Assembly of single-shelled cores and double-shelled virus-like particles after baculovirus expression of major structural proteins P3, P7 and P8 of *Rice dwarf virus*

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Expression of the core capsid protein P3 of *Rice dwarf virus* in a baculovirus system resulted in the formation of single-shelled core-like particles in insect cells in the absence of any other capsid proteins. Double-shelled virus-like particles were also observed upon mixing or co-expression of P3 and the major outer capsid protein P8, suggesting that P3 and P8 have the ability to form double-shelled particles both *in vivo* and *in vitro*. Core protein P7 expressed in a similar manner was incorporated into the virus-like particles.

Rice dwarf virus (RDV), which belongs to the genus *Phytoreovirus* in the family *Reoviridae*, has six structural proteins, P1, P2, P3, P5, P7 and P8, which are encoded by the S1, S2, S3, S5, S7 and S8 segments of the dsRNA genome, respectively (Omura & Yan, 1999). The outer shell of the RDV capsid is composed of P2 and P8, while the core consists of P1, P5 and P7 proteins enclosed by the P3 core capsid protein. The P3 core and P8 outer capsid proteins constitute the major framework of the architecture of RDV. P7, a nucleic acid-binding protein, which also binds to P3, is an important contributor to the conformation of RDV particles (Ueda & Uyeda, 1997). Results of a recent study of the structure of RDV suggested that some of the P8 trimers, anchored by interactions between P3 and P8 at positions on the threefold axes of symmetry, might form the seeding centre for assembly of the symmetry-mismatched inner and outer shells (Wu et al., 2000). A study of transgenic rice that expressed P3 and P8 also suggested that the P3–P8 interaction is necessary for the formation of a structure that closely resembles that of RDV particles (Zheng et al., 2000). However, the ability of the major core protein P3 to produce a single-shelled core and the mechanisms for sorting and organizing capsid proteins and segments of genome dsRNA remain to be clarified. In this study, we expressed the P3, P7 and P8 proteins in insect cells using a baculovirus system to determine the way in which the basic structural proteins of the RDV particle are generated by these major structural proteins.

Virus was propagated in our laboratory and viral dsRNA was purified from virions with a QIAquick PCR Purification Kit (Qiagen), according to the manufacturer’s instructions. To generate the cDNAs from S3, S7 and S8, which included full-length open reading frames for the P3, P7 and P8 proteins of RDV, respectively, we used the following pairs of primers: 5′-CCCATTGAGTGGACGTACGCGAGCATATGACG-3′ and 5′-CATGGTCATAGAGGGCTGATCTCATTGCGG-3′ (S3F-NcoI/S3R-NcoI); 5′-AGATCTTTGGCCCGC-ACATG-3′ and 5′-CCATGGCGGTAGTAATGACCCAA-3′ (S7F-NcoI/S7R-NcoI); and 5′-ATACTATGTCACGGCACG-ATGTTGTTGACAC-3′ and 5′-AAGCTTCTAAATTTGTTTGGATGATTATCTTCCAAATAC-3′ (S8F-BglII/S8R-HindIII).

The primers were based on the terminal RNA sequences of the S3, S7 and S8 segments, respectively (Kano et al., 1990; Nakashima et al., 1990; Omura et al., 1989) and restriction sites are underlined. cDNAs were generated by RT-PCR as described previously (Hagiwara et al., 2001). After digestion by appropriate restriction enzymes, the products of PCR were ligated into the transfer vector pBlueBacIII (Invitrogen). Restriction analysis and DNA sequencing were performed to confirm that the coding sequence of each segment was oriented appropriately with respect to the baculovirus polyhedrin promoter.

We found that the amino acid sequences predicted from the nucleotide sequences of S3 and S7 cDNAs contained some substitutions when compared with previously reported sequences (Kano et al., 1990; Nakashima et al., 1990). These alterations were located at residues 183 (His to Gln), 184 (Gly to Arg) and 1014 (Gly to Glu) in S3, and at residue 231 (Ala to Thr) in S7. The S8 cDNA encoded a protein with an amino acid insertion (Ala) at position 179 when its product contained one additional amino acid (Ala to Thr) in S7. The S8 cDNA encoded a protein with an amino acid insertion (Ala) at position 179 when its product was compared with the sequence of S8 reported previously (Omura et al., 1989).
Linearized AcNPV DNA and resultant transfer plasmid prepared to express P3 were used to co-transfect cultured Sf-9 cells in the presence of CellFECTIN in accordance with the manufacturer’s instructions (Invitrogen). To solubilize the expressed P3 protein, recombinant baculovirus-infected Sf-9 cells were mixed with BugBuster Protein Extraction Reagent (Novagen) and incubated for 30 min at 25°C; the supernatant was collected after centrifugation for 5 min at 30,000 g. After 10–40% sucrose density gradient centrifugation (SDGC) of the supernatant for 70 min at 94,500 g, the material at a position close to the predicted position of RDV cores was collected and pelleted by centrifugation for 60 min at 155,000 g. Many core-like particles were observed in this fraction after expression of P3 alone (Fig. 1a). To confirm that the core-like particles were constructed from P3 protein, immunogold staining of the particles was performed using P3-specific antibody according to the method described by Lin (1984). As shown in Fig. 1(b), core-like particles were specifically labelled with gold particles in electron microscopy. These results indicate that P3 protein itself has the ability to form a single-shelled core without the assistance of any other structural proteins of RDV.

