Molecular analysis of the pothos latent virus genome

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Pothos latent virus (PoLV) is an isometric virus with a positive-sense, single-stranded RNA genome of 4415 nt. The genome contains five open reading frames (ORF), coding for five proteins with approximate molecular masses of 25, 84, 40, 27 and 14 kDa, respectively. In vitro synthesized PoLV RNA was infectious to Nicotiana benthamiana plants and protoplasts, but could not support replication of the defective interfering (DI) and satellite RNAs associated with Cymbidium ringspot tombusvirus. No DI RNA related to PoLV was generated after repeated passaging with infected sap. Mutagenesis studies defined the role of the ORF 4 product (27 kDa) as a movement protein, and the ORF 5 product (14 kDa) as being responsible for symptom severity. Moreover, it was shown that the coat protein (CP) is important in regulating synthesis of the 14 kDa protein, excess production of which is lethal to infected plants. CP mutants defective in capsid formation infected plants systemically and induced severe necrotic symptoms. Conversely, CP mutants able to form apparently normal virus particles induced symptoms indistinguishable from those elicited by wild-type virus.

Introduction

Pothos latent virus (PoLV) is an icosahedral plant virus isolated from symptomless plants of Scindapsus aureus (pothos, family Araceae) growing in hydroponic culture. Natural infection of other plants was not found (Sabanadzovic et al., 1995). Mechanical inoculation of PoLV to most herbaceous hosts results in chlorotic or necrotic spots on inoculated leaves 2–3 days post-inoculation (p.i.). Only Nicotiana benthamiana and N. clevelandii can become systemically infected. In N. benthamiana, systemic symptoms appear about 3–5 days p.i., and consist initially of a mosaic and distortion of the leaf blade, followed (in 2–3 days) by necrotic spots in the interveinal tissue that may expand to the entire leaf. Systemically infected leaves, including the small apical ones, may desiccate completely. Nevertheless, many infected plants recover and the new vegetation may show only mild chlorotic symptoms or be symptomless.

PoLV genomic RNA has been cloned and its complete sequence determined (Rubino et al., 1995a). The 4415 nt genome contains five open reading frames (ORF) (Fig. 1). ORF 1 encodes a protein with a molecular mass of 25 kDa. Readthrough of the amber stop codon of ORF 1 would yield a protein of 84 kDa (ORF 2). ORF 3 is located centrally and encodes a protein of 40 kDa. ORFs 4 and 5 encode proteins of 27 kDa and 14 kDa, respectively, and are in nearly the same position at the 3′ end of the genome. ORF 5 is completely nested within ORF 4, but in a different reading frame. In vitro translation studies showed that ORF 1 (and most likely ORF 2) are expressed from genomic RNA, whereas proteins encoded by ORFs 3, 4 and 5 are synthesized from two 3′ co-terminal subgenomic (sg) RNAs of approximately 2·0 kb (sg1) and 0·8 kb (sg2) (Rubino et al., 1995a). Amino acid sequence alignments of proteins encoded by PoLV with those of several plant RNA viruses revealed significant similarities between the readthrough domain of ORF 2 and the protein encoded by ORF 3 with the corresponding regions of tombusviruses. On the other hand, the proteins encoded by ORFs 1, 4 and 5 were only distantly related to the comparable tombusvirus proteins (Rubino et al., 1995a).

Notwithstanding these striking similarities between tombusviruses and PoLV, no definitive taxonomic status has yet been assigned to PoLV (Rubino et al., 1995a). To define the position of PoLV in the family Tombusviridae, further molecular characterization of this virus was undertaken by synthesizing a full-length infectious clone and mutating it at several positions. Further experiments were carried out to determine whether PoLV could act as helper for the replication of defective interfering (DI) and satellite (sat) RNA molecules associated with Cymbidium ringspot tombusvirus (CymRSV) infections (Burgyan et al., 1991; Gallitelli & Hull, 1985). The results of these studies are reported in this paper.

