Expression of the *Peanut stunt virus* Coat Protein Gene Is Essential and Sufficient for Production of Host-Dependent Ribbon-Like Inclusions in Infected Plants

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ABSTRACT

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We previously have reported that infection of tobacco protoplasts or leaf tissue with the cucumovirus *Peanut stunt virus* (PSV) induced the production of unusual cytoplasmic ribbon-like inclusions. The formation of these novel inclusions is strain-specific, because infection of tobacco with subgroup II PSV strains, but not subgroup I strains, induced the production of inclusions. Furthermore, we have demonstrated that induction of the ribbon-like inclusions maps to PSV subgroup II RNA3, which codes for the coat protein (CP) and movement protein (MP). We have now extended these studies using chimeric constructs containing CP and MP open reading frames (ORFs) from PSV strains ER and W that belong to subgroups I and II, respectively. Additionally, recombinant *Potato virus*

X (PVX) vectors containing translatable and untranslatable PSV CP ORF were constructed. Plants inoculated with infectious chimeric PSV or recombinant PVX transcripts were analyzed for CP expression by enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction and for inclusion production by electron microscopy. The results of these experiments indicated that translation of the CP ORF alone is essential and sufficient for inclusion production. In immunogold labeling experiments using an antiserum to PSV virions, abundant gold labeling of the inclusions was observed, suggesting that PSV CP is probably a major component of the inclusions. Because inclusion production is host specific, a host factor is likely to be involved. In addition to their diagnostic importance, these novel inclusions may also prove valuable in identifying the host factors that interact with PSV CP.

Additional keywords: *Nicotiana benthamiana*, vector, viral expression.

Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* in the family *Bromoviridae* (23). Other members of the genus are *Tomato aspermy virus* (TAV) and the type member *Cucumber mosaic virus* (CMV). PSV is an economically important pathogen of legumes worldwide (19,32). Like other cucumoviruses, PSV has a tripartite genome of positive-strand RNAs, designated RNAs 1, 2, and 3, that are packaged in isometric particles of about 28 nm in diameter. RNAs 1 and 2 encode the 1a and 2a proteins, respectively, which along with host components, are required for replication. RNA3 is dicistronic and encodes a putative movement protein (MP) and the coat protein (CP), which is expressed from subgenomic RNA4 (9,13,14). RNA4 is packaged together with RNA3. A small overlapping gene (2b), encoded by RNA2, is present in all cucumoviruses sequenced to date and is expressed through subgenomic RNA4A (7). CMV 2b protein, essential for systemic virus spread in cucumber, has been identified as a suppressor of posttranscriptional gene silencing, and thus a pathogenicity determinant (1).

Strains of PSV have been classified into two major subgroups (subgroups I and II) based on serology and percent nucleotide sequence identity (9,11). Furthermore, a naturally occurring reassortant (PSV-BV-15) between the two subgroups has been reported and characterized at the molecular level (10).

We have recently reported that infection of tobacco protoplasts or leaf tissue with PSV subgroup II strains induced the production

of unusual cytoplasmic ribbon-like (RL) inclusions (24). The inclusion structures appeared as long, thin, densely staining sheets, which were prevalent within the cytoplasm, accumulating most commonly near vacuoles. The formation of these novel RL inclusions appears to be subgroup-specific, because infection of tobacco with subgroup II PSV strains, but not subgroup I strains, induced the inclusions. Furthermore, inclusion formation was shown to be host-specific because the inclusions were not detected in either of two leguminous host species infected with PSV subgroup II strains (24). Using tobacco protoplasts electroporated with various assortments of infectious RNA transcripts derived from cDNA clones of the genomes of two distinct strains, it was demonstrated that induction of the RL inclusions maps to PSV subgroup II RNA3 (24). Because PSV RNA3 is dicistronic, encoding an MP and a CP, molecular genetic analysis of PSV RNA3 was utilized in the present study to determine whether the MP, CP, or both are the genetic determinants of inclusion induction.

