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Efficient generation of *cowpea mosaic virus* empty virus-like particles by the proteolytic processing of precursors in insect cells and plants

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ABSTRACT

To elucidate the mechanism of formation of *cowpea mosaic virus* (CPMV) particles, RNA-2-encoded precursor proteins were expressed in *Spodoptera frugiperda* cells. Processing of the 105K and 95K polyproteins in *trans* to give the mature Large (L) and Small (S) coat proteins required both the 32K proteinase cofactor and the 24K proteinase itself, while processing of VP60, consisting of the fused L–S protein, required only the 24K proteinase. Release of the L and S proteins resulted in the formation of virus-like particles (VLPs), showing that VP60 can act as a precursor of virus capsids. Processing of VP60 expressed in plants also led to efficient production of VLPs. Analysis of the VLPs produced by the action of the 24K proteinase on precursors showed that they were empty (RNA-free). This has important implications for the use of CPMV VLPs in biotechnology and nanotechnology as it will permit the use of noninfectious particles.

Introduction

Cowpea mosaic virus (CPMV) is the type member of the genus comovirus within the family Comoviridae. CPMV has a genome consisting of two molecules of positive-strand RNA (RNA-1 and RNA-2), which are separately encapsidated in icosahedral particles of approximately 28-nm diameter. Preparations of CPMV can be resolved into three components, Top (T), Middle (M), and Bottom (B) by density gradient centrifugation. These represent empty (RNA-free) particles, particles containing RNA-2 and particles containing RNA-1, respectively. Although the amount of T component varies, it rarely represents more than 10% of a virion preparation. Particles from all 3 components contain 60 copies each of a Large (L) and Small (S) coat protein arranged with pseudo $T=3$ ($P=3$) symmetry (Lomonossoff and Johnson, 1991; Lin et al., 1999). The L and S proteins are situated around the 3- and 5-fold symmetry axes and contain two and one β-barrels, respectively. The S protein can exist in two forms, fast (Sf) and slow (Ss), depending on whether the C-terminal 24 amino acids are present (Taylor et al., 1999). Detailed knowledge of the structure of the CPMV particle, coupled with its robustness, has led to it being extensively used in biotechnological and nanotechnological applications (for a recent review, see Steinmetz et al., 2009).

Both CPMV genomic RNAs are expressed through the synthesis and subsequent processing of large precursor polyproteins (for a review, see Goldbach and Wellink, 1996). RNA-1 encodes the proteins involved in protein processing and RNA replication. The RNA-1-encoded polyprotein self-processes in *cis* through the action of the 24K proteinase domain embedded within it to give the 32K proteinase cofactor, the 58K helicase, the VPg, the 24K proteinase, and the 87K RNA-dependent RNA-polymerase. RNA-2 is translated to give a pair of polyproteins, the 105K and 95K proteins, as a result of initiation at two different AUG codons at positions 161 and 512 on the RNA (Holness et al., 1989). These polyproteins are processed by the RNA-1-encoded 24K proteinase in *trans* at 2 sites to give the 58K/48K pair of proteins (which differ only at their N-termini) and the mature L and S coat proteins (Fig. 1A). In vitro translation studies have shown that processing at the glutamine/methionine (Q/M) site between the 58/48K proteins and the L protein also requires the presence of the RNA-1-encoded 32K "proteinase cofactor" (Vos et al., 1988).

Although much is known about the structure and properties of the mature CPMV particle, relatively little is known about the mechanism of virus assembly. It has, to date, proved impossible to develop an in vitro assembly assay since the L and S proteins isolated from virions are insoluble in the absence of denaturants (Wu and Bruening, 1971). CPMV virus-like particles (VLPs) can be formed in both cowpea protoplasts (Wellink et al., 1996) and *Spodoptera frugiperda* (*Sf*21) insect cells (Shanks and Lomonossoff, 2000) by the coexpression of the individual L and S coat proteins. However, in both cases, the yield of assembled particles was low and the system clearly does not mimic the situation that occurs during a virus infection where the mature L and S proteins are both produced by proteolytic processing of the RNA-2-encoded polyproteins by the RNA-1-encoded 24K proteinase

