overview of T=3 icosahedral plant viruses will be provided with a special focus on virus particle dynamics and the role of the CP N-terminal arm.
1.2 Plant virus transmission

As mentioned earlier, there are no reported examples of plant virus entry by utilizing host cell surface molecules as their receptors. Infection of a new plant can only be initiated by entering the host cell via a wound. Plant viruses are transmitted in nature either mechanically by vegetative propagation, infected sap, seed or by specific association with specific invertebrate and fungal vectors (Table 1.1) (for a collection of recent reviews on vector transmission of plant viruses see 139, 68, 163). Vector transmission is a highly specific process in which the specificity is determined at different levels such as the type, family, genus, species and biotype of both the vector and the virus (83). Successful vector transmission requires: 1) acquisition - a specific association between the virus and the vector; 2) retention – the ability of the virus to remain infectious during vector association; and 3) inoculation – dissociation of the virus from the vector and subsequent infection of a new host.

Understanding the concepts and terminology of plant virus vector transmission is valuable in epidemiological studies since it conveys an important property of the vector’s ability to retain the virus. Watson and Roberts (204) proposed the first system of classification, which was based on early biological studies on aphid and leafhopper transmission. They classified viruses into two groups, non-persistent and persistent, based on the duration in which the virus remains inoculative in the vector (125). As our understanding of the biological and molecular aspects of vector transmission of plant viruses increased over the years, the classification of plant virus transmission has been revised and some new terms have been added. In this literature review, I have followed the terminology used by Gray and Banerjee (66) and Gray and Rochon (67). This
<table>
<thead>
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<td><em>Tenuivirus</em></td>
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1 This table was adapted and modified from reference # 66
terminology is based upon two important criteria: 1) the ability of the virus to cross the vector cell membrane and subsequent internalization and; 2) virus retention time in the vector. The following are the different types of transmission mechanisms observed in viruses transmitted by invertebrate vectors.

*Non-circulative transmission (externally borne):* In this type of transmission, the virus does not cross the vector cell membrane. Instead, it is carried externally on the surface of the vector such as mouthparts. Non-circulative transmission can be conveniently divided into two types, *semi-persistent* and *non-persistent*. In both types the virus is associated specifically to either the cuticular lining of the mouthparts or the foregut of the vector. The acquisition feeding time is very short (a few seconds to minutes), and the acquired virus is transmitted immediately by the vector. In semi-persistent transmission, the virus remains infectious for slightly longer periods in the vector.

*Circulative transmission (internally borne):* In circulative transmission, the virus can cross the vector cell membrane and becomes internalized. All circulative viruses are retained by the vector even after moulting. Acquisition and inoculation periods are usually longer (hours to days). Circulative transmission can be further divided into *propagative (replicative)* and *non-propagative (non-replicative)*, based upon the ability of the virus to replicate in the vector body upon internalization.
The mechanisms of virus transmission by fungi and the terminology used are slightly different from virus transmission by invertebrates and will be discussed in the section 1.2.4.3.

1.2.1 Arthropod transmission

Arthropods are the most important group of plant virus vectors, both in terms of the number of plant viruses transmitted and the economic importance of the disease caused. Arthropod vectors (197) transmit more than 70% of the viruses infecting plants. Approximately 99% of all arthropod vectors are insects (72). Among 29 orders that are described in the sub-phylum Insecta, the orders Homoptera, Coleoptera (beetles) and Thysanoptera (thrips) contain the important plant virus vectors. Over 70% of all insect vectors belong to the order Homoptera, which includes aphids, leafhoppers, whiteflies and mealybugs (83, 125). Mouthparts of these insects are of the piercing and sucking type, which is well-suited for the plant virus transmission. In addition, many of these insects are tissue-specific feeders often feeding on phloem, where many plant viruses are located (125).

1.2.1.1 Aphid transmission

Aphids belong to the order Homoptera and are the most important group of plant virus vectors. Approximately 20% of the 4000 aphid species described are known to be vectors of plant viruses (125). The following are the three different stages of the aphid transmission cycle (83, 201): Acquisition phase. This refers to the phase in which the
vector aphid feeds on an infected plant to acquire a virus for subsequent transmission; *Latent period.* This is the period of time in which the virus is associated with the vector aphid before it can be transmitted; *Retention time.* This is the length of time during which the viruliferous aphid can transmit the virus to a susceptible host.

The aphid transmission section of this literature review is described under three subcategories: 1) non-circulative and non-persistent; 2) non-circulative and semi-persistent; and 3) circulative and non-propagative.

### 1.2.1.1.1 Non-circulative, non-persistent aphid transmission

Most of the aphid transmitted plant viruses are transmitted in a non-persistent manner (83). In this type of transmission the virus is acquired rapidly by aphids after feeding on an infected plant for a very short time. The acquired vector transmits these viruses immediately, since the aphid rapidly loses (usually within hours) its ability to transmit the infectious virus (201). According to Pirone and Blanc, (137), the non-persistent viruses can be divided into two types based on the molecular mechanisms involved: viruses following a capsid strategy and viruses a following helper component strategy.

#### 1.2.1.1.1.1 Capsid strategy

Several groups of viruses including cucumoviruses, alfamoviruses and carlaviruses do not need a helper component for successful aphid transmission. These viruses are grouped under the non-circulative, non-persistent viruses using the capsid strategy, since the virus particles sufficient for vector transmission. Much of the
information on the molecular mechanisms behind this type of transmission comes from the work on cucumovirus transmission.

_Cucumber mosaic virus_ (CMV) is transmitted by a wide variety of aphids (134). With the help of transcapsidation studies using a poorly transmissible CMV-6 and a highly transmissible CMV-T, Gera _et al._, (60), showed that the coat protein contains the determinants for aphid transmission. Further transcapsidation studies by Chen and Francki, (43) showed that _Tobacco mosaic virus_ RNA is aphid transmissible only if it is encapsidated _in vitro_ by an aphid transmissible _Tomato aspermy virus_ strain V coat protein, confirming the role of the CP as the sole determinant in aphid transmission. Amino acid comparison of the coat proteins of the highly transmissible Fny isolate of CMV and the poorly transmissible M-CMV revealed that aa 129 and 162 are important for the aphid transmission (132). Repeated mechanical passage resulted in a mutant that is transmissible by only one of the two aphids that usually transmits CMV, indicating that different regions of the viral coat protein influence the transmission of CMV by different aphids (133). Recently Liu _et al._ reported that a conserved capsid surface domain of CMV consisting of a negatively charged βH-βI loop is important for aphid transmission. Mutations introduced in this loop affected aphid transmission without grossly affecting virion structure or virus infectivity. Liu _et al._ predicted that the change in the surface charge caused by the mutations in the βH-βI could be responsible for the transmission deficiency.
1.2.1.1.2 Helper strategy

Viruses belonging to the genera Potyvirus, Caulimovirus, and Waikavirus are known to require a virus encoded helper component in addition to coat protein for successful vector transmission (65, 66, 67). Helper component is a non-structural protein that is believed to mediate the virus-vector interactions by forming a molecular bridge between the virus particle and its putative receptor in the aphid mouthparts (137). Much of our understanding of the molecular basis for helper dependent aphid transmission has come from poty- and caulimoviruses. The following section briefly describes the helper dependent aphid transmission of potyviruses.

The potyviruses are filamentous particles made up of nearly 2000 subunits of the coat protein (145). In many potyviruses the coat protein has been implicated in aphid transmission. An N-terminal segment (30-90 aa in length) of the coat protein that is exposed on the particle surface, has been shown to be important for aphid transmission. In several potyviruses, mild treatment of virus particles with trypsin results in loss of this exposed N-terminal segment and a corresponding loss of aphid transmission (145). Based upon amino acid sequence comparisons of several aphid transmissible and non-aphid transmissible potyviruses, it was suggested that a conserved aspartate-alanine-glycine (DAG) motif located in the N-terminal region of the coat protein is involved in aphid transmission (116, 128, 136). Atreya et al., (8), provided direct evidence for the involvement of the DAG motif in aphid transmission. These studies showed that a substitution of Gly to Lys in the DAG motif of Tobacco vein mottling virus (TVMV) completely abolished aphid transmissibility. There are several reports indicating the pivotal role of the DAG motif in aphid transmission of potyviruses (8, 145). Recently it
has been reported that the flanking aa sequences of the DAG motif also play an important role in aphid transmission (9, 10). *In vitro* protein overlay studies have shown that in TVMV the DAG motif interacts with the viral encoded helper component (HC-Pro) (19).

Govier and Kassanis, (64) first reported the role of the helper component in (HC-Pro) potyvirus transmission. They showed that aphid non-transmissible *Potato virus-C* (PVY$^c$) and *Potato acuba mosaic virus* (PAMV, now classified as Potexvirus) are transmitted only when aphids are fed previously on aphid transmissible PVY-infected plant extracts. They also concluded that the helper component might act as a bridge between the aphids and the virus particles. Cell-free translation experiments provided the evidence that HC-Pro is of viral origin (80). Potyvirus helper component is a papain-like protease and Carrington *et al.*, (37), showed the first indication of its proteolytic activity. Based upon amino acid sequence comparisons of the helper components of aphid transmissible and non-aphid transmissible isolates of PVY and PVY$^c$, it was suggested that a lysine-isoleucine-threonine-cysteine (KITC) motif may be important for helper activity (192). Later, the role of the KITC motif in the helper activity was reinforced by site-directed mutagenesis experiments (145). Based on *in vitro* protein overlay experiments and immunogold labelling studies it has been proposed that this KITC motif might be involved in binding to the aphid stylets (20, 131). Comparison of wild type (WT) and PAT (poorly aphid transmissible) strains of *Zucchini yellow mosaic virus* (ZYMV) revealed that a highly conserved proline-threonine-lysine (PTK) motif is also important for helper activity (99, 145). It has been shown that this PTK motif binds to the ZYMV CP in dot blot experiments, suggesting that the PTK motif may be a part of the virion-binding domain of HC-Pro (131).
1.2.1.1.2 Non-circulative, semi-persistent aphid transmission (Helper strategy)

Semi-persistent transmission is very similar to non-persistent transmission except that the acquisition, inoculation and retention times are slightly longer. There is no conclusive evidence that molecular mechanisms involved in semi-persistent transmission are different from non-persistent transmission (21). Much of the information on semi-persistent transmission comes from aphid transmission of caulimoviruses (for recent reviews see, 21, 70).

*Cauliflower mosaic virus* (CaMV) is transmitted by the aphids *Myzus persicae* and *Brevicoryne brassicae*. Like potyviruses, aphid transmission of CaMV involves helper components. Pirone and Megahead, (135), first demonstrated the requirement of the helper component. They showed that the purified virus was unable to be transmitted by aphids. Lung and Pirone (111) confirmed the presence of a helper component by transmitting the purified non-transmissible Campbell strain of CaMV using aphids that were fed first on a transmissible cabbage strain CaMV-infected plant. This helper component is called the aphid transmission factor (ATF). Several mutagenesis and gene replacement studies showed that the CaMV gene-II product (P2 protein) is the aphid transmission factor (7, 208). Later it was shown that the lack of aphid transmissibility of Campbell and CM1841 isolates of CaMV was due to a single aa substitution in P2 at residue 94 from glycine to arginine (71). Blanc *et al* (18) unequivocally demonstrated the role of P2 as the helper factor by transmitting several non-transmissible CaMV isolates using recombinant P2 protein produced in the baculovirus expression system. In addition, they showed that the aphids that were incubated with P2 still could not transmit purified CaMV particles, suggesting that still an additional transmission factor may be
needed. The CaMV gene III product (P3 protein) was identified as an additional aphid transmission factor (100). It was shown that the P2 incubated aphids were able to transmit purified CaMV only when they were also pre-incubated with bacterially expressed P3 protein.

The presence of two transmission factors has prompted the idea that the bridge between the CaMV and the aphid mouthparts could be made of both P2 and the P3 proteins. A large C-terminal domain spanning aa 61-110 of P3 protein was shown to contain determinants for virion binding (101). In addition, the N-terminal region of P3 was shown to contain a binding domain for P2. The current proposed model for the aphid transmission of CaMV is: 1) P2 protein binds to the putative receptor in the aphid alimentary canal; and 2) the N-terminal region of the P3-virion complex that is formed in infected plants binds to the C-terminal region of P2; thus completing the bridge (21, 70).

1.2.1.1.3 Circulative, non-propagative aphid transmission

Viruses that are transmitted in a circulative manner are internalized by the vector. The translocation of virus across the cell membranes inside the vector is very important for successful transmission. Circulative, non-propagative transmission of luteoviruses by aphids is one of the well-studied virus/vector interactions both at the biological and molecular level. The following section describes recent advances in luteovirus-aphid interactions.

*Vector determinants:* The general circulative pathway is similar for both propagative and non-propagative viruses. Virus is acquired via the food canal and then
released into the haemocoel, after passing through the foregut, midgut and hindgut. Eventually the virus must be associated with the salivary glands to be transmitted to the plant host (66). Based on ultrastructural studies, it has been proposed that receptor-mediated endocytosis and exocytosis may be involved in the virus crossing the barriers of the aphid gut and salivary gland epithelial linings (197). It has been reported that the determinant for vector specificity does not lie at the gut barrier, since different luteoviruses are acquired into the haemocoel irrespective of whether the carrying aphid is a vector or not (61, 66). The haemocoel acts as a reservoir in which acquired viruses remain infective without undergoing replication. Following acquisition and entry into the haemocoel, the virus diffuses through the haemolymph until it encounters the aphid’s salivary glands (26). Luteoviruses are exclusively associated with the accessory salivary gland (66, 68). The specificity is believed to be determined at the level of the accessory salivary gland (62).

Recently Li et al., (103) have identified the possible receptors for Barley yellow dwarf virus (BYDV) in its vector aphid Sitobean avenae. With the help of virus overlay assays they showed that BYDV binds to two proteins (33 kDa and 50 kDa) from the head tissues of the vector aphid Sitobean avenae; but not from the non-vector aphid Rhopalosiphum padi. Anti-idiotypic antibody produced against a monoclonal antibody (MAV-4) of BYDV-MAV was able to bind several proteins including the 33 and 50 kDa proteins from the head tissue of Sitobean avenae. These data support the presence of receptor or receptor-like molecules in the aphid salivary gland for the virus.
**Viral determinants:** Rochow (164) showed the involvement of the coat protein in aphid transmission using virus transcapsidation studies on MAV and RPV isolates of BYDV. The aphid *R. padi* transmits the RPV isolate but not the MAV isolate of BYDV. During co-infection in a single plant, virus containing the MAV genome encapsidated in RPV coat protein could be transmitted by *R. padi*. MAV replication was detected in the new host plant, but the virus purified for this infection (MAV genome encapsidated by MAV coat protein) could not be transmitted by *R. padi*. The importance of the coat protein in luteovirus transmission also comes from studies of transmission of umbraviruses. Umbraviruses do not encode a coat protein (182) and are transmitted by aphids only if plants are co-infected by a luteovirus which provide the coat protein necessary for transmission. The type of luteovirus providing the coat protein determines which aphid will transmit the umbravirus (125).

The 24 kDa major coat protein of luteoviruses is encoded by ORF-3. Sequence analysis showed that there is a larger ORF (ORF-5) following ORF-3. Due to occasional translational suppression of the coat protein stop codon, ORF-5 is expressed, resulting in a 74 kDa readthrough (RT) protein. The RT protein is exposed on the particle surface and is not necessary for particle assembly (42, 92, 151), providing evidence for the requirement of the RT protein in aphid transmission. Moreover, a mutant of a PAV isolate of BYDV that does not have RT protein, is not aphid transmissible. Sequence comparisons of the RT proteins of several luteoviruses revealed that the N-terminal region is highly conserved, whereas the C-terminus is variable. Purified preparations of several luteoviruses that are aphid transmissible revealed that the RT protein is present in a truncated form with its C-terminal variable region being proteolytically cleaved *in vivo.*
These studies indicate that the C-terminal region of the BYDV RT protein is not important for aphid transmission (202). In addition, mutant BYDV particles that do not contain the N-terminal region of the RT protein are not aphid transmissible (53, 68). The N-terminal region of the RT protein contains important determinants for the aphid transmission. Several point mutations in the N-terminal region of the RT protein of Beet western yellows virus (BWYV) resulted in reduced transmission (25). Moreover, it was shown that aphid transmission of the RPV isolate of BYDV could be prevented by mixing the purified virus with the antibodies raised against the N-terminal region of the RT protein before aphid feeding, whereas antibodies raised against C-terminal region RT protein did not prevent transmission (119). Virions that did not contain the RT protein were able to be taken up by aphids and were observed in haemocoel, indicating that the coat protein contains sufficient determinants to cross the aphid gut barriers to reach the haemocoel (42, 66, 148). It also suggests that the role of the RT protein probably lies in facilitating the virus entry from the haemolymph to the accessory salivary gland (66).

**Role of endosymbiotic bacteria in aphid transmission:** Aphids harbour endosymbiotic bacteria that belong to the genus *Buchnera* in their haemocoel. The bacteria provide essential amino acids that are not synthesized by the aphids. In addition, they also produce large quantities of symbionin, a chaperonin protein, which is a homologue of *E.coli* chaperonin Gro-EL (15). The chaperonins are a class of proteins that play an important role in protein folding and translocation across membrane barriers (See 66). It has been shown that symbionin binds to purified luteoviruses and the RT proteins *in vitro* (54). In addition the N-terminal half of the RT protein of BWYV was shown to
bind to symbionin. (196). It has been suggested that the RT protein on the viral capsid is involved in binding to symbionin and thereby helping the virus to move from the aphid haemolymph to the accessory salivary gland. However, the role of symbionin in vector specificity has not yet been proven, since luteoviruses bind to symbionin from both vector and non-vector aphid species with relatively similar affinities (196).

1.2.2 Thrips transmission

Viruses in the genus *Tospovirus* are transmitted by thrips in a circulative and propagative manner. *Tomato spotted wilt virus* (TSWV) is the type member of this genus and much of the information (molecular and biological) on transmission comes from TSWV and its vector *Franklineilla occidaentalis*. At least 10 species of thrips have been reported to transmit tospoviruses (194). TSWV and its vector, *F. occidaentalis* have a unique relationship, in that the larvae but not the adults must acquire the virus in order to be transmitted. Virus acquisition capacity rapidly declines as immature larva develop into adults (195). Four potential barriers have been recognized for the virus to cross in its thrips vector: 1) entry into the midgut; 2) escape from the midgut; 3) entry into the salivary gland; and 4) escape from the salivary gland (194). TSWV has been reported to be associated with cellular membranes both in its thrips vector and the plant host wherein the viral glycoproteins, GP1 and GP2 are involved. Immunolocalization experiments have shown that TSWV is associated with the Golgi complex in thrips and with the endoplasmic reticulum (ER) in the plant cell (58, 193). Several lines of evidence show that GP1 and GP2 are the viral attachment proteins in the thrips body. Electron microscopic
studies revealed that the TSWV GP1 binds to the apical membrane in the midgut of the thrips larvae. The GP2 contains an arginine-glycine-aspartic acid (RGD) motif, which is a highly conserved motif among several animal virus attachment proteins and known to play an important role in recognizing viral receptors (197). Recently, with the help of gel overlay assays, Bandla et al., (12) showed that both GP1 and GP2 bind to a 50 kDa protein in total thrips protein extracts. Viral GPs did not recognize the same band in similar assays using non-vector and adult thrips, which reflects the virus-vector association in nature. Anti-idiotypic antibodies to the viral GPs selectively bind to 50 kDa protein in western blots, indicating the specificity of the interaction. Electron microscopic studies showed the association of anti-idiotypic antibodies of viral GPs with the larval midgut membrane. This data suggests that the GP1 and GP2 are viral attachment proteins. In addition, it also suggests that the 50 kDa protein is a potential receptor in thrips larva for TSWV. Viral GP2 also binds to a 94 kDa protein, which is abundant in the thrips body, but is not in the midgut. Moreover this 94 kDa protein was recognized in both vector and non-vector thrips (96). It has been suggested that this protein may be involved in circulation of the virus in the thrips vector.

1.2.3 Nematode transmission

Some nematodes are root-infecting ectoparasites of plants. Plant viruses belonging to Nepo- and Tobravirus genera are transmitted by nematodes. Currently 12 nepoviruses and three tobraviruses are transmitted by nematodes belonging to the families Longidoridae and Trichodoridae respectively (114). Species within the genus Longidorus, Paralongidorus and Xiphinema of the family Longidoridae and species
within the genus *Paratrichodorus* and *Trichodorus* of the family *Trichodoridae* are identified as vectors of plant viruses. Virus transmission by nematodes is of the non-circulative and semi-persistent type, since: 1) virus is retained in the vector for several weeks; 2) viruses do not replicate in the vector; and 3) virus is not retained after moulting (114, 115). Based on specificity studies, Brown and Weischer (30) proposed two concepts in nematode transmission: 1) exclusivity, in which the vector nematode species transmits only one virus or one serologically distinct virus strain; and 2) complementarity, in which the vector nematode species transmits more than one virus or serologically distinct strains of a virus. Successful transmission of virus by nematodes depends on five phases. They are acquisition, adsorption, retention, release and transfer of the virus. The specificity of the virus and nematode interaction probably lie at the adsorption, retention and release phases of the transmission process (114).

**Viral determinants:** Tobr- and nepoviruses are positive-sense, single stranded RNA viruses with two genomic RNAs, a larger RNA1 and a smaller RNA2, which are encapsidated into separate virus particles (113). Tobravirus particles are rod-shaped and nepovirus particles are spherical. Pseudo-recombination experiments involving viral genomic RNAs from differentially transmissible isolates of the same virus have shown that RNA2 of both tobra- and nepoviruses contain important determinants for vector transmission (73, 138).

RNA2 of nepovirus encodes a coat protein along with two other proteins called 2A and 2B. Gene replacement studies between *Grapevine fanleaf virus*, transmitted by *X. index*, and *Arabis mosaic virus* (*ArMV*), transmitted by *X. diversicaudatum* have shown
that 2A and 2B proteins are not necessary for transmission, indicating the CP as a sole
determinant of vector specificity (16). RNA2 of tobraviruses also produces a coat protein
along with three other proteins, 2B, 2C and a small 9 kDa protein. The C-terminal 20 to
30 aa of the coat protein is exposed on the surface of the particle and is relatively
unstructured (22). Deletion of 15 aa in this region has resulted in loss of transmission in
*Pea early browning virus* (PBEV) isolate Ppa56 and *Tobacco rattle virus* isolate PpK20.
Replacement of the coat protein gene of a poorly transmissible SP5 isolate of PEBV with
the coat protein gene of the highly transmissible Ppk20 isolate of TRV, did not result in
high transmissibility, indicating the role of other proteins encoded by RNA2 in vector
transmission apart from the coat protein. Deletion of the 2C gene reduced transmission in
PEBV, but not in TRV (81). Deletion of 2B from both PEBV and TRV resulted in loss of
vector transmission (81, 112). In addition, it has been shown that the 2B protein can act
*in trans* so that a TRV transmission mutant lacking the 2B gene can be transmitted when
co-inoculated with WT TRV (198). Yeast two-hybrid and immuno-gold labelling studies
have shown that the 2B protein interacts with the coat protein C-terminus, probably
acting as a helper component (200). Moreover, a single aa substitution in the PEBV 2B
gene completely abolished vector transmission. A predicted coiled-coil region of 2B
protein is speculated to be involved in the interaction with CP (199). It has been
suggested that the 2B protein might form a bridge between TRV and the oesophageal
lining of nematode mouthparts. Also, the specificity of tobravirus transmission could be
determined by the 2B protein (115).
Vector determinants: The site of virus retention in the vector nematode mouthparts varies either from the inner surface of the odontostyle, the region between odontostyle and cuticular lining of the guiding sheath or the oesophageal tract depending on the vector species. (114). A discontinuous layer of carbohydrate staining material was observed on the oesophageal tract of *X. diversicaudatum* and *Paratrichodorus pachydermus* (29). Adsorption of ArMV and *Strawberry latent ringspot virus* (SLRSV) particles was found only in the presence of carbohydrate lining material in the oesophageal tract of *X. diversicaudatum* (155). These studies provide some preliminary evidence for the involvement of specific recognition molecules in nematode transmission. It has been suggested that the nepovirus CP may have lectin-like properties in recognizing carbohydrates (29).

1.2.4 Fungus transmission

The fungi that are known to transmit plant viruses are root-infecting, zoosporic, obligate parasites on plants (34). Two species belonging to Phylum *Chytridiomycota* (*Olpidium bornovanus* and *Olpidium brassicae*) and three species belonging to the plasmodiophorids (*Polymyxa graminis*, *P.betae* and *Spongospora subterranea*) are recognized as vectors of several plant viruses (Table 1.2 and 1.3) (4, 34,163). Plasmodiophorids were considered as fungi for a long time. But recently, based on molecular studies, their taxonomic status has been changed. They are now considered to be protozoans (24, 203). Although chytrids and plasmodiophorids are taxonomically different, they share several characteristics with regard to their life cycles: 1) both are
zoosporic and obligate parasites of plants; 2) survival and infection occurs in nature via resting spores; 3) both are holocarpic (i.e., the entire thallus is converted into a zoosporangium) (34). Because of the similarities in their life cycles and their relation to virus transmission, several reviews discussed chytrids and plasmodiophorids as fungal vectors of plant viruses (2, 4, 34). However, they are now referred to as fungal and plasmodiophorid vectors of plant viruses.