MATERIALS AND METHODS

Plasmid construction. Plasmids pW1 and pW2, containing full-length cDNA of PSV-W RNA1 and RNA2, respectively, were described previously (11). Plasmids pE3 and pW3, containing full-length cDNA of PSV-ER RNA3 and PSV-W RNA3, respectively, (11) were used to generate the chimeric RNA3 constructs. pEW3 was constructed by first subcloning the *EcoRI/SpeI* fragment from pE3 into a pUC19 cloning vector. The resultant plasmid, pE3-MP, contained the region from the start of the T7 promoter to nucleotide 1023 of the PSV-ER RNA3 cDNA, ending at the *SpeI* restriction endonuclease site. The 3' end sequence of the PSV-W RNA3 cDNA, extending from *SpeI* to the *PstI* site at the

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3' end of the cDNA sequence, was inserted into pE3-MP between the *SpeI* and *PstI* sites to produce pEW3 (Fig. 1). Plasmid pWE3 was constructed by exchanging the *MluI/PstI* fragment from pW3 with the corresponding fragment from pE3 (Fig. 1). Plasmid pW3-MP, which was used as a template for preparing hybridization probes specific for PSV-W MP coding sequence, was constructed by subcloning the *EcoRI/SpeI* fragment from pW3 (Fig. 1) into pUC19.

Preparation and electroporation of protoplasts and RNA analysis. Protoplasts were prepared from cultured tobacco suspension cells (*Nicotiana tabacum* cv. Xanthi-nc) as described by Passmore et al. (22). Procedures for protoplast electroporation, RNA extraction from electroporated protoplasts, and northern hybridization analysis were performed as described previously (24). Hybridization probes specific for PSV-W RNAs 1 and 2 were synthesized using plasmids pW1 and pW2 as templates with the nick-translation system of Gibco-BRL (Life Technologies, Gaithersburg, MD). Probes specific to PSV-W MP and PSV-ER MP coding sequences were similarly synthesized, but using plasmids pW3-MP and pE3-MP, respectively.

Construction of recombinant *Potato virus X* vector and expression of PSV-W CP. A pair of polymerase chain reaction (PCR) primers, a forward primer, PSV-W CPf1 (5'-ggcggatcATGGCATCTAGATCTGG-3'), and a reverse primer, PSV-W CPr (5'-ggggtatcgtcgacTTAGACCGGGAGCTTGG-3'), were used to amplify the PSV-W CP open reading frame (ORF) using plasmid pW3 as a template. A mutated version of the CP ORF that rendered it in effect untranslatable was generated by the introduction of two stop codons (printed in bold in primer CPf2 below), two and seven codons downstream of the ATG initiation codon. For this purpose, the forward primer PSV-W CPf2 (5'-ggcggatcATGGCATCTT**GT**ATCTGGTAACGGTT**G**ACGC-3') and the reverse primer PSV-W CPr were used. The restriction sites *EcoRV* and *SalI* (underlined in the sequence of primers CPf1 and CPr, respectively) were incorporated at the 5' and 3' end of the CP cDNA, respectively, to facilitate cloning into the *Potato virus X* (PVX) expression vector pPVX2C2S (4). PCR was carried out in a 50- μ l reaction mixture containing 1 $\times$ *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 2.3 pmol of each primer, 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 100 ng of plasmid pW3 DNA, and 1.25 units of *Taq* DNA polymerase. The initial denaturation was performed at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The reaction was completed with a final extension step for 7 min at 72°C. The resulting PCR-amplified CP cDNAs containing translatable and mutated (untranslatable) CP coding sequences were digested with *SalI* and *EcoRV* and ligated into similarly digested pPVX2C2S to generate PVXCP^{PSV-W} and PVXmutCP^{PSV-W}, respectively.

In vitro transcription and plant inoculations. Capped RNA transcripts were synthesized from *SpeI*-linearized PVXCP^{PSV-W}, PVXmutCP^{PSV-W}, and pPVX2C2S as described by Chapman et al. (4). The reaction products were extracted by phenol/chloroform and further purified by ethanol precipitation. The products of the 10- μ l reaction were resuspended in 35 μ l of RNase-free water containing 50 mM sodium phosphate, pH 7.2, and 5 μ g of bentonite per μ l. Young *N. benthamiana* and tobacco seedlings were dusted with Carborundum and 20 μ l of the inoculum was rubbed onto each leaf.

Reverse transcription-PCR and enzyme-linked immunosorbent assay testing of infected tobacco and *N. benthamiana* plants. For RT-PCR, total RNA was isolated from PVX2C2S-, PVXCP^{PSV-W}-, PVXmutCP^{PSV-W}-, or PSV-W-infected plants with a commercial RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The first strand cDNAs were subjected to PCR using the PSV-W primers CPf1 (CPf2, in case of cDNA derived from the PVXmutCP^{PSV-W}-infected plants) and

CPr. The conditions for the PCR reactions and thermocycling program were as described previously. The PCR products were fractionated by gel electrophoresis on 1% agarose gels.