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**Methods**

**Preparation of a full-length cDNA clone of PoLV.** PoLV was propagated in *N. benthamiana*. Virus purification and RNA extraction were previously described (Sahadzovic et al., 1995; Rubino et al., 1995a). To obtain synthetic infectious transcripts of PoLV, a full-length cDNA copy of genomic RNA was cloned in two steps, downstream of the bacteriophage T7 RNA polymerase promoter.

Genomic viral RNA (1 pmol; 1.5 µg) was denatured by heating at 70°C for 10 min in the presence of 150 pmol of the oligonucleotide 5' GATACTAGAGGCCTACATCGCAGAAGC 3', which contains a sequence complementary to the last 18 nt of the PoLV genome and restriction sites for EcoRV (underlined) and XbaI (bold italic). cDNA was synthesized with 200 U Superscript reverse transcriptase (BRL) at 42°C for 30 min, digested with ribonuclease H, purified through a QiAquick column (Qiagen) and amplified by 35 cycles of PCR with, as a second primer, oligonucleotide 5' GGGGAAAAAGACGGGCAATT 3', which is homologous to nt 1412–1429 in the PoLV genome, and Vent DNA polymerase (New England BioLabs). The melting, annealing and polymerizing steps were carried out at 94°C (1 min), 45°C (1 min) and 72°C (4 min). The last polymerization step was extended by 10 min. Double-stranded DNA was extracted with chloroform, concentrated with ethanol, resuspended in 20 µl 10 mM Tris–HCl (pH 7.6), 1 mM EDTA, blunt-ended with Klenow, gel-purified, ligated to Smal-digested, dephosphorylated pUC18 and cloned in *Escherichia coli* strain DH5α.

The 5’ region was cloned by priming first-strand synthesis with the oligonucleotide 5' TCTCTACATGCTTACCAG 3', complementary to nt 2186–2203 of PoLV genomic RNA. cDNA was amplified by PCR with, as a second primer, oligonucleotide 5' ATCGATAATTGACTCGATGACTAGTATGAAATTACTATAAG 3', which contained nt 2–17 of the PoLV genome fused to 17 nt of the bacteriophage T7 RNA polymerase promoter consensus sequence (underlined) and 5 nt contributing to the formation of a Clal restriction site (italic). The first nt of the PoLV genome sequence (A) (Rubino et al., 1995a) was substituted by a G (bold) to allow efficient transcription by T7 RNA polymerase (Dunn & Studier, 1983). The PCR product was treated and cloned as before.

For full-length clone construction, the 5’ clone was digested with SavI (in the vector polylinker), blunt-ended, phenol–chloroform extracted, digested with *Bam*HI (position 1589 in the virus sequence), dephosphorylated and gel-purified. The 3’ clone was digested with *Bam*HI and EcoRV. The fragment containing the last 2826 nt of the PoLV genome sequence, plus an extra 7 nt at the 3’ end, was ligated to the *Bam*HI–SavI digested 5’ clone and cloned in *E. coli* DH5α.

**Construction of mutant clones.** To prepare deletion clone DBg/BS, the BglII site at position 1459 of the full-length clone was eliminated by mutating from AGATC to AGATA (isoleucine codons underlined), which is a silent mutation in the coding sequence of ORF 2. The modified full-length clone was then digested with *Bst*II (nt 2525) and *Bst*EI (nt 3454), and the termini were blunt-ended prior to religation and bacterial transformation.

Clone DBg/BS/GUS was constructed by inserting the β-glucuronidase (GUS) gene, contained in a BglII and BamHI fragment from clone pTL-7SN3-GUS/BglI (Restrepo et al., 1990), between the BglII and *Bst*EI sites of the above-modified full-length PoLV clone. pTL-7SN3-GUS/BglI was first digested with *Bam*HI (in the vector polylinker), blunt-ended and then digested with BglII. The modified PoLV clone was first digested with *Bst*EI, blunt-ended and then digested with BglII. Finally, the GUS-coding sequence was ligated to the PoLV clone prior to transformation.

