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

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Received 30 September 2002/Accepted 18 December 2002

Despite the importance of vectors in natural dissemination of plant viruses, relatively little is known about the molecular features of viruses and vectors that permit their interaction in nature. Cucumber necrosis virus (CNV) is a small spherical virus whose transmission in nature is facilitated by zoospores of the fungus Olpidium bornovanus. Previous studies have shown that specific regions of the CNV capsid are involved in transmission and that transmission defects in several CNV transmission mutants are due to inefficient attachment of virions to the zoospore surface. In this study, we have undertaken to determine if zoospores contain specific receptors for CNV. We show that in vitro binding of CNV to zoospores is saturable and that vector zoospores bind CNV more efficiently than nonvector zoospores. Further studies show that treatment of zoospores with periodate and trypsin reduces CNV binding, suggesting the involvement of glycoproteins in zoospore attachment. In virus overlay assays, CNV binds to several proteins, whereas CNV transmission mutants either fail to bind or bind at significantly reduced levels. The possible involvement of specific sugars in attachment was investigated by incubating CNV with zoospores in the presence of various sugars. Two mannose derivatives (methyl α-D-mannopyranoside and α-D-mannosamine), as well as three mannose-containing oligosaccharides (mannotriose, α3,6-mannopentaose, and yeast mannan) and L-(-)-fucose, all inhibited CNV binding at relatively low concentrations. Taken together, our studies suggest that binding of CNV to zoospores is mediated by specific mannose and/or fucose-containing oligosaccharides. This is the first time sugars have been implicated in transmission of a plant virus.

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 form Frankliniella occidentalis, the main thrip vector for Tomato spotted wilt virus (genus Tobravirus), is a potential receptor. Symbionin, a homologue of Escherichia coli GroEL chaperonin 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.
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).

In vitro zoospore binding assay. 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).

Trypsin, periodate, and phospholipase C treatment of O. bornovanus zoospores. One milliliter of O. bornovanus zoospores (5 × 10^7/ml) was incubated in either 10 mM sodium periodate–0.1% trypsin (Sigma) or 5 mM of phospholipase C (Sigma) for 15 min. Periodate oxidation 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 × 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 virosomes 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).

Virus overlay assays. Virus overlay assays were conducted essentially as described by Salas-Benito and del Angel (24) with some modifications. A total of 2 × 10^6 zoospores (20 μl) in denaturating 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 fluorescein isothiocyanate-labeled concanavalin-A (ConA–FITC; Sigma) or 50 μg of FITC-labeled Tetragonolobus pupurea agglutinin (TPA–FITC; Sigma) were incubated for 10 min with washed O. bornovanus zoospores (2 × 10^5 to 2 × 10^6) in 1 ml of binding buffer. The zoospores were visualized with a Zeiss Axiopt epifluorescence microscope using an excitation wavelength of 450 to 490 nm. The specificity of the labeling 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.

RESULTS

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 × 10^6 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 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—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. Figure 1B 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 [Fig. 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 [Fig. 1C]). These results 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.

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, 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-(−)–sorbitose, D-(−)–fructose, methyl α-D-mannoside, yeast mannan, α-D-mannose, α-D-mannopentaose, mannotriose-bio[α-D-glucosaminyl], and N-acetyl-D-mannosamine. The sugars were initially tested for inhibitory activity using 14-fold serial dilutions beginning with 0.2 M sugar. Dilutions of α-D-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 mannosyl 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.

Labeling of O. bornovanus zoospores with FITC-labeled lectins. Sixteen micrograms of fluorescein isothiocyanate-labeled concanavalin-A (ConA–FITC; Sigma) or 50 μg of FITC-labeled Tetragonolobus pupurea agglutinin (TPA–FITC; Sigma) were incubated for 10 min with washed O. bornovanus zoospores (2 × 10^5 to 2 × 10^6) in 1 ml of binding buffer. The zoospores were visualized with a Zeiss Axiospt epifluorescence microscope using an excitation wavelength of 450 to 490 nm. The specificity of the labeling 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.

The above 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.
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 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.

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 × 10⁶ zoospores (Fig. 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 (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 Fig. 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.