Double-antibody sandwich, enzyme-linked immunosorbent assay (ELISA) (8) was used for detection of PSV antigen in PVXCP^{PSV-W}- and PVXmutCP^{PSV-W}-infected plants. PSV-W- and PVX-infected plants served as positive and negative controls, respectively. One-hundred-milligram leaf tissue samples from test plants were used. The level of PSV-W CP accumulation in infected plants was estimated by including purified PSV-W virion preparations of known concentration as standards.

Fig. 1. Peanut stunt virus (PSV) cDNA constructs used for generating chimeric RNA3. (Top panel), Schematic representations of PSV-W RNA3 cDNA constructs from which transcripts were generated and used along with PSV-W RNAs 1 and 2 transcripts to electroporate tobacco protoplasts. W-RNA3 (top diagram): wild-type RNA3 and EW-RNA3 (middle diagram): contains the 5'-half of PSV-ER RNA3 including the ER-movement protein (MP) gene, and the 3'-half of PSV-W RNA3 containing PSV-W coat protein (CP) gene; WE-RNA3: bottom diagram is derived from the 5'-half of PSV-W RNA3 retaining the PSV-W MP gene and the 3'-half of PSV-ER RNA3 containing PSV-ER CP gene. (Bottom panel), Accumulation of PSV RNA in tobacco protoplasts electroporated with RNA transcript sets containing PSV-W RNAs 1 and 2 with W-RNA3, EW3 RNA3, or WE3 RNA3. Lanes labeled "Mock" represent protoplasts electroporated with buffer alone, and without RNAs. Nucleic acids were extracted from protoplasts 4 days postelectroporation and subjected to northern hybridization analysis. The resultant blots were hybridized with ³²P-labeled probes specific to A, W RNAs 1 and 2, B, strain W MP gene, and C, strain ER MP gene. Arrowheads indicate migration positions of RNAs.

Electron microscopy and immunogold labeling. Protoplast samples (approximately 2×10^5) were prepared for electron microscopy as described previously (24). For Spurr embedding of leaf tissue, *N. benthamiana* or *N. tabacum* leaf pieces, 1×2 mm in size, were fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C. Following several rinses in the same cacodylate buffer, the samples were transferred to a second fixative (1% OsO₄ in 0.1 M cacodylate buffer, pH 7.2) and kept for 2 h at 4°C. After fixation, the samples were rinsed several times in water to remove all traces of buffer and kept overnight in an aqueous uranyl acetate solution (saturated uranyl acetate/water [1:1, vol/vol]) at room temperature. This was followed by dehydration in ethanol series and embedding in Spurr's medium (27).

For embedding in LR White medium, the leaf pieces were kept overnight in a fixative containing 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Dehydration was limited to 50 and 70% ethanol, and infiltration was accomplished by adding LR White in small amounts over a 2-h period and leaving the material in pure LR White overnight. The leaf pieces were embedded in fresh LR White in capped gelatin capsules at 55°C overnight.

Thin sections (approximately 90 µm) were cut with a diamond knife on an LKB III Ultratome (LKB Instruments, Rockville, MD) and stained with lead citrate (in the case of the LR White-embedded material, the sections were also stained with uranyl acetate). Stained sections were viewed with an electron microscope (Hitachi H-600; Hitachi Scientific Instruments, Pleasanton, CA).

For immunogold labeling, LR White thin sections on nickel grids were floated on blocking solution (2% bovine serum albumin in TBST: 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Tween 20) for 10 min at room temperature and incubated for 16 h with primary antibody (rabbit immunoglobulin G [IgG] derived from an antiserum to PSV-W, 20 µg/ml). The sections were then washed with TBST and incubated for 1 h with the secondary antibody (goat anti-rabbit IgG conjugated with 15 nm of gold particles, 1:25 in TBST). This was followed by washing with TBST, washing with water, and staining in uranyl acetate and lead citrate. Two types of controls were included. In one, sections from plants inoculated with the PVX vector alone were subjected to the same labeling procedure, and in the second, sections from plants inoculated with

transcripts derived from the recombinant vector PVXCP^{PSV-W} were treated with IgG from a preimmune serum instead of that derived from the PSV-W antiserum.