Frameshift mutants BstB and Nhe were generated by digestion with either BstBI or NheI (at nt 3128 or nt 3146, respectively), followed by blunt-ending and religation.

Mutants D219 and D233, as well as the BglII mutant described above, were constructed by site-directed mutagenesis. This was done according to Kunkel et al. (1987) by using a Mutagenesis Kit (Bio-Rad) and following the manufacturer’s instructions, except that *E. coli* strains RZ1032 and TG1 were employed as ung- and ung+ hosts, respectively. The mutations were introduced in restriction fragments, which were sequenced before subcloning back in to the full-length clone. Mutagenic oligonucleotides were generally 20 nt long with the mismatched nucleotide, or the looped-out region, located in the centre.

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**Fig. 1. Structure of wild-type and mutant genomes of PoLV.** (a) Schematic representation of the wild-type PoLV genome showing molecular masses (in kDa) of proteins encoded by the respective ORFs (open boxes). (b) Structure of wild-type (wt) and mutant CP. The major structural domains (R, a, S and P) and relevant restriction sites (Bgl II, BstB; N, NheI and Bs, BstEI) are shown. Black boxes correspond to regions where a frameshift has changed the structure of ORF 3. Lines are deleted sequences (not to scale for mutants D219 and D233). In clone DBg/BS/GUS the region deleted in clone DBg/BS was substituted by the GUS gene (hatched box). (c) Nucleotide and amino acid sequences of the R domain of PoLV CP. The arrow indicates the site of insertion of a U residue for premature termination (mutant TAA). In-frame AUG codons are underlined; the mutants AI, All and Alll were obtained by progressively changing the AUGs to AUC codons.
In vitro transcription, inoculation and analysis of progeny 
RNA. Wild-type and mutant plasmids (approximately 2 µg) were linear-
ized with XbaI, and run-off transcripts, containing an extra 5 nt at the 3’ 
end, were synthesized using T7 RNA polymerase (BioLabs). Transcripts 
were inoculated to N. benthamiana plants and protoplasts, and RNA 
was extracted from infected leaf tissues as previously described (Dalmary 
et al., 1993). For Northern blot analysis, RNA was denatured with 
formamide and formaldehyde, electrophoresed in 12% agarose gels and 
transferred to nylon membranes (Sambrook et al., 1989). Virus-related 
RNA was detected by using a 32P-labelled probe prepared by nick-
translation of a clone representing the 3’-terminal 400 nt of the viral 
genomic RNA.

Sequence analysis of the encapsidated progeny RNA of mutants in 
the 5’ region of the coat protein (CP) gene was done by preparing a 
cDNA, with an oligonucleotide complementary to nt 2850–2872, then 
amplifying this region by PCR, with oligonucleotide primers comple-
mentary to nt 2493–2510 and homologous to nt 2301–2320. The PCR 
product was cloned as above and sequenced.

In vitro transcripts of CymRSV DI and sat RNAs, prepared as in 
Burgyan et al. (1992) and Rubino et al. (1992), respectively, were 
inoculated in the presence of the homologous helper virus (CymRSV) or 
carnation Italian ringspot virus (CIRV) a tombusvirus similar but not 
genetic to CymRSV (Rubino et al. 1995) or PoLV. Tissue extracts were 
analysed by Northern blotting with a 32P-labelled probe prepared by nick-
translation of clones representing DI or sat RNA sequences (Rubino et al. 
et al., 1990).

To test for the generation of DI RNA associated with PoLV 
infections, N. benthamiana plants were inoculated with PoLV RNA in vitro 
transcripts, and further serial passages were made by sap inoculation at 
7–10 day intervals. Analysis of infected tissues was done by Northern 
blotting, with the PoLV 3’-end probe, and RT–PCR (Burgyan et al., 

Protein extraction and analysis. Purified virus preparations were 
mixed with an equal volume of dissociation buffer, boiled for 3 min, 
electrophoresed by discontinuous SDS–PAGE in a 12% gel (Laemmli, 
1970), and stained with Coomassie brilliant blue. Molecular mass markers 
were from Sigma.

