Sequence Analysis and Genome Organisation of Poinsettia Mosaic Virus (PnMV) Reveal Closer Relationship to Marafiviruses Than to Tymoviruses

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Sequence comparison and genome organisation of poinsettia mosaic virus (PnMV), a putative member of the tymoviruses, revealed a closer relationship to marafiviruses. The complete nucleotide sequence of PnMV was determined. The 6099-nt RNA genome encodes a putative 221-kDa polyprotein that lacks a stop codon between the replicase and the coat protein genes, as in most tymovirus RNAs. The genomic RNA has a poly(A) tail at its 3'-terminus in contrast to the tRNA-like structure found in the RNA of most tymoviruses, and no homology was observed to the conserved noncoding region of the tymoviral 3'-termini. The tymobox of PnMV, a 16-nt region of the subgenomic RNA (sgRNA) promoter shared by most tymoviruses, differs in 3 nt from the RNA sequence of tymoviruses but is identical to the sequence of marafiviruses. At least three sgRNAs were found in PnMV-infected Euphorbia pulcherrima and in isolated PnMV particles; one that is 650 nt long encodes the 214-kDa coat protein, and the others are about 3.5 and 1.7 kb and contain the 5'- and the 3'-terminal parts of genomic RNA, respectively. Like tymoviruses, PnMV particles sediment as top and bottom components. The particles of the top component contain the sgRNA (650 nt) encoding the coat protein, and those of bottom component contain both genomic and sgRNAs.

INTRODUCTION

In Euphorbia pulcherrima plants, poinsettia mosaic virus (PnMV) induces symptoms ranging from inapparent to light mottling, depending on cultivation (Plennestiel et al., 1982). PnMV occurs worldwide and frequently in commercial cultivars of Poinsettia (Bertaccini et al., 1996; Chiko, 1983; Lesemann et al., 1983; Fulton and Fulton, 1980), often in association with poinsettia cryptic virus (PnCV) and a phytoplasma (Koenig and Lesemann, 1980; Lee et al., 1997). The latter has recently been shown to be responsible for the free-branching phenotype (Lee et al., 1997). Plants free of viruses and phytoplasmas can be obtained by somatic embryogenesis (Preil et al., 1982), but the offspring are restricted in branching. For horticulture, it is desirable to obtain plants that contain phytoplasma as branching agents but that lack PnMV. Learning more about the molecular biology of PnMV would open gentechnological approaches to protect Poinsettia from virus infection specifically.

The PnMV isometric virions are approximately 26–26 nm in diameter and sediment as top and bottom components. The top component is noninfectious, in contrast to the bottom component (Fulton and Fulton, 1980). The virions contain one single-stranded plus-sense RNA, and the capsid is composed of 21.7-kDa protein subunits (Plennestiel et al., 1982).

PnMV was originally classified as a tentative member of the genus Tymovirus because of its morphology, mechanical transmissibility, and high titer in infected plants (Fulton and Fulton, 1980). A hallmark of members of the genus Tymovirus is their replication in invaginations built by both membranes of the chloroplast envelope (Lesemann, 1977; Matthews, 1991). In the case of PnMV, vesicles are built by the inner membrane at the chloroplast periphery (Lesemann et al., 1983) without any evidence of replication (D.-E. Lesemann, personal communication). However, no serological relationship has been found between PnMV and tymoviruses (Lesemann et al., 1983).

Recently, marafiviruses such as oat blue dwarf virus (OBDV) were grouped into the tymo-like lineage of α-like plant viruses because of characteristic sequence similarities in the methyltransferase (MTR), helicase (HEL), and polymerase (REP) domains (Edwards et al., 1997). Marafiviruses and tymoviruses are diverse genera of structurally similar RNA plant viruses (Koenig et al., 1995) that share some similarities in genome organisation and expression. The genomic RNA of OBDV contains a single open reading frame (ORF) coding for a protein with strong sequence homologies to the replicase-associated
protein of tymoviruses. Also, the coat protein (CP) sequence is located at the extreme C-terminal part of the replicase-associated polyprotein, and the CP either is expressed from subgenomic RNA (sgRNA) or results from protease processing of the polyprotein. The putative sgRNA promoter in OBDV RNA is strikingly similar to the tymobox in tymovirus RNA (Edwards et al., 1997; Ding et al., 1990).