RESULTS

Delineation of the genetic determinants of RL inclusions formation. Tobacco protoplasts were electroporated with infectious transcripts generated from full-length cDNA clones of PSV-W RNAs 1 and 2 combined with transcripts from full-length cDNAs of the wild-type W RNA3, the chimeric EW RNA3, or the chimeric WE RNA3 constructs (Fig. 1, upper panel). Northern hybridization analysis of total RNA from infected protoplasts, using ³²P-labeled probes specific for PSV-W RNAs 1 and 2 (Fig. 1, panel A), strain W MP gene (panel B), and strain ER MP gene (panel C) confirmed the identity of the predicted progeny viral RNAs. Electron microscopic examination of thin sections from infected protoplasts revealed the presence of the RL inclusions in protoplasts previously electroporated with the wild-type PSV-W RNA transcript set as well as those inoculated with the chimeric EW RNA3 transcript, but not with the chimeric WE transcript, in place of the wild-type PSV-W RNA3 (data not shown). The finding that inclusions were detected in protoplasts inoculated with W RNAs 1 and 2 plus the chimeric EW RNA3, but not with the chimeric WE RNA3, suggested that inclusion production maps to PSV-W CP gene.

Expression of PSV CP ORF from a PVX vector. To determine whether expression of PSV-W CP gene alone is sufficient for induction of the RL inclusions, the CP ORF or a mutated (untranslatable for practical purposes) CP ORF was cloned between the *EcoRV* and *SalI* sites of the PVX vector pPVX2C2S to generate pPVXCP^{PSV-W} and pPVXmutCP^{PSV-W}, respectively (Fig. 2). Infectious transcripts, generated from these plasmid constructs, were used to inoculate *N. benthamiana* and *N. tabacum* seedlings. In all cases, mild mosaic and mottling symptoms developed within 10 days postinoculation. The production of the pertinent PSV-W CP mRNA in PVXCP^{PSV-W}- and PVXmutCP^{PSV-W}-infected plants was verified by RT-PCR, because the predicted PCR product of 650 bp was evident in both cases (Fig. 3). ELISA assessment of PSV-W CP accumulation in the test plants indicated that only PVXCP^{PSV-W}-infected plants contained the CP antigen (Table 1).

Fig. 2. Potato virus X (PVX) constructs used for expression of Peanut stunt virus (PSV) coat protein (CP). The diagrams show the major features of the PVX vector (pPVX2C2S) and derivatives used in this study. Features of pPVX2C2S shown are the PVX RNA-dependent RNA polymerase (RdRp); virus genes encoding movement proteins (M1, M2, and M3); the virus coat protein gene (CP); and the promoter from T7 bacteriophage (T7), the position of the restriction site *SpeI* used in plasmid linearization. The PVX derivative constructs pPVXCP^{PSV-W} and pPVXmutCP^{PSV-W} contain in addition translatable and mutant (in effect untranslatable) cDNA inserts of full-length PSV CP coding sequences, respectively.

We determined at the outset of this study that RL inclusions similar to those reported earlier from PSV-W-infected *N. tabacum* (24) were readily detected in thin sections from PSV-W-infected *N. benthamiana* (Fig. 4A). The RL inclusions were also detected in *N. benthamiana* previously inoculated with PVXCP^{PSV-W} transcripts (Fig. 4B). Numerous typical RL inclusions were seen interspersed among the PVX-induced laminate inclusion component (LIC), which is unique to PVX infection (Fig. 5). LIC consists of protein sheets that are closely associated with virus particles and ribosome-like beads. No RL inclusions were detected in *N. benthamiana* inoculated with transcripts of PVXmutCP^{PSV-W}, and the thin sections were indistinguishable from those obtained with infections with the PVX vector alone (data not shown).

Immunogold labeling of the RL inclusions with antibodies specific for PSV CP. Abundant gold labeling of the RL inclusions was observed when thin sections from the PVXCP^{PSV-W}-infected plants were immunogold labeled with antibodies derived from an antiserum to PSV-W virions (Fig. 6A). No labeling or negligible gold labeling was detected in control thin sections treated with antibodies from a preimmune serum (Fig. 6B). Likewise, no labeling or negligible gold labeling was detected in thin sections derived from plants previously inoculated with the PVX vector alone and immunogold labeled with PSV-W antibodies (Fig. 6C).