SDS-soluble proteins were extracted from infected tissue by grinding 
20–100 mg in 10 vols dissociation buffer. Samples were boiled for 3 min, 
and insoluble material was removed by centrifugation. Extracts were 
analysed for the presence of PoLV CP by SDS–PAGE and Western 
blotting. After transfer to Immobilon P nylon membranes (Millipore), the 
proteins were incubated with an antiserum raised against intact virus 
particles produced in rabbit (Sabanadzovic et al., 1995). The anti-
gen–antibody complexes were detected by incubation with alkaline 
phosphatase-linked goat anti-rabbit IgG, followed by incubation with 
5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro blue tetra-
zolium (NBT) (Sigma).

Electron microscopy. A drop of purified virus or infected crude 
sap was applied to carbon-coated grids and negatively stained with 2% 
uranyl acetate. Samples were examined with a Philips 201 C electron 
microscope.

Results

Infecitivity of full-length in vitro transcripts of PoLV and 
synthesis of subgenomic RNAs

In vitro synthesized PoLV RNA was as infectious as viral 
RNA, as symptoms developed with the same intensity and 
rapidity on all inoculated plants. The clone carrying the 
mutation deleting the BglII site at position 1459 was equally 
infectious.

Total RNA was extracted from inoculated and systemic 
leaves of N. benthamiana plants 3, 6 and 10 days p.i. and 
analysed by Northern blotting. Blots of extracts made 3 days 
p.i. showed one major virus-specific RNA species, which 
corresponded to genomic RNA (4415 nt), and two smaller 
species, which corresponded to sg1 and sg2 RNAs (Fig. 2, 
lanes 1 and 2). The sg RNAs were still present 6 days p.i. but 
in different concentrations, sg2 being less abundant (Fig. 2, 
lanes 3 and 4). At 10 days p.i. sg2 had decreased to the point 
of being barely detectable (Fig. 2, lanes 5 and 6). In general, the 
bands corresponding to sg1 and sg2, at 6 and 10 days p.i., were 
more diffuse than at 3 days p.i. suggesting that both sg RNAs 
were partially degraded.

When RNA was prepared from virus particles purified from 
inoculated plants 5 days p.i. only the genomic RNA band and a 
faint band corresponding to sg1 were detected (Fig. 2, lane 7), 
indicating that PoLV subgenomic RNAs are either poorly or 
not encapsidated (sg1 and sg2, respectively).

Specificity of PoLV replicase

Neither CymRSV DI or sat RNA replicated when co-
inoculated with PoLV, whereas replication of both molecules 
was supported by CymRSV or CIRV (Fig. 3a, b).

To test whether DI RNA-like molecules were produced in 
PoLV infections, a passage experiment was performed. North-
ern blot analysis of RNA extracts from infected plants at each 
of 12 sub-inoculations did not reveal the presence of DI RNA 
molecules (Fig. 3c shows the pattern of PoLV RNAs in the last 
three passages); neither were DI RNA-like molecules detected 
by RT–PCR in any of the serial passages. None of the sap-
inoculated plants displayed attenuated symptoms. On the 
contrary, symptoms became more and more severe due to the 
increasing concentration of inoculum.

Role of the protein encoded by ORF 3

Alignment of the amino acid sequence of PoLV CP with 
those of tombusviruses revealed that it could be divided into
structural domains, random (R), arm (a), shell (S) and protruding (P) and that the most conserved region was in the S domain, whereas the other domains were much less conserved (Rubino et al., 1995a).