In contrast to tymoviruses, marafiviruses lack a stop codon separating the polymerase gene from the CP gene as well as an overlapping ORF coding for a putative movement protein (OP). Furthermore, marafiviruses are restricted to phloem and are not transmitted mechanically like tymoviruses (Lockhart et al., 1985).

RESULTS AND DISCUSSION

CP analysis

PnMV particles were purified on sucrose gradients and fractions analysed by SDS–PAGE. In Serva-violet-stained gels, two proteins of 21 and 33 kDa were detected (Fig. 1A). Western blot analysis with PnMV antiserum revealed that the 21-kDa protein corresponds to the PnMV coat protein (Fig. 1B). The upper bands are presumably protein dimers of the 21-kDa protein (Fig. 1B, fractions 5 and 6). The PnMV antigens were distributed throughout the sucrose gradient (Fig. 1B, fractions 5–14) with two peak fractions representing the top (Fig. 1A, fraction 1A, fraction 6) and the bottom (Fig. 1A, fraction 1A, fraction 10) component of the viral particles. The top component is present in higher amounts than the bottom component. The second protein visible in the Serva-violet-stained gel of 33 kDa (Fig. 1A, fractions 1A, fraction 10–12) reacted with antiserum against PnCV (data not shown). PnMV was mechanically transmitted to N. benthamiana plants. It induced mild chlorosis in systemically infected leaves. Sucrose density gradient analysis of viruses revealed results similar to those shown above (data not shown).

Cloning and sequencing of PnMV genome

Polyadenylation of genomic PnMV RNA was suggested by poly(dT) chromatography on total RNA of infected E. pulcherrima and Northern blot analysis (Fig. 2). 3′-Rapid amplification of cDNA ends (RACE) confirmed the presence of a 3′-terminal poly(A) tract with a length of 17 nt at minimum. As in eukaryotic mRNAs, PnMV-RNA carries a putative polyadenylation signal sequence (AAUAAA, Fig. 3). Such polyadenylation signal sequences are common among plant viruses with poly(A) tails. 32P-pCp end-labeling experiments showed that the poly(A) tract is the true end of OBDV (Edwards et al., 1997).

The only known member of the tymoviruses with a poly(A) tail at its 3′-terminus is Dulcamara mottle virus (EMBL: AF035634), but recent results with 32P-pCp end-labeling indicated a 3′-C residue with the polyadenylated sequence upstream of the 3′-end (T. W. Dreher, personal communication). Tymoviruses usually carry a tRNA-like structure at the 3′-end. The secondary structure of the 3′-noncoding region is the most conserved one among tymoviruses, and for TYMV RNA, it was shown that the 3′-terminal 28 nt, including the pseudoknot structure, contains the (−)strand RNA promoter (Deiman et al., 1997) with its main determinant lying in the free 3′-terminal ACC(A) end (Deiman et al., 1998).
The CP can be either cleaved from the precursor or expressed from sgRNA. Translation from the first AUG-codon present at position nt 5445 would result in a 21.4-kDa protein, which is consistent with the CP size estimated by SDS–PAGE.

Additional ORFs with coding capacities of 10–17 kDa were identified in (+)-strand RNA, and one ORF of 30 kDa was identified in the (–)-strand RNA.

No ORF corresponding to the tymovirus OP was found (Fig. 3). This protein putatively functions as a movement protein of TYMV (Bozarth et al., 1992). In contrast to tymoviruses but in accordance with OBDV, PnMV lacks this gene as well as the stop codon separating the polymerase and the capsid protein genes.