DISCUSSION

We presented several lines of evidence in support of the conclusion that translation of PSV CP ORF alone is essential and suffi-

cient for the production of the RL inclusions in tobacco and *N. benthamiana*. We have previously shown that inclusion production is strain specific because strains belonging to subgroup II, but not subgroup I, can induce the production of the RL inclusions (24). We also demonstrated that inclusion production maps to the dicistronic RNA3. The present study established that the determinant for RL inclusions maps to the CP gene from subgroup II strains because inocula containing chimeric RNA3 in which the CP ORF was derived from subgroup II strains, but not subgroup I, induced the production of RL inclusions. Furthermore, the finding that PVX vector-expressed PSV-CP induced the production of the RL inclusions provided conclusive evidence that translation of the CP gene alone is essential and sufficient for induction of inclusion formation.

Results from immunogold labeling experiments (this study) suggested that PSV CP is probably a major component of the RL inclusions. We have previously shown that when PSV-specific-antibodies were used to immunogold label thin sections of PSV-W-infected tobacco protoplasts, gold labeling was observed within the cytoplasm in association with virus-like particles and along the surface of the RL inclusions (24). However, we were unable to distinguish between labeling that may have been associated with viral particles adjacent to the surface of the RL inclusions or labeling that may have been directly associated with the inclusions. This uncertainty has now been resolved because of the availability of the PVXCP^{PSV-W}-infected plants, which are devoid of PSV-W particles and which express only the PSV CP gene. Abundant gold labeling of the RL inclusions was clearly observed in thin sections from such plants. It is known that cucumovirus capsid assembly and maintenance of virion stability are dependent on protein-RNA interactions (21). Unlike viruses that are dependent on protein-protein interactions for their assembly and stability, cucumoviruses do not form empty capsids *in vivo* or *in vitro*, because the presence of viral RNA is required for directing capsid assembly (12). Furthermore, it appears that the ratio of protein to RNA is rather strictly controlled during assembly because all cucumovirus nucleoprotein component particles have similar morphology and sedimentation properties and no components that package less than approximately 3-kb RNA (equivalent to RNA1, RNA2, or RNA3 plus RNA4) are known to occur in nature (21). This may explain why no virus-like particles were observed in the PVXCP^{PSV-W}-infected plants even though an RNA containing PSV CP ORF (0.65 kb) is present. In addition, such RNA also lacks PSV-specific 5' and 3' untranslated regions that may contain encapsidation signals.

The reason why the CP of PSV subgroup II strains, but not subgroup I strains, induce the production of the RL inclusions is not known. As far as we are aware, RL inclusions have not been observed with infections by any other cucumoviruses, and thus may serve as a valuable diagnostic tool for identification of PSV subgroup II strains. Aggregates and crystalline inclusions of virus

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA from *Nicotiana benthamiana* infected with transcripts from three *Potato virus X* (PVX) constructs. Lane 1, 1-kb DNA ladder (Gibco-BRL, Gaithersburg, MD); lanes 2 through 4, amplification of total RNA from plants infected with pPVXCP^{PSV-W}, pPVXmutCP^{PSV-W}, and pPVX2C2S transcripts, respectively. The primer pairs used for amplification were *Peanut stunt virus* (PSV)-CPf1 and PSV-CPr (specific for PSV CP open reading frame; lanes 2 and 4) and PSV-CPf2 and PSV-CPr (specific for the mutant PSV-CP coding sequence; lane 3). The RT-PCR products (approximately 650 bp; arrowhead) were visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

TABLE 1. Accumulation of *Peanut stunt virus* (PSV) strain W coat protein (CP), as assessed by enzyme-linked immunosorbent assay (ELISA), in *Nicotiana benthamiana* and *N. tabacum* previously inoculated with infectious transcripts derived from *Potato virus X* (PVX) vector constructs

Host	Inoculum ^a	ELISA values ($A_{405\text{ nm}}$) ^b
<i>N. benthamiana</i>	PVXCP ^{PSV-W}	1.751
<i>N. benthamiana</i>	PVXmutCP ^{PSV-W}	0.035
<i>N. benthamiana</i>	PVX2C2S	0.043
<i>N. benthamiana</i>	Mock	0.059
<i>N. tabacum</i>	PVXCP ^{PSV-W}	1.600
<i>N. tabacum</i>	Mock	0.022
Purified PSV-W virions (100 ng/ml)		1.732

^a Carborundum-dusted leaves were rub-inoculated with transcripts from the PVX constructs (20 μ l per leaf) or mock-inoculated with inoculation buffer (0.05 M sodium phosphate buffer, pH 7.2).