To construct double-shelled particles, cells that expressed P3 or P8 were mixed, treated with BugBuster Protein Extraction Reagent, and processed in the same manner for the core particle preparation. After SDGC, the band material was pelleted by high-speed centrifugation as mentioned above. As shown in Fig. 1(c), double-shelled virus-like particles that resemble in both size and appearance RDV particles purified from RDV-infected rice plants (Fig. 1d) were observed by electron microscopy. Co-expression of P3 and P8 in Sf-9 cells also produced similar structures (data not shown); however, there were no such structures when P8 alone was expressed. These results suggest that recombinant P3 and P8 have the ability to generate double-shelled particles both in vivo and in vitro.

To determine whether core protein P7 could be enclosed inside the core particles, recombinant P7 was co-expressed with P3 in insect cells; P8 was then added and virus-like particles were purified as mentioned above. The proteins in the purified particles were analysed by SDS-PAGE (10% polyacrylamide) and Western blotting with P3-, P7- and P8-specific antibodies. As shown in Fig. 2, P3 (lanes 4 in (a) and (b)), P7 (lanes 4 in (a) and (c)) and P8 (lanes 4 in (a) and (d)) were clearly detected in the purified virus-like particles. It has been reported that proteins with mobility higher than P8 are also encoded by RDV segment 8 (Suzuki & Sugawara, 1991; Mao et al., 1998), suggesting that the smaller bands detected in Fig. 2(d) may be such a protein. The band with molecular size bigger than P7 in Fig. 2(c) could be a band specific for baculovirus expression, but no details are known.

To confirm that the P7 protein is located inside the virus-like particles, purified core-like particles composed of P3 and P7 were observed after immunogold labelling using P7-specific antibody. The antibody did not react with core-like particles (data not shown), in contrast to gold labelling of core-like particles when reacted with P3-specific antibody (refer to Fig. 1b). To further rule out the possibility that the expressed free P7 was located at the same position as double-shelled virus-like particles in SDGC, particles purified by SDGC were further subjected to 30% CsCl equilibrium centrifugation for 24 h at 190,400 g. After such centrifugation, a single band formed at the middle portion of the centrifugation tube. Single-shelled core-like particles were observed under the electron microscope in this band. This result suggests that a large proportion of the P8 outer capsid proteins were removed, leaving the P8 that had bound strongly to core particles (T-trimer; Wu et al., 2000) in CsCl equilibrium centrifugation. As shown in Fig. 3, P7 was detected in the re-purified particles, suggesting that the protein is not simply co-purifying in SDGC. Our results thus support the hypothesis that P7 is packaged inside viral particles.

**Fig. 1.** Electron micrographs of uranyl acetate-stained RDV particles. (a) Core-like particles composed of P3 proteins purified from recombinant baculovirus-infected Sf9 cells; (b) immunogold electron micrograph of core-like particles composed of P3; (c) virus-like-particles obtained by mixing recombinant P3 and P8; (d) RDV particles purified from RDV-infected rice plants. Bars represent 100 nm.
with the genomic RNA, since P7 binds to RNA (Ueda & Uyeda, 1997).

The present study showed that P3 has the intrinsic ability to form single-shelled core particles. This demonstrates that P3 has the conformational properties that enable bi-lateral binding of the homologous proteins which results in self-assembly of a spherical particle. Co-expression of P3 with a core protein such as P7 resulted in the location of P7 inside the core particles specifically. These results suggest that core proteins and the genomic RNAs that bind P7 might form aggregates before the formation of RDV core. Our study in vitro clearly demonstrated that the outer capsid protein P8 can adhere to core particles after the formation of the P3 layer, although the possibility remains that P3–P8 is the unit that is important for assembly of capsids in vivo. Observations described in this study will provide significant insight into the mechanism of assembly of the capsid proteins and the sorting of the genome segments of reoviruses.

REFERENCES