The tymbox region of PnMV was identical to the putative subgenomic promoter of the marafiviruses and differed from those of tymoviruses (Figs. 3 and 4A). The 5′-terminal sequences of most tymoviral sgRNAs start with the sequence AAU/G, the initiation box (Ding et al., 1990). The putative starting point in PnMV sgRNA lies 9 nt downstream of the tymbox region (AAG) (Fig. 4A).

sgRNA. Northern blot analysis revealed several sgRNAs (Figs. 2D and 2E). One sgRNA of 650 nt hybridised exclusively to 3′-terminal probes (Fig. 2D), whereas the two other prominent subgenomic RNAs of 1.7 and 3.5 kb hybridised with 5′- and 3′-probes (Figs. 2D and 2E). From these data, we predict that the 650-nt sgRNA represents the CP encoding sgRNA produced by the subgenomic promoter. The other two RNAs are probably defective molecules. In general, defective RNAs (D-RNAs) contain portions of the parental virus genome, maintaining cis-acting elements that contain virus replication and encapsidation signals (Roux et al., 1991; White et al., 1991). Whether these defective RNAs of PnMV interfere with the parental helper remains to be clarified. No such sgRNAs were detected in OBDV-infected oat (Edwards et al., 1997).

To determine which RNAs are packaged, nucleic acids were isolated from each fraction of the sucrose gradient of PnMV-infected Poinsettia and analysed by Northern blotting. The top component contains the 650-nt sgRNA (Fig. 1C, fractions 5–7), whereas the bottom component (Fig. 1C, fractions 9–12) contained the genomic RNA as well as residual amounts of sgRNAs of different sizes. Similarly, top-component particles of TYMV contained subgenomic CP mRNA, fragments of the genomic RNA, or host tRNAs (Noort et al., 1982; Szybiak et al., 1978).

Sequence comparison

OBDV and PnMV have similar genome organisations (Fig. 4B) and nucleotide (60% identity) and encoded amino acid (48% identity for replicase proteins, 35% for CP) sequences. OBDV and PnMV should therefore be regarded as members of the tymo-like lineage of α-like plant viruses (Koonin et al., 1993) as proposed by Ed-
FIG. 3. Nucleotide sequence of the PnMV genomic RNA together with the amino acid sequence of the predicted 221-kDa polyprotein. MTR, HEL, and REP motifs (Edwards et al., 1997; Rozanov et al., 1992) are indicated, and the most conserved amino acids among α-like plant viruses (supergroup 3) are shaded in these parts (Koonin and Dolja, 1993). The putative HEL/REP papain-like protease cleavage site is designated by an arrow, and the predicted start codon of the CP is boxed. The tymobox region of PnMV is designated and boxed in black. The cysteine and histidine residues required for TYMV protease activity are indicated (Bransom and Dreher, 1994; Rozanov et al., 1995).
wards et al. (1997). The degree of relationship to carlaviruses, potexviruses, and tymoviruses is shown in Fig. 4C for the conserved replicase-associated polyprotein omitting the CP sequence.

PnMV resembles tymoviruses because of its high titer in infected plants, its mechanical transmissibility, the induction of invaginations at the chloroplast membrane, and the expression of only one CP. However, its genome organisation, sequence homology, and polyadenylated genomic RNA and the identical sequence of the subgenomic promoter (“tymobox”) argue in favour of PnMV as a member of the marafiviruses.

FIG. 4. (A) Sequence alignment of the putative tymoviral and marafiviral sgRNA promoter region. The regions of the conserved tymobox and initiation site for sgRNA transcription are boxed (Ding et al., 1990). OBDV harbours two sequences downstream the tymobox (underlined), which may represent the initiation box (Edwards et al., 1997). (B) Schematic overview of genome organisation and coding strategies of OBDV (marafivirus), TYMV (tymovirus), and PnMV. *Stop codon present between the replicase-associated polyprotein gene and the capsid protein gene in tymoviral genome. The large box represents the ORF encoding the replicase-associated polyprotein, and the small box represents the ORF coding for the CP. The hatched box represents the overlapping protein. In the case of OBDV, the 24- and 21-kDa capsid protein genes are in-frame and coterminal at the 3'-end. (C) Dendrogram (Clustal X, Tree View) based on the overall similarity of the replicase-associated polyproteins of PnMV, OBDV, and representatives of the trichoviruses, carlaviruses, and potexviruses. The EMBL accession numbers for marafiviruses and tymoviruses used in phylogenetic analysis are apple chlorotic leaf spot virus [ACLSV (X99752)], eggplant mosaic virus [EPMV (J04374)], erysium latent virus [ErLV (AF098523)], kenneuya yellow mosaic virus [KYMV strain Jervis Bay (D00637)], oat blue dwarf virus [OBDV (U87832)], ononis yellow mosaic virus [OYMV (J04375)], physalis mottle virus [PhyMV (Y16104)], turnip yellow mosaic virus [TYMV (X07441), TYMC cDNA infective clone (X16378), and TYMVA Australian isolate (J04373)]. For carlaviruses, potexviruses, and trichoviruses, the EMBL accession numbers are sour cherry green ring mottle virus [CGRMV (AF017780)], grapevine trichovirus B [GVB (X75448)], shallot virus X [SHVX (Q04575)], garlic virus A [GV-A (AB010300)], potato virus X [PVX (X55802)], and strawberry mild yellow edge-associated virus [SMYEv (P28897)].
Moreover, PnMV lacks the overlapping protein found in tymovirus RNA (Fig. 4B), which is thought to be necessary for cell-to-cell movement of TYMV (Bozarth et al., 1992). The OP proteins are not as conserved as the replicase proteins (Ding et al., 1990), and no homology exists to other viral proteins (Morch et al., 1988). TYMV full-length RNA transcripts with mutations in the OP initiation codon replicated poorly in vivo (Weiland and Dreher, 1989). However, despite that fact, PnMV is not, as are marafiviruses, restricted to phloem tissue. Further in vitro translation of PnMV virion RNA will clarify whether an OP equivalent is expressed.