^b Values are means of four replicates.

particles within the cytoplasm and vacuoles have often been reported in conjunction with infections by cucumoviruses (18). PSV CPs from subgroup II strains can be differentiated from those from subgroup I strains based on serology and percent nucleotide/amino acid sequence identity (9,11,20). Since the three-dimensional structures of CMV and TAV have recently been solved by X-ray crystallography and found to be similar (16,26), it is reasonable to assume that PSV structure would also be similar to these two cucumoviruses. The availability of these structures makes it possible to compare the specific regions of CP predicted to be involved in host interactions and determine whether PSV subgroup II strains could be differentiated from other cucumoviruses. For this purpose, multiple alignments of CP amino acid sequences from PSV, CMV, and TAV strains were examined for differences in primary structures (Fig. 7). The region extending from amino acid position 129 to 149 (Fig. 7, CMV numbering; boxed) was identified as having the greatest structural differ-

ence between the backbones of TAV and CMV and predicted to confer host specificity unique to each virus (16). The conserved proline at position 129 in CMV is of particular interest, because variants at this position are known to cause strikingly different disease symptoms (21). Although PSV-W has a deletion at this position, the presence or absence of proline at position 129 is probably not relevant to inclusion formation, because PSV-B, another subgroup II inclusion-forming strain, as well as the PSV subgroup I strains and CMV have prolines at this position. On the other hand, the presence of proline at position 141 (shaded) in PSV subgroup II strains (instead of lysine or alanine in other cucumoviruses) may contribute to conformational differences, because it occurs in a region predicted to form a helix (EF α -helix) in CMV and TAV (16,26). The significance of the projected conformational changes to inclusion formation could be addressed by mutational analysis in future studies. The stretch of acidic amino acids in the external H-I loops of the CP subunits (Fig. 7, amino

Fig. 4. **A**, Electron micrograph of a thin section of *Nicotiana benthamiana* mesophyll tissue infected with *Peanut stunt virus* strain W. Arrowheads indicate densely stained ribbon-like inclusions. Bar = 500 nm. **B**, A thin section of *N. benthamiana* mesophyll tissue inoculated with transcripts from the *Potato virus X* (PVX) construct pPVXCP^{PSV-W}. Densely stained ribbon-like inclusions (arrowheads) are observed with the PVX-induced inclusions known as a laminar inclusion component (LIC). **C** = chloroplast. Bar = 500 nm.

acid positions 190 to 198, CMV numbering; boxed) is also of interest because this highly conserved negative cluster is predicted to be first to contact components of the host plant or insect vector (16). The change from glutamate to glutamine in PSV subgroup II

CP (Fig. 7, amino acid position 195, shaded) is not expected to significantly change the net negative charge in this region projected to function in ion-binding or in formation of inter-subunit salt linkages.

Fig. 5. Electron micrograph of thin sections of *Nicotiana benthamiana* mesophyll tissue infected with transcripts from the *Potato virus X* (PVX) construct pPVXCP^{PSV-W}. Densely stained ribbon-like inclusions (arrowheads) are observed interspersed with the PVX-induced laminate inclusion component (LIC). LIC consists of protein sheets that are closely associated with virus particles (*) and ribosome-like beads (arrow). M = mitochondrion and C = chloroplast. Bar = 1,000 nm.

Fig. 6. Thin sections of *Nicotiana benthamiana* mesophyll tissue infected with pPVXCP^{PSV-W} transcripts and immunogold labeled with immunoglobulin G (IgG) derived from an antiserum to *Peanut stunt virus* (PSV)-W virions or from a preimmune serum. **A**, Gold particles (≈ 15 nm) are seen associated with the ribbon-like (RL) inclusions (arrowheads) within the cytoplasm in a thin section treated with PSV antiserum. **B**, Control thin section of PVXCP^{PSV-W}-infected tissue treated with IgG derived from a preimmune serum. RL inclusions are indicated by arrowheads. **C**, Control thin section of PVX2C2S-infected tissue treated with IgG derived from an antiserum to PSV-W virions. RL inclusions are indicated with arrowheads. LIC = laminate inclusion component. Bar = 500 nm.