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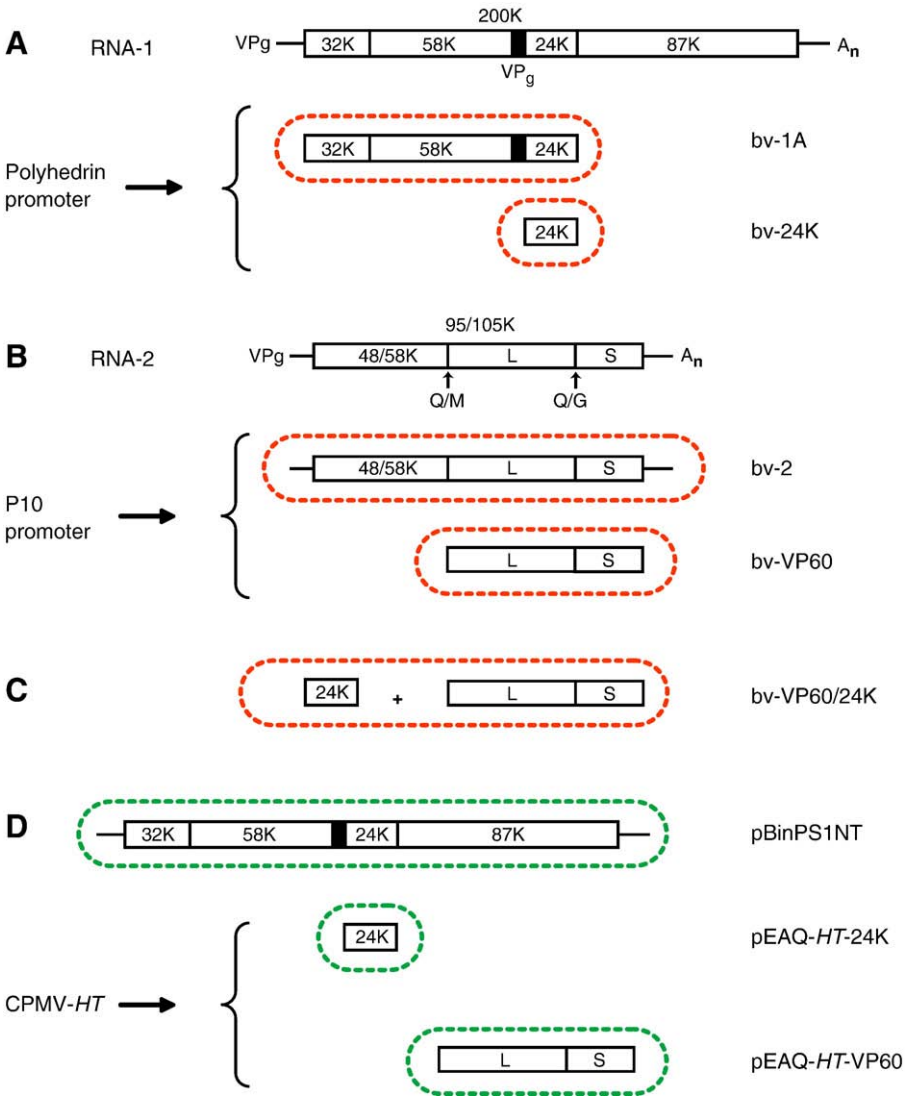

Fig. 1. Diagrammatic representation of constructs used to express CPMV proteins in insect cells and plants. The constructs for expression in insect cells and plants are shown surrounded by red and green dotted lines, respectively. (A) Organization of CPMV RNA-1 with the portions of the RNA expressed using baculovirus constructs bv-1A and bv-24K shown below. Expression in insect cells was driven by the polyhedrin promoter. (B) Organization of CPMV RNA-2 with the portions of the RNA expressed using baculovirus constructs bv-2 and bv-VP60 shown below. Expression in insect cells was driven by the P10 promoter. (C) Structure of baculovirus construct bv-VP60/24K designed to express both the 24K proteinase (under the control of the polyhedrin promoter) and VP60 (under the control of the P10 promoter). (D) Structure of constructs expressed in plants via agro-infiltration. pBinPS1NT is a full-length copy of CPMV RNA-1 while pEAQ-*HT*-24K and pEAQ-*HT*-VP60 were designed to express the 24K proteinase and VP60, respectively, using the CPMV-HT expression system (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2009). Abbreviations: 32K, 32K proteinase cofactor; 58K, helicase; VPg, viral protein genome-linked; 24K, 24K proteinase; 87K, RNA-dependent RNA polymerase; 95/105K – polyproteins produced by translation of full-length RNA-2; 48/58K, N-terminal proteins produced by cleavage of the RNA-2 polyproteins at the Q/M site; L, Large coat protein; S, Small coat protein.

acting in *trans* (Franssen et al., 1982). Cleavage at Q/M site between the 58/48K proteins and the L protein leads to the production of a 60-kDa L–S fusion protein (termed VP60), which has been proposed as the immediate precursor of the mature L and S proteins (Franssen et al., 1982; Wellink et al., 1987). However, experiments with plants transgenic for VP60 showed that this protein could not, by itself, assemble into structures resembling virus particles (Nida et al., 1992) and its role in particle morphogenesis has remained unclear. Attempts to demonstrate that VP60 plays a precursor role in particle formation have been hampered by the fact that it accumulates to only very low levels during infection of plants (Rezelman et al., 1989) and the observation that cleavage at the glutamine/glycine (Q/G) site between the L and S proteins occurs only at very low hemin concentration in reticulocyte lysates (Bu and Shih, 1989). Expression in protoplasts of an artificial precursor consisting of the sequence of the 24K proteinase linked to VP60, although apparently leading to the efficient release of the mature L and S proteins through cleavage in *cis*, did not produce VLPs. This led to the suggestion that the conformation of the coat proteins produced in this manner may have been aberrant, thereby preventing assembly (Wellink et. al, 1996). Alternatively, it may indicate that the processing of the artificial precursor was insufficiently precise, since processing by the 24K proteinase is less specific in *cis* than in *trans* (Clark et al., 1999). Whatever the cause, these experiments once more failed to demonstrate the formation of VLPs following proteolytic processing of a precursor and left the role of VP60 in doubt.

To determine whether CPMV VLPs can be formed when the mature L and S proteins are produced by proteolytic processing in *trans*, we initially examined the processing of RNA-2-encoded precursors by the RNA-1-encoded 24K proteinase in insect cells. This system was selected because it had previously been shown to support the efficient processing of RNA-1-encoded precursors by the 24K proteinase (van Bokhoven et al., 1990; 1992). We show in this manuscript that VLPs are efficiently produced when the L and S proteins are released from