The function of PoLV CP in replication, movement and pathogenesis was investigated by using a number of deletion and site-directed mutants (Fig. 1b, c; Table 1). Clone DBg/Bs had a large deletion in ORF 3 between nt 2525 and nt 3454. The CP portion that could still be synthesized was a large part of the R domain (51 out of 56 amino acids). Clone DBg/Bs/GUS was prepared by inserting the GUS gene between the BglII and BstEII sites, resulting in linkage of the residual R domain to the amino terminus of GUS. CP mutants BstB and Nhe were constructed by introducing a frameshift, so that CP synthesis terminated either four amino acid residues before or three amino acid residues after the beginning of the P domain (BstB and Nhe, respectively). Therefore, both clones essentially lacked a P domain. Mutants in the S domain were prepared by deleting either of two pairs of contiguous amino acids, proline–lysine (positions 219–220) or glycine–glycine (positions 233–234), to obtain defective clones D219 and D233, respectively. All these clones were infectious and spread systemically in N. benthamiana, with the exception of DBg/Bs, which remained localized in the inoculated leaves.

The appearance of systemic symptoms varied from 4 (D219), to 10–15 (BstB and Nhe), to 21 (D233) days p.i., but the responses elicited by all these mutants were essentially the same. In particular, symptoms consisted of a mosaic and distortion of the systemic leaves, followed by dark necrotic patches, which were initially confined to the leaf veins and adjacent tissues, but extended shortly afterwards to the petioles and stems. Eventually the leaves wilted and collapsed, still containing large portions of green tissue, the stem bent at the point(s) of necrosis and the plants withered and died within a few days.

Electron microscopy observations of leaf dips failed to reveal the presence of virus particles in the case of mutants BstB and Nhe, whereas Western blot analysis showed the presence of a truncated protein (ca. 28 kDa) (Fig. 4a, lanes 2 and 3), smaller than wild-type (Fig. 4a, lane 1). Mutants D219 and D233 produced a protein of ca. 40 kDa (Fig. 4b, lanes 3, 4

Table 1. Properties of PoLV CP mutant clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>CP*</th>
<th>Virions†</th>
<th>Symptoms‡</th>
<th>sg2§</th>
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<tr>
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<td>NS</td>
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* CP detected by Western blot analysis or Coomassie brilliant blue staining of polyacrylamide gels. +, present; −, absent; ND, not detected.
† Virions were detected by electron microscopy in negatively stained preparations of purified virus and/or leaf dips from infected plants. +, present; −, absent.
‡ Symptoms in systemic leaves similar to wild-type (WT) or more severe (S). NS, no systemic infection.
§ The concentration of sg2 RNA was estimated visually in Northern blots of RNA extracted from infected leaves whether similar to wild-type (WT) or higher (High). NS, no systemic infection.
Northern blot analysis of progeny viral RNA from mutants BstB and Nhe (Fig. 5a, lanes 2 and 4), D219 and D233 (Fig. 5b, lanes 1 and 2), and DBg/Bs/GUS (Fig. 5c, lanes 1 and 2) showed a pattern of viral RNAs different from wild-type in that the amount of sg2 was consistently higher than in the wild-type (Fig. 5a, lane 6; Fig. 5b, lane 4 and Fig. 5c, lane 6). Genomic and sg1 RNAs from clone DBg/Bs/GUS were smaller than the construct used as inoculum, indicating that a deletion had occurred in the inserted foreign gene, particularly in systemic leaves (Fig. 5c, lanes 1 and 2). However, in extracts of inoculated leaves, viral RNA of the size corresponding to input inoculum was still detected in addition to deleted forms (not shown), and, in fact, GUS activity was present in inoculated, but not systemically invaded leaves (not shown). Finally, clone DBg/Bs replicated in inoculated leaves (Fig. 5c, lanes 3 and 4), but not in the upper leaves (Fig. 5c, lane 5).

Mutations were introduced in the N-terminal region of the PoLV CP either to completely inhibit its synthesis or to allow synthesis of a CP lacking the R domain (see Fig. 1c). In clone TAA, a T residue was introduced, between nt 2386 and nt 2387 of the full-length clone, to form a UAA stop codon four amino acids after the initiation codon. The next in-frame AUG codon was at position 2402. In clone AI, the first AUG codon was changed to AUC; in clone AII, the first and the second AUGs (position 2402) were changed to AUC; and in clone AIII, the first, second and third (position 2459) AUGs were changed to AUC. With clone AI, CP synthesis begins at AUG 2402, thus eliminating nine N-terminal amino acids, and with clone AII, synthesis begins at AUG 2459 to produce a protein 28 amino acids shorter. With clone AIII, the first available in-frame AUG is at position 2837, and CP synthesis produces a truncated CP lacking 154 amino acids, i.e. the entire R domain and most of the S domain.