An unusual feature of CMV and TAV structure is that the N-termini of the CP subunits (Fig. 7, residues 29 to 48, CMV numbering; underlined) form helices directed into the interior of the virus where they interact with encapsidated RNA (16,26). The regions preceding these helices (residues 1 to 28 for CMV) are disordered and contain the N-terminal basic arm with its characteristic arginine-rich motif, which probably interact with RNA (16, 25). From examination of the sequence alignments of the N-terminal regions of PSV strains in comparison with CMV and TAV (Fig. 7) and from considerations of secondary structure predictions (data not shown) using the Gor IV method (6), it is expected that PSV CP subunits form comparable helices attached to their N-termini, which are located inside the virions. This conclusion is consistent with our assumption that the viral RNA does not contribute to inclusion formation, which is a function of free CP and is independent of RNA-binding that takes place inside the virions.

strains subgroup II contain only 3 arginines (Fig. 7, shaded). It is of interest that the amino acid sequence at the N-terminal end of PSV CP from strain Mi, proposed to belong to a third subgroup of PSV strains (33), is similar to that of subgroup I (Fig. 7). The N-terminal Arg-rich cluster of some viral proteins is known to act as a cellular localization signal to direct the protein into specific regions in the cytoplasm (17). The Arg-rich region near the N terminus of cucumovirus CPs may also function within the context of adjacent sequence as a localization signal to target the CP to specific sites in the infected cell. The detection of CMV CP in the nucleus of infected tobacco tissue (15) is of interest in this regard. It is possible that, in the absence of the full complement of Arg residues, as in the case of PSV subgroup II strains, the CP may exhibit different cellular distribution and its accumulation in the cytoplasm and interaction with host components may lead to the formation of the RL inclusions. Taken together, considerations of CP amino acid sequence and structure suggest that inclusion formation might result from differences in conformation or cellular distribution of CP between PSV subgroup II strains and other cucumoviruses.

PSV CP, like that of CMV and many other RNA plant viruses, is predicted to be multifunctional (2). In addition to virion assem-

Fig. 7. Multiple alignment of the amino acid sequences of the coat proteins (CPs) of strains of *Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), and *Tomato aspermy virus* (TAV). The conserved arginine cluster at positions 10 to 20 in CMV CP as well as the corresponding arginines in PSV and TAV CPs are shaded. The N-terminal region (amino acid positions 29 to 48 in CMVfny), projected to form an α -helix, is underlined. The regions extending from amino acid positions 129 to 149 and 190 to 198, considered relevant in virus–host interactions, are boxed. The CP amino acid sequences (indicated in the one-letter code) were aligned using the Clustal X Multiple Alignment Program. Asterisk (*) indicates positions which have a single fully conserved residue; a colon (:) indicates that one of the following “strong” groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW; and a dot (.) indicates that one of the following “weaker” groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY.

bly and RNA-binding to encapsidate the viral genome, PSV CP is expected to play a role in aphid transmission, cell-to-cell and systemic movement, and induction of host resistance response (3,5, 28–31). Present evidence (21) suggests that the individual CP subunits rather than the assembled virions may play a role in some of these functions (e.g., cell-to-cell movement). The formation of RL inclusions, as discussed earlier, is also proposed to be a function of the free CP subunits. The fact that PSV CP still forms the typical RL inclusions in the presence of PVX inclusions (this study) is indicative of its strong capacity as a functional component rather than mere deposition of CP material. Because PSV CP (subgroup II strains) was established as the only viral component necessary and sufficient for induction of the RL inclusions and since inclusion production is host specific (24), a host factor is likely to be involved. Thus, the identification of CP as a major constituent of the RL inclusions should facilitate the development of appropriate procedures, e.g., immunoaffinity-based methodology, to isolate and characterize the host factors implicated. It has been previously documented that the CPs of cucumoviruses have active host-specific functions. For example, Taliansky and Garcia-Arenal (30) presented conclusive evidence that the CP of cucumoviruses has host-specific determinants for long-distance movement and suggested that a specific interaction of CP and some host components is required for this process to occur. The nature of the interaction and the host components involved, however, are unknown. The RL inclusions present a workable system to gain insight into PSV CP–host interactions.

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LITERATURE CITED

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