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 virions. Figure 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.
CNV coat protein subunit, have reduced 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 (lane 4) shows that LLC5 binds with reduced efficiency (40, 39, and 21% of WT CNV binding, respectively) (12, 22). Experiments with preincubation of CNV with several serial 10-fold dilutions of sugar prior to adding virus to the zoospores. Sugars showing relatively significant inhibition (arbitrarily defined as those sugars with a 50% effective concentration [EC50] 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 1 shows that among 15 sugars tested, mannose, α3,6-mannopentaose, and methyl α-ᴅ-mannopyranoside showed EC50s of <10 mM. The EC50s for these sugars varied, with mannose showing the strongest relative inhibitory activity (EC50 = 128 μM) and D-mannosamine showing the weakest (2.7 mM) (Table 1). Inhibition curves for each of these sugars

Inhibitors

<table>
<thead>
<tr>
<th>Sugar</th>
<th>EC50 (mM)</th>
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<tbody>
<tr>
<td>Mannose</td>
<td>0.128</td>
</tr>
<tr>
<td>α3,6-Mannopentaose</td>
<td>0.157</td>
</tr>
<tr>
<td>Methyl α-ᴅ-mannopyranoside</td>
<td>1.9</td>
</tr>
<tr>
<td>Maenan</td>
<td>2.0</td>
</tr>
<tr>
<td>L(-)-Fucose</td>
<td>2.3</td>
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<tr>
<td>D-Mannosamine</td>
<td>2.7</td>
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Noninhibitors

<table>
<thead>
<tr>
<th>Sugar</th>
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<tbody>
<tr>
<td>D(+)-Glucose</td>
</tr>
<tr>
<td>L(+)-Arabinose</td>
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<tr>
<td>D(+)-Galactose</td>
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<tr>
<td>N-Acetyl-D-glucosamine</td>
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<tr>
<td>L(-)-Sorbose</td>
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<tr>
<td>N-Acetyl-D-mannosamine</td>
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<tr>
<td>D(+)-Xylose</td>
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<tr>
<td>D(+)-Manuose</td>
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<td>D(-)-Fructose</td>
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a Inhibitors are arbitrarily classified as those sugars with EC50s of <10 mM.
are presented in Fig. 5. Among the sugars tested for inhibitory activities, no monosaccharides except methyl α-D-mannoside, α-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.

Surfaces of *O. bornovanus* 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 glucose, and TPA-FITC, which recognizes fucose, were the only two lectins that gave detectable binding (Fig. 6). ConA and TPA lectins bound densely and uniformly over the surface of the zoospore body and flagella. Labeling 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 α-D-(+)-mannose and/or α-D-(+)-glucose and l-(−)-fucose on the surfaces of *O. bornovanus* zoospores.

**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 (Fig. 1), two criteria used to demonstrate receptor-mediated attachment of animal viruses to their receptors (28). Extrapolation from the saturation binding curve indicates that \(2.7 \times 10^4\) binding sites are present on zoospores (i.e., 0.018 pmol per \(4 \times 10^5\) zoospores). This num-
ber is within the range of virus receptor sites normally found on eukaryotic cells (34). The saturation binding curve in Fig. 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).

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 mannann or mannool-\( \alpha \)-d-mannopyranoside dramatically reduced CNV binding (Fig. 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 1). Interestingly, two mannose derivatives (\( \text{methyl-\( \alpha \)-d-mannopyranoside} \) and \( \text{D-mannosamine} \), as well as three mannose-containing oligosaccharides (mannotriose, \( \alpha \)-3,6-mannopentaose, and yeast mannann), were found to be strong inhibitors, with \( EC_{50} \) ranging from 128 \( \mu \)M for mannotriose to 2.7 \( \text{mM} \) for D-mannosamine. \( \text{L-(-)Fucose} \) was also found to be an efficient inhibitor, with an \( EC_{50} \) of 2.3 \( \text{mM} \). \( \text{D-Mannose} \) 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 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 nM (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 (Fig. 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 \( O. bornovanus \) 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 case 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 has 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 Fig. 6 show that \( O. bornovanus \) zoospores contain both \( \text{L-(-)Fucose} \) and \( \text{D-(+)Mannose} \) 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.

**ACKNOWLEDGMENTS**

We thank Ron Reade for many helpful discussions during the course of this research, and we also thank Steve Orban, Amanda Brown, and Paul Neilsen for excellent technical assistance. We are grateful to Dave Theilmann for reviewing the manuscript prior to submission. Part of this research was supported by the Natural Sciences and Engineering Research Council of Canada (RG 43640).

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