Plants infected with mutants TAA and AI displayed symptoms indistinguishable from wild-type and apparently normal virus particles were readily detected in purified preparations (not shown). The CP extracted from these virus and 5), indistinguishable from wild-type (Fig. 4b, lane 1). Generally, the concentration of CP in mutant infections was lower than wild-type. No CP was detected in tissue extracts from plants infected with mutants DBg/Bs or DBg/Bs/GUS.

Northern blot analysis of progeny viral RNA from mutants BstB and Nhe (Fig. 5a, lanes 2 and 4), D219 and D233 (Fig. 5b, lanes 1 and 2), and DBg/Bs/GUS (Fig. 5c, lanes 1 and 2) showed a pattern of viral RNAs different from wild-type in that the amount of sg2 was consistently higher than in the wild-type (Fig. 5a, lane 6; Fig. 5b, lane 4 and Fig. 5c, lane 6). Genomic and sg1 RNAs from clone DBg/Bs/GUS were smaller than the construct used as inoculum, indicating that a deletion had occurred in the inserted foreign gene, particularly in systemic leaves (Fig. 5c, lanes 1 and 2). However, in extracts of inoculated leaves, viral RNA of the size corresponding to input inoculum was still detected in addition to deleted forms (not shown), and, in fact, GUS activity was present in inoculated, but not systemically invaded leaves (not shown). Finally, clone DBg/Bs replicated in inoculated leaves (Fig. 5c, lanes 3 and 4), but not in the upper leaves (Fig. 5c, lane 5).

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Fig. 4. Western blot analysis of wild-type and deletion mutants of PoLV CP in sap extracted from plants infected with wild-type (a and b, lanes 1), mutants Nhe and BstB (a, lanes 2 and 3, respectively), and D233 (b, lanes 3 and 4) and D219 (b, lane 5). Protein extraction was done 20 days p.i. for mutants Nhe and BstB; 7 days p.i. for mutant D219, and 7 and 20 days p.i. (b, lanes 3 and 4) for mutant D233. Lane 2 in (b) contains extract from an uninfected control plant. Prestained protein size markers (kDa) (Sigma) are indicated on the left-hand side.

Fig. 5. Northern blot analysis of wild-type PoLV genomic (G) and subgenomic (sg1 and 2) RNAs extracted from plants infected with wild-type and CP mutants. (a) Inoculated leaves (lanes 1 and 3) and systemic leaves (lanes 2 and 4) infected with Nhe (lanes 1 and 2) and BstB (lanes 3 and 4) mutants and a systemic leaf infected with wild-type (lane 6). RNA extraction was done 3 weeks p.i., from desiccating leaf tissue for Nhe and BstB mutants and chlorotic leaf tissue for wild-type. Lane 5 contains RNA from an healthy control plant. (b) Systemic leaves infected with mutants D219 (lane 1), D233 (lane 2) or wild-type (lane 4). RNA extraction was done 4 (D219) and 20 (D233 and wild-type) days p.i. Lane 3 contains RNA from a healthy control plant. (c) Systemic leaves infected with mutant DBg/Bs/GUS (lanes 1 and 2) or wild-type (lane 6), inoculated leaves (lanes 3 and 4) and systemic leaves (lane 5) infected with DBg/Bs. RNA extraction was done 2 weeks p.i.
particles, and analysed by SDS–PAGE, proved to be a single protein species migrating slightly faster than wild-type (Fig. 6a, lanes 2 and 3). A region between nt 2301 and nt 2510 of the encapsidated viral RNA was amplified by RT–PCR and sequenced, showing that the introduced mutation had been maintained in the progeny of both TAA and AI clones. This led to the conclusion that protein synthesis had begun at the second available AUG (position 2402). The deduced molecular mass of the mutant protein is 39 079 Da, i.e. 1041 Da smaller than wild-type (40 120 Da), which is in line with its electrophoretic behaviour.