1.2.4.1 Zoospore structure and the life cycle of *Olpidium* spp.

*O. bornovanus* and *O. brassicae* are obligate, root-infecting parasites, which are extremely common in nature. These are symptomless parasites on the host but, are economically important because of their ability to act as vectors for several important plant viruses (163). Much of the information on the structure of the *Olpidium* zoospore and life cycle (Figure 1.1 and 1.2) comes from the work on *O. brassicae* by Temmink and Campbell (191). *O. brassicae* zoospores are small, about 2-3 µm in diameter, with an oval shaped body surrounded by a plasmalemma (188). Zoospores of *O. bornovanus* are also oval shaped but are 6-7 µm, slightly larger than that of *O. brassicae*.

Possession of a single posterior, whiplash flagellum is a typical characteristic of *Olpidium* species. A membranous sheath that is continuous with the plasma membrane of the zoospore body also covers the flagellum. Zoospores contain a prominent nucleus, several mitochondria, vacuoles, multivesicular bodies and lipid globules. Endoplasmic reticulum is distributed throughout the zoospore body as short tubules, and ribosomes are present in high number (189). Several chytrid zoospores are known to contain a
Figure 1.1. Schematic representation of the ultrastructure of the *O. brassicae* zoospore*.

Abbreviations used: A, axoneme; AS, axonemal sheath; ER, endoplasmic reticulum; K, kinetosome; L, lipid globules; M, mitochondrion; MVB, multivesicular body; N, nucleus; R, rhizoplast; RV, rhizoplast vesicle; V, vacuole; ZE, zoospore ectoplast.

* This diagram was adapted from reference # 190.
prominent cell coat (45). The cell coat is present on the flagellum as well as on the zoospore body. Cytochemical studies have demonstrated that the cell coat is predominantly made of carbohydrates, especially glucosyl and/or mannosyl residues (141).

Primary zoospores are produced from resting spores upon favourable environmental conditions. These primary zoospores swim to the host plant root hairs, where they encyst. During encystment the flagellum is projected away from the host surface. Chytrid zoospores become sticky during encystment, indicating a change in the composition of cell surface material (141). Upon encystment, the zoospore retracts its flagellum and in several chytrids, it has been suggested that the axoneme coils and is released into the zoospore cytoplasm. The axonemal sheath wraps around the zoospore body and ultimately fuses with the plasma membrane (141). In the case of *O. brassicae*, Temmink, (191) proposed a “reeling in” mode of flagellar retraction in which, the axonemal sheath along with the axoneme is released into the zoospore cytoplasm. Once the flagellum is retracted, a cyst wall appears around the zoospore body and a papillum develops in between the host cell and the cyst. Then, a vacuole appears in the cyst and the zoospore cytoplasm along with surrounding ectoplast move into the host cell via the papillum (190). The thallus increases in size as it undergoes mitotic divisions becoming a mature zoosporangium. A cell wall appears around the mature thallus and it is therefore believed that virus transmission to the root cell must occur before the thallus wall is formed (188). As thalli mature, zoospores are formed inside and are released to the outside through exit tubes upon contact with moisture. During unfavourable conditions the thalli may develop into resting spores.
Figure 1.2. Life cycle of *O. brassicae* (adapted from reference # 191)
1.2.4.2 Life cycle of plasmodiophorids

The plasmodiophorid life cycle consists of two phases: 1) the primary or sporangial phase, which results in zoosporangia and the release of secondary zoospores; and 2) the secondary or sporogenic plasmodium, which produces thick-walled resting spores (24, 95). All plasmodiophorids zoospores are biflagellate with anteriorly located whiplash flagella (6). The resting spores of plasmodiophorids are called sporosori. Upon favourable conditions, these sporosori germinate and release primary zoospores. The primary zoospores swim to the host root hair or epidermal cells where they encyst. A tubular structure called “Rohr” that contains a dagger-like body called a “Stachel” is formed within the cyst. Later the contents of zoospores along with the Rohr and the Satchel are injected into the host cell. Inside the cell, the contents of primary zoospores undergo cruciform nuclear divisions to form a multinucleate plasmodium. This plasmodium can develop into either of the two phases described above. During the sporangial phase, the nucleus undergoes several cycles of non-cruciform mitotic divisions resulting in an aggregate of zoosporangia. Later these zoosporangia develop exit tubes to release secondary zoospores either to the outside of the root or into adjacent root cells. During the sporogenic phase, the nucleus undergoes non-cruciform mitotic divisions before forming unicellular thick-walled resting spores.

1.2.4.3 Modes of fungus transmission

Campbell, (31) introduced terminology to fungus-transmitted viruses. It is slightly different from the terminology used for invertebrate transmitted viruses because of the differences in life cycles of fungi and invertebrates. The terminology was based upon
virus acquisition and location of virion relative to resting spores. The *in vitro* mode of transmission involves virus acquisition by vector outside the host and the virus is not located within the resting spore. In the *in vivo* mode of transmission, virus is acquired by vector within the infected plant and the virus is persistent in resting spores. Later the terms non-persistent (externally borne) and persistent (internally borne) were suggested to align the terminology with that of aphid transmission (2, 4, 187). Non-persistent transmission was applied viruses that were carried externally on the surface of the zoospores (*in vitro*). Whereas, the term persistent transmission was used when virus was acquired and carried internally (*in vivo*). In this thesis I have followed the terminology used by Campbell (31).

1.2.4.3.1 *In vitro* transmission

Evidence for this type of transmission comes from the work on TNV transmission by *O. brassicaceae* (191). Virus can be transmitted by mixing purified virus from infected crude sap with virus-free zoospores. Virus-free zoospore cultures can be obtained by air drying the doubly-infected roots for several weeks or months during which time virus loses infectivity but resting spores retain the capacity to initiate fungus infection. Virus-free culture can also be obtained by treating roots containing resting spores with 20% trisodium phosphate (Na$_3$PO$_4$) or 5N HCl prior to root inoculation. Viruses transmitted in the *in vitro* manner lose infectivity following this treatment and were suggested to be outside rather than inside the spores (34). Studies showed that adding virus specific antisera to virus/zoospore suspensions immediately upon virus/zoospore mixing could prevent transmission of virus. If antiserum was added much later (after 10-15 minutes)
transmission of virus could not be prevented. This indicated that virus adsorption to zoospores is occurring within 5 to 10 minutes of mixing (34, 163). As mentioned earlier, two species of Olpidium (O. bornovanus and O. brassicae) have been shown to transmit viruses by the in vitro method (Table 1.2 and 1.3). All the viruses that are known to be transmitted by in vitro method are isometric particles belonging to the family Tombusviridae (Table 1.2; 63). Under natural conditions, virus and vector zoospores are released independently into the soil from the roots of infected plants. Virus particles are then adsorbed onto the zoospore plasmalemma and flagellar sheath. Upon flagellar retraction, virus is believed to enter the zoospore cytoplasm along with the flagellar sheath (181, 191).

**Specificity:** Virus and zoospore interactions are very specific and there is a direct correlation between the ability of the virus to adsorb to the zoospore surface and transmission efficiency. Electron microscopic studies (191) have shown that TNV, which is transmitted by O. brassicae zoospores, adsorbed to O. brassicae zoospore more efficiently than it did to non-vector O. bornovanus zoospores. Transmission of TNV by O. brassicae zoospores also depends on the isolate of fungus. Lettuce and tomato isolates of O. brassicae transmit TNV, whereas the mustard isolate does not. CNV adsorbed onto O. bornovanus zoospores more efficiently than that of non-vector O. brassicae zoospores (191). Moreover different O. bornovanus isolates differ in their ability to transmit Cucumber leaf spot virus (CLSV), Melon necrotic spot virus (MNSV), Cucumber soil borne virus (CSBV), Squash necrosis virus (SqNV) and CNV (33). These specificity
Table 1.2. Viruses transmitted by *Olpidium* vectors<sup>1</sup>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acronym</th>
<th>Genus</th>
<th>Fungal vector</th>
<th>Acquisition mode</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tombusviridae</strong></td>
<td></td>
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</tr>
<tr>
<td><em>Cucumber necrosis virus</em></td>
<td>CNV</td>
<td>Tombusvirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Cucumber leaf spot virus</em></td>
<td>CLSV</td>
<td>Aureusvirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Cucumber soilborne virus</em></td>
<td>CSBV</td>
<td>Carmovirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Melon necrotic spot virus</em></td>
<td>MNSV</td>
<td>Carmovirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Squash necrosis virus</em></td>
<td>SqNV</td>
<td>Carmovirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Red clover necrotic mosaic virus</em></td>
<td>RCNMV</td>
<td>Dianthovirus</td>
<td><em>O. bornovanus</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Chenopodium necrosis virus</em></td>
<td>ChNV</td>
<td>Necrovirus</td>
<td><em>O. brassicae</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Lisianthus necrosis virus</em></td>
<td>LNV</td>
<td>Necrovirus</td>
<td><em>O. brassicae</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Tobacco necrosis virus-A</em></td>
<td>TNV-A</td>
<td>Necrovirus</td>
<td><em>O. brassicae</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><em>Tobacco necrosis virus-D</em></td>
<td>TNV-D</td>
<td>Necrovirus</td>
<td><em>O. brassicae</em></td>
<td>In vitro</td>
</tr>
<tr>
<td><strong>Viruses in unassigned families</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Mirafiori lettuce virus</em></td>
<td>MiLV</td>
<td>Ophiovirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
<tr>
<td><em>Tulip mild mottle mosaic virus</em></td>
<td>TMMMV</td>
<td>Ophiovirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
<tr>
<td><em>Freesia leaf necrosis virus</em></td>
<td>FLNV</td>
<td>Varicosavirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
<tr>
<td><em>Lettuce big vein virus</em></td>
<td>LBVV</td>
<td>Varicosavirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
<tr>
<td><em>Lettuce ring necrosis virus</em></td>
<td>LRNV</td>
<td>Varicosavirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
<tr>
<td><em>Tobacco stunt virus</em></td>
<td>TSV</td>
<td>Varicosavirus</td>
<td><em>O. brassicae</em></td>
<td>In vivo</td>
</tr>
</tbody>
</table>

<sup>1</sup>This table was adapted from reference # 163
Table 1.3. Viruses transmitted by plasmodiophorid vectors

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acronym</th>
<th>Genus</th>
<th>Fungal vector</th>
<th>Acquisition mode</th>
</tr>
</thead>
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<tr>
<td>Potyviridae</td>
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<td>Barley mild mosaic virus</td>
<td>BaMMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Barley yellow mosaic virus</td>
<td>BaYMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Oat mosaic virus</td>
<td>OMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Rice necrosis mosaic virus</td>
<td>RNMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Wheat spindle streak mosaic virus</td>
<td>WSSMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Wheat yellow mosaic virus</td>
<td>WYMV</td>
<td>Bymovirus</td>
<td>P. graminis</td>
<td>In vivo</td>
</tr>
<tr>
<td>Viruses in unassigned families</td>
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<td></td>
</tr>
<tr>
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<td>BNYVV</td>
<td>Benyvirus</td>
<td>P. betae</td>
<td>In vivo</td>
</tr>
<tr>
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<td>BSBMV</td>
<td>Benyvirus</td>
<td>P. betae</td>
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<td>IPCV</td>
<td>Pecluvirus</td>
<td>P. graminis</td>
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<td>PCV</td>
<td>Pecluvirus</td>
<td>P. graminis</td>
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<td>Furovirus</td>
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<td>OGSV</td>
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<tr>
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<td>Furovirus</td>
<td>P. graminis</td>
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</tr>
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<td>Soilborne cereal mosaic virus</td>
<td>SBCMV</td>
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<td>P. graminis</td>
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<td>Soilborne wheat mosaic virus</td>
<td>SBWVM</td>
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<td>P. graminis</td>
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<td>SrCSV</td>
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<td>Potato mop top virus</td>
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<td>Pomovirus</td>
<td>S. subterranea</td>
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<td>BSBV</td>
<td>Pomovirus</td>
<td>P. betae</td>
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<td>AWMV</td>
<td>?</td>
<td>P. graminis</td>
<td>In vivo</td>
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<td>Watercress yellow spot virus</td>
<td>WYSV</td>
<td>Tombusvirus?</td>
<td>S. subterranean</td>
<td>Not known</td>
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<td></td>
<td></td>
<td></td>
<td>f.sp. nasturtii</td>
<td></td>
</tr>
</tbody>
</table>

1 This table was adapted from reference #163
studies strongly suggest the involvement of receptor or receptor-like molecule(s) on the zoospore surface (2, 34, 154, 163)

**Viral determinants:** Based on specificity studies on viruses that are transmitted via the *in vitro* fashion, it was speculated that specific receptors on *Olpidium* zoospores are involved in recognizing the viral coat protein (2, 191). MacLean et al., (121) first demonstrated the involvement of coat protein in the *in vitro* mode of transmission. Reciprocal exchanges of CP genes were made between infectious cDNA clones of the non-transmissible cherry strain of *Tomato bushy stunt virus* (TBSV-Ch) and transmissible CNV. Virions containing the TBSV-Ch genome encoding the CNV CP were found to be readily transmitted by *O. bornovanus*. However, transmission did not occur using virions containing the CNV genome encoding the TBSV-Ch CP. Robbins et al., (153) provided further evidence of the involvement of the coat protein in CNV transmission by *O. bornovanus*. A naturally occurring CNV transmission defective mutant (LL5) was isolated by repeated mechanical passage of CNV on *Nicotiana clevelandii*. Further sequence analyses showed that the loss of transmissibility was due to a single amino acid substitution (glutamic acid (E) to lysine (K)) in the CP shell domain (see Chapter 2). Further, with the help of *in vitro* virus/zoospore binding assays, it was concluded that the poor transmissibility of LL5 was at least partially due to its inability to bind to vector zoospores efficiently. Amino acid sequence comparisons of the CPs of several viruses of *Tomusviridae* have shown that viruses transmitted by *O. bornovanus* are more closely related to each other than they are to the genus to which they belong (163).
Vector determinants: Robbins (154) provided some preliminary evidence for the presence of receptor or receptor-like molecules on the surface of *O. bornovanus* zoospores for CNV. These studies showed that virus/zoospore binding is specific and saturable suggesting typical receptor mediated interactions. *In vitro* binding studies showed that CNV, CLSV and MNSV, which are transmitted by *O. bornovanus*, bound to *O. bornovanus* zoospores more efficiently than TNV, which is transmitted by *O. brassicae*. Similarly, TNV bound to *O. brassicae* zoospore more efficiently than CNV, CLSV and MNSV (see Chapter 3). *In vitro* binding studies have also shown that CNV/zoospore interactions are time and pH dependent (154). In addition, FITC labelled lectin binding studies have shown the presence of glucose/mannose and fucose on the surface of *O. bornovanus* and *O. brassicae* zoospores. It was speculated that these sugars could be a part of the putative viral receptor on the zoospore surface (154).

Virus delivery to host plant: As mentioned earlier, it has been postulated that virus bound to the surface of zoospores enters the zoospore cytoplasm along with the flagellar sheath and axoneme during flagellar retraction as virus particles have been observed between the whorls of flagellar sheath membrane inside the zoospore cytoplasm (181). In order for virus to infect a root cell it must cross the plasma membrane of the young fungal thallus (before it develops a thick wall) (188). It is intriguing that, even though the virus can be found in the zoospore cytoplasm, it is unable to be incorporated into resting spores. Two possible reasons suggested by Rochon *et al.*, (163) are: 1) during co-infection, virus may not be present in the same tissue as the resting spores develop and; 2) Virus may not be able to cross the membrane of the thallus from the root cell...
cytoplasm. More experimental study is needed in this area of the *in vitro* mode of transmission.

1.2.4.3.2 *In vivo* transmission

In the *in vivo* or internally-borne mode of transmission, virus is acquired during vector development inside the host plant. Virus is also carried inside the resting spore, and presumably also within zoospores (4, 34, 163). One *Olpidium* species (*O. brassicae*) and three plasmodiophorids (*P. graminis, P. betae* and *S. subterranea*) are reported to transmit plant viruses in the *in vivo* fashion. All viruses that are known to transmit in this mode are either rod-shaped or filamentous particles with multipartite genomes (Table 1.3; 163). Viruses belonging to Ophio- and Varicosaviruses are transmitted by *O. brassicae*. Viruses belonging to the *Bymo-, Beny-, Porno-, Furo* and *Pecluvirus* genera are transmitted by plasmodiophorids (Table 1.3; 63).

Several lines of evidence are available to show the persistence of virus inside the resting spores. *Lettuce big vein virus* (LBVV) was shown to remain for long periods in air-dried as well as chemically treated resting spores (31, 32). Persistence of several other viruses in their respective vector resting spores has been reported (4). Immuno-gold labelling studies have provided the evidence for the presence of *Barley mild mosaic virus* (BaMMV) in zoospores and zoosporangia of *P. graminis* (38). Similar results were obtained in *Beet necrotic yellow vein virus* (BNYVV)/*P. betae* infected plants (4). There are very few reports providing direct evidence for the presence of virus in resting spores, probably because of technical difficulties, such as the thick cell wall of resting spore and low number of viruses per spore (163). Filamentous virus-like bundles of *Barley yellow*
mosaic virus (BYMV) were observed in resting spores of *P. graminis* (39). *Potato mop top virus* (PMTV) -like particles were observed in fractured resting spores of its vector *S. subterranea* (123). Recently Driskel and Verchow (50) provided the evidence for the presence of the movement protein and viral RNA, but not the coat protein of *Soil-borne wheat mosaic virus* (SBWMV) in resting spores of *P. graminis*. It has been suggested that the viral RNA/movement protein complex may be the infectious component (50, 163).

**Vector determinants:** The role of viral encoded proteins in plasmodiophorid transmission has been well studied. BNYVV, which is transmitted by *P. betae*, has five RNA components. RNA4 and RNA2 have been shown to be important for fungus transmission (183, 184). RNA2 encodes the coat protein as well as a CP readthrough (RT) product. The RT domain is thought to play an important role in particle assembly and its C-terminus is probably exposed on the particle surface (171). Tamada and Kusume (184) have reported that serial passage of BNYVV in the absence of vector has resulted in deletions at the C-terminal end of the RT domain, which in turn, resulted in complete loss of fungus transmission, indicating the RT is essential for vector transmission. Further work has shown that a KTER motif located at the N-terminus of the RT domain is important for fungus transmission (185). PMTV also produces a CP RT which has been shown to be essential for its transmission by *S. subterranea* (147). Read-through proteins of several other viruses were also shown to be important for fungus transmission (4, 163).

A 39 kDa protein expressed by *Peanut clump virus* (PCV) RNA2 and a P2 protein encoded by bymovirus RNA-2 are reported to be essential for fungus transmission (117).
Unlike RT proteins, these two proteins are expressed independently from the coat protein. The P2 protein was also shown to be associated with inclusion bodies in infected tissues. It has been suggested that these two proteins might act as helper components bridging the CP and vector as in aphid transmission of poty and caulimoviruses (163). Adams et al, (3) have recently identified two trans-membrane domains (TM1 and TM2) in the CP-RT of beny-, furo- and pomoviruses genera and in the P2 protein of bymoviruses. The TM2 is either absent or disrupted in naturally occurring deletion mutants that are not fungally transmitted. A strong role for these two trans-membrane domains has been suggested in translocation of virus from the host cell cytoplasm to fungal plasmodia (3).

1.3 Virus/receptor interactions

Most animal, insect and bacterial viruses require attachment to the host cell surface for successful infection. This attachment is mediated by a viral attachment protein (VAP) (either envelope glycoprotein or one or more capsid proteins) and a host cell surface molecule (s) acting as virus receptor (14, 79, 118, 165, 210, ). Similarly, many plant viruses may require cellular receptors on their vectors for successful transmission to susceptible hosts (163, 197). Recognition of a cellular receptor by a virus is the first and crucial step in the infection process. Binding to the host cell surface receptor usually results in either direct fusion between the viral lipid envelope and host cell plasma membrane, receptor-mediated endocytosis or conformational changes in the viral capsid, all of which lead to the transfer of the viral genome or viral nucleoprotein into the host cell (140, 210). The nature, number and expression of host cell receptors play an important role in determining host range and tissue tropism (102). Virus/receptor
interactions are often complex and are often followed by extensive structural rearrangements in either the VAP or the viral capsid. Understanding the structural details of virus/receptor interactions has considerable significance in terms of designing drugs that inhibit virus entry (14).

Virus particles are known to attach to cell surfaces non-specifically through electrostatic interactions. Viruses can also adhere non-specifically to many substances including inert materials (186). In order to distinguish between receptor mediated attachment and non-specific interaction, Tardieu et al., (186) have proposed three standard criteria: 1) Binding of viruses to the host cell must be saturable, indicating a discrete number of binding sites; 2) virus binding to cells that do not contain specific binding component(s) should not be saturable; and 3) unlabelled binding of virus should be competitively inhibited by labelled virus or by closely related viruses (assuming they share the same receptor).

1.3.1 Virus receptors

Viruses take advantage of a wide variety of cell surface molecules as their receptors. A virus receptor can be defined as the host cell surface component(s), that interacts specifically with the virus and facilitates viral entry and subsequent infection (79, 176). Several virus receptors have been recognized and characterized (Table 1.4 and 1.5). The receptors belong to different families of proteins, carbohydrates and lipids that are involved in important cellular functions such as signal transduction, cell adhesion, immune modulation and enzymatic activities (14). Viral receptors can be identified by various techniques. These include the use of specific monoclonal antibodies raised
against the cell surface molecules, gene transfer techniques, mutant cell lines, virus overlay assays and the use of carbohydrate digesting enzymes (210).

A comparison of viruses and their cellular receptors reveals no apparent correlation between the virus family and receptor structure and function (14). For example, viruses belonging to *Picornaviridae* share common structural features in their capsids, but they use different molecules such as integrins, glycoproteins of the immunoglobulin superfamily, decay accelerating factor (CD55) and sialic acid as their receptors (Table 1.4 and 1.5). Sometimes a virus like HIV may use different receptor types to enter different cell lines or even to infect the same cell type (210). Several viruses belonging to different families may use the same molecule as their cellular receptor. For example, integrin αvβ3 serves as receptor for adenovirus and coxsackie virus A9, whereas poliovirus receptor (PVR) is used by poliovirus and two herpesviruses (Table 1.4). Some of the factors that influence the choice of the receptor are: 1) close proximity of the receptor molecule to the viral attachment site (210); 2) nature, availability and abundance of the receptor molecule on cell surface (102), and 3) ability of the receptor molecule to trigger further events that prime the virus for the subsequent steps in infection process (14, 210). In several viruses, it was reported that a single amino acid substitution in the VAP or the capsid often results in the change of receptor recognition and cellular tropism (14). For example, a substitution of Leu226 to Gln in the receptor binding pocket of haemagglutinin (HA) glycoprotein of influenza virus changes its receptor specificity from NeuAc α2-6 Gal to NeuAc α2-3 Gal (205). Based on the above observations it has been suggested that the structure and function of the virus
Table 1.4. Viral protein receptors and co-receptors

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus^2</th>
<th>Receptor^3</th>
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</thead>
<tbody>
<tr>
<td>Retroviridae</td>
<td>HIV</td>
<td>CD4, CXCR4, CCR5, CCR3, CCR2B, CCR8, STRL-33, TYMSTR, BOB/GPR15</td>
</tr>
<tr>
<td></td>
<td>SIV</td>
<td>CCR5, STRL-33, TYMSTR, GPR-1, CD4</td>
</tr>
<tr>
<td></td>
<td>GALV/FeLV/SSAV</td>
<td>Pit-1</td>
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<td></td>
<td>MLV-E</td>
<td>MCAT-1</td>
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<td></td>
<td>MLV-A</td>
<td>Pit-2</td>
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<td></td>
<td>ALV-A</td>
<td>TVA</td>
</tr>
<tr>
<td></td>
<td>BLV</td>
<td>BLVR</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Poliovirus</td>
<td>PVR</td>
</tr>
<tr>
<td></td>
<td>Coxasckie-B</td>
<td>CAR</td>
</tr>
<tr>
<td></td>
<td>Coxasckie-A9</td>
<td>avβ3^4</td>
</tr>
<tr>
<td></td>
<td>Major rhinoviruses</td>
<td>ICAM-1</td>
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<tr>
<td></td>
<td>Minor rhinoviruses</td>
<td>LDLR, α2MR, LRP</td>
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<tr>
<td></td>
<td>Echoviruses</td>
<td>α2β1^4, CD55</td>
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<tr>
<td>Herpesviridae</td>
<td>BHV-1</td>
<td>PVR, Prr1, HveC</td>
</tr>
<tr>
<td></td>
<td>HSV-1, HSV-2</td>
<td>Prr2, HveB, HveC, Prr1</td>
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<td>HHV-7</td>
<td>CD4</td>
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<td>Reovirus</td>
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<td>Ad-2, Ad-5</td>
<td>CAR</td>
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<td></td>
<td>Adenovirus</td>
<td>αvβ3^4, αvβ5^4</td>
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<td></td>
<td>Coronavirus-229E</td>
<td>Aminopeptidase-N</td>
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<tr>
<td>Togaviridae</td>
<td>Sindbis</td>
<td>Laminin receptor</td>
</tr>
</tbody>
</table>

1This table was adapted and modified from reference # 210.


