Outer-capsid P8 proteins of phytoreoviruses mediate secretion of assembled virus-like particles from insect cells

Naoyuki Miyazaki,\(^1,2,3\) Kyoji Hagiwara,\(^1\) Taiyun Wei,\(^1\) Hongyan Chen,\(^1\) Atsushi Nakagawa,\(^2\) Li Xing,\(^3\) R. Holland Cheng\(^3\) and Toshihiro Omura\(^1\)

1National Agricultural Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan
2Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan
3Department of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA

Phytoreoviruses are composed of two concentric capsid layers that surround a viral genome. The capsids are formed mainly by the inner-capsid P3 protein and the outer-capsid P8 protein. During the infection of insect-vector cells, these play important roles in packaging the viral genome and the enzymes required for its transcription. P3 and P8 proteins, when co-expressed in Spodoptera frugiperda cells, co-localized in cells and were released as spherical clusters. In contrast P3 proteins expressed in the absence of P8 protein were associated with the cells when they were examined by confocal microscopy. Cryo-electron microscopy revealed that the secreted clusters, composed of P3 and P8 proteins, were double-layered virus-like particles that were indistinguishable from intact viral particles. Our results indicate that P8 proteins mediate the secretion of assembled virus-like particles from S. frugiperda insect cells and, therefore, most probably from insect-vector cells also.

Phytoreoviruses in the family Reoviridae, such as rice dwarf virus (RDV) and rice gall dwarf virus (RGDV), have a double-layered capsid that encapsidates and protects their genomes from the environment and serves to transport each genome and the enzymes required for its transcription from cell to cell. The core-capsid protein forms a smooth, thin, continuous layer, which is a structure that has been found in all viruses in the family Reoviridae analysed to date (Miyazaki et al., 2008 and references therein).

The outermost capsid shell of reoviruses appears to play important roles in maintaining the stability of the thin, innermost capsid shell and in sequestering the dsRNA genome, as well as in conferring host specificity and mediating entry into host cells. In the case of RDV, the outer capsid shell is composed of the major capsid-protein P8, which plays an important role in the infection of insect-vector cells (Omura & Yan, 1999), and the minor outer-capsid proteins P9 and P2, which are required for adsorption of the virus to host insect cells (Tomaru et al., 1997; Omura et al., 1998). In our earlier studies of core-like particles composed solely of the P3 core-capsid protein of RDV and expressed using a baculovirus expression system in cells of the insect Spodoptera frugiperda (Sf\(^9\) cells; Hagiwara et al., 2004), we detected and purified core-like particles from infected cells but we failed to find them in the culture medium.

Infectious and intact RDV and RGDV particles that contained both the P3 core- and the P8 outer-capsid proteins were, however, detected in the culture medium of vector cells grown in monolayers (VCM; Wei et al., 2008). These results and the cell association of P3 proteins noted above suggested that the outer capsid proteins of phytoreoviruses might play a role in the transmembrane transport of viral particles in insect-vector cells.

Intact viral particles of RDV and RGDV are icosahedral, double-layered particles of approximately 70 nm in diameter (Omura & Mertens, 2005). Each double-layered capsid consists of 780 molecules of the major outer-capsid P8 protein and 120 molecules of the inner-capsid P3 protein, enclosing the genome and inner protein components that are required for transcription (Iwasaki et al., 2008). The core-capsid protein P3 of RDV has the intrinsic ability to assemble into single-shelled core-like particles and double-layered virus-like particles (VLPs) when the P3 protein is co-expressed with outer-capsid P8 protein in Sf\(^9\) cells (Hagiwara et al., 2003). Furthermore, the P8 protein of RGDV, which is 51 % identical at the amino acid level to the P8 protein of RDV (Noda et al., 1991), is able to form VLPs when RDV P3 and RGDV P8 proteins are co-expressed in Sf\(^9\) cells (Miyazaki et al., 2005). Thus the major capsid proteins of phytoreoviruses have the inherent
ability to self-assemble in vivo in the absence of any other viral proteins. In the present study, we examined the localization of P3 and P8 proteins in cultured Sf9 cells that had been infected with recombinant baculovirus that encoded either one or both of the viral proteins. We aimed to determine the way in which the virus transports its genome and transcriptional enzymes across the membrane of insect-vector cells to enable the spread of the phytoreovirus within the host.