MATERIALS AND METHODS

Plant material

Euphorbia pulcherrima Willd. ex Klotsch (cv. ‘Angelika’) plants were cultivated in an insect-free glasshouse at 18°C (day) and 12°C (night) to induce virus symptoms. E. pulcherrima was processed through somatic embryogenesis as described previously (Preil, 1982). These plants were generally phytoplasma free and restricted in branching. Some progeny plants were found to be virus infected but also restricted in branching (see Results). Nicotiana benthamiana DOMIN plants were grown under the same conditions. Young leaves of PnMV-infected E. pulcherrima plants were ground in 0.03 M phosphate buffer (pH 7.3) and Carbogran (Lonzia; mesh size, 320), and the extract was rubbed on leaves of 4-week-old seedlings of N. benthamiana.

Virus and viral RNA preparation

PnMV particles were purified as described by Leemann et al. (1983). Systemically infected leaves from E. pulcherrima or N. benthamiana plants were triturated in 2 ml/g of 0.07 M phosphate buffer (pH 7.3) containing 0.1% mercaptoethanol. The extract was filtered through Miracloth (Calbiochem, San Diego, CA), and the filtrate was mixed with n-butanol to 8% (v/v). After 30 min at room temperature, the mixture was subjected to one round of differential centrifugation (Sorvall SS34 rotor; 20 min, 12,000 g; Beckman SW 28 rotor, 4 h, 100,000 g; 4°C), and the final pellet was resuspended in 0.03 M phosphate buffer, pH 7.3 (1 ml/5 g of leaf tissue). RNA was extracted from this pellet and treated with DNase I, or the virus particles were further purified by centrifugation through a 10–40% (w/v) linear sucrose density gradient (Beckman SW 40 rotor; 4.5 h, 85,000 g; 4°C). Fractions of 500 μl were collected from the top of the gradient.

RNA was isolated from these fractions after treatment with DNase I (10 U; 1 h at 37°C) by SDS addition to 1% phenol/chloroform extraction and precipitation with ethanol. Before electrophoresis, RNAs were denatured by 1 M glyoxal in 50% DMSO and 10 mM sodium phosphate buffer (pH 7.0) at 50°C for 1 h. Electrophoresis was as described by Sambrook et al. (1989). The RNA sizes were determined using the digoxigenin-labeled RNA molecular weight marker I (Boehringer-Mannheim Biochemicals, Indianapolis, IN: 0.3, 0.4, 0.6, 1.0, 1.6, 1.9, 2.8, 5.3, and 7.4 kb).

Polyadenylated RNA of PnMV-infected, free-branching plants was purified from total cellular RNA using the batch procedure according to the Oligotex mRNA kit (Qiagen, Studio City, CA). RNA was Northern blotted and hybridised against a digoxigenin-labeled probe that was homologous to the 3’-terminal part of the PnMV genome (nt 5400–6099). To analyse subgenomic RNA components, total cellular RNA was run on an agarose gel and Northern blotted as described above. Blots were either hybridised with DNA probes homologous to the 5’- (nt 747–1609) or 3’- (nt 5400–6099) ends of the PnMV genome or hybridised with a mixed probe of cDNA clones covering the PnMV genomic RNA.