3Abbreviations of receptors and coreceptors: BLVR, bovine leukemia virus receptor; CAR, coxsackie adenovirus receptor; CCR, CC-chemokine receptor; CR, complement receptor; CXCR, CXC-chemokine receptor; GPR, G-protein coupled receptor; Hve, herpesvirus entry protein; ICAM, intracellular adhesion molecule; JAM, junction adhesion molecule; LDLR, low density lipoprotein receptor; LRP, lipoprotein receptor related protein; α2MR, α2-macroglobulin receptor; MCAT, murine cationic amino acid transporter; MHVR, mouse herpesvirus receptor; Pit, inorganic phosphate transporter; Prr, poliovirus receptor related; PVR, poliovirus receptor; TYMSTR, T-Lymphocyte-expressed seven-transmembrane domain receptor;

4Integrins
Table 1.5. Viral carbohydrate receptors

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coronaviridae</strong></td>
<td>Human coronavirus</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td></td>
<td>Bovine coronavirus</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td><strong>Herpesviridae</strong></td>
<td>HSV, Human CMV</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td><strong>Orthomyxoviridae</strong></td>
<td>Influenza A</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td></td>
<td>Influenza C</td>
<td>9-O-acetylsialic acid</td>
</tr>
<tr>
<td><strong>Paramyxoviridae</strong></td>
<td>Sendai</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td><strong>Parvoviridae</strong></td>
<td>Canine parvovirus</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td><strong>Papovaviridae</strong></td>
<td>Murine polyomavirus</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td><strong>Reoviridae</strong></td>
<td>Reovirus-3</td>
<td>Sialic acid-containing oligosaccharides</td>
</tr>
<tr>
<td><strong>Retroviridae</strong></td>
<td>HIV</td>
<td>Heparan sulphate</td>
</tr>
</tbody>
</table>

1This table was adapted and modified from reference # 210

2Abbreviations of virus names: HIV, Human immunodeficiency virus; HSV, Herpes simplex virus; CMV, Cytomegalovirus.
receptor is not a major determinant in the evolution of virus structure within a family. Instead, viruses have evolved under different selection pressures to recognize different receptors, while maintaining the similarities in their genomes and keeping the key three-dimensional structural motifs in their capsids and receptor attachment proteins (47, 48).

1.3.2 Secondary receptors

In addition to primary receptors, several viruses use other cell surface molecules as secondary receptors. Secondary receptors can be divided into two types: 1) Initial attachment receptors and 2) co-receptors. Usually virus affinity to the secondary receptor(s) is low compared to the primary receptor (69, 79).

Initial attachment receptors for several animal viruses have been reported. In many cases, heparan sulphate, an extracellular matrix associated proteoglycan, was identified as initial attachment receptor. It has been suggested that heparan sulphate recruits large amounts of virus to the cell surface, allowing virus to find adhesion strengthening, high affinity receptors that facilitate virus entry into the cell (79, 170).

Co-receptors are often involved in post primary receptor binding. Co-receptors for several viruses have been identified. These include integrins for adenoviruses, chemokine receptors (CC and CXC variety) for HIV, SIV and poxviruses and fibroblast growth factor receptor for adeno-associated virus-2 (Table 1.4; 170). In HIV, co-receptor binding is tightly coupled with primary receptor binding. The primary receptor CD4 induces conformational changes in the virion glycoprotein gp120, so that the chemokine receptor binding site is exposed. Binding to this chemokine receptor induces further
conformational changes which facilitates virus-cell membrane fusion by exposing the fusion peptide (51).

1.3.3 Conformational changes involved in virus particles during cell entry

After binding to receptors, viruses adopt at least four major mechanisms to enter cells: 1) pH independent fusion at the cell surface, 2) pH dependent fusion in acidic endosomes, 3) receptor-mediated endocytosis and 4) receptor-mediated conformational changes in the virion at the cell surface and subsequent genome delivery (170). In all these entry mechanisms, the virus capsid or the VAP needs to undergo conformational change that allows the exposure of internally located or hidden regions of the VAP or the capsid. Conformationally restructured virions are always hydrophobic and proteolytically sensitive. In enveloped viruses, the hidden hydrophobic fusion peptide becomes exposed and lodged into the cellular membrane causing viral and cellular membrane fusion. In non-enveloped viruses, internally located N-terminal hydrophobic sequence becomes externalized leading to pore formation or destabilization of the cellular membrane (51, 48, 82, 107). Due to their hydrophobic nature, the fusion peptides of enveloped viruses and the N-terminal CP segments of nonenveloped viruses are always protected and hidden in unbound virus, so a signal is needed to expose these hydrophobic regions at an appropriate time. The trigger that causes conformational changes is either a receptor or changes in pH or ionic environment (140). For receptor induced conformational changes, a higher affinity interaction is needed between the virus and the receptor (48).
1.3.3.1 Enveloped viruses

Enveloped viruses bind to cellular receptors with their surface glycoproteins, which subsequently results in viral and cellular membrane fusion. The membrane fusion could either occur with plasma membrane at neutral pH (HIV, measles and herpes simplex viruses), or with endosomal membrane in clathrin coated pits at low pH (influenza, dengue and vesicular stomatitis viruses) (51, 130). Viral membrane fusion mechanisms are well studied in influenza and HIV. Although, these two viruses belong to two entirely different families with different physical morphologies, genome organizations and replication strategies, they share a lot of common features with regard to membrane fusion and cell entry mechanisms (51). Fusion proteins of both these viruses are trimers and synthesized as single chain precursors. Later, these are cleaved by host proteases making them metastable. These metastable fusion proteins are separated from the stable form by an energy barrier. Upon receiving the activation signal, pre-fusogenic fusion proteins undergo extensive conformational changes releasing free energy, which is used for the fusion of viral and cellular membranes (40).

The influenza virus haemagglutinin: The influenza virus envelope glycoprotein, haemagglutinin (HA), is synthesized as HA0 in infected cells and it is proteolytically cleaved to HA1 and HA2 by host proteases. HA1 and HA2 are connected by disulfide bonds. Native HA is a 135Å long trimer consisting of a globular head, made of HA1 and a stem region made of a part of HA1 and all of HA2. The globular head of HA is a distorted jellyroll structure containing a receptor (sialic acid) binding pocket at the tip (205). The stalk contains a trimeric coiled coil structure in which the amino terminal
fusion peptide is buried inside. The approximate distance between the fusion peptide in native HA and the host cell membrane is 100Å. Large conformational changes are needed in order for the fusion peptide to reach the host cell membrane. As discussed earlier, low pH is the trigger needed to convert HA from a native state to a fusogenic state. Low pH activated HA is proteolytically sensitive and hydrophobic in nature. Residues 55 to 75, which are maintained as an extended loop in native HA, are converted in a helical structure. Due to this conformational rearrangement, the central trimeric-coiled coil becomes extended, allowing the buried fusion peptide to reach the host cell membrane (51, 175).

**HIV envelope glycoprotein:** The envelope glycoprotein of HIV (gp160) is synthesized in the ER and cleaved by host proteases in the Golgi apparatus to yield gp120 (surface glycoprotein, SU) and gp41 (transmembrane glycoprotein, TM). The gp120 and gp41 are connected by noncovalent interactions. The gp120 consists of an inner domain that interacts with gp41 and an outer domain, which is exposed on the surface of the trimer. These two domains are connected by a β-sheet called the bridging sheet. The gp41 consists of an ectodomain and a membrane spanning domain. The ectodomain consists of a trimeric coiled coil structure in which the N-terminus fusion peptide is hidden (209).

Upon binding to CD4, gp120 undergoes major conformational changes involving the inner and outer domain shift. Due to this change the CD4/gp120 unit bends in such a way that it exposes the chemokine (coreceptor) receptor-binding site. Several lines of evidence show the refolding of up to 100 aa residues in gp120 upon CD4 binding (209, 40). The chemokine receptor binding induces further conformational changes in the
gp41 ectodomain, resulting in the insertion of the fusion peptide into the host cell membrane.

The trimeric coiled coil structure is a common motif found in several other viral fusion proteins and cellular vesicle fusion proteins (51).

1.3.3.2 Nonenveloped viruses

The host cell entry of nonenveloped viruses does not involve fusion, since they lack a lipid bilayer. Instead, multiple copies of one or more capsid proteins are involved in causing a pore or disruption through which viral nucleic acid or nucleoprotein complex enters the host cell cytoplasm (41, 140). Most of the nonenveloped viruses enter the host cell via receptor-mediated endocytosis (130). The activation signals needed for virus uncoating at the endosomal membrane are not well characterized in nonenveloped viruses except in picornaviruses, where the virion disassembly is triggered upon receptor interaction (82).

Virus entry mechanisms of nonenveloped viruses are well-studied in picorna (polio- and rhinoviruses) and reoviruses. Although these viruses are distinct from each other in terms of virus architecture, genome organizations and replication strategies, they share many common features with regard to entry mechanisms (41, 107).

**Picornavirus entry:** Picornaviruses are icosahedral particles 30 nm in diameter, composed of 60 protomers. Each protomer is made of four polypeptides, VP1, VP2, VP3 and VP4. The particle surface is made up of VP1, VP2 and VP3, all of which contain approximately 240 to 290 aa residues. VP4 is a shorter polypeptide (~70 aa) lying across
the inner surface of the capsid. VP2 and VP4 are cleaved products of VP0. The VP4 functions as a separated N-terminal extension of VP2, and contains a covalently linked N-terminal myristic acid group, which is predicted to play an important role in the penetration of the cellular membrane (82, 144). It is known that receptor binding sites of several picornaviruses are present in a depression at the base of the particle icosahedral 5-fold axis known as the canyon (166).

The poliovirus particle (160S), upon binding to its cellular receptor (PVR) undergoes conformational changes to convert it into a 135S “A” particle. This 135S particle is hydrophobic and proteolytically sensitive compared to the 160S native virion (59). It has been suggested that, upon receptor binding, the virus particle undergoes expansion in which the VP1, VP2 and VP3 at the particle 3-fold axis move outwardly, creating significant gaps between subunits at the base of the canyon. This movement of subunits is similar to movement of subunits during swelling of several isometric plant viruses (76, 82). During this structural transition, the N-terminal ends of VP1 and VP4 come out through the openings at the base of icosahedral five-fold axis to form an amphipathic helical bundle. Insertion of this helical bundle causes a pore, or disruption in the host cell membrane, through which viral RNA enters the cell (82, 178).

Reovirus entry: Reovirus virions are large nonenveloped icosahedral particles 85 nm in diameter. The virion architecture is complex, containing a T=1 viral core harbouring a 10 segment dsRNA genome. The viral core surface is formed by a major core protein λ1, a second core protein σ2 and a third core protein λ2. The λ2 projects from the surface at each five fold axis in the form a turret through which viral mRNA
passes into the host cell. A trimeric viral attachment protein σ1 is associated with each turret (107, 41, 150). The viral attachment protein consists of an elongated tail domain and a globular head domain. The tail domain binds to a carbohydrate receptor, sialic acid, and the globular head binds to a protein receptor, junction adhesion molecule (JAM) (57).

The viral core is layered by a membrane penetration protein μ1 and its protector protein σ3. There are 200 heterohexameric complexes of these two proteins in the form of T=13 cover over the viral core (41, 107). Reovirus undergoes a series of structural transitions during the host cell invasion: 1) shedding of σ3 due to proteolytic cleavage, converting the native virion into Inter Sub Virion Particles (ISVPs); 2) an autolytic cleavage at the N-terminal myristoylated ends of penetration protein μ1 trimer. These two events convert μ1 trimer into a proteolytically sensitive, hydrophobic, metastable structure, which is ready to penetrate the host cell membrane. Upon receiving the activation signal in the endosome, helical domains of μ1 trimer separate from each other at the particle three fold axis, inserting N-terminal myristilated ends of μ1. The insertion of μ1 in to the endosomal membrane changes λ2 turret conformation, releasing σ1 attachment protein from the particle and viral RNA is released into the cell cytoplasm (41, 107). The activation signal that is triggering these conformational changes has not yet been determined. It has been suggested that an interaction with the lipid head group or the conditions in the lumen of the endosome could act as possible triggers (107).

1.4 Cucumber necrosis virus

Cucumber necrosis virus was first identified in 1952 on greenhouse cucumber plants in southern Ontario, Canada (122). Infected plants showed a marked malformation
of leaves and systemically infected leaves exhibited chlorosis and necrosis. The number of fruits produced from an infected plant is greatly reduced and fruits showed an occasional conspicuous green mottling. Under experimental conditions, only 10 to 20% of CNV infected plants showed systemic infection (122). Although the natural host range of CNV is very narrow, its experimental host range includes several dicotyledonous plants.

Initially, CNV was thought to be a member of the Necrosis virus genus, but later Rochon and Tremaine (156) reported that it belongs to the tombusvirus group. CNV is a spherical virus 30 nm in diameter that contains a monopartite single-stranded, positive sense RNA genome comprised of approximately 4,700 nt. CNV is serologically distinct from other tombusviruses probably due to the variable nature of the protruding domains of tombusvirus coat proteins (156). CNV is the only definitive member of the tombusvirus genus that is transmitted by a soil-borne fungus, but several other viruses that belong to the family Tombusviridae are transmitted by fungi (Table 1.2)

1.4.1 Genomic organization

The entire genome of CNV has been cloned and sequenced (157). The genome contains five long open reading frames (ORFs) encoding 33, 92, 41, 21 and 20 kDa proteins (Figure 1.3). The 92 kDa protein is produced via translational readthrough of the UAG stop codon of ORF1, which produces a 33 kDa protein. Two 3' co-terminal sub genomic RNAs (sgRNA1 and sgRNA2) of sizes 2.1 and 0.9 kb are produced from CNV RNA during infection. SgRNA1 serves as a template for translation of the 41 kDa protein from ORF3. sgRNA2 is a bifunctional mRNA encoding 21 and 20 kDa proteins from
**Figure 1.3.** Genomic organization of CNV. The five open reading frames (ORFs) present on the CNV genome and the sizes of their corresponding proteins are indicated. The 33 and 92 kDa proteins are produced from genomic length RNA. Subgenomic RNAs 1 and 2 serve as templates for the 41 and 21/20 kDa proteins, respectively. Subgenomic RNA 2 is bicistronic wherein expression of the 20 kDa protein occurs via a leaky scanning mechanism (91).
distinct overlapping open reading frames (ORFs 4 and 5 respectively) (159). Expression of the ORF for the 20 kDa protein occurs via a leaky scanning mechanism (91).

1.4.2 Functions of CNV encoded proteins.

**p33.** p33 is proposed to be involved in viral replication based upon the data available on ORF1 products of tombusviruses (162). In several other tombusviruses, p33 along with p92 have been proposed to be a part of the replication complex and are known to be associated with membranes (172). The level of accumulation of p33 is 20 fold more than that of p92 in TBSV-infected plants and protoplasts (172). The p33 protein encoded by Cymbidium ring spot virus (CymRSV) has been shown to localize to peroxisomal membranes and a 7 kDa segment of p33 has been suggested to contain the peroxisome membrane targeting signal (126).

**p92.** p92 is produced as a result of translational readthrough of the p33 stop codon. Initial amino acid sequence comparisons by Rochon and Tremaine (157), showed that CNV p92 contains the glycine-aspartate-aspartate (GDD) motif, which is characteristic of the RNA dependent RNA polymerases (RdRp) of several positive-stranded RNA viruses (94, 157). Recently Nagy and Pogany (124) have isolated and characterized the CNV RdRp and showed that CNV p92 does indeed contribute to viral replicase activity. In addition, an arginine/proline rich sequence termed the RPR motif was found to be important for viral RNA replication (129).

**p41.** Amino acid sequence comparisons and immunoprecipitation studies have shown that p41 is the coat protein (CP) (89, 157). A coat protein mutant (PD-) which lacks the sequence coding for the CP protruding domain (PD) was found to replicate well
and move systemically on mechanically inoculated *N. benthamiana* plants. Neither CNV virions nor CP subunits were found in PD (-) infected plant tissues due to further deletion of the CNV CP ORF (120). Frameshift and deletion mutations in the protruding domain were also proven to be deleterious to particle formation (173). These studies demonstrate that the CNV P domain is important for virus assembly probably by promoting CP dimer formation, which is an important initial step in the assembly of several T=3 icosahedral viruses (77, 142, 177, 179). CNV CP was also shown to be important for fungus transmission (for details see Section 1.2.4.3.1). For details on the CNV CP subunit and particle structure, see Section 1.5.4.

**p20 and p21.** A CNV mutant, which does not express the p20 gene showed highly attenuated symptoms on mechanically inoculated plants even though levels of RNA replication in *N. clevelandii* indicating that p20 has a major influence on symptom induction (159). Recently it has been shown that the equivalent protein of other tombusviruses (p19) acts as a suppressor of gene silencing (173). More recently, CNV p20 has also been shown to act as a suppressor of gene silencing (Rochon and Yu Xiang, personal communication). CNV p21 is a product of ORF4 and is essential in cell-to-cell movement (90). The manner in which p21 facilitates viral movement has not yet been elucidated.

### 1.4.2 Defective interfering RNAs (DI RNAs) in CNV

Defective interfering (DI) RNAs are frequently found in tombusvirus-infected plants (146, 162). DI RNAs are naturally occurring deletion mutants of the viral genome which are unable to replicate in the absence of viral genome. It is believed that DI RNAs
are produced as a result of errors made during replication of the viral genome. DI RNAs obtained during CNV infections have been isolated and characterized. CNV DI RNAs retained 5’ untranslated and 3’ terminal regions along with small portion of the ORF for the 92 kDa replicase protein (55). A CNV mutant that does not express p20 has been shown to generate DI RNAs rapidly (158). Moreover, CNV coat protein is not required for the efficient generation of DI RNAs (161).

1.4.4 Structural aspects of CNV

The CNV coat protein subunit consists of 380 aa. Putative structures of the CNV coat protein subunit, asymmetric unit and particle have been obtained by homology modelling (see Chapter 2; 93a) based on the crystal structure of the closely related Tomato bushy stunt virus, TBSV (74). The CNV subunit folds into three distinct domains termed the RNA binding (R; 58 aa), shell (S; 167 aa) and protruding (P; 116 aa) domains. The R and S domains are connected by a 34 aa arm, and the S and P domains are connected by a 5 aa hinge (Figure 1.4). The R and arm domains are located internal to the particle; the S domain forms the shell of the particle and the P domain projects outward from the shell. The P domain interacts in pairs across the two-fold axis to form protrusions on the surface (Figure 1.7).

The CNV particle, by analogy to TBSV and several other plant viral capsids is arranged in the form of T=3 icosahedral symmetry. The 180 CP subunits that comprise the T=3 structure are chemically identical, but in order to satisfy quasi-equivalence requirements, subunits adopt slightly different three-dimensional structures. These conformationally
Figure 1.4. Linear and three dimensional structure of the CNV CP subunit (C-type). (A) The different CP structural domains are depicted in different colors and are designated as follows: RNA binding (R, white); arm (a, yellow); shell (S, blue); hinge (h, red); and protruding (P, gray). The number of amino acids in each domain are indicated below individual domains. Also shown are flanking sequences in the CNV genome. (B) Surface representation of the homology modelled CNV subunit (C-type). The different structural domains are represented in the same colors as in (A). The disordered R domain is not shown.
Figure 1.5. Ribbon diagrams of homology modelled CNV CP subunits and their location on the particle icosahedral axes. I) A subunit (red), II) B Subunit (blue) and III) the ordered C subunit (green). IV) Subunits A, B and C constitute the icosahedral asymmetric unit (Q3). Pentamers of A subunit are located on the particle icosahedral five-fold axis (5) and B and C subunits constitute the hexamers at the icosahedral three-fold axis (3). The C/C subunits interact at the icosahedral two fold axis (2). The oligomer was generated using the oligomer generator at: http://mmtsbg.scripps.edu/viper/util.php (149). Images of the CNV CP subunit and oligomer were manipulated using WebLab Viewer Lite software (Molecular Simulations, Inc.).
Figure 1.6. Surface representation of the CNV β-annulus at the particle 3-fold axis viewed from the interior of the capsid. The B and C-type subunits are coloured in gray and white respectively. The extended arms of C-type subunits are depicted in red, blue and yellow for the purpose of clarity.
Figure 1.7. Structure of the CNV particle. A, B and C-type subunits are represented in red, blue and green respectively. The different icosahedral axes are labelled as 5 (five-fold), 3 (three-fold), 2 (two-fold) and Q3 (quasi three-fold). (A) Diagrammatic representation of the CNV particle. The cutaway section shows the region that the disordered R domain may occupy in the particle interior. This diagram was adapted from reference # 74. (B) Surface representation of homology modeled CNV particle. The oligomer was generated using the oligomer generator at: http://mmtsbc.scripps.edu/viper/util.php (149). The image of the CNV particle was manipulated using Web Lab Viewer Lite software (Molecular Simulations, Inc.)
by Harrison et al (74). The A and B subunits are identical but the C subunit acquires a slightly different hinge conformation so that the S and P domains are quite differently oriented. In addition, the arm region is ordered in the C subunit but is disordered in the A and B subunits. The ordered 34 aa C subunit arm is divided into an 18 aa ‘β’ and 16 aa ‘ε’ regions. The ordered arms make a U-turn, fold along the inner edge of the shell domain, and extend towards the particle 3-fold axis. The β regions of the three-fold symmetry related C subunits inter-digitate to form a 54 aa annular structure called the β-annulus. The β-annular network of all C subunits forms a scaffold inside the particle, which is believed to give stability to the particle (Figure 1.6).

In the CNV particle there are 12 icosahedral five-fold axes (a total of 60 copies of the CP subunit) consisting of pentamers formed from A subunits. The B and C subunits interact at the icosahedral three-fold axis to form 20 hexamers (120 copies). As in other T=3 viruses, the icosahedral asymmetric unit consists of A, B and C subunits, which interact at the particle quasi three-fold axis (Figure 1.5 and 1.7).

1.5 Role of N-terminal arm in T=3 icosahedral plant viruses

In T=3 icosahedral viruses, the arrangement of CP subunits follows quasi-equivalent interactions as suggested by Casper and Klug (36). To achieve this quasi-equivalence, subunits in virus shells adopt slightly different conformations. These different conformations are often obtained with the help of segments of the CP polypeptide acting as molecular switches (78, 85). In several small plant and insect RNA viruses these molecular switches are N-terminal segments that are ordered in only one or
two of the three independent subunits. In some animal viruses, like polyomavirus, the C-terminal segment acts as a molecular switch (180). In the case of Flock house virus (FHV), an insect virus, the N-terminal 20 to 30 aa of the C subunit along with the RNA acts as molecular switch (56, 85). In several plant viruses the N-terminal segments contain basic aa residues which are shown to be important in RNA binding and assembly (44, 109, 167, 143). Deletion of this N-terminal ordered arm in many T=3 plant viruses has resulted in T=1 particles devoid of RNA (52, 109, 169).

Crystal structures of several T=3 plant viruses have been determined (1, 35, 74, 97, 98, 110, 127, 177, 179). In several of these viruses, it is evident that a part of the internally located N-terminal region of the coat protein subunit is ordered only in C, or B and C subunits. A part of this ordered N-terminal segment is involved in an annular network of β-strands at the particle three-fold axis. The interaction of ordered N-terminal segments at the particle three-fold axis is one of the common structural themes found in T=3 plant viruses (86). In the case of many tombus-, sobemo- and necroviruses only the C subunit arms are ordered and they interact at the particle three-fold axis to form the β-annulus. In tymo- and bromo viruses, N-terminal segments of both the B and C subunits are ordered and interact with each other at the particle three-fold axis to form an annular structure called the β-hexamer.

It is believed that assembly of several T=3 icosahedral viruses starts from dimers of the coat protein subunit (77, 142, 177, 179). An individual dimer can exist in two conformational states: one where the arms are ordered and dimer structure is flat and the other where the arms are disordered and the dimer structure is curved. In the particle the former structure is designated as the C/C dimer and the latter is called the A/B dimer.
Harrison and his co-workers (77, 177) have proposed that assembly of TBSV and TCV initiates by the formation of the $\beta$-annulus from a trimer of the C/C subunit dimers. Further assembly occurs by the addition of the dimers (either C/C or A/B) with no further distinct intermediate structures. As dimers are being added onto the initiation structure, the N-terminal arm of the C subunit will predetermine the structural state of the incoming dimer (77). Thus the local rules theory (17) applies here wherein the conformation of the incoming protein unit is determined by the structure of the protein to which it attaches. Similarly, in the case of bromo-and tymoviruses, it has been proposed that the $\beta$-hexamer may be the initiation structure during assembly (35, 87, 179). Recent work on Flock house virus (FHV), Cowpea chlorotic mottle virus (CCMV) and Physalis mottle virus (PhMV) has shown that the ordered N-terminal segment or $\beta$-annular structure is not necessary for virus assembly (49, 168, 206). These studies suggest that virus assembly could be initiated from the 5-fold axis by a pentamer of the A/B dimers (211). Although it seems that the $\beta$-annular structure and order/disorder mechanism is not required for correct assembly, the evolutionary conservation of these structures in several T=3 plant viruses suggests that they might still be important in making the assembly process more efficient and stabilizing the assembled virion (106).