We investigated the localization of the recombinant capsid proteins in insect cells using a baculovirus expression system in Sf9 cells. In addition to the previously constructed baculovirus system for expressing the outer-capsid P8 proteins of RDV and RGDV and the P3 core-capsid protein of RDV (Hagiwara et al., 2003; Miyazaki et al., 2005), we also constructed a baculovirus expression system for the P3 protein of RGDV, as described previously (Miyazaki et al., 2005). After appropriate incubation of Sf9 cells, we removed the cells from the culture medium by centrifugation for 10 min at 30,000 × g. We then analysed supernatants from the culture media of cells that had been infected with various combinations of recombinant baculoviruses by SDS–PAGE (10% polyacrylamide) and Western blotting.

Recombinant P3 proteins of RDV and of RGDV were expressed and detected within Sf9 cells but were not detected in the culture media. In contrast, P8 proteins of RDV and RGDV were expressed and detected both within Sf9 cells and in the culture media. The results for RDV P3 and RGDV P8 proteins are shown in Fig. 1. In this case RDV-P3-specific and RGDV-P8-specific antibodies were used to detect the respective proteins. RDV P3 and RGDV P3 were detected both within Sf9 cells and in the culture media, as were RDV P8 and RGDV P8 in the combinations RDV-P3/RDGVP8, RDV-P3/RDGVP8 and RGDV-P3/RGDV-P8. Interestingly, heterogenic protein interactions, namely between RDV P3 and RGDV P8 (RDV-P3/RGDV-P8), also resulted in the secretion of P3 proteins (Fig. 1), confirming a previous finding that these proteins can assemble into chimeric VLPs when co-expressed in Sf9 cells (Miyazaki et al., 2005). For unknown reasons, we failed to obtain VLPs from the combination of RGDV P3 and RDV P8. Our results suggested that inner-core P3 proteins, bound to outer-capsid P8 proteins, had been secreted from infected Sf9 cells into the culture media. Such co-secretion implies that foreign proteins can be secreted from the insect cells together with P8 proteins if these proteins can form a stable complex with P8 proteins.

To determine the way in which VLPs might exit Sf9 cells, we examined the subcellular locations of P3 and P8 proteins by confocal microscopy. We expressed the P3 and P8 proteins of RDV in Sf9 cells and stained the cells with RDV-P3-specific antibodies conjugated to FITC and with RDV-P8-specific antibodies conjugated to rhodamine. We then examined the cells as described previously (Wei et al., 2006c). When the P3 and P8 proteins were co-expressed, they co-localized in spherical clusters that were located inside and outside the cells (Fig. 2). In contrast, fluorescence due to FITC from cells that expressed only RDV P3 was found exclusively inside the cells. These results suggested that VLPs composed of P3 and P8 proteins had been released from the Sf9 cells as spherical clusters, whose appearance resembled that of the vesicular compartments.

**Fig. 1.** Identification by SDS-PAGE and Western blotting of P3 protein of RDV and P8 protein of RGDV in recombinant-baculovirus-infected Sf9 cells. (a) Samples were separated by SDS–PAGE and proteins were stained with Coomassie brilliant blue R (Nacalai Tesque). Lane 1, purified RDV particles; lane 2, RGDV particles; lane 3, Sf9 cells that expressed RDV P3 protein; lane 4, that expressed RGDV P8 protein; lane 5, that co-expressed RDV P3 and RGDV P8 proteins. The same amount of cell lysate was loaded in each lane. (b) Samples were separated by SDS-PAGE and proteins were detected by Western blotting with RDV P3-specific (upper panel) or RGDV P8-specific (lower panel) antibodies. Lane 1; purified RDV particles; lane 2, purified RGDV particles; lanes 3 and 6, Sf9 cells and the culture media, respectively, that expressed RDV P3 protein; lanes 4 and 7, cells and the culture media, respectively, that expressed RGDV P8 protein; lanes 5 and 8, cells and the culture media, respectively, that co-expressed RDV P3 and RGDV P8 proteins. Since RGDV P8-specific antibodies cross-reacted with RDV P8 protein, a weak band representing the RDV P8 protein in purified RDV particles was also detected in lane 1. The same respective amounts of cell lysate and culture supernatant were loaded in each lane. Although the level of expression of P3 protein was much higher than that of P8 protein, the P3 protein was not detected in the culture supernatant (lane 6). However, the P3 protein was detected in the supernatant when it was co-expressed with the P8 protein.
that are exploited by authentic RDV particles for their transport and release from infected insect-host cells (Wei et al., 2008, 2009a). Our results suggest that assembled VLPs, generated in non-host Sf9 cells, are released from these cells via vesicular compartment-like structures in a manner similar to that of intact viral particles (Wei et al., 2008).