Serology

Viral proteins were analysed by SDS–PAGE in 12.5% gels according to Laemmli (1970). Gels were stained with 1% Serva-violet (Boehringer-Mannheim) or blotted onto nitrocellulose membranes (Towbin et al., 1979). Sizes of proteins were determined using the molecular weight standard LMW (Pharmacia, Piscataway, NJ: 94, 67, 43, 30, 20.1, and 14.4 kDa).

PnMV (124/25.06.80; T. 1024) and PnCV (119/14.02.80; T. 64) polyclonal rabbit-antisera were kindly provided by Prof. Dr. Renate Koenig (Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany). For Western blots, antisera were diluted 1:5000 in PBS buffer (137 mM NaCl, 2.7 M KCl, 7.9 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2) supplemented with 2.5% BSA and 0.02% NaN3. Immune reactions and detection of primary antibodies by goat anti-mouse IgG-alkaline phosphatase were performed according to Harlow and Lane (1988).

Random primed cDNA library of PnMV

RNA from virus particles purified by sucrose density gradient centrifugation was denatured for 10 min at 65°C and reverse-transcribed for 1 h at 37°C with MuMLV reverse-transcriptase (Boehringer-Mannheim) using random hexamer oligonucleotides as primers. Second-strand synthesis was performed with the second-strand synthesis mix from TimeSaver cDNA Synthesis Kit (Pharmacia Biotech) according to the manufacturer’s recommendations. After phenol/chloroform extraction, the second-strand reaction was purified from excess oligonucleotides using microconcentrators (Microcon-50; Amicon, Beverly, MA). An EcoRI–NotI adaptor was added to the cDNA ends by using T4 DNA ligase, and excess adaptor was removed (Microcon-50; Amicon). The 5’-
<table>
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<th>Name</th>
<th>Position in PnMV genome</th>
<th>5’-Oligonucleotide sequence-3’</th>
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<tr>
<td>PnMV1</td>
<td>415–432</td>
<td>AGCATCTTCTCAACTACC</td>
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<tr>
<td>PnMV3</td>
<td>983–965</td>
<td>GGCAGTGGTGTAAGTAAAC</td>
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<tr>
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<td>1531–1549</td>
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<td>PnMV21</td>
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Reverse transcription-polymerase chain reaction (RT–PCR) and cloning

To close gaps between cDNA library clones, PnMV sequence-derived primers were designed for RT–PCR as shown in Table 1.

PCR amplification was carried out using 1–5% of the cDNA suspension in a 50-μl reaction containing 20 pmol of primers, 0.1 mM each dNTP, 1.5 mM MgCl2, 10× reaction buffer, and 1 U of Taq polymerase (Eurobio). The reaction was heated for 5 min at 95°C and subjected to 30 amplification cycles (1 min at 95°C, 1 min at 55°C, 1.5 min at 72°C). PCR products were gel-purified by agarose gel electrophoresis and glass milk absorption (NucleoTrap; Macherey & Nagel) and blunt end-cloned into pBluescript II KS (+).

Terminal sequence analysis of PnMV genomic RNA

The genomic RNA 5’- and 3’-ends of isolated PnMV particles were determined by RACE using the SMART-RACE Kit (Clontech, Palo Alto, CA). For 3’-RACE, RT was done using an oligo(dT) primer (3’-CDS) and 5’-RACE was done using PnMV-specific primer (GSP 1: 5’-CACG-GTGGAGTGGGAGACTG-3’). PnMV-genome positioning at nt 500–476). The SMART oligo provided with the kit served as an extended template for RT. The SMART sequence is also part of the 3’-CDS primer. For amplification of the 5’-end or the 3’-end, GSP 1 or GSP 2 (5’-CGGCTCTCACCACAGAAGACG-3’) was used together with the kit universal primer mix (UPM), which recognizes the SMART sequence. PCR fragments were gel-purified, and cDNA ends were polished by treatment with Klenow fragment and cloned into the EcoRI site of pHBluescript KS (+).

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