1.6 Virus particle dynamics in T=3 icosahedral plant viruses

Both animal and plant viruses are highly flexible in solution and have a propensity to undergo conformational changes under physiological conditions (88, 207). In the case of several animal viruses it has been shown that this ability to undergo
conformational changes plays an important role in the process of infection (82, 107, 175). Recently we have shown that conformational changes that are occurring in CNV upon binding to Olpidium zoospores play a key role in its vector-mediated infection (93). In many viruses it has been demonstrated that internally located CP polypeptide segments are occasionally exposed to the outer surface, indicating the dynamic nature of viruses in solution (23, 88, 104).

Several plant virus capsids contain divalent cations, which play an important role in maintaining the structural integrity of the virus. X-ray crystal structures of several plant viruses have revealed the presence of calcium or magnesium ions between the subunits (105, 110, 127). In TBSV, there are two calcium ions per subunit which link neighbouring subunits in the CP asymmetric unit (74). These calcium ions interact with carboxylate groups from the side chains of glutamate and aspartate residues. It has been suggested that an important function of these ions is to provide a means of disassembly during the initial stages of the infection process (105). Many small spherical plant viruses undergo a reversible change in their structural conformation in the presence of metal chelating agents at elevated pH (207). This process is often referred to as swelling. During this process, the metal ions are chelated and due to the repulsion between negatively charged aspartate residues, the particle undergoes expansion. Incardona and Kaesberg (84) first observed this dynamic behaviour in BMV. Later Bancroft and co-workers studied this phenomenon extensively in CCMV (11) The crystal structure of the swollen form of TBSV (152) has revealed that during expansion the particle opens at the quasi three-fold axis between A, B and C subunits (where calcium ions are located). There are 60 openings appearing in the particle and each opening is about 18 to 20 Å in
diameter. The hexamers along with the β-annuli and the pentamers essentially remain structurally unchanged due to their extensive interactions with other CP subunits thus preventing complete dissociation of the swollen particle (88). In addition, contacts between A/B subunits at the particle quasi two-fold axis are strengthened due to the formation of a new β-strand resulting from a six residue ordering in A and B subunits (152). As a result, in the expanded structure, the icosahedral two-fold axis and quasi two-fold axis become more similar. Moreover, the protruding domains of C/C subunits are rearranged due to structural changes occurring elsewhere in the virus. It has also been shown that a part of the internally located R and arm domains of the A and B subunits are extruded through the large openings becoming exposed on the outer surface of the particle and making swollen virus proteolytically sensitive (63, 75, 93). The atomic structure of the expanded form of CCMV has also been determined (179) at 28 Å by X-ray crystallography and cryo electron microscopy. Although the compact form of TBSV and CCMV differ significantly, the expanded structures share a relatively higher degree of similarity. This indicates common structural transitions occurring during expansion of T=3 plant viruses (88). It is possible that in solution, the unfolded A and B arms may “breath out” of the virion only occasionally (207) but, the addition of EDTA at higher pH favours the equilibrium towards the conformation in which arms are exposed more frequently. This also means that most of the T=3 plant viruses where expansion has been reported are probably programmed to undergo this conformational transition in nature.

As mentioned earlier, the swollen conformation of plant viruses has been implicated in virion disassembly during the initial stages of the infection process (5, 27, 28, 152). But, Albert et al. (5) reported that a CCMV mutant, which is deficient in
swelling *in vitro*, is as infectious as the wild type virus indicating that, in this case swelling may not be required for disassembly. We have recently demonstrated that the swollen-like conformation of CNV is important in the vector transmission by zoospores of the fungus *O. bornovanus* (93).

### 1.7 Summary of Thesis Objectives

The overall goal of this thesis is to characterize the molecular determinants (viral and vector) required for CNV transmission by zoospores of its vector *O. bornovanus*. The thesis objectives are divided into three parts.

1. To identify specific sequences and regions on the CNV coat protein and capsid that are important for fungus transmission.

   **A. Analysis of naturally occurring CNV transmission-deficient mutants.** Serial mechanical passage of plant viruses in the absence of vector often results in mutants which have lost their ability to be transmitted by their vector. This approach was successfully employed by Robbins *et al* (153) to isolate naturally occurring transmission-deficient CNV mutants. In one such mutant LL5, it was found that a single aa substitution in the shell domain was responsible for inefficient zoospore binding and transmission. The same approach and methodology was followed to further characterize regions on the CNV capsid that are important for fungus transmission.

   **B. Location of mutated amino acids on homology-modelled CNV subunit and capsid.** CNV is structurally closely related to *Tomato bushy stunt virus*. Three-
dimensional structures of the CNV CP subunit, asymmetric unit and particle were obtained by homology-modelling in order to locate the position of mutated amino acids of CNV transmission mutants on the CNV subunit and capsid. Results of these studies are described in Chapter 2.

C. Site-directed mutational analysis. Site-specific in vitro mutagenesis of the CNV CP subunit was conducted to identify CP regions that are important for fungus transmission focussing on regions that were previously identified as being important for transmission (see Chapter 2; 92a). In addition, four prolines in the arm domain and two prolines in the hinge were also mutated to determine the importance of the arm and the hinge in CNV transmission. Results of these experiments are described in Chapters 4 and 5.

2. Biochemical characterization of putative receptor(s) for CNV on O. bornovanus zoospores.

A. Pre-treatment of zoospores with periodate, trypsin and phospholipase C.

Previous studies in our laboratory (154) suggested the presence of specific recognition molecules (receptors) on the surface of O. bornovanus zoospores for CNV. In order to determine the basic biochemical nature of putative receptor(s), zoospores were treated with trypsin, sodium periodate and phospholipase C and used in in vitro binding assays using CNV.

B. Virus overlay assays. CNV overlay assays using total zoospore proteins were conducted in order to determine whether any zoospore protein(s) or glycoprotein(s) are involved in CNV attachment.
C. Sugar inhibition studies. Previous work in our lab (154) showed that the surface of *O. bornovanus* zoospores contains mannose-/glucose- and fucose- containing sugars. Sugar inhibition studies were conducted using virus overlay assays and enzyme-linked zoospore binding assays to determine the role of different sugars in CNV binding to zoospores. The results of the above experiments were described in Chapter 2.

3. To determine if zoospore-bound CNV is conformationally different from unbound virus and whether conformational changes are important for vector transmission of CNV.

Several animal virus particles undergo conformational changes as apart of their entry into the host cell. Several plant viruses undergo swelling in the presence of EDTA at alkaline pH. Experiments therefore were conducted to assess the possible significance of swelling in CNV attachment to zoospores. Protease digestion experiments using zoospore-bound, swollen and unbound (native) CNV were conducted to determine if CNV undergoes conformational changes upon binding to zoospores and whether conformationally altered zoospore-bound CNV is structurally similar to swollen CNV.

Two highly conserved prolines in the arm region of CNV CP that are predicted to be important for particle conformation were mutated to determine if conformational changes in CNV particles are important for fungus transmission. The results of these experiments are described in Chapter 4.
1.8 References


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91. **Johnston, J. C., and D. M. Rochon.** 1996. Both codon context and leader length contribute to efficient expression of two overlapping open reading frames of a cucumber necrosis virus bifunctional subgenomic mRNA. Virology **221**:232-239.


CHAPTER TWO

Identification of Specific Cucumber Necrosis Virus Coat Protein Amino Acids Affecting Fungus Transmission and Zoospore Attachment*

2.1 Introduction

Efficient transmission of the majority of plant viruses requires distinct invertebrate or fungal vectors. In most cases, transmission has been shown to be a highly specific process in which only certain vectors can transmit certain viruses (for reviews, see references 4, 6, 13, 14, 23, 35). These observations suggest that virus particles as well as vectors contain specific sites that mediate their recognition. The coat protein (CP) of a plant virus has been shown to play an important role in transmission, and particular amino acids within the CP have been shown to be essential for this process (for reviews, see references 4, 6, 13, 14, 23, 35). However, for the most part, the exact role of these amino acids in transmission including their potential role in vector attachment, is not known. Recent work with cucumber necrosis virus (CNV) has suggested that attachment of virions to vector zoospores is an important aspect of the transmission process (24).

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CNV, a member of the genus *Tomusvirus*, is a 30-nm spherical virus with a monopartite positive-sense RNA genome (25). Transmission of CNV in nature occurs via zoospores of the *Chytridiomycete* fungus, *Olpidium bornovanus* (6, 9, 24). Zoospores and virus particles are released independently into the soil from the roots of infected plants. Virus is adsorbed onto the plasma membrane of zoospores and then enters into roots upon zoospore encystment. Studies of CNV transmission by *O. bornovanus*, and *Olpidium* transmission of several other small spherical plant viruses, have shown that the transmission process is highly specific (1, 6). For example, *O. bornovanus* transmits CNV but not *Tobacco necrosis necrovirus* (TNV), and conversely, *O. brassicae* transmits TNV but not CNV (10, 34). Moreover, different isolates of *O. bornovanus* transmit different viruses with varying efficiency (7), and different strains of TNV are transmitted with varying efficiency by the same *O. brassicae* isolate (17, 33, 34). Electron microscopy studies have shown that adsorption of virus to the zoospore plasmalemma is specific and reflects the virus-vector associations observed in nature (34). Together, these studies indicate the existence of a specific recognition mechanism between virus and vector zoospores.

Previous work has shown that the CNV CP contains determinants that specify its interaction with zoospores of *O. bornovanus* (20, 24). Reciprocal exchanges between the CP gene of CNV and that of the nontransmissible cherry strain of *Tomato bushy stunt virus* (TBSV) in infectious full-length cDNA clones showed that particles obtained from the TBSV genome containing the CNV CP were transmissible but particles from the CNV genome containing the TBSV CP were not. Also, a single amino acid mutation
(Glu to Lys) in the CNV CP shell domain results in lowered transmission efficiency of CNV by *O. bornovanus*. *In vitro* binding studies demonstrated that this mutant bound zoospores less efficiently than CNV, indicating that specific regions of the CNV coat protein can mediate zoospore adsorption (24). In this study, we have isolated and characterized several distinct naturally occurring CNV transmission mutants. In each mutant, transmission deficiency was found to be due to a single amino acid substitution in the CNV CP. Moreover, each transmission mutant bound zoospores less efficiently than CNV, suggesting that the altered amino acids affect features of the CNV capsid involved in vector attachment.

### 2.2 Materials and Methods

#### 2.2.1 Isolation of CNV transmission mutants

CNV transmission mutants were obtained following serial passage of virus essentially as described previously (24) except that cucumber cotyledons were used as the local lesion host for isolation of individual mutants.

#### 2.2.3 Virus purification

A miniprep procedure (24) similar to the procedure described below was employed to partially purify CNV and CNV mutants for use in the initial screenings for transmission mutants. For all other experiments, virus was purified by differential centrifugation as follows. Infected leaves were ground in 2 volumes of 100 mM sodium acetate (pH 5.0) containing 5 mM β-mercaptoethanol and allowed to stand on ice for 30 to 60 min. The slurry was then filtered with Miracloth (Calbiochem) and centrifuged at 8,000 g in a GSA rotor. The supernatant was adjusted to 8% polyethylene glycol
(molecular weight, 8,000; Sigma) and stirred at 4°C for 1 to 2 h. Virus was pelleted by centrifugation at 8,000 g in a GSA rotor, resuspended in 10 mM sodium acetate (pH 5.0), and subjected to high-speed centrifugation (145,000 g for 2.5 h in a Ti 50.2 rotor) at 4°C. Virus pellets were resuspended as before and centrifuged at 20,800 g in an Eppendorf microcentrifuge. The supernatant was collected and passed through a 0.2-μm-pore-size filter. Concentration of virus was determined spectrophotometrically using an extinction coefficient of 4.5 (absorption of 4.5 at 260 nm is equal to one milligram of virus in a millilitre). The concentration of virus purified by the miniprep procedure was determined by electrophoresis of several dilutions of virions through 1% agarose gels buffered in 45 mM Tris-45 mM borate, (pH 8.3) followed by ethidium bromide staining in buffer containing 1 mM EDTA. Dilutions of purified virus with known concentrations was used as standard for determining the mass of mini-prepped virus loaded on the gel.

2.2.4 Fungus transmission assay

Purified virions were tested for transmission by *O. bornovanus* zoospores as previously described (5, 7, 20). Virus (1 μg) was incubated with 10 ml of zoospores (10⁴/ml in 50 mM glycine, pH 7.6). After a 15-min acquisition period, the mixture was poured onto pots containing 12- to 16-day-old cucumber seedlings. Five days later, roots of cucumber seedlings were tested for the presence of virus by double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) using polyclonal antisera raised to CNV particles (20). Absorbance readings greater than fivefold over background (i.e., 0.1 at A₄₀₅) were considered positive. Each transmission experiment included a wild-type (WT) CNV control, a test to determine any background level of CNV transmission in the absence of zoospores and a test for the presence of contaminating virus in zoospore.
preparations. Transmission in the absence of zoospores was not detectable in any of the experiments.

2.2.5 Cloning and sequence analysis of transmission mutants

Double-stranded cDNA copies of the CP coding regions of transmission mutants were obtained by reverse transcription-PCR (RT-PCR) (30). The template was total RNA extracted from either infected leaves or purified virus particles. The plus-sense primer (CNV oligonucleotide 81, 5'AAGAGGTTGAATTCTGTCAGG3') corresponded to CNV nucleotides 2148 to 2168 upstream up the CNV CP open reading frame (ORF) and included a unique EcoRI site (underlined). The minus-sense primer (CNV oligonucleotide 7, 5'TGTTCCCTAGCGTCGC3') corresponded to the complement of CNV nucleotides 3854 to 3869 and lies downstream of the CP ORF. Following amplification, the RT-PCR product was digested with EcoRI and NcoI (both enzymes cut at regions flanking the CP ORF) and ligated into similarly digested pK2/M5, a full-length cDNA clone of WT CNV (26). The sequence of the transferred region of each transmission mutant was determined by cycle sequencing using dye-labeled terminators and AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems). Samples were sequenced using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

The double mutant LL5K8 was prepared by digestion of LLK8 with BglII and NcoI (which cleave at unique sites surrounding the LLK8 mutation) followed by insertion of the gel-purified fragment into BglII/NcoI-digested LL5 (24). The presence of both the LLK8 and LL5 mutations was verified by sequence analysis.

2.2.6 In vitro transcription and inoculation of plants
Preparation of T7 polymerase runoff transcripts and inoculation of plants were as described previously (26).

2.2.7 In vitro binding assay

The assay used was a modification of the one described by Robbins et al. (24). One hundred micrograms of purified virus was incubated with $5 \times 10^5$ *O. bornovanus* zoospores in 1 ml of 50 mM sodium phosphate buffer (pH 7.6) for 1 h. Following incubation, zoospores were pelleted by centrifugation at 5,000 rpm for 7 min in an Eppendorf microcentrifuge. The pellet was washed with 1.5 ml of binding buffer and then resuspended in sterile water. The zoospore pellet was assayed for the presence of virus by either Western blot or slot blot analysis using CNV monoclonal antibody 57-2 (24) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The quantity of virus in the pellet was determined by densitometric analysis of exposed film using the ImageQuant program (Molecular Dynamics). The amount of CNV that pelleted in the absence of fungus was subtracted from the amount of CNV that pelleted in the presence of fungus. Antibody 57-2 was confirmed to react equally to WT virus and mutants in slot blot analysis using denatured virus.

2.2.8 Homology modelling

The three-dimensional coordinates of the CNV proteins were modeled after the published coordinates of TBSV, a virus with a known similar structure (PDB entry 2TBV) (22). The virus has icosahedral symmetry with three, independent quasi-equivalent structural positions, A, B, and C. Each protein was modeled after its cognate structural homolog with the program Modeler (29) on a Silicon Graphics computer.
Images of the modeled CNV subunit and trimer were manipulated using WebLab ViewerLite software (Molecular Simulations, Inc.). Surface representations were obtained using the "solvent surface" option. The CNV-TBSV alignment was from a multiple alignment using the CPs of several members of the *Tombusviridae*, including artichoke mottled crinkle virus (PIR2:S24926), carnation Italian ringspot virus (PIR2:S52718), cucumber leafspot virus (21), cymbidium ringspot virus (PIR1:VCVGC), melon necrotic spot virus (PIR1:VCVEMN), pelargonium leaf curl virus (PIR1:A48355), pothos latent virus (SP_VI:Q84832), type TBSV PIR2:S07259, and the cherry strain of TBSV (PIR1:VCVGTB). The program Pileup (version 10.1; Genetics Computer Group) (8) was used to create the multiple alignment.

### 2.3 Results

#### 2.3.1 Isolation of transmission mutants from mechanically passaged CNV

CNV was mechanically passaged 12 times through *Nicotiana clevelandii*, and individual local lesions were isolated following inoculation of cucumber cotyledons. The CP ORFs and flanking regions of six putative transmission mutants (as determined by reduced transmissibility [data not shown]) were amplified by RT-PCR and cloned in place of the WT CNV CP ORF in an infectious CNV cDNA clone. The cloned region was then sequenced to determined the presence of mutations. Transcripts of each of the clones were inoculated onto plants, and purified virus from infected plants was tested for transmissibility. Of 87 local lesions analyzed, 7 were ultimately found to contain virus with reduced transmission. Results of the transmission tests (Table 2.1) show that cloned mutants designated LLK8, LLK10, LLK63, LLK82, LLK84, and LLK85 were less
Table 2.1. Transmission and *in vitro* binding efficiencies of CNV mutants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Transmission¹</th>
<th>% Binding²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CNV</td>
<td>49/51 (96)</td>
<td>100.0</td>
</tr>
<tr>
<td>LLK00</td>
<td>10/10 (100)</td>
<td>130.0 ± 8.5</td>
</tr>
<tr>
<td>LLK8</td>
<td>3/14 (21)</td>
<td>68.3 ± 17.5</td>
</tr>
<tr>
<td>LLK10</td>
<td>4/15 (27)</td>
<td>39.0 ± 22.3</td>
</tr>
<tr>
<td>LLK26</td>
<td>1/10 (10)</td>
<td>ND</td>
</tr>
<tr>
<td>LLK63</td>
<td>3/21 (14)</td>
<td>20.8 ± 17.2</td>
</tr>
<tr>
<td>LLK82</td>
<td>15/20 (75)</td>
<td>64.3 ± 24.1</td>
</tr>
<tr>
<td>LLK84</td>
<td>10/20 (50)</td>
<td>53.0 ± 7.5</td>
</tr>
<tr>
<td>LLK85</td>
<td>19/25 (76)</td>
<td>53.0 ± 12.3</td>
</tr>
<tr>
<td>LL5K8</td>
<td>0/8 (0)</td>
<td>21.7 ± 6.6</td>
</tr>
</tbody>
</table>

¹ Number of pots showing transmission/number of pots tested. Values in parentheses indicate percentages of pots showing transmission. The data represent a compilation of at least three separate experiments for each virus.

² Percentage of virus bound in an *in vitro* binding assay relative to the amount of WT CNV binding (average ± standard deviation of three separate experiments for each mutant). ND, not determined.

transmissible than WT CNV (transmission efficiency, 96%). LLK8, LLK10, and LLK63 transmitted at lower efficiencies (i.e., 21, 27, and 14%, respectively), whereas LLK82, LLK84, and LLK85 transmitted at higher efficiencies (75, 50, and 76%). An uncloned mutant (LLK26) also transmitted with reduced efficiency (10%). Sequence analysis of LLK26 showed that it is identical to LLK8 (see below).
We wished to examine the infectivity and level of accumulation of each mutant in order to determine whether the reduced transmission efficiency was due to reduced ability of virus to accumulate in plants following transmission. LLK8, LLK10, LLK63, LLK82, LLK84, and LLK85 virions were inoculated onto *N. clevelandii*, and plants were monitored for symptoms and for RNA and virion accumulation. All mutants produced symptoms typical of WT CNV on *N. clevelandii*, resulting in large necrotic lesions on inoculated leaves and subsequent systemic necrosis and death of the plants within 10 to 14 days postinoculation (dpi) (data not shown). Agarose gel electrophoresis of total RNA extracts of infected plants at 3 dpi indicated that each mutant accumulated to approximately the same level as WT CNV (Figure 2.1). In addition, in three separate experiments, DAS-ELISA of leaf extracts at 5 dpi indicated that, on average, virions of LLK8, LLK10, LLK63, LLK82, and LLK85 accumulated to approximately the same level as WT CNV (data not shown). LLK84 virions accumulated to approximately 50% of the WT CNV level. All mutants were also capable of infecting cucumber and produced equivalent-sized necrotic lesions on inoculated cotyledons (data not shown). In addition, virion accumulation in cucumber was monitored by agarose gel electrophoresis, and all mutants, including LLK84, accumulated to approximately the same level as WT CNV (data not shown). The integrity of virus particles used for transmission tests was also assessed. Virus particles of each of the transmission mutants were analyzed by agarose gel electrophoresis and found to migrate as discrete bands (Figure 2.2). LLK8 and LLK10 particles comigrated with WT CNV, whereas particles of LLK63 and LLK84 migrated
Figure 2.1. Agarose gel electrophoresis of total leaf RNA extracts from plants infected with CNV transmission mutants. *N. clevelandii* plants were inoculated with equal amounts of WT CNV or the indicated mutant virions, and total RNA was extracted from inoculated leaves 3 dpi. Equal amounts of total RNA were loaded onto a nondenaturing 1% agarose gel. The gel was stained with ethidium bromide.
Figure 2.2. Agarose gel electrophoresis of particles of CNV fungus transmission mutants. The indicated viruses (500 ng of each) were electrophoresed through a 1% agarose gel buffered in Tris-borate (pH 8.3). Virions were visualized by ethidium bromide staining in the presence of 1 mM EDTA.
slightly slower than WT CNV and those of LLK82 and LLK85 migrated faster. The greater mobility of LLK82 and LLK85 particles is likely due to the higher net negative charge of the mutated CP (Gly to Glu and Asn to Asp, respectively [see below]). The basis for the slightly slower mobility of LLK63 and LLK84 is not known, but possibly these particles have a slightly expanded conformation, as previously suggested for the CNV LL5 transmission mutant (24). The ability of LLK8, LLK10, LLK63, LLK82, and LLK85 to accumulate to approximate WT levels in infected plants suggest that factors other than transmissibility do not likely contribute substantially to their reduced transmission frequencies. DAS-ELISA values for LLK84-infected leaves were approximately twofold less than that of WT CNV. As discussed below, it is possible that the lower accumulation of LLK84 may contribute to the lower transmission frequency of this mutant.

2.3.2 Mutations in CNV transmission mutants map to either the CP shell or protruding domain

Based on the structure of the related TBSV CP, the CNV CP contains three major structural domains: the R domain, which in the capsid faces the interior; the S domain, which forms the shell of the capsid; and the P domain, which projects outward from the capsid. The linear arrangement of these domains on the CNV CP as well as their predicted structures in the particle subunit and capsid are shown in Figure 2.3D. The CP ORFs as well as flanking sequences used in the construction of cloned transmission mutants described above were sequenced to determine the location and nature of the mutations responsible for the reduced fungus transmission (Figure 2.4). In addition to the unique mutations present in each clone, all transmission mutants also contained a T-to-G
Figure 2.4. Locations of mutations in CNV fungus transmission mutants. The portion of the mutant genome analyzed for mutation is shown. EcoRI and NcoI restriction enzyme sites used for cloning the mutant CP gene and flanking sequences are indicated for WT CNV. R, a, S, h, and P correspond to the different structural domains of the CNV CP (Fig. 3D). p92, p20, and p21 indicate flanking CNV ORFs. The two mutations present in the transmissible LLK00 and in all CNV transmission mutants are shown by asterisks and are described in detail for LLK00. Mutations in LL5 are also shown. LL5 was made by in vitro mutagenesis of our WT CNV infectious clone and does not contain the two substitutions present in the other mutants. Details of mutations including nucleotide position in the CNV genome, nucleotide change, amino acid position in the CNV CP, and amino acid change are given for each mutant.
mutation at CNV nucleotide 2824, which results in a Phe-to-Cys change at amino acid position 66 in the CP arm domain, and a silent G-to-T mutation at nucleotide 3674 in the coding region of the CP protruding domain (LLK00 [Figure 2.4]). These same mutations were noted in the previously described CNV transmission mutant LL5 (24), and studies ruled out any effect of the amino acid substitution in the arm domain mutation in the low transmission efficiency of LL5. In addition, these studies showed that the LL5 shell domain mutation was sufficient to induce the loss of transmissibility. To determine if the arm and protruding domain mutations together affect CNV transmission, particles produced from transcripts of a cDNA clone containing only these two mutations (LLK00) were tested for transmission. The results (Table 2.1) demonstrated that these mutations do not affect transmission efficiency. Subsequent sequence analysis of two other CNV clones from passaged virus showed that both mutations were present in both clones (data not shown). Therefore, it appears that these two mutations arose spontaneously following mechanical passage of the original full-length CNV cDNA clone and probably represent the predominant form of the WT transmissible virus from which subsequent transmission mutants arose. The following discussion of the transmission mutants is based on mutations unique to these viruses.