To determine the structure of the P3 and P8 complex that was secreted from the Sf9 cells, we purified the particles as follows. We removed the Sf9 cells from the culture medium by centrifugation for 10 min at 30,000 g and then the particles were pelleted by centrifugation for 60 min at 155,000 g. We suspended the pellet in a 0.1 M histidine, 0.01 M MgCl2 solution (pH 6.2, His–Mg solution). We then mixed the suspension with 20 % (v/v) CCl4 for 1 min and centrifuged the mixture for 10 min at 30,000 g. After sucrose density-gradient centrifugation (10–40 %) of the supernatant for 60 min at 94,500 g, we collected the banded material and pelleted particles by centrifugation for 60 min at 155,000 g. The final pellet, containing the assembled particles, was suspended in His–Mg solution.

Particles composed of P3 and P8 were then examined by electron microscopy (EM; Hitachi H-7000). Their morphology was indistinguishable from that of intact RDV and RGDV (Hagiwara et al., 2003). Secreted particles composed of RDV P3 and RGDV P8 also formed similar VLPs (Fig. 3a), whose 3D structure we determined by cryo-EM and 3D reconstruction. Particles were observed with a cryo-electron microscope (JEM-2100F; JEOL), operated at 200 kV with a nominal magnification of 30,000 × and data were recorded with a 4096 × 4096 pixel charge-coupled device. The imaging procedures were done as described previously (Miyazaki et al., 2010). The final reconstruction of the VLP was computed from 460 particles at 2.7 nm resolution, which was assessed using the 0.5 threshold in the Fourier shell correlation between two reconstructions calculated from the two halves of the dataset. As shown in Fig. 3(b), the structure of the VLP revealed a T=13 icosahedral symmetry (left). A central cross-section of the VLP is also shown (right). The P8 proteins are designated P, Q, R, S and T trimers according to their positions in the T=13 icosahedral lattice.
Mertens, 2005). We have, as yet, no evidence for the secretion of packaged proteins within the capsid shell. However, RDV P7 protein is incorporated into the cavity of inner-core particles when the P7 protein is co-expressed with the P3 core-capsid protein (Hagiwara et al., 2003). Therefore, the P7 protein might be incorporated into the virus-like particles and released from cells when P3, P7 and P8 proteins are co-expressed in Sf9 cells, although the packaging signal remains to be identified.

Co-expression of the P3 core-capsid and the P8 outer-capsid proteins in Sf9 cells resulted in both the production of double-shelled particles and the secretion of these particles from the cells into the culture medium. However, these results were obtained in non-host Sf9 cells and observations with Sf9 cells might not accurately reflect events in leafhopper host cells. Such secretion into the medium differs from the case of rotavirus since rotavirus-core-like particles, composed of baculovirus-expressed VP2 and VP6 and corresponding to the P3–P8 VLPs of phytooreoviruses, have been reported to be associated with cells (Sabara et al., 1991). However, recombinant VP7 protein and rotavirus-like particles, generated by the simultaneous expression of recombinant VP6 and VP7 proteins and lacking the innermost capsid layer composed of 120 molecules of VP2, were secreted from infected cells. On the basis of these results, we propose that the component of the outermost layer of the viruses, namely the VP7 protein of the rotavirus in the third layer and the P8 protein of RDV in the second layer, might function similarly in the transport of viral particles across the membrane of host cells. Thus, as one of multiple ways in which the virus might spread, capsid proteins of phytooreoviruses that form the outermost capsid layer might confer the ability on mature virus particles to penetrate the cell membrane of the host insect and to infect surrounding uninfected cells. In the case of RDV, another pathway involves the non-structural protein Pns10, as reported previously (Wei et al., 2006; Katayama et al., 2007). In this pathway, Pns10 proteins form tubular structures that contain viral particles. These tubular structures protrude from infected cells and penetrate neighbouring uninfected cells, allowing safe virus transfer from cell to cell. Similarly, bluetongue virus is released from infected cells via multiple pathways and one of its non-structural proteins, NS3, is responsible for intercellular trafficking and release of viral particles from cells (Hyatt et al., 1993; Celma & Roy, 2009).

Both RDV and RGDV are unusual in their ability to multiply in both plant cells and insect-vector cells. Recent studies have revealed various functions for the viral proteins and their roles during viral replication: for example, in adsorption to and penetration of insect-vector cells (Yan et al., 1996; Wei et al., 2007), in propagation in the viroplasm (Wei et al., 2006c) and in intracellular movement of the virus within infected insect-vector cells (Wei et al., 2006b, 2009b). The present study demonstrates that the viruses also appear to require a function that is associated with the P8 outer-capsid protein for the transport of infectious viral particles out of infected insect-vector cells.

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References