Figure 2.4 shows that each transmission mutant (LLK8, LLK10, LLK63, LLK82, LLK84, and LLK85) contains a single amino acid substitution in the CP and that these occur in either the CNV CP shell or protruding domains; no amino acid changes were found in the R and arm domains, which are located in the particle interior. Two of the transmission mutants, LLK85 and LLK84, contain single amino acid substitutions in the shell domain, whereas the remaining transmission mutants contain single changes in the
protruding domain (Figure 2.4). As described above, mutants LLK26 and LLK8 contained identical protruding domain mutations.

Additional nucleotide substitutions that do not affect the CP amino acid sequence were found in LLK10, LLK82, and LLK85. In LLK10, two silent substitutions were found: one in the 3'-terminal region of CNV p92 ORF (the putative RNA-dependent RNA polymerase) (25) and the other in the arm region of the CNV CP ORF. LLK85 contained a silent substitution in the coding region of the CNV CP protruding domain. These mutations were not further investigated since they do not affect the protein sequence and are not present in areas of the genome which have known regulatory nucleotide sequences. LLK82 contains a T-to-C change in the core promoter for the subgenomic mRNA2 that encodes proteins involved in cell-to-cell movement (p21) and symptom induction (p20) (16, 26). However, as described above, several analyses of LLK82 accumulation levels failed to indicate that the T-to-C change affects virus accumulation (see above).

2.3.3 CNV transmission mutants show decreased binding to zoospores in vitro

We have previously shown that CNV binds to zoospores in vitro and that the CNV, transmission mutant LL5 shows reduced in vitro zoospore binding (24). These data suggested that the LL5 CP lacks an important determinant for attachment to zoospores. We wished to assess the possibility that reduced transmission of LLK8, LLK10, LLK63, LLK82, LLK84, and LLK85 is due to inefficient ability of mutant particles to bind zoospores. One hundred micrograms of each transmission mutant was incubated with 5 × 10^5 zoospores for 1 h, followed by low-speed centrifugation to pellet zoospores and
washing to remove unbound or nonspecifically bound virus. The amount of bound virus in the pellet was determined by Western blot or slot blot analysis. Table 2.1 shows that each transmission mutant binds to zoospores less efficiently than WT CNV, with binding efficiencies ranging from approximately 21 to 68% of that of WT CNV. These results suggest that the reduced transmission of CNV mutants is at least partly due to their reduced abilities to attach to zoospores during the transmission process.

2.3.4 An artificial double mutant transmits and binds to zoospores at a lower efficiency than either of the individual mutants

An artificial double mutant (LL5K8 [Figure 2.4]) containing the mutations present in both LLK8 and the previously described LL5 mutant (24) was constructed and assessed for transmission. Table 2.1 shows that this mutant is less transmissible (0%) than either LLK8 (21% transmission) or LL5 (20% transmission) (24). Corresponding results were obtained in in vitro binding studies, i.e., LL5K8 binds zoospores less efficiently (22%) than either LLK8 (68%) (Table 2.1) or LL5 (50%) (24). When the double mutant was tested for its ability to infect and accumulate in N. clevelandii and cucumber, no substantial decrease in the level of RNA accumulation (Figure 2.1) or particle accumulation as determined by ELISA (data not shown) was observed. In addition, particles appeared intact, as determined by agarose gel electrophoresis (Figure 2.2). These results reinforce the role of both the LLK8 and LL5 mutations in the attachment and transmission processes.

2.4 Discussion

We have isolated and characterized several naturally occurring CNV mutants deficient in transmission by O. bornovanus. Each mutant contains amino acid
substitutions in the CP, reinforcing previous studies on the role of this protein in fungus transmission (20, 24). All of the CP mutations occurred in either the shell or protruding domain. These portions of the CP, unlike the R and arm domains, form the surface of the particle, which raises the possibility that the affected amino acids may serve as attachment sites for interaction of CNV with a putative zoospore receptor (see below).

In vitro binding studies showed that each transmission mutant bound to zoospores less efficiently than WT CNV (Table 2.1). These data suggest that zoospore binding plays an important role in transmission of these mutants, although other unidentified viral or host factors likely contribute to the transmission process.

All transmission mutants accumulated in cucumber to approximately the same level as WT CNV, indicating that virus particles are stable and that defects in transmission cannot be attributed to an inability of particles to accumulate in cucumber following transmission. With the exception of LLK84, which accumulated to approximately 50% of the WT CNV level, all transmission mutants also accumulated to WT CNV levels in *N. benthamiana* (Figures 2.1 and 2.2). The basis for the slightly reduced accumulation of LLK84 in this host is not known, but considering the location of the LLK84 mutation in the trimer interface, it is possible that the particles are partly defective in assembly or disassembly. We note that accumulation data were taken from both inoculated and systemic tissue of infected *N. benthamiana* but only from inoculated leaves of cucumber. It is possible that the lower accumulation levels observed in *N. benthamiana* are due to decreased ability of LLK84 to move systemically.
LLK8 and LLK10 contain mutations corresponding to amino acids that are immediately next to each other in the linear structure of the CP P domain (amino acids 294 and 295, respectively [Figure 2.4]). Amino acids from other mutants did not cluster on the primary CP structure. However, it was of interest to assess whether the other mutations clustered in the secondary or tertiary structure of the subunit or capsid and whether these sites are potentially exposed on the surface. To do this, homology modelling of the CNV CP subunit was conducted using the known high-resolution X-ray crystal structure of the related TBSV CP subunit (15). Figures 2.3A and B show ribbon and surface representations, respectively, of the modeled CNV subunit, and Figure 2.3C shows a surface representation of the modeled CNV CP trimer (the asymmetric unit). The surface representation models predict that with the exception of LLK10, all of the mutated sites (including the previously identified site in LL5) are exposed on the surface of the subunit or trimer. In addition, six of seven of the mutated sites (i.e., LLK82, LLK8, LLK84, LL5, and LLK85) are preferentially located on one side of the CP subunit (Figure 2.3B). Mutated amino acids in LLK8, LLK10, and LLK82 are all located on the outer wall of the protruding domain dimer, and those in LLK84 and LL5 are near each other in a region of subunit-subunit interaction in the trimer (Figures 2.3A and B). The fact that the mutations map to distinct regions on the capsid is compatible with multiple mechanisms for transmission and binding defects. Nevertheless, the modeled CNV CP trimer predicts that most of the mutated sites (LL5, LLK8, LLK10, LLK82, and LLK84) are in or near a cavity formed by the trimer on the particle quasi-threefold axis. It is therefore possible that the trimer cavity represents an important site for recognition of a putative zoospore receptor. If these mutations disrupt binding to a receptor, it would
Figure 2. Locations of mutated amino acids on the CNV CP subunit and trimer in CNV transmission mutants. (A) Ribbon diagram of the homology modeled CNV CP subunit (subunit C) showing locations of mutated sites (in white in ball-and-stick form) in each of the transmission mutants. The mutated site in LLK10 is shown in red to distinguish it from the adjacent LLK8 mutation. Locations of the P, S, and a domains are indicated (see panel D for details). The disordered R domain is not shown. (B) Surface representation of the CNV CP subunit (subunit C) showing locations of mutated sites in white. The position of the buried LLK10 mutation is indicated by the white dotted lines. The LLK63 mutation is not visible in this orientation. (C) Surface representation of the CNV trimer (asymmetric unit) showing locations of mutated sites in each transmission mutant. The red, blue, and green areas correspond to the A, B, and C subunits. The asterisk shows the quasi-threefold axis of symmetry (D). (D) Diagrammatic representation of the structure of TBSV used for reference to the CNV structure. (a) Linear order of the different CP domains is shown along with the number of amino acids comprising each CNV domain (R, RNA binding domain; a, arm; S, shell domain; h, hinge; P, protruding domain). (b) Subunit structure with locations of indicated domains. (c) Particle structure with the A subunit in red, B in blue, and C in green. The cutaway section shows the region that the disordered R domain may occupy in the particle interior. (This diagram was adapted from reference 3).
suggest that the receptor has complementary symmetry. Alternatively, the affected amino acids in these mutants may affect subunit-subunit interactions and virion conformation, thereby indirectly affecting virion attachment and subsequent transmission. The slower electrophoretic mobility of mutants LL5 (24), LLK63, and LLK84 (and LL5K8) (Figure 2.2) is consistent with the notion that reduced binding and transmission efficiencies may be due to conformational changes in particle structure as a result of the amino acid substitution. As stated above and shown in Figure 2.3C, LLK84 and LL5 mutations lie in a region of subunit contact and could therefore affect subunit interactions. Similarly, the mutation in LLK63 lies in a region of protruding domain dimer interactions and could affect particle conformation by interfering with protruding domain contacts.

The mutation in LLK10 reduces transmission to about 27% of the WT CNV level and decreases binding to 39% as a result of a Val-to-Ala change at amino acid 295 in the CP protruding domain. This substitution lies immediately next to the mutated site in LLK8. The modeled CNV subunit does not predict that the affected LLK10 amino acid is exposed on the particle surface. It is possible that replacement of Val by Ala indirectly affects transmission and binding by changing the accessibility of other exposed amino acids in this region.

The structure of the shell domain of the tombusvirus CP subunit is similar to that of the picornavirus particle (27), which raises the question as to whether the putative zoospore attachment sites on CNV correspond to any of the known cellular receptor attachment sites on picornaviruses. In foot-and-mouth disease virus, an RGD motif in the G-H loop of VP1 has been implicated in receptor attachment (11, 19). Interestingly, the
Gly-to-Val mutation in the CNV mutant LLK84 lies within the structurally analogous G-H loop and is located within an SGD triplet. Mutagenesis studies may help in the final identification of this region of the CNV capsid in zoospore attachment.

Virus attachment sites on animal viruses are for recognition of receptors that lie on host cells infected by the virus. Plant viruses do not recognize receptors for host cell attachment, but certain plant viruses are likely to possess attachment sites for recognition of the vector that transmits the virus to its host. In other cases, a virus-encoded helper factor is believed to mediate interaction between the vector and the virus particle (23). Specific virus attachment sites for cellular receptors have been identified for several animal viruses, including poliovirus, foot-and-mouth disease virus, and influenza virus (12, 28, 31). However, such sites have not yet been identified in plant viruses, despite their importance in the establishment and dissemination of plant virus disease. Specific regions of the capsid involved in transmission have been identified in several plant viruses (4, 6, 13, 14, 23, 32, 35), but to our knowledge no experiments have been conducted to determine if these sites are involved in the vector attachment stage of transmission. In tomato spotted wilt virus, an RGD motif has been identified in one of the viral structural proteins (18) and has been implicated but not proven to be involved in vector attachment. Our studies represent the initial stages of work that aims to identify features of virion architecture required for attachment to a vector. It is hoped that further work will provide information on evolutionarily conserved features of virus particles that are involved in receptor attachment. In addition, virus attachment mutants should aid in the identification of virus vector receptors about which very little is known.
2.5 References


3 CHAPTER THREE

Evidence that Binding of Cucumber Necrosis Virus to Vector Zoospores Involves Recognition of Oligosaccharides*

3.1 Introduction

Animal viruses gain entry to host cells by using host cell surface molecules as receptors (11, 16, 23, 26). However, plant viruses generally gain entry into plants through specific invertebrate or fungal vectors which breach the otherwise impermeable cell wall. In most cases, transmission of plant viruses has been shown to be a highly specific process in which only certain vectors can transmit certain viruses (4, 9, 19, 31). These observations suggest that vectors contain specific sites that are recognized by virus particles. The coat proteins of several plant viruses have been shown to be important in the transmission process (4, 9, 19, 31). However, there are only few reports of the possible involvement of receptors in or on vectors that mediate transmission. Li et al. (15) have recently identified two proteins from head tissues of an aphid vector as potential receptor candidates for Barley yellow dwarf virus (family Luteoviridae). In addition, Bandla et al., (3) have reported that a 50-kDa midgut protein from Frankliniella occidentalis, the main thrip vector for Tomato spotted wilt virus (genus Tospovirus), is a potential receptor. Symbionin, a homologue of Escherichia coli GroEL chaperonin

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protein which is highly abundant in aphid hemolymph (31), has been shown to bind to luteoviruses and to play an important role in the transmission process. Certain other viruses, such as potyviruses, caulimoviruses, and tobraviruses, require additional virus-encoded "helper factor" proteins for transmission. In the cases of potyviruses and caulimoviruses, the helper factor likely serves as a bridge between virus particles and attachment sites in the vector (9, 10, 31). Several small spherical viruses within the family Tombusviridae are transmitted by zoospores of the Chytrid fungus Olpidium (either Olpidium bornovanus or Olpidium brassicae) (4). It has been proposed that transmission occurs following the release of zoospores and virus from different plants into the soil and subsequent adsorption of virus particles onto the surfaces of zoospores. Bound virus then enters the cells of plants following the encystment of zoospores on roots (1, 4). Several studies have shown that the transmission process is highly specific (1, 4). For example, O. brassicae transmits the necrovirus Tobacco necrosis virus (TNV-A) but not the tombusvirus Cucumber necrosis virus (CNV), and conversely, O. bornovanus transmits CNV but not TNV-A (7, 30). Moreover, different O. bornovanus isolates transmit either CNV, Melon necrotic spot virus (MNSV), or Cucumber leaf spot virus (CLSV) with various efficiencies (5), and different necrovirus species are transmitted with different efficiencies by the same O. brassicae isolate (13, 29, 30). Electron microscopy studies have shown that adsorption of virus to the zoospore plasmalemma is specific and reflects the virus-vector associations observed in nature (30). Together, these studies indicate the existence of a specific recognition mechanism(s) between virus and vector zoospores.

Previous work has shown that the CNV coat protein contains determinants for the specificity of transmission by O. bornovanus (18, 22). More recently, it has been shown
that a cavity at the quasi-threefold axis is important in CNV attachment and transmission (12). In this study, we wished to determine whether the acquisition and subsequent transmission of CNV by *O. bornovanus* involves specific zoospore receptors.

### 3.2 Materials and Methods

#### 3.2.1 Virus isolates and purification

CNV, CLSV, and TNV-A were maintained by mechanical passage in *Nicotiana benthamiana* or *Nicotiana clevelandii*, and MNSV was similarly maintained in *Cucumis sativis*. The viruses were purified by differential centrifugation as previously described (12).

#### 3.2.2 Maintenance of fungal cultures

*O. bornovanus* (isolate SS196) was maintained on cucumber roots (*C. sativis* cv. Poinsette 76), and *O. brassicae* (isolate SS58) was maintained on lettuce roots (*Lactuca sativa* cv. White Boston) as described by Campbell et al. (5).

#### 3.2.3 In vitro zoospore binding assays

*In vitro* zoospore binding assays were conducted as previously described (12) using Western blot analysis followed by densitometry for quantification of the amount of bound virus. Monoclonal antibody 57-2 was used for detection of CNV, and the respective polyclonal antibodies were used for all other viruses. The monoclonal antibody was prepared in mice using CNV particles as the immunogen (M. Robbins and D. Rochon, unpublished data). Saturation binding data was analyzed using nonlinear
regression analysis and the "one-site binding method" in the GraphPad Prism software package (http://www.graphpad.com).

3.2.4 Trypsin, periodate, and phospholipase C treatment of O. bornovanus zoospores

One milliliter of O. bornovanus zoospores (5 x 10^5/ml) was incubated in either 10 mM sodium periodate-0.1% trypsin (Sigma) or 5 mU of phospholipase C (Sigma) for 15 min. Periodate oxidation was done in deionized water, trypsin digestion was done in 100 mM sodium phosphate buffer (pH 7.6), and phospholipase C treatment was done in 10 mM Tris (pH 7.6)-5 mM CaCl2. Following treatment, the zoospores were pelleted at 2,000 x g for 7 min, resuspended in 1 ml of binding buffer (50 mM sodium phosphate buffer, pH 7.6), and then used in an in vitro binding assay as described above. CNV virions were assessed for resistance to residual trypsin digestion by incubating 100 µg of CNV in 0.002 to 0.2% trypsin for 40 min in binding buffer. The integrity of the virus was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and agarose gel electrophoresis. Degradation of the CNV coat protein subunit was not observed following SDS-PAGE, and the virus particle appeared intact following agarose gel electrophoresis (data not shown).

3.2.5 Virus overlay assays

Virus overlay assays were done essentially as described by Salas-Benito and del Angel (24) with some modifications. A total of 2 x 10^6 zoospores (20 µl) in denaturation buffer (14) were loaded per lane on an SDS-12% PAGE gel (14). The proteins were blotted onto nitrocellulose membranes (0.45-µm pore size; Bio-Rad) and then renatured overnight in phosphate-buffered saline (PBS)-4% bovine serum albumin at 4°C. The blots
were washed three times for 10 min each time in PBS and then blocked in PBS containing 5% dry milk powder for 1 to 2 h. After being washed as described above, the blots were incubated with 100 μg of virus in 10 ml of 50 mM sodium phosphate buffer (pH 7.6) for 3 h. The blots were washed and then incubated for 1 h with CNV monoclonal antibody 57-2. The antigen-antibody complexes were detected using peroxidase-labeled goat anti-mouse antibody (Jackson Immuno Research Laboratories) and the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech). Monoclonal antibody 57-2 was confirmed to react equally to wild type (WT) CNV and to the CNV transmission mutants in slot blot analysis using undenatured virions.

Sugar inhibition studies were conducted by preincubating CNV virions in 10 ml of binding buffer (pH 7.6) and either 0.1 M d-(-)-mannose, 0.1 M methyl α-d-mannoside, or 1 mg of mannan/ml for 45 min at room temperature prior to the addition of virions to the blots.

3.2.6 Microtiter plate binding assays

A modification of a previously described microtiter plate-based binding assay utilizing lectins (17) was used. Polystyrene microtiter plates (96 well; Libro/Titerek) were coated with 100 ml of *O. bornovanus* zoospores (2.5 x 10^6 zoospores/ml) in 50 mM potassium phosphate buffer, pH 7.0, and incubated overnight at 37°C. After incubation, the plates were blocked at 37°C for 2 h in PBS containing 5% milk powder and 5% bovine serum albumin. After being washed with distilled water, the plates were incubated for 1 h at 37°C with either 8 μg of CNV in 100 μl of 50 mM sodium phosphate (pH 7.6) binding buffer or 8 μg of CNV preincubated with sugar solution (see below) in binding
buffer. The plates were washed as described above, and the amounts of CNV bound were
determined using CNV monoclonal antibody 57-2 followed by detection with goat anti-
mouse antibody conjugated to alkaline phosphatase. The relative amounts of bound virus
were determined 10 to 40 min following the addition of substrate by measuring the
absorbance at 405 nm. Under these conditions, the relationship between bound virus and
absorbance at 405 nm was linear, as determined by a dilution series of CNV virions.

The following sugars were tested for the ability to inhibit CNV binding to
*O. bornovanus* zoospores: D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, L-(-)-arabinose, L-
(-)-fucose, N-acetyl-d-glucosamine, D-(+)-xylose, L-(-)-sorbose, D-(-)-fructose, methyl α-d-
mannoside, yeast mannan, d-mannosamine, α3,α6-mannopentaose, mannotriose-bis[N-
acetyl-d-glucosaminyl], and N-acetyl-d-mannosamine. The sugars were initially tested for
inhibitory activity using 14 10-fold serial dilutions beginning with 0.2 M sugar. Dilutions
of α3,α6-mannopentaose and mannotriose were as described above, beginning with 1,067
and 538 μM solutions, respectively. Sugars that showed inhibition were then further
tested within the inhibitory range using several threefold dilutions. Inhibition in the case
of yeast mannan was determined using a starting concentration of 15 mg/ml (equivalent to
~0.1 M in terms of the mannose residue concentration) followed by a series of threefold
serial dilutions. CNV-sugar solutions were incubated for 45 min at room temperature
prior to being added to the zoospores.

### 3.2.7 Labelling of *O. bornovanus* zoospores with FITC-labeled lectins

Sixteen micrograms of fluorescein isothiocyanate-labeled concanavalin-A (ConA-
FITC; Sigma) or 30 μg of FITC-labeled *Tetragonolobus pupurea* agglutinin (TPA-FITC;
Sigma) were incubated for 10 min with washed *O. bornovanus* zoospores (2 × 10⁵ to 2 × 10⁶) in 1 ml of binding buffer. The zoospores were viewed with a Zeiss Axiophot epifluorescence microscope using an excitation wavelength of 450 to 490 nm. The specificity of the labelling reaction was determined by preincubating the lectin with either 0.5 M D-(+)-mannose (in the case of ConA) or 0.15 M L-(-)-fucose (in the case of TPA) prior to adding it to the zoospores.

### 3.3 Results

#### 3.3.1 Binding of CNV to zoospores is saturable and specific.

Two major criteria for viral recognition sites as receptors are saturability and specificity (28). An *in vitro* binding assay (12, 22) was used to determine whether CNV binding to *O. bornovanus* zoospores is saturable. Increasing amounts of CNV (2.2 to 55 pmol) were incubated with 4 × 10⁵ *O. bornovanus* zoospores in binding buffer. Following a 1-h incubation, the virus-zoospore suspensions were centrifuged at low speed, the pellets were washed, and the amounts of virus bound to pelleted zoospores were determined using Western blot analysis followed by densitometry. Figure 3.1A shows that binding of CNV to zoospores is indeed saturable, becoming apparent at ~20 pmol of CNV.

To further assess the possibility that *Olpidium* zoospores contain specific virus attachment sites, CNV, MNSV, and CLSV—each known to be transmitted by *O. bornovanus* (but not by *O. brassicae*)—and TNV-A—known to be transmitted by *O. brassicae* (but not by *O. bornovanus*)—were examined for the ability to bind to zoospores of either *O. bornovanus* or *O. brassicae* using the above-described binding assay. Figures
Figure 3.1. Virus-zoospore binding assays. (A) Saturation binding of CNV to *O. bornovanus* zoospores. Increasing amounts of CNV were added to $4 \times 10^5$ zoospores, and the amounts of bound virus were determined using Western blot analysis followed by densitometry. The amount of virus bound to washes of uninfected roots was also determined for each concentration of virus, and this value was subtracted from the amount of virus bound to zoospores. All values represent the average of triplicate samples from one representative experiment. (B and C) Ten picomoles of either CNV, MNSV, CLSV, or TNV-A was incubated with $10^6$ *O. bornovanus* (B) or *O. brassicae* (C) zoospores, and the amount of virus bound was determined as for panel A. The results are the averages of triplicate treatments from two separate experiments plus standard deviations. (This figure was derived from work done by former graduate student, Marjorie Robbins.)
3.1 B and C shows that CNV, MNSV, and CLSV each bind *O. bornovanus* zoospores more efficiently than TNV-A (2.6-, 2.8-, and 17-fold, respectively [Figure 3.1B]) and that TNV-A binds *O. brassicae* zoospores more efficiently than either CNV, MNSV, or CLSV (3.3- and 30-fold, with no detectable CLSV binding [Figure 3.1C]). These experiments show that specificity indeed occurs in the attachment of these viruses to *Olpidium* zoospores and, moreover, that the specificity observed *in vitro* reflects previously described biological specificities (4, 5). These results, in conjunction with the saturation binding experiments, support the possibility that specific zoospore receptors are involved in the acquisition and transmission of these viruses in nature.

### 3.3.2 Periodate, trypsin, and phospholipase C treatment of *O. bornovanus* zoospores.

To initially characterize the biochemical nature of the molecule(s) on the surface of *O. bornovanus* zoospores involved in CNV attachment, zoospores were treated with either 10 mM sodium periodate, 0.1% trypsin, or 5 mU of phospholipase C. The treated zoospores were washed and subsequently used in an *in vitro* binding assay with CNV virions. Figure 3.2 shows that periodate treatment of zoospores decreased CNV binding by 72%, suggesting that carbohydrates are important for CNV binding. Trypsin digestion of zoospores reduced virus binding by 84%, indicating that proteins are also important for attachment. No decrease in CNV binding was observed using phospholipase C-treated zoospores. Together, these results suggest that proteins and/or glycoproteins on the zoospore surface play an important role in CNV binding.
Figure 3.2. Effects of trypsin, sodium periodate, and phospholipase C digestion on CNV binding to O. bornovanus zoospores. Zoospores were treated with 0.1% trypsin, 10 mM sodium periodate, or 5 mU of phospholipase C and used in an in vitro binding assay with 100 \( \mu \text{g} \) (10 pmol) of CNV particles. The amounts of virus bound were determined as for Fig. 1, using densitometry, and the results are expressed as the percentage of WT CNV binding. The results shown are the averages of triplicate samples (plus standard deviations) and are representative of three independent experiments for periodate and trypsin treatments and two independent experiments for phospholipase C.
3.3.3 CNV binds to specific-size proteins in *O. bornovanus* zoospore extracts.

Virus overlay assays were conducted to further investigate the possibility that CNV recognizes specific proteins or glycoproteins present on zoospores. Total proteins from $2 \times 10^6$ zoospores (Figure 3.3, lane 9) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with CNV virions. Bound virus was detected using a monoclonal antibody raised to CNV virions. Figure 3.3 (lane 2) shows that CNV bound predominantly to five low-molecular-mass proteins with estimated masses of 40, 39, 36, 34, 30, and 15 kDa and to several high-molecular-mass proteins of 119 and 63 kDa, along with a group of proteins ranging from 88 to 92 kDa. This binding pattern was observed repeatedly with different batches of zoospore preparations, but slight variations in the relative banding intensity were observed, as well as small variations in the number of high-molecular-mass proteins resolved. This was particularly true of the three proteins of ~88 to 92 kDa shown in Figure 3.3 (lane 2), which sometimes resolved as only one or two species. The complexity of the banding pattern suggests that CNV virions may be recognizing a group of related proteins or a common residue on multiple proteins, such as a specific carbohydrate moiety.

3.3.4 CNV transmission mutants bind with reduced efficiency in virus overlay assays.

To determine the specificity of the interaction between CNV and zoospore proteins in virus overlay assays, we used three previously characterized CNV transmission mutants (LL5, LLK10, and LLK63 [12]). The mutants differ from WT CNV by a single amino acid substitution in the CNV coat protein subunit, have reduced
Figure 3.3. Binding of CNV and CNV transmission mutants to total zoospore extracts using virus overlay assays. Total *O. bornovanus* zoospores (2 x 10^6 zoospores [zoos]/lane) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with 100 μg (10 pmol) of either WT CNV, LL5, LLK10, or LLK63 (lanes 2, 4, 6 and 8, respectively). Bound virus was detected using a monoclonal antibody specific to CNV. Lanes 1, 3, 5 and 7 each contain 200 ng of either CNV, LL5, LLK10, or LLK63 virions used as an internal detection standard respectively. Lane 9, SDS-PAGE of *O. bornovanus* zoospores (2 x 10^6 zoospores) stained with Coomassie blue.
transmission efficiencies (30, 27, and 14% of that of WT CNV, respectively), and have reduced binding efficiencies in solution binding assays (40, 39, and 21% of WT CNV binding, respectively) (12, 22). Figure 3.3 (lane 4) shows that LL5 binds with reduced efficiency in virus overlay assays and that little or no binding is observed when LLK10 (lane 6) and LLK63 (lane 8) are used. The reduced abilities of virions of CNV transmission mutants to bind to proteins in zoospore extracts suggests that the multiple proteins detected by WT virions are due to a specific interaction important for zoospore recognition during transmission.

3.3.5 CNV binding to *O. bornovanus* zoospores is competitively inhibited by several mannose-containing sugars.

The possibility that CNV may be recognizing a carbohydrate moiety present on multiple glycoproteins in zoospore extracts was examined by preincubating CNV virions with specific sugars prior to adding virus in a virus overlay assay. Figure 3.4 shows that preincubation of CNV with 0.1 M methyl α-D-mannoside or 1 mg of mannan/ml abolished detectable CNV binding to *O. bornovanus* proteins, whereas 0.3 M D-(+)-mannose showed only a slight reduction in CNV binding. The specific inhibition of CNV binding by methyl α-D-mannoside and mannan suggests that these (or closely related) sugars may represent components of several glycoproteins that CNV binds to in overlay assays.

To facilitate the analysis of the inhibitory potentials of several other sugars, we modified a previously described microtiter plate-based lectin binding assay (17) in which CNV acts as the lectin. In the modified assay, zoospores are bound to microtiter plates, and virus, in the presence or absence of a specific sugar, is incubated with bound
Figure 3.4. Virus overlay assays using CNV incubated with mannose, methyl α-D-mannoside or yeast mannan. *O. bornovanus* zoospores (2 \( \times \) 10^6 zoospores [zoos]/lane) were subjected to SDS-PAGE, blotted, and incubated with either 100 µg (10 pmol) of CNV without sugar (lane 2) or 100 µg of CNV in the presence of either 0.3 M D-(+)-mannose (lane 4), 0.1 M methyl α-D-mannoside (lane 6), or 1 mg of yeast mannan/ml (lane 8). Bound virus was detected using a CNV monoclonal antibody. Lanes 1, 3, 5, and 7 each contain 200 ng of CNV used as an internal detection standard.
zoospores. The binding of virus to the zoospores is then quantified using a CNV-specific monoclonal antibody in an enzyme-linked immunosorbent assay-based assay.

Fifteen sugars were tested for inhibitory potential (Table 3.1) by preincubating CNV with several serial 10-fold dilutions of sugar prior to adding CNV to the zoospores. Sugars showing relatively significant inhibition (arbitrarily defined as those sugars with a 50% effective concentration \([\text{EC}_{50}]\) of <10 mM) were further analyzed for inhibitory potential by preincubation of virus with serial threefold dilutions of the sugar in the concentration range where inhibition was observed using the 10-fold dilutions. Table 3.1 shows that among 15 sugars tested, mannotriose, \(\alpha3,\alpha6\)-mannopentaose, methyl \(\alpha\)-D-mannopyranoside, mannan, \(l\)-(−)-fucose, and D-mannosamine showed \(\text{EC}_{50}\)s of <10 mM. The \(\text{EC}_{50}\)s for these sugars varied, with mannotriose showing the strongest relative inhibitory activity (\(\text{EC}_{50} = 128 \mu\text{M}\)) and D-mannosamine showing the weakest (2.7 mM) (Table 3.1). Inhibition curves for each of these sugars are presented in Figure 3.5. Among the sugars tested for inhibitory activities, no monosaccharides except methyl \(\alpha\)-D-mannoside, D-mannosamine, and fucose were able to inhibit CNV binding to zoospores. Sugars containing three or more mannose residues showed strong inhibition at very low concentrations. These results, like those obtained using overlay assays, suggest that mannose-containing oligosaccharides may play an important role in CNV attachment to zoospores.

3.3.6 Surfaces of *O. bornavanus* zoospores contain fucose and mannose and/or glucose residues.

Zoospores were assessed for the presence of specific sugar residues using a variety of FITC-labeled lectin probes. ConA-FITC, which recognizes mannose and/or
Figure 3.5. Inhibition of CNV binding to *O. bornovanus* zoospores by several sugars. Several threefold dilutions of the indicated sugars were incubated with 8 µg (0.8 pmol) of CNV, and the amounts of CNV bound to plated *O. bornovanus* zoospores were determined using a microtiter plate assay. The absorbance at 405 nm is plotted as a function of the concentration of sugar using nonlinear regression analysis. Each data point represents the average (± standard deviation) of triplicate treatments. The results shown were obtained in at least one other independent experiment.
Table 3.1. Sugars classified as inhibitors and noninhibitors in virus-zoospore binding assays

<table>
<thead>
<tr>
<th>Sugar</th>
<th>EC\textsubscript{50} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitors\textsuperscript{1}</strong></td>
<td></td>
</tr>
<tr>
<td>Mannotriose</td>
<td>0.128</td>
</tr>
<tr>
<td>(\alpha_3\alpha_6)-Mannopentaose</td>
<td>0.157</td>
</tr>
<tr>
<td>Methyl (\alpha)-D-mannopyranoside</td>
<td>1.9</td>
</tr>
<tr>
<td>Mannan</td>
<td>2.0</td>
</tr>
<tr>
<td>L-((-))-Fucose</td>
<td>2.3</td>
</tr>
<tr>
<td>D-Mannosamine</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Noninhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>D-((+))-Glucose</td>
<td></td>
</tr>
<tr>
<td>L-((+))-Arabinose</td>
<td></td>
</tr>
<tr>
<td>D-((+))-Galactose</td>
<td></td>
</tr>
<tr>
<td>(N)-Acetyl-D-glucosamine</td>
<td></td>
</tr>
<tr>
<td>L-((-))-Sorbose</td>
<td></td>
</tr>
<tr>
<td>(N)-Acetyl-D-mannosamine</td>
<td></td>
</tr>
<tr>
<td>D-((+))-Xylose</td>
<td></td>
</tr>
<tr>
<td>D-((+))-Mannose</td>
<td></td>
</tr>
<tr>
<td>D-((-))-Fructose</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Inhibitors are arbitrarily classified as those sugars with EC\textsubscript{50}s of \(<10\) mM.
glucose, and TPA-FITC, which recognizes fucose, were the only two lectins that gave detectable binding (Figure 3.6). ConA and TPA lectins bound densely and uniformly over the surface of the zoospore body and flagella. Labelling was eliminated or reduced when the lectins were preincubated with either mannose (in the case of ConA) or fucose (in the case of TPA) (data not shown), indicating that lectin binding is a result of a specific interaction. These results indicate the presence of D-(+)-mannose and/or D-(+)-glucose and L-(−)-fucose on the surfaces of *O. bornovanus* zoospores.

### 3.4 Discussion

Despite the importance of vectors in the natural spread of plant viruses, little or nothing is known about the components of vectors that viruses recognize. In this study, we have examined the possibility that a fungal vector of a plant virus contains specific receptors for virus recognition and further examined the biochemical nature of the putative receptor. Toward this end, we have found that the binding of CNV to zoospores is both saturable and specific (Figure 3.1), two criteria used to demonstrate receptor-mediated attachment of animal viruses to their receptors (28). Extrapolation from the saturation binding curve indicates that \( \sim 2.7 \times 10^4 \) binding sites are present on zoospores (i.e., 0.018 pmol per \( 4 \times 10^5 \) zoospores). This number is within the range of virus receptor sites normally found on eukaryotic cells (34). The saturation binding curve in Figure 3.1 was also used to determine the dissociation constant (\( K_d \)). A \( K_d \) of \( 5.7 \times 10^{-9} \) M was obtained, indicating a very tight association between virus and zoospores. \( K_d \) values of \( 10^{-5} \) to \( 10^{-9} \) are typically found for binding of animal viruses to their cellular receptors (32).
Figure 3.6. Labeling of zoospores with FITC-labeled ConA and TPA. FITC-labeled ConA (a and b) or TPA (c and d) were incubated with zoospores and photographed using either differential interference contrast microscopy (a and c) or fluorescence microscopy (b and d). Scale, 5 mm ≈10 μm. (This figure was derived from work done by former graduate student, Marjorie Robbins.)
Reduced CNV binding following trypsin and periodate treatment of O. bornovanus zoospores, along with the results of virus overlay assays, suggests the involvement of multiple proteins and/or glycoproteins in CNV attachment. Phospholipase C did not affect CNV binding, but further experiments are required to fully explore the possibility that membrane interactions are not involved in CNV attachment. The addition of protease inhibitors to zoospores during zoospore release from fungus-infected roots did not affect the number of bands observed (data not shown), suggesting that proteolytic degradation following zoospore release is likely not a factor in the generation of multiple bands. Incubation of virus with blots under high-stringency conditions (up to 0.55 M sodium salt) also did not affect the complexity or the intensity of the signal (data not shown), suggesting that nonspecific binding of CNV is not responsible for the multiple bands. Interestingly, incubation of CNV with mannose or methyl-\(\alpha\)-D-mannopyranoside dramatically reduced CNV binding (Figure 3.4). These experiments therefore suggested that the multiple zoospore proteins that CNV recognizes in virus overlay assays may be due to the species possessing a common oligosaccharide component.

To assess the possibility that oligosaccharides play a role in virus attachment, we tested several sugars for the ability to inhibit CNV binding (Table 3.1). Interestingly, two mannose derivatives (methyl-\(\alpha\)-D-mannopyranoside and \(\alpha\)-mannosamine), as well as three mannose-containing oligosaccharides (mannotriose, \(\alpha\)-3,\(\alpha\)-6-mannopentaose, and yeast mannan), were found to be strong inhibitors, with EC\(_{50}\)s ranging from 128 \(\mu\)M for mannotriose to 2.7 mM for \(\alpha\)-mannosamine. \(\alpha\)-(-)-Fucose was also found to be an efficient inhibitor, with an EC\(_{50}\) of 2.3 mM. \(\alpha\)-Mannose did not show inhibition, suggesting that
specific features of mannose-containing sugars are required for efficient virus-sugar interaction. Taken together, our studies suggest that CNV may have lectin-like (lectins are carbohydrate binding proteins of plant, animal and viral origin) properties that contribute to its ability to bind oligosaccharides on its vector. This observation also raises the question of whether CNV may also require oligosaccharides in or on plant cells for successful infection or multiplication in plants.

The binding of individual lectins to monosaccharides (monovalent binding) is very weak, with affinities in the range of 0.1 to 10 mM (33). Analysis of saturation binding curves of CNV to zoospores suggests that the affinity of CNV for zoospores is ~5.7 nM. The apparent high affinity of CNV for zoospores may indicate the involvement of more specific receptor or receptor-like interactions that do not exclusively involve carbohydrates. On the other hand, it is now recognized that highly avid (nanomolar range) lectin interactions can exist at cell surfaces due to multivalent binding (21, 33) between lectins and oligosaccharide receptors.

CNV, MNSV, CLSV, and TNV-A virions bind to vector zoospores more efficiently than to nonvector zoospores (Figure 3.1). TNV-A differs from the other three viruses in that it lacks the C-terminal P domain. This raises the possibility that the P domain of CNV, MNSV, and CLSV enhances binding to O. bornavanus zoospores and, further, that the presence of this domain may interfere with binding to O. brassicae zoospores.

The CNV mutants LL5, LLK10, and LLK63 contain single amino acid mutations in the coat protein which decrease transmissibility and zoospore binding (12, 22), as well
as binding to zoospore species in virus overlay assays. The mutation in LL5 is in the shell
domain at the particle quasi-threefold axis, and the mutations in LLK10 and LLK63 are in
the P domain, with the LLK10 mutation facing the quasi-threefold axis. The inability of
these mutants to react in virus overlay assays may suggest the involvement of the specific
mutations or their potential conformational effects in binding oligosaccharides and/or
glycoproteins; however, further experiments will be required to assess this potential role.

Several animal viruses are known to bind host cells via oligosaccharide or
proteoglycan receptors (11, 26). In the case of influenza virus, which binds sialic acid-
containing oligosaccharides, the interaction with the sugar is sufficient for cell attachment
(27). In the cases of several other animal viruses that bind proteoglycans, it is thought that
the proteoglycan is used as an initial attachment receptor before further higher-affinity
receptors strengthen the attachment (25). Whether acquisition of CNV requires more than
one type of receptor for stable attachment remains to be determined.

Cytochemical and structural analysis of the zoospores of certain Chytrid species
have indicated that the zoospore is surrounded by a polysaccharide-containing cell coat
and that a common component of the coats of some chytrids is mannose (8, 20). The
lectin binding studies in Figure 3.6 show that O. bornovanus zoospores contain both l-(-)-
fucose and d-(+)-mannose and/or -glucose. Indeed, several other lectins with a variety of
different sugar specificities did not react with zoospores (data not shown). Thus, CNV
appears to utilize sugars that are prominent on the zoospore surface for attachment.

Structural studies of the coat protein subunit of tomato bushy stunt virus, a close
relative of CNV, have shown that the shell domain folds into a jellyroll-type structure
typical of several plant and animal virus coat protein subunits (6). Interestingly, the lectin ConA also folds into a jellyroll-type structure (6). Indeed, it has been suggested (2) that the overall similarity in structural topology between the tombusvirus capsid and ConA may indicate that tombusviruses have evolved from lectins. The studies described here support the hypothesis that CNV has lectin-like properties that may play a key role in the recognition of its vector.
3.5 References


CHAPTER FOUR

Evidence that Vector Transmission of a Plant Virus Requires Conformational Change in Virus Particles*

4.1 Introduction

Transmission of plant viruses in nature often involves invertebrate or fungal vectors and can be a highly specific process in which only certain vectors transmit certain viruses. (8, 10, 13, 14, 34, 46). The specificity of the transmission process has suggested that virus particles as well as vectors contain specific sites that mediate their interaction, (25, 26) and, moreover, that there may be some similarity between the way plant viruses attach to vectors and that of animal virus/host cell interactions. The coat proteins (CPs) of several plant viruses, as well as specific amino acid residues within the CP, have been shown to play important roles in transmission (5, 8, 10, 13, 14, 25, 28, 34, 35, 46). However, for the most part, the exact role of these amino acid residues in transmission, including their potential role in specific stages of the transmission process, is not known.

Cucumber necrosis virus (CNV), a member of the Tombusviridae in the genus Tombusvirus, is naturally transmitted by zoospores of the fungus, Olpidium bornovanus. (10, 12) It is believed that CNV particles are adsorbed to the surface of

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motile zoospores following independent release of virus and zoospores from the roots of infected plants. The virus then enters root cells upon zoospore encystment. (1, 10). Studies of CNV transmission by *O. bornovanus* have suggested that specific amino acid residues in the CP are important for transmission efficiency, and that a cavity at the particle quasi-3-fold axis may be an important virus attachment site (25). More recently, we have provided evidence that recognition of zoospores by CNV may involve glycoprotein receptors on the zoospore surface (26).

CNV is a 30 nm $T=3$ icosahedron consisting of 180 copies of a single CP subunit. The structure of the CNV particle and CP subunit is similar to that of tomato bushy stunt virus (TBSV), the type member of *Tombusvirus* genus. Putative structures of the CNV CP subunit and trimer have been obtained using homology modelling (25) based on the X-ray crystal structure of TBSV (17). The CNV CP subunit folds into three distinct regions termed the RNA binding (R), shell (S) and protruding (P) domains. The R and S domains are connected by a 34 amino acid residue arm, and the S and P domains are connected by a small hinge (h) (Figure 4.1(A)). The R and arm domains are located internally in the particle, the S domain forms the shell of the virus and the P domain projects from the surface. Three ordered C subunit arms are interconnected at the particle 3-fold axis to form an internal network called the $\beta$-annulus (Figure 4.1(C)). The $\beta$-annulus stabilizes the particle and is believed to determine particle curvature during assembly.

Treatment of CNV (as well as several other small spherical plant viruses) with metal-chelating agents at alkaline pH results in particles with a swollen conformation (49, 132).
Figure 4.1. Location of Pro73 and Pro85 on the CNV CP subunit and β-annulus. (A) Location of Pro73 and Pro85 in the arm of the linear CP subunit. The different CP structural domains are indicated in different colours and are designated as follows: RNA binding (R, white); arm (a, yellow); shell (S, light gray); hinge (h, blue); and protruding (P, gray). The location of Pro73 and Pro85 in the arm are indicated with red arrows. Also shown are the location of the Lys53 and Arg95 trypsin cleavage sites in swollen wild-type CNV. EcoRI, BglII and Ncol sites used for cloning the mutant CP genes and flanking sequences in the CNV genome are indicated. (B) Surface representation of homology modeled CNV CP subunit (subunit C). The different structural domains are represented in the same colours as in (A). The locations of Pro73 and Pro85 are indicated in red. The disordered R domain is not shown. (C) Representation of the CNV β-annulus showing the location of Pro73 and Pro85 residues. B and C correspond to the locations of the B an C subunits relative to the β-annulus.
The swollen conformation of TBSV is due to repulsion of subunits at the particle quasi-3-fold axis and is believed to be accompanied, in part, by movement of all or part of the R and arm domains of the A and B subunits to the outside of the particle (18). It has been suggested that the swollen conformation of plant viruses may be an important part of the uncoating process during the initial stages of virus infection (7, 36, 47).

It is known that several animal viruses undergo conformational change upon receptor attachment (11, 20, 23, 41, 51,). We wished to investigate if attachment of CNV to putative receptor(s) on vector zoospores involves conformational change. We provide evidence that zoospore-bound CNV is conformationally different from native CNV. In addition, we report that a poorly transmissible CNV mutant capable of binding zoospores in vitro fails to undergo conformational change. We discuss the possible role that conformational change may play in the CNV transmission process.

4.2 Materials and Methods

4.2.1 Virus purification

A miniprep procedure was employed to partially purify particles of CNV and CNV mutants for use in initial transmission tests (35). For all other experiments, the virus was purified by differential centrifugation as described (25).

4.2.2 Maintenance of O. bornovanus cultures

O. bornovanus isolate SS196 was maintained on cucumber roots (Cucumis sativis cv. Poinsette 76) essentially as described (9).
4.2.3 Agarose gel electrophoresis of purified virus

Virus particles were electrophoresed through 1% (w/v) agarose gels in TB buffer (40 mM Tris–borate, pH 8.3) as described (25). Virions were stained with ethidium bromide and photographed under ultraviolet illumination.

4.2.4 In vitro mutagenesis

Oligonucleotide directed in vitro mutagenesis was used to produce CNV CP mutants with altered Pro73 and Pro85 residues (Table 4.1). To produce mutants Pro73Gly and Pro85Gly, an EcoRI/ NcoI fragment, encompassing the CNV CP and flanking regions (Figure 4.1(A)) in a full-length infectious cDNA clone of CNV (PK2/M5) (39) was subcloned into EcoRI/NcoI-digested pT7 Blue (Novagen) and used as a template for in vitro mutagenesis. Oligonucleotide primers used for mutagenesis are described in Table 4.1. Mutants were screened by sequencing. Selected plasmid DNA was then digested with EcoRI/NcoI and the fragment containing the mutation was cloned into similarly digested pK2/M5. pK2/M5 was used as template for the production of Pro73Ala, Pro73Cys and Pro73Leu mutants (Table 4.1). Following mutagenesis, plasmid DNA was digested with EcoRI/BglII and the mutated fragment was cloned back into pK2/M5 to obtain pPro73Ala, pPro73Cys and pPro73Leu. The regions between the EcoRI/NcoI sites for pPro73Gly, pPro85Gly and the EcoRI/BglII sites for pPro73Ala, pPro73Cys and pPro73Leu were sequenced to confirm that spurious mutations were not introduced.
### Table 4.1. Oligonucleotides used for constructing Pro73 and Pro85 mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence&lt;sup&gt;1&lt;/sup&gt;</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro73 mutants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro73Gly</td>
<td>ATTCCCGCGGCGATAGGCCAG (-)</td>
<td>(2848-2827)</td>
</tr>
<tr>
<td>Pro73Ala</td>
<td>ATTGCCGCGGCGATAGGCCAG (-)</td>
<td>(2848-2827)</td>
</tr>
<tr>
<td>Pro73Cys</td>
<td>ATGCACCGGCGGCGATAGGCCAG(-)</td>
<td>(2848-2827)</td>
</tr>
<tr>
<td>Pro73Leu</td>
<td>ATTAGCGCGGCGGCGATAGGCCAG (-)</td>
<td>(2848-2827)</td>
</tr>
<tr>
<td>Oligo# 117&lt;sup&gt;3&lt;/sup&gt;</td>
<td>CTCTTATGCCTATGCAGTTAAG (+)</td>
<td>(2849-2871)</td>
</tr>
<tr>
<td><strong>Pro85 mutant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro85Gly</td>
<td>CTACCTTTCTTCTTTAACCGCATAG (-)</td>
<td>(2883-2855)</td>
</tr>
<tr>
<td></td>
<td>GTTTCAACAGCAAAAAGGATCTGTG (+)</td>
<td>(2884-2909)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Sequences of primers are shown 5'-3'. Underlined nucleotides correspond to mutated positions and (-) and (+) antisense or sense relative to the CNV genome, respectively.

<sup>2</sup>Nucleotide positions are relative to the complete CNV genome (Rochon & Tremaine, 1989).

<sup>3</sup>Oligo#117 was used as (+) sense primer for all Pro73 mutants.
4.2.5 *In vitro* transcription and inoculation of plants

Preparation of phage T7 polymerase run-off transcripts and inoculation of plants were done as described (39).

4.2.6 Fungus transmission assays

Purified virions were tested for transmission by *O. bornovanus* zoospores essentially as described but with slight modification (25). A sample (1 μg) of virus was incubated with 10 ml of $1 \times 10^5$ zoospores/ml in 50 mM glycine (pH 7.6). After 15 minutes, the virus/zoospore suspension was poured onto 12–16 day old cucumber seedlings. Five days later, roots of cucumber seedlings were tested for the presence of virus by DAS-ELISA, using polyclonal antisera raised against CNV particles(30). Absorbance readings five times higher than background level were considered as positive for transmission. Each transmission experiment included a wild-type CNV control, a test to determine any background level of CNV transmission in the absence of fungus, and a test for the presence of contaminating virus in zoospore preparations. Transmission in the absence of zoospores was not detectable in any of the experiments.

4.2.7 *In vitro* binding assays

*In vitro* binding assays were conducted as described (25). Briefly, 100 μg of purified virus was incubated with $5 \times 10^5$ zoospores in 1 ml of 50 mM sodium phosphate buffer (pH 7.6) for one hour. Following incubation, zoospores were centrifuged at 2700g for seven minutes in an Eppendorf microcentrifuge. Unbound virus was carefully aspirated and the zoospore pellet was washed in 1.5 ml of 50 mM sodium phosphate buffer, pH 7.6. The zoospore pellet was assayed for the presence of virus by Western blot.
analysis using a CNV polyclonal antibody (RAD) raised against the R and arm domains of CNV CP (see below. Bound antibody was detected with goat anti-rabbit/peroxidase conjugate and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The quantity of virus in the pellet was determined by densitometric analysis of exposed film using the ImageQuant program (Molecular Dynamics). The amount of virus pelleted in the absence of the fungus was subtracted from the amount pelleted in the presence of the fungus. RAD polyclonal antibody was confirmed to react equally to wild-type CNV and Pro73Gly CP subunit in Western blot analysis.

To obtain zoospore-bound viruses for the trypsin digestion experiments, ten in vitro binding experiments were conducted separately. Following the first centrifugation, pellets were resuspended in buffer and then all ten pellets were pooled. The volume of the combined pellets was adjusted to 1.5 ml, and the mixture was centrifuged for seven minutes at 2700g as above. The final pellet was resuspended in sodium phosphate buffer, pH 7.6.

4.2.8 Production of polyclonal antisera

RAD antibody was produced from a synthetic CNV CP fragment corresponding to the R and arm domains. The R and arm domain coding region (corresponding to CP amino acid residues 1–106) was cloned into pET24d (Novagen) adjacent to a polyhistidine tag. Transformed BL21 cells were induced and fusion protein was purified by cobalt chelate chromatography. Approximately 500 µg of protein was used for injection of rabbits. Serum from the fifth boost was used for purification of RAD IgG.
4.2.9 *In vitro* swelling of virions

CNV or Pro73Gly virions (either 600 ng of virus/10 μl of reaction volume or 10 μg of virus/10 μl of reaction volume) were swollen in 50 mM sodium phosphate buffer (pH 7.6), 25 mM EDTA. The swelling reaction was allowed to proceed at room temperature for 30–40 minutes.

4.2.10 Limited proteolysis of native, swollen and zoospore-bound virus

Pilot experiments were conducted to determine the amount of trypsin required to distinguish native and swollen forms of CNV when digestions were conducted in a 10 μl volume using 600 ng of virus. Approximately 600 ng of native, swollen and zoospore-bound forms of CNV or Pro73Gly were digested in 50 mM sodium phosphate buffer (pH 7.6) at room temperature with 100 ng of trypsin (porcine pancreas; Sigma) in a final volume of 11 μl. Aliquots of 1 μl were removed at 2, 4, 8, 16, 32 and 64 minutes time-points. Digestion was terminated by the addition of 1 μl of 50 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Two separate 1 μl aliquots were removed prior to the addition of trypsin and incubated for two and 64 minutes to serve as controls.

Approximately one-sixth of each sample (equivalent to 10 ng of starting material) was subjected to SDS-12% PAGE gels and subsequently transferred onto PVDF membranes. CP was detected by RAD polyclonal antibody as above, using an enhanced chemiluminescence detection system (Amersham/Pharmacia Biotech.).

Limited proteolysis was also conducted using a higher concentration of virus: 100 μg of virus was swollen as above in a 100 μl reaction volume and 10 μl aliquots were incubated with 20 ng of trypsin for two to 64 minutes at room temperature. Digestions
were terminated as above, electrophoresed through an SDS/PAGE gel, and then stained with Coomassie brilliant blue.

4.2.11 Amino acid sequence analysis of trypsin-digested swollen virions

CNV virions (10 μg) were swollen in 50 mM sodium phosphate buffer (pH 7.6), 25 mM EDTA in a 10 μl reaction volume for 30 minutes at room temperature. Swollen virions were then treated with 100 ng of trypsin for five minutes at room temperature in an 11 μl volume. The digestion products were then electrophoresed through 4–12% NuPAGE Bis–Tris gels (Invitrogen) and then transferred to a PVDF membrane (Sequiblot; BioRad). The membrane was stained with Coomassie brilliant blue, and the 33.5 kDa and 36 kDa digestion products were excised from the membrane. The amino-terminal sequence was determined using Edman degradation (Nucleic Acid and Protein Synthesis Proteomics Facility, University of British Columbia, Vancouver, BC, Canada).

4.3 Results

4.3.1 Proteolytic digestion patterns of zoospore-bound CNV differ from those of native CNV

As several animal virus capsids undergo conformational change upon binding to their cellular receptors, we wished to determine whether CNV undergoes conformational change upon binding to its zoospore vector. CNV was incubated with zoospores in an in vitro binding assay as described (25). Limited proteolysis was then conducted over a 64 minute time-course. A similar time-course experiment was conducted using native CNV virions and results were analyzed by Western blot analysis. As can be seen in Figure
zoospore bound CNV is susceptible to digestion by trypsin as early as 2 minutes post-incubation, resulting in prominent 36 kDa and 33.5 kDa digestion products. In contrast, and as expected, native CNV is resistant to trypsin digestion up to 64 minutes post-incubation (Figure 4.2(A)). The differential sensitivity of zoospore-bound and native CNV to digestion by trypsin suggests strongly that CNV particles undergo conformational change upon zoospore binding.

Previous studies with TBSV and several other small spherical plant viruses have shown that the swollen forms of virus particles are proteolytically sensitive under conditions where native virions are not (15, 18). The proteolytic sensitivity of zoospore-bound CNV alerted us to the possibility that the bound form of CNV may resemble the swollen state of CNV. To assess this possibility, trypsin digestion time-course experiments were conducted as described above, using CNV virions swollen in vitro. Figure 4.2 shows that the proteolytic digestion pattern observed for swollen CNV is similar to that observed using zoospore-bound CNV (compare Figure 4.2 (C) and (B)) in that 36 kDa and 33.5 kDa fragments are observed as the major proteolytic digestion products in both. This suggests that zoospore-bound CNV may resemble the swollen state of CNV. However, as can been seen in Figure 4.2, the 36 kDa and the 33.5 kDa products in zoospore-bound CNV appear to be less stable than in swollen CNV, i.e. the 36 kDa and the 33.5 kDa bands are only barely visible in zoospore-bound CNV following longer incubation times, whereas they remain visible in swollen CNV (compare Figure 4.2(B) and (C)). In addition, swollen CNV digestions frequently contain a minor 39 kDa product (see Figure 4.2(C)), whereas zoospore-bound CNV only occasionally shows a 39 kDa
Figure 4.2. Time-course of trypsin digestion of native, swollen, and zoospore-bound CNV and Pro73Gly. Approximately 600 ng each of (A) native, (B) zoospore-bound and (C) swollen forms of CNV or Pro73Gly were digested with 100 ng of trypsin for two to 64 minutes. Equal volumes of digested material (equivalent to 10 ng of starting material) were loaded on to an SDS/polyacrylamide gel, blotted, and detected with a polyclonal antibody specific to the CNV R and arm domains (RAD). The digestion times (in minutes) are indicated below the lanes. Lanes 1–6 are trypsin-treated (+T) samples and lane 7 and 8 are mock treated (-T) samples for (2 and 64 minutes, respectively). (D). Trypsin digestion of CNV and Pro73Gly using 10 μg of virus and 20 ng of trypsin over a 64 minute time-course. The numbers at the left correspond to the relative molecular masses (in kDa) of the major trypsin digestion products. The asterisks (*) correspond to full-length CNV CP.
product (data not shown). The results of these experiments therefore suggest that zoospore-bound CNV is similar to swollen CNV, but not identical.

4.3.2 Effect of mutations of Pro73 and Pro85 on CNV particle formation

To further assess the potential importance of the conformational state of CNV in zoospore binding and transmission, a panel of CNV mutants was constructed with alterations in either Pro73 or Pro85, which are highly conserved residues located within the CP arm. We chose to alter these residues because it had been suggested that these residues may be important for proper particle conformation (2). In addition, both residues are located in the interior of the particle and therefore would not likely directly affect attachment to zoospores via their specific binding to a zoospore receptor. Figure 4.3 shows an alignment of the arm region of several Tombusviridae members demonstrating conservation of both proline residues among several plant virus genera. Based on the structure of TBSV,(2) it has been suggested that the conserved proline residue corresponding to CNV Pro73 may be important for formation of the β-annulus, since it forms a hook at the start of the β-annulus (Figure 4.1(B)) and therefore may be required for proper oligomerization of the three C-subunit arms. Similarly, it was suggested that the Pro85 residue might be important for quasi-equivalence by serving as a molecular switch to regulate the position of the arm.

Four CNV Pro73 mutants were constructed (pPro73Gly, pPro73Ala, pPro73Cys and pPro73Leu) which contain either Gly, Ala, Cys or Leu, respectively, in place of Pro73. One CNV Pro85 mutant was constructed (pPro85Gly), which has Gly in place of Pro85 (Table 4.1). Transcripts of each of the mutants were inoculated onto plants to
Figure 4.3. Location of conserved proline residues in the CP arm of several small spherical plant viruses. The CP arm sequences of several small spherical plant viruses are aligned. The acronym for the virus species is indicated at the left along with the name of the genus to which it belongs. The location of the conserved Pro residues in the arm is indicated with an arrow; the numbering of the residue is based on its location in the CNV CP subunit. Underlined amino acid residues are part of the arm involved in β-annulus formation in TBSV-BS and RYMV. Abbreviations and references for sequences are as follows: CNV, cucumber necrosis virus; (38). MNSV, melon necrotic spot virus; (37). CLSV, cucumber leaf spot virus; (32). RCNMV, red clover necrotic mosaic virus; (50). TBSV-Ch, cherry isolate of tomato bushy stunt virus; (16). TBSV-BS, tomato bushy stunt virus; (24). TCV, turnip crinkle virus; (22). SBMV, southern bean mosaic virus; (40). SeMV, sesbania mosaic virus; (3). RYMV, rice yellow mottle virus; (33). TNV-A, tobacco necrosis virus-A; (31).
assess their ability to initiate infection and produce virions in plants (Table 4.2). All mutants established infections on *Nicotiana benthamiana* and virus particles could be purified from the leaves of infected plants (Table 4.2). The yield of particles was very low in the case of Pro73Ala (about 10% that of wild-type CNV) and even lower for Pro73Cys, Pro73Leu and Pro85Gly (each about 2%) compared to that obtained from leaves infected with wild-type CNV. However, particle accumulation in Pro73Gly-infected *N. benthamiana* appeared to be approximately equal to that of wild-type CNV (Table 4.2).

To further examine properties of Pro73Gly, we conducted infectivity tests and we measured accumulation in plants using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA). The dilution end point of wild-type CNV on *Nicotiana clevelandii* was determined by inoculating plants with different concentrations of purified CNV virions (three leaves per plant; nine plants per treatment) and then observing plants for the development of systemic symptoms. Six, three and zero plants became infected when using 50, 16.6 and 5.5 pg of CNV inoculum, respectively, per leaf. Similar results were obtained with Pro73Gly virions, wherein seven, five and one plant became infected. These data therefore suggest that Pro73Gly can establish infections in *N. clevelandii* as well as CNV does. To test the level of accumulation in cucumber (the host used for transmission studies), Pro73Gly was inoculated onto cucumber cotyledons. Necrotic local lesions similar in size to those produced by CNV were observed; however, DAS-ELISA of leaf extracts indicated that Pro73Gly virions accumulated to approximately 40% of wild-type CNV (data not shown). Together, these data suggest that both Pro73 and Pro85 have important roles in CNV virion accumulation. In addition, the
Table 4.2. Properties of CNV Pro73 and Pro85 mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Infectivity</th>
<th>Virus Particles</th>
<th>Virus Yield</th>
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<tr>
<td>Pro73 mutants</td>
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<tr>
<td>Pro73Gly</td>
<td>+</td>
<td>yes</td>
<td>Similar to WT CNV</td>
</tr>
<tr>
<td>Pro73Ala</td>
<td>+</td>
<td>yes</td>
<td>~10% of WT CNV</td>
</tr>
<tr>
<td>Pro73Cys</td>
<td>+</td>
<td>yes</td>
<td>~2% of WT CNV</td>
</tr>
<tr>
<td>Pro73Leu</td>
<td>+</td>
<td>yes</td>
<td>~2% of WT CNV</td>
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<tr>
<td>Pro85 mutant</td>
<td></td>
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<tr>
<td>Pro85Gly</td>
<td>+</td>
<td>yes</td>
<td>~2% of WT CNV</td>
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1Refers to the ability of infectious clones of the mutants to infect *N. benthamiana*.
2Virus particle formation was determined by agarose gel electrophoresis following “mini-prep” purification from inoculated leaves.
3Yields were assessed by comparison of equal volumes of purified virus from an equivalent mass of infected tissue.
data suggest that Pro73 can be replaced by glycine but that virion accumulation in cucumber is reduced by approximately 60%.

4.3.3 Transmission efficiency of Pro73Gly virions by *O. bornovanus* is reduced dramatically

Transmission assays were conducted using Pro73Gly virions to determine its transmission efficiency relative to wild-type CNV. Transmission efficiency was scored by determining the number of pots infected *versus* the number of pots inoculated. Figure 4.4 shows that 23 of 25 pots (92% efficiency) inoculated with CNV/zoospore mixtures became infected, whereas only one of 25 pots (4% efficiency) became infected when Pro73Gly/zoospore mixtures were used. Thus, the Pro to Gly mutation at amino acid position 73 of the CNV arm appears to affect transmission efficiency much more dramatically than its effect on particle accumulation in mechanically inoculated plants.

4.3.4 Binding efficiency of Pro73Gly to zoospores is similar to that of wild-type CNV

We have previously shown that the transmission deficiency of several naturally occurring CNV transmission mutants is, at least partially, due to a reduced ability to bind to vector zoospores (25, 35). We wished to assess the possibility that reduced transmission of Pro73Gly is due to its inability to bind to zoospores during the transmission process using an *in vitro* virus/zoospore binding assay. Figure 4.5 shows that Pro73Gly binds to zoospores with approximately the same (or increased) efficiency (90–130%) as wild-type CNV. These results suggest that reduced transmissibility of Pro73Gly is not a result of its inefficient binding to zoospores during the transmission process.
Figure 4.4. Summary of fungus transmission assays using Pro73Gly virions. Transmission assays were conducted using 1 μg of virus (either CNV or Pro73Gly) in 10 ml of zoospores ($1\times10^5$ zoospores/ml). The percentage of pots showing transmission is indicated on the X-axis. The numbers on the bars indicate the number of pots showing transmission versus the number of pots tested. The data represent a compilation of five separate experiments.
Figure 4.5. Summary of Pro73Gly in vitro virus/zoospore binding assays. A sample (100 μg) of either Pro73Gly or CNV virions were incubated with $5 \times 10^5$ zoospores in an in vitro binding assay. The amount of bound virus was determined using Western blot analysis followed by densitometry. Binding efficiency was determined as the percentage of virus bound relative to the amount of WT CNV binding. The results are the average of two separate experiments (± the standard deviation) using triplicate samples of each virus per experiment.
4.3.5 The swollen conformation of Pro73Gly is different from that of CNV

The location of Pro73 in the β-annulus of virus particles suggested that the Gly mutation might influence transmissibility through its effect on the conformation of particles, since the β-annulus is known to play an important role in virus stability. Pro73Gly particles were therefore observed by electron microscopy; however, no obvious difference in morphology in comparison to CNV particles was apparent (data not shown). In addition, agarose gel electrophoresis, which can be used to monitor conformational changes in virus particles, did not reveal differences in electrophoretic mobility of Pro73Gly and CNV viruses (Figure 4.6, compare lanes 1 and 2). However, as can be seen in Figure 4.6, swollen Pro73Gly migrates slightly slower than swollen CNV, and resolves as a more discrete band. These results suggested that the swollen conformation of Pro73Gly may be different from that of CNV, and that this may be the feature that results in loss of transmission.

Limited proteolysis of native and swollen virus was therefore conducted to further assess potential differences in the native and swollen forms of Pro73Gly and CNV. Figure 4.2 shows the results of a proteolysis experiment where it can be seen that native Pro73Gly virions remain intact throughout the incubation time, similar to what was observed with native CNV particles (Figure 4.2(A)). However, the proteolytic digestion profile of swollen Pro73Gly appears different from that of swollen CNV (Figure 4.2(C)). In particular, and most evident, is the observation that trypsin digestion of swollen CNV results in prominent 36 kDa and 33.5 kDa products, whereas little or no 36 kDa or 33.5 kDa product is observed in trypsin-digested swollen Pro73Gly. In addition, a comparison
**Figure 4.6.** Agarose gel electrophoresis of native and swollen CNV and Pro73Gly. Approximately 500 ng of native or swollen virions was electrophoresed through a 1% (w/v) agarose gel buffered in Tris–borate (pH 8.3). Virions were visualized by ethidium bromide staining in the presence of 1 mM EDTA.
of the relative levels of CP in swollen Pro73Gly virions treated with trypsin for 64 minutes (lane 6) versus mock-treated, swollen Pro73Gly (lane 8) with the corresponding treatments of swollen CNV particles revealed that relatively lower levels of Pro73Gly are digested. To further assess the possibility that swollen Pro73Gly particles are more resistant to trypsin digestion, a time-course experiment was conducted using a lower mass ratio of trypsin to virus (i.e. 20 ng of trypsin and 10 μg of virus). Figure 4.2(D) shows that, indeed, swollen Pro73Gly particles are more resistant to proteolysis than are swollen CNV particles. From these studies, we conclude that swollen Pro73Gly particles are different from swollen CNV particles.

4.3.6 The N-terminal region of swollen CNV particles is accessible to trypsin digestion

In many small spherical viruses, conversion to the swollen state involves translocation of the N-terminal region of the CP to the particle exterior (36). In view of the observed differences in the swollen states of wild-type and Pro73Gly particles, we wished to determine the location of the 36 kDa and 33.5 kDa trypsin cleavage sites in swollen CNV particles. Edman degradation analysis was therefore conducted using purified 36 kDa and 33.5 kDa protein products. It was found that the 36 kDa product consisted of primarily one protein species that begins with Lys53, which is just upstream from the CP arm domain. The 33.5 kDa species appeared to be a mixture of similar-sized products, with the most prominent cleavage product corresponding to digestion at Arg95, which is located just after the arm domain (Figure 4.1(A)). Thus, as with other small spherical plant viruses, at least part of the CNV CP N-terminal domain is exposed on the particle surface. In addition, the near absence of these protein species in trypsin-treated
Pro73Gly particles indicates that a major structural difference between swollen wild-type and Pro73Gly particles is the near absence of the N-terminal coat protein region on the surface of particles.

4.3.7 Zoospore-bound Pro73Gly does not undergo conformational change

Our *in vitro* binding experiments in Figure 4.5 indicate that the poor transmissibility of Pro73Gly cannot be attributed to inefficient binding to zoospores. However, the observation that the swollen conformation of Pro73Gly is different from that of CNV (Figure 4.2), along with our observation that CNV undergoes a conformational change similar to that of swollen CNV upon zoospore binding (Figure 4.2), raised the possibility that Pro73Gly may be poorly transmitted due to an inability to form the proper conformation upon zoospore binding. To test this possibility, we conducted trypsin digestion experiments on zoospore-bound Pro73Gly, as was conducted on wild-type zoospore-bound CNV (Figure 4.2). Figure 4.2(B) shows that, unlike zoospore-bound wild-type CNV, zoospore-bound Pro73Gly particles are resistant to trypsin digestion throughout the 64 minute digestion reaction. These results suggest that the inability of Pro73Gly to be transmitted may be a result of its inability to undergo conformational change upon zoospore binding.

4.4 Discussion

Previous work in our laboratory has shown that specific amino acid residues in the CNV CP S and P domains are important for zoospore attachment and subsequent transmission by *O. bornavarius* (7). Here, we have examined the possible involvement of the internally located CP arm in fungus transmission, focusing on two proline residues in
the arm region. Substitution of either residue results in decreased particle accumulation in most cases (Table 4.2), suggesting the importance of the proline residue in either particle assembly, disassembly or stability. However, substitution of proline 73 with glycine had a less deleterious effect, and had no observable effect in *N. clevelandii*. Pro73 forms part of the β-annulus, which is an important intermediate in assembly of particles, possibly by facilitating formation of trimers of dimers at the particle 3-fold axis (42). The observation that Pro73Gly particles accumulate to wild-type levels in *N. clevelandii* and to 40% of wild-type levels in cucumber (Table 4.2) suggests that substitution with Gly does not have a strongly detrimental effect on β-annulus formation, or that the β-annulus may not be absolutely required for virus assembly (48).

It is believed that Pro85 may be important for virus assembly by controlling the arm position and acting as a molecular switch to regulate the ratio of the two types of conformations required of the C and A/B subunits to satisfy quasi-equivalence (2). It is possible that the Pro to Gly substitution in Pro85Gly reduces the efficiency of particle formation by engendering an unacceptably high number of conformational states in the arm and thereby restricting efficient oligomerization during particle assembly.

The data presented here suggest that successful transmission of CNV requires conformational change of virus particles during or following the attachment phase of transmission. We believe that this is the first demonstration that a plant virus undergoes conformational change during acquisition by its vector. It also is the first time that *in vivo* evidence has been obtained for the biological significance of the swollen state of a small spherical plant virus.
Our studies indicate that zoospore-bound CNV resembles CNV particles swollen *in vitro*. It is believed that many viruses have a highly dynamic character *in vivo* (27, 4) and conformational changes in capsid structures are purported to be associated with the initial stages of infection in many viruses (11, 23, 51, 41, 20). In the well-studied poliovirus system, receptor attachment initiates a series of conformational changes that allow the virus to attach to membranes, to form a pore in the membrane and to release viral RNA from the capsid (23). It has been noted that the structural states associated with the various transitions of poliovirus particles are highly analogous to the expanded states of structurally similar plant viruses. In particular, the rotation and outward movement of poliovirus VP2 and VP3 along the 3-fold axis is analogous to the expansion at the quasi-3-fold axis in plant virus capsids. In addition, the outward movement of the amino-terminal region of VP1 at the 5-fold axis is analogous to the extrusion of the plant virus A and B subunit N termini (23, 19). Indeed, these similarities have previously prompted the suggestion that the swollen state of plant virus capsids may correspond to an important intermediate during the initial stages of infection. Our observation that zoospore-bound CNV virions are structurally different from native virus, and that they resemble virions swollen *in vitro*, suggests that the biological significance of the swollen state may lie, at least in part, in the vector attachment process. Although further experiments are required to determine the extent of similarity between the swollen and bound forms of CNV, our results clearly show that conformational change is associated with zoospore attachment. Zoospore-bound CNV is proteolytically more sensitive than native virions (Figure 4.2(A) and (B)). This might reflect the dynamics of externalization in the native virus and the possibility that the arms of bound virus are trapped in a more exposed state.
Previous studies with other spherical plant viruses have shown that virions swollen \textit{in vitro} are more readily translated in cell-free translation systems (7, 47, 6), suggesting that swelling \textit{in vivo} may be associated with viral uncoating and concomitant translation of the genome. It is possible that the virus uncoating mechanism \textit{in vivo} occurs on membranes within cells and is mechanistically similar to the vector attachment process.

Interestingly, exposure of Pro73Gly particles to \textit{in vitro} swelling conditions results in particles with reduced mobility on agarose gels similar to that observed with swollen wild-type CNV particles (Figure 4.6). The reduced mobility of swollen CNV particles is likely due to the increase in particle size following repulsion of the three subunits at the quasi-3-fold axis. The observation that Pro73Gly particles also migrate more slowly suggests that they too undergo expansion. However, as shown in Figure 4.2, trypsin digestion of swollen Pro73Gly particles does not result in the same high-level accumulation of the 36 kDa and 33.5 kDa products seen in swollen CNV digestions, although these products can be observed when large amounts of swollen mutant particles are digested (see Figure 4.2(D), lanes 5 and 6). Thus, although Pro73Gly particles are expanded at the quasi-3-fold axis, it appears as though the N-terminal region of a significant proportion of the CP subunits have not translocated to the particle exterior.

Pro73Gly particles bind zoospores efficiently (Figure 4.5) but are largely insensitive to trypsin digestion (Figure 4.2(B)). This suggests that reduced transmission of Pro73Gly virions is due to a defect in the ability of particles to undergo some aspect of conformational change during zoospore attachment. As described above, binding of
poliovirus to its cellular receptor has been shown to result in distinct conformational changes and it has been suggested that the receptor acts as a catalyst to promote the required changes (45). In addition, in the case of human rhinovirus, the ability of the virus to undergo conformational change has been related directly to its ability to infect cells (29). Previous work in our laboratory has suggested that specific oligosaccharides and/or glycoproteins present on the zoospore surface act as receptors for CNV attachment (26). In analogy to that observed in poliovirus, we hypothesize that binding of CNV to its zoospore receptor may facilitate conformational change in CNV, and that this conformational change is required to complete subsequent stages of the transmission process. Our experiments did not assess if bound Pro73Gly particles have undergone the expansion aspect of swelling, but the observation that they do expand under in vitro conditions supports the notion that binding to zoospores fails to induce particles to extrude the N-terminal domain. Thus, at a minimum, extrusion of the N-terminal arms appears to be highly essential for transmission.

It is believed that the main function of the zoospore in CNV transmission is to provide a means for entry of CNV into otherwise impermeable root cells. In consideration of the proposed parallels between poliovirus entry into host cells and CNV attachment to zoospores, it is possible that zoospores may play a more significant role than has been recognized so far. For example, attachment may contribute to root cell infection by "priming" particles for translation in the root cytoplasm. Alternatively, CNV may enter zoospores prior to root cell infection. Indeed, electron microscopy of CNV/zoospore-infected plants has suggested that CNV uptake by O. bornovanus zoospores involves endocytosis (43). In addition, it is known that transmission of some
plant viruses by Olpidium brassicae is believed to involve uptake of virus particles in the co-infected plant (10).

Our finding that CNV undergoes conformational change upon zoospore attachment raises the possibility that a similar change might be important for other aspects of the virus infection cycle. For example, it is possible that the uncoating process may occur on membranes within an initially infected cell. Further experiments are required to investigate this possibility.

In summary, the results of our experiments suggest strongly that CNV undergoes conformational change as part of the transmission process. Moreover, our data suggest that the basis for the reduction in transmission of Pro73Gly particles is due to the inability of the A and B subunit arms to be translocated to the surface. Thus, as has been suggested in poliovirus, extrusion of the arms of CNV may be important for interaction with the zoospore membrane and possibly for entry of the virus or viral nucleic acid into zoospores.
4.5 References


5 CHAPTER FIVE
GENERAL DISCUSSION

This thesis explored some of the molecular determinants (viral and vector) required for vector transmission of CNV by its fungal vector O. bornovanus. One goal was to determine if any specific sequences on the CNV coat protein are important in recognizing zoospores during the attachment phase of the transmission process. Previous work in this lab showed that the CP plays an important role in determining the specificity of CNV transmission by O. bornovanus. It has been shown that continuous passage of plant viruses in the absence of the vector would result in variants that are deficient in vector transmission. Robbins et al. (1997) isolated and characterized LL5, one such naturally occurring transmission deficient mutant. It was shown that specific regions on the shell domain of CNV CP play an important role in fungus transmission.

To further identify the regions important for CNV transmission the methodology developed for the characterization of LL5 was followed. We isolated and characterized six distinct, naturally occurring CNV transmission mutants. Each mutant was found to contain an amino acid substitution either in CP shell or protruding domains which is responsible for transmission deficiency. Transmission efficiencies of these mutants were variable (Table 2.1) and in vitro virus-zoospore binding assays showed that each mutant binds zoospores less efficiently than WT CNV, suggesting that reduced transmissibility is at least partially due to inefficient zoospore binding. An artificial double mutant containing mutations present in both protruding and shell domains was shown to bind and transmit less efficiently by zoospores, reinforcing the role of both mutations in
attachment and transmission processes. In order to determine whether these mutated amino acids are exposed and clustered on the coat protein tertiary and quaternary structures, homology modelling of the CNV CP subunit, asymmetric unit and particle was conducted based on the closely related X-ray crystal structure of TBSV (Figure 2.3). Mapping of these mutated amino acids indicated most of the mutants are exposed on the surface of the particle. Several of these mutated amino acids are located in or near a cavity formed by the trimer at the particle quasi three-fold axis, suggesting that this region may be important in recognizing a putative receptor on the zoospore surface. It is not known whether these mutations contribute to reduced transmissibility by affecting interaction with the putative receptor or indirectly by changing the virion conformation. Isolation and characterization of more naturally occurring transmission mutants and mutagenic analysis of conserved amino acids in the quasi three-fold axis cavity in CNV or other related T=3 fungally transmitted viruses (CLSV, MNSV and TNV) might be useful in determining the further importance of this region in fungus transmission.

Previous work from our lab has provided the preliminary evidence for the presence of receptor or receptor-like molecules on the zoospore surface (8). It was shown that binding of CNV to zoospores is saturable, indicating a discrete number of recognition molecules on zoospores for CNV. In addition, in vitro binding studies have demonstrated efficient binding between viruses and their respective vector zoospores and that this reflects their association in nature (8, 9).

In an attempt to further characterize the biochemical nature of the molecule(s) on the surface of O. bornovanus zoospores involved in CNV attachment, zoospores were treated with periodate, trypsin and phospholipase-C. Reduced CNV binding to pre-treated
zoospores suggested that glycoproteins on the zoospore surface may mediate CNV attachment. CNV bound to several specific-sized proteins in virus overlay assays, whereas CNV transmission mutants failed to bind or bound at significantly reduced levels. The possible involvement of specific sugars in virus binding was investigated using sugar inhibition studies. It was found that mannose-containing oligosaccharides and fucose inhibited CNV binding at relatively low concentrations. These studies suggest that binding of CNV to zoospores is mediated by specific mannose-/or fucose-containing oligosaccharides or glycoproteins. Interestingly previous studies from our lab revealed the presence of mannose/glucose and fucose containing sugars on the surface of the zoospores, reinforcing the possible involvement of these sugars as receptors or a part of receptors for CNV attachment (8). In addition chytrid zoospores are known to contain a prominent cell coat predominantly made of mannose-containing sugars (6).

Carbohydrates play an important role as biological recognition molecules in critical cellular functions such as signal transduction, immune modulation and enzyme catalysis (2). Carbohydrates are excellent carriers of information due to their complex nature and structural diversity (11). The ability of cell surface carbohydrates to act as receptors is usually dependent on: 1) the presence of a particular sugar; 2) accessibility; and; 3) mode of presentation (for example, linkages in oligosaccharides) (11). Oligosaccharides are used as primary, as well as secondary receptors by different animal viruses (Table 1.5). Several animal viruses that recognize oligosaccharides as their entry receptors are considered as viral lectins. Lectins are carbohydrate binding proteins of plant and animal origin that recognize oligosaccharides present on cell surfaces. The tail spike protein (TSP) of Salmonella phage 22, haemagglutinin (HA) of influenza viruses,
VP1 of polyomaviruses and the VP4 of rotaviruses, are all known to contain a lectin-like fold in their proteins which harbour an oligosaccharide binding site (13, 3). The ability of several mannose-containing oligosaccharides to inhibit CNV binding to zoospores suggests that CNV has lectin-like properties that may play an important role in recognition of its fungal vector. Interestingly, the lectin Con-A has a jellyroll topology, which is a common structural motif found in several viral capsids. It has been suggested that there is broad structural similarity between Con-A and the tombusvirus capsid (1). This may indicate that tombusviruses have evolved from lectins. Taken together, these studies support the hypothesis that CNV has lectin-like properties that may play an important role in the recognition of its vector. In the case of some animal viruses that bind to proteoglycans, it is thought that the proteoglycan is used as initial attachment receptor before a further high affinity receptor strengthens the attachment (4, 10). Whether mannose-containing oligosaccharides are acting as primary or secondary receptors for CNV attachment remains to be determined.

Future work may involve the testing of these sugars as inhibitors of transmission of CNV and other related viruses by Olpidium. Also, structural determination of CNV complexed with a sugar (mannotriose or α3-, α6-mannopentaose) either by X-ray crystallography or by cryo-electron microscopy image reconstruction techniques will be useful in determining the sugar binding region on the CNV capsid. Further identification of zoospore proteins involved in CNV recognition could involve 2-D gel electrophoresis of zoospore proteins (either total or membrane-associated) followed by overlay assays using CNV virions as a probe. Proteins that specifically bind to CNV can then be digested with trypsin and identified using mass spectrometry. It may be possible to obtain
c DNA clones corresponding to the proteins and to use those clones or expressed proteins to further assess their role as receptor(s) for CNV.

Limited trypsin digestion of zoospore-bound, swollen and native (unbound) CNV revealed that zoospore-bound CNV is conformationally different and resembles the swollen conformation. In addition we have shown that reduced transmission of Pro73Gly, a CNV transmission-deficient mutant was due to its inability to undergo this conformational change. Together, these results suggest that conformational changes in virus particles are important for fungus transmission of CNV.

During poliovirus entry into the host cell, the virus particle undergoes expansion upon binding to the receptor in which CP subunits VP1, VP2 and VP3 move outwardly creating significant gaps at the base of five-fold axis. The internally located N-terminal regions of VP1 and VP4 come out through these openings to form an amphipathic helical bundle, which not only promotes virion binding to the cellular membrane, but also causes a pore or disruption through which viral RNA enters the cell (5). Similar to the poliovirus system, our data shows that CNV undergoes a swollen-like conformation in which the N-terminal arms of the A and B subunits are exposed. This structural similarity could indicate functional homology between CNV/zoospore interaction and poliovirus/host cell interaction. In the case of zoospore-bound CNV, the externalized arms could interact with the zoospore membrane resulting in stabilization of the virus/zoospore interaction and/or release of viral RNA into the zoospore during transmission. Interestingly, parallel studies in our lab have suggested the presence of a putative transmembrane domain in the arm region of the CNV CP. Green fluorescent protein (GFP) tagging experiments revealed that the arm is specifically targeted to chloroplast membranes (D. Rochon, Y. Xiang, &
R. Reade, unpublished observations). These studies suggest that externalization of the arm may promote CNV/zoospore membrane interactions. It would be interesting to investigate whether the conformational changes induced by zoospore binding are also important for critical steps of virus infection inside the plant cell, such as disassembly. It is also possible that the requirement for conformational change in virus particles could be a common phenomenon in plant virus/vector interactions. In the case of potyviruses these conformational changes could be induced by virus interaction with helper factor, since it has been suggested that helper factor play a critical role in determining the specificity of aphid transmission.

It is generally well-accepted that virions bind to the outside of zoospores as a means for zoospore assisted entry into root cells. However, exactly how bound virus enters the root cytoplasm is not clear. Temminck and Campbell (12) suggested that virus attached to the flagellar sheath is transferred to the zoospore cytoplasm upon flagellar retraction and that CNV enters root cytoplasm via injection of the zoospore cytoplasm. However, this would require that CNV crosses the zoospore membrane to enter the host cell. It seems more likely that virus bound to the zoospore plasmalemma is injected into the root cytoplasm during injection of the zoospore protoplast. In this case, virus would be outside of the protoplast and within the root cytoplasm. The final stages of viral uncoating would then take place in the root cytoplasm.

A major significance of the work described in this thesis is the emerging similarities between plant virus/vector interactions and animal virus/host cell receptor interactions. In addition, the work in this thesis is the first to demonstrate: 1) that specific regions of the virus capsid are important for vector attachment and transmission; 2) the
role of specific sugars in vector attachment and; 3) the importance of conformational
to the work described in this
changes in the virus particle for vector transmission. Based on the work described in this thesis we propose the following model (9, Figure 5.1) for CNV binding to *O. bornovanus* zoospores during transmission: 1) CNV recognizes a glycoprotein or oligosaccharide
receptor on the surface of the zoospore wherein the cavity at the CNV particle quasi
three-fold axis contains important sugar binding elements; 2) upon binding to the putative receptor, the CNV particle acquires a swollen or swollen-like conformation in which the A, B, and C subunits move away from each other to form openings through which disordered arms of the A and B subunits are translocated to the exterior of the particle; and 3) the hydrophobic portions of the A and B arms interact with the zoospore membrane resulting in stabilization of virus/zoospore interaction (9; Fig. 5.1).
Figure 5.1 Model for CNV binding to O. bornovanus zoospores. (A) A portion of the CNV particle showing the icosahedral five-fold and quasi three-fold axis. The A, B and C subunits are represented in red, blue and green colors respectively. Yellow circles represent the predicted positions where the A and B subunit arms come out during expansion. (B) Possible steps involved in the interaction between CNV and zoospore during the attachment phase of the transmission process. Only the A and B subunits are shown. This figure was adapted and modified from reference #9.
5.1 References


6 CHAPTER SIX
APPENDIX

6.1 Introduction

Isolation and characterization of several naturally occurring CNV transmission mutants showed that specific regions of the CNV capsid are important for transmission by *O. bornovanus*. Two regions, one on the protruding domain facing the cavity at the particle quasi three-fold axis and the other region on the particle shell at the quasi three-fold axis could play an important role in recognizing zoospores during the attachment phase of transmission (2; see Chapter 2). We have also showed that CNV acquires a swollen-like conformation upon binding to the zoospores, and the extrusion of the internally located *arms* during this conformational change is important for fungus transmission (3).

In the present preliminary study, we utilized *in vitro* mutagenesis to further assess the importance of the region on the CP protruding domain that is predicted to be important for fungus transmission. Two non-conserved proline residues in the *arm* and two additional proline residues, (one, a conserved proline at the base of the hinge, and another in the middle of the hinge) were also mutated to assess their effect on fungus transmission (Figure 6.1).
Figure 6.1. Surface representation of the CNV CP subunit (subunit C) in different orientations showing location of mutated amino acids. Different structural domains are indicated in different colors as follows: arm (yellow); shell (light gray); hinge (blue) (not visible in B); and protruding (P, gray). The disordered R domain is not shown. (A) Location of Pro63, Pro67, Pro85, Pro259, Pro262, Ser338 and Ser339 (B) Location of Thr292, Leu353, Trp296, and Gln297 on the protruding domain. All mutated amino acids are colored in red except Ser338 and Trp296, which are represented in pink to distinguish from adjacent Ser339 and Gln297 respectively.
6.2 Materials and Methods

6.2.1 Virus purification

Partially purified virus particles of wild type CNV and CNV mutants were obtained by a miniprep procedure (4).

6.2.2 Maintenance of *O. bornovanus* cultures

*O. bornovanus* isolate SS196 was maintained on cucumber roots (*Cucumis sativus* cv. Poinsette 76) essentially as described (1).

6.2.3 *In vitro* mutagenesis

Oligonucleotide-directed *in vitro* mutagenesis was used to produce CNV CP mutants (Table 6.2). To produce mutants Thr292Ala, Asp293Ala, Trp296Ala, Gln297Ala, Ser338Ala, Ser338Phe, Ser338Thr, Ser339Gly, Leu353Ala, Pro63Gly, Pro63Ala, and Pro85Ala, an *EcoRl*/*Neol* fragment encompassing the CNV CP and flanking regions (Figure 4.1(A)) in a full-length infectious cDNA clone of CNV (pK2/M5) (5) was subcloned into *EcoRl*/*Neol*-digested pT7 Blue (Novagen) and used as a template for *in vitro* mutagenesis. Oligonucleotide primers used for mutagenesis are described in (Table 6.1). Mutants were screened by sequencing. Selected plasmid DNA was then digested with *EcoRl*/*Neol* and the fragment containing the mutation was cloned into similarly digested pK2/M5. pK2/M5 was used as the template for the production of Pro67Gly, Pro67Ala, Pro259Gly, Pro259Ala, Pro262Gly, Pro262Ala (Table 6.2). Following mutagenesis, plasmid DNA was digested with either *EcoRl*/BglII (in the case
### TABLE 6.1. Oligonucleotide primers used for constructing various CNV CP mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro63Gly</td>
<td>TGTCCGTGGGCTATGAGAGC (-)</td>
<td>(2818-2719)</td>
</tr>
<tr>
<td>Pro63Ala</td>
<td>TGTGC\textsubscript{G}GTGGGCTATGAGAGC (-)</td>
<td>(2818-2719)</td>
</tr>
<tr>
<td>Oligo#178\textsuperscript{3}</td>
<td>GGT\textsubscript{T}TTTCTGGGGCTATCGCCCG (+)</td>
<td>(2819-2842)</td>
</tr>
<tr>
<td>Pro67Gly</td>
<td>CCAC\textsubscript{C}AAAAGCCTGTGGGTCGGGC (-)</td>
<td>(2830-2808)</td>
</tr>
<tr>
<td>Pro67Ala</td>
<td>CCAG\textsubscript{A}AAAGCCTGTGGTGTCGGGC (-)</td>
<td>(2830-2808)</td>
</tr>
<tr>
<td>Oligo#121\textsuperscript{3}</td>
<td>GGT\textsubscript{C}ATCGCGCGCGCAATC (+)</td>
<td>(2831-2849)</td>
</tr>
<tr>
<td>Pro85Ala</td>
<td>CTAG\textsubscript{C}TTTCTTCTTACGCCAGATAG (-)</td>
<td>(2883-2855)</td>
</tr>
<tr>
<td></td>
<td>GTT\textsubscript{T}CAAACAGCAAAAAAGGATCTGTCG (+)</td>
<td>(2884-2909)</td>
</tr>
<tr>
<td>Pro259Gly</td>
<td>GTTC\textsubscript{C}CTGTGGCTCAAAACAGATC (-)</td>
<td>(3406-3383)</td>
</tr>
<tr>
<td>Pro259Ala</td>
<td>GTG\textsubscript{C}CTGTGGCTCAAAACAGATC (-)</td>
<td>(3406-3383)</td>
</tr>
<tr>
<td>Oligo#190\textsuperscript{3}</td>
<td>GTC\textsubscript{G}CCTTTCTTCTGGAATCG (+)</td>
<td>(3407-3425)</td>
</tr>
<tr>
<td>Pro262Gly</td>
<td>AGAG\textsubscript{C}CCCGACGTTGGTTGTGC (-)</td>
<td>(3415-3395)</td>
</tr>
<tr>
<td>Pro262Ala</td>
<td>AGAG\textsubscript{C}CCCGACGTTGGTTGTGC (-)</td>
<td>(3415-3395)</td>
</tr>
<tr>
<td>Oligo#193\textsuperscript{3}</td>
<td>TCT\textsubscript{G}GAATCGTTGGTTCGGAGAGGAG (+)</td>
<td>(3416-3437)</td>
</tr>
<tr>
<td>Thr292Ala</td>
<td>AAGG\textsubscript{T}TCGGCGAAGCGCAACCTC (-)</td>
<td>(3509-3486)</td>
</tr>
<tr>
<td></td>
<td>GT\textsubscript{A}TGGCAGCAACGTTGTCG (+)</td>
<td>(3510-3528)</td>
</tr>
<tr>
<td>Trp296Ala</td>
<td>GTAG\textsubscript{G}CGCAAGCGACCTGTGCCCG (+)</td>
<td>(3510-3531)</td>
</tr>
<tr>
<td>Gln297Ala</td>
<td>GTAT\textsubscript{G}GGCGACCGTGTCGCCCG (+)</td>
<td>(3510-3533)</td>
</tr>
<tr>
<td>Oligo#128\textsuperscript{4}</td>
<td>AAGG\textsubscript{T}TCGGTGACGCAGAGCCAC (-)</td>
<td>(3509-3489)</td>
</tr>
<tr>
<td>Ser338Ala</td>
<td>AGAG\textsubscript{G}GCTCGGCGGCTGTGCCT (-)</td>
<td>(3643-3618)</td>
</tr>
<tr>
<td>Ser338Phe</td>
<td>AGAG\textsubscript{A}ATCCGGCGGCTGTGCAAC (-)</td>
<td>(3643-3622)</td>
</tr>
<tr>
<td>Ser338Thr</td>
<td>AGAG\textsubscript{G}TCCGGGCGGCTGTCTC (-)</td>
<td>(3643-3618)</td>
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<tr>
<td>Ser339Gly</td>
<td>AGCG\textsubscript{T}TCGGGCCGGCGTCTCAC (-)</td>
<td>(3643-3622)</td>
</tr>
<tr>
<td>Oligo#112\textsuperscript{5}</td>
<td>GC\textsubscript{A}ATATGTTGGCAACACATCATA (-)</td>
<td>(3644-3668)</td>
</tr>
<tr>
<td>Leu353Ala</td>
<td>ACT\textsubscript{C}GCGTTGGCAATTCACGCATAG (-)</td>
<td>(3689-3664)</td>
</tr>
<tr>
<td></td>
<td>CCT\textsubscript{T}TGGGCTACCGGGAGCC (+)</td>
<td>(3690-3710)</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Sequences of primers are shown 5' to 3'. Underlined nucleotides correspond to mutated positions, and the antisense (-) or sense (+) orientation relative to the CNV genome.

\textsuperscript{2} Nucleotide positions are relative to the complete CNV genome (5).

\textsuperscript{3} Oligos 178, 121, 190 and 193 were used as (+) sense primers for Pro63, Pro67, Pro259 and Pro262 mutants, respectively.

\textsuperscript{4} Oligo\#128 was used as (-) sense primer for Trp296 and Gln297 mutants.

\textsuperscript{5} Oligo\#112 was used as (+) sense primer for Ser338 and Ser339 mutants.
of Pro67Gly and Pro67Ala) or EcoRI/NcoI (in the case of Pro259Gly, Pro259Ala, Pro262Gly and Pro262Ala) and the mutated fragment was cloned back into pK2/M5 to obtain a full-length clone containing the mutation.

6.2.4 In vitro transcription and inoculation of plants

Preparation of T7 polymerase run-off transcripts and inoculation of plants were done as described (6).

6.2.5 Fungus transmission assay

Partially purified virions were tested for their ability to be transmitted by O. bornovanus zoospores as described previously (3; Chapter 4).

6.3 Results and conclusions

Mutations introduced into the CNV CP are listed in Table 6.1. The location of mutations in the three-dimensional structure of the CP subunit is shown in Figure 6.1. All the mutated amino acids in the P domain and hinge are predicted to be exposed on the surface of the particle except ser338 which is buried in the P/P domain contact region. Pro63, Pro76, Pro85 are present on the internally located arm domain. Transcripts corresponding to each of the mutants (Table 6.2) were inoculated onto N. benthamiana to assess their ability to produce virions in plants. All the mutants were found to be capable of producing symptoms in plants. Agarose gel electrophoresis of partially purified virus preparations (data not shown) of all mutants showed that virus particles could be purified from all mutants, except Asp293Ala, Trp296Ala, Ser338Phe, Ser339Gly and Leu353Ala
Figure 6.2. Summary of transmission efficiencies of CNV mutants. Transmission assays were conducted using 1 µg of virus in 10 ml of zoospores (1x10^5 zoospores/ml) as described in reference #3 and Chapter 4. The percentage of pots showing transmission is indicated on the X-axis. The numbers on the bars indicate the number of pots showing transmission versus the number of pots tested. The data represented for CNV, Pro63Gly, Pro259Gly and Pro259Ala is a compilation of at least three experiments. For the rest of the mutants, the data presented are from one experiment.
Table 6.2. Properties of CNV mutants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Particle formation&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Fungus transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro63Gly</td>
<td>arm</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pro63Ala</td>
<td>arm</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro67Gly</td>
<td>arm</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro67Ala</td>
<td>arm</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro85Ala</td>
<td>arm</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro259Gly</td>
<td>S</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pro259Ala</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro262Gly</td>
<td>hinge</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro262Ala</td>
<td>hinge</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thr292Ala</td>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trp296Ala</td>
<td>P</td>
<td>-</td>
<td>Not tested</td>
</tr>
<tr>
<td>Gln297Ala</td>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ser338Ala</td>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ser338Phe</td>
<td>P</td>
<td>-</td>
<td>Not tested</td>
</tr>
<tr>
<td>Ser338Thr</td>
<td>P</td>
<td>-</td>
<td>Not tested</td>
</tr>
<tr>
<td>Ser339Gly</td>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leu353Ala</td>
<td>P</td>
<td>-</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Abbreviations used: S, shell domain; P, protruding domain

<sup>1</sup>Virus particle formation was determined by agarose gel electrophoresis following “mini-prep” purification of virus from inoculated leaves
(Table 6.2). The yield of particles appeared to be approximately equal to that of WT CNV. Transmission assays were conducted for all the mutants that produced virus particles to assess their transmission efficiency relative to WT CNV. Transmission efficiency was measured by determining the number of pots infected versus the number of pots inoculated. Figure 6.2 shows that Pro63Gly transmitted with very low efficiency (10%), whereas Pro259Gly was not transmitted by zoospores. WT CNV and all other mutants were transmitted with higher efficiency (92% to 100%).

These results show that the majority of the mutants (Table 6.2) are capable of being transmitted by *O. bornovanus*. In the case of Ser338 it was previously shown that a mutation to glycine (LLK63) allowed for particle formation but interfered with transmission (2; Chapter 2). Here it is shown that changing Ser338 to Ala did not significantly affect particle formation or transmissibility. However, mutation to Thr or Phe prevented particle formation. Ser338 is located in a region wherein P/P domain contact occurs and it is therefore likely that the substitution with the bulky aromatic Phe or the larger Thr prevents particle formation by interfering with P/P domain interaction.

Mutation of Pro259 and Pro63 to Ala or Gly did not interfere significantly with particle formation (Table 6.2). However, transmission was affected when Pro259 was mutated to Gly. It is possible that the greater conformational freedom engendered by Gly substitution does not support the proper conformational changes required for successful transmission as previously suggested for Pro73Gly (3; Chapter 4). Further infectivity studies are required to assess the fitness of the Pro259 and Pro63 mutants and experiments need to be designed to assess potential changes in conformation induced by these substitutions. Future work could involve testing whether these mutants: 1) bind to
zoospores efficiently using the *in vitro* binding assay; and 2) whether the mutants fail to undergo conformational changes upon zoospore binding as the possible reason(s) for reduced transmission.

As far the other remaining transmissible mutants, further mutagenesis experiments are required to assess the importance of these particular amino acids in fungus transmission.
6.4 References


Co-authorship Statement

Mr. Kakani played a major role in the design and execution of experiments in Chapter 2, 3, 4 and 6 which form the bulk of the research described in this thesis. He also contributed substantially to data analyses and manuscript preparation for each Chapter. The contributions of co-authors are listed below:

1. Dr. Jean-Yves Sgro (Chapter 2) conducted the homology modelling and subsequent three-dimensional structural representation of the CNV particle described in Section 2.2.8.

2. Dr. Marjorie Robbins designed, conducted and analyzed the experiments described in Fig. 3.1 and Fig. 3.6 in Chapter 3.

3. Ron Reade assisted in the development of procedures to analyze structural changes in CNV virions as a result of fungal zoospore attachment thereby contributing, in part, to Figure 4.2, excluding the parts of figures corresponding to Pro73Gly. Ron Reade also assisted in the determination of the location of trypsin cleavage sites in swollen CNV particles (Section 4.3.6).

4. D’Ann Rochon was the supervising scientist. She developed the broad goals and assisted in the design and interpretation of experiments as well as manuscript preparation.

D’Ann Rochon: .................................................
(Supervisor and senior author)

Naga Kishore Kakani: ...

(Thesis author)