Plants inoculated with AII and AIII initially showed symptoms intermediate between those infected with mutants forming (TAA and AI) or not forming virions (D219, D233, Nhe, BstB and DBgGUS). A few plants showed small necrotic patches on the petioles and stems, but these survived and developed symptoms like wild-type. Virions were present in these plants (not shown), the CP of which was either of two distinct sizes (AII) (Fig. 6a, lane 4) or of only one size (AIII) (Fig. 6a, lane 5), but none was identical to wild-type (Fig. 6a, lane 1). Virus purified from AII infected plants was infectious and gave rise to progeny virus with the same two types of CP (Fig. 6b, lane 3).

Sequencing of ten clones from the AII mutant progeny showed that three reversion events had occurred. In two sequenced clones, the fourth CP triplet (GUG; valine) had reverted to AUG; in two others, both the 4th and the 13th triplets (AUU; isoleucine) had reverted to AUG; in the remaining six, only the isoleucine codon had reverted to AUG. AII mutants were therefore expected to synthesize proteins of two sizes with molecular masses of 39 805 Da and 38 764 Da, respectively, which correspond to the bands seen after SDS–PAGE.

Sequencing of AIII progeny showed reversion of the 4th CP triplet GUG to AUG, which became the new start codon giving rise to a protein slightly smaller (39 807 Da) than wild-type. It was also noted that in four out of ten sequenced clones a mutation at position 20 had also occurred from UUA (leucine) to UCA (serine).

Northern blot analysis showed that the proportion of genomic and sg RNAs in leaves infected with mutants TAA, AI, AII and AIII (Fig. 7, lanes 2, 3, 4 and 5, respectively) was the same as wild-type (Fig. 7, lane 1).

**Roles of proteins encoded by ORF 4 and ORF 5**

The putative role of the protein encoded by ORF 4 is that of a virus movement protein, since it contains a short sequence of amino acids similar to the conserved motifs of the ‘30K superfamily’ of movement proteins (Mushegian & Koonin, 1993; Rubino *et al.*, 1995a). To ascertain whether the protein product of ORF 4 was indeed necessary for cell-to-cell spread of PoLV, its initiation codon was mutated to AUC to obtain clone D27. The first available AUG in this mutant was at position 4026, i.e. after the initiation codon of the protein encoded by ORF 5 (position 3722). Plants inoculated with this clone did not show symptoms for several weeks p.i., and Northern blots of inoculated and upper leaves failed to show any viral RNA (not shown). On the other hand, clone D27 replicated in *N. benthamiana* protoplasts to the same level as wild-type (Fig. 2, lanes 8 and 9).

The protein encoded by PoLV ORF 5 has no significant
Discussion

The translated ORF 1 and the readthrough domain of ORF 2 of PoLV aligned with the corresponding regions of all sequenced tombusviruses, suggesting that the expression products of these two ORFs are directly involved in viral RNA synthesis (Rubino et al., 1995a). However, failure of PoLV to support CymRSV DI and sat RNAs indicates that PoLV replicase may have specific recognition sequences different from those of tombusviruses. It is worth recalling that tombusvirus DI and sat RNA can be replicated by heterologous tombusviruses (White & Morris, 1994; Havelda & Burgyan, 1995; Gallitelli & Hull, 1985; this paper).

The 27 kDa and 14 kDa proteins, encoded by the 3' nested ORFs 4 and 5, are responsible for virus movement and symptom expression, respectively. Inactivation of the 27 kDa protein abolished cell-to-cell movement, since no viral RNA could be detected in inoculated and systemic leaves. Inactivation of the 14 kDa protein abolished the necrotic response to infection.

CP mutants that did not synthesize encapsidation-competent CP were able to infect plants systemically (with the exception of clone DBg/Bs) and elicited symptoms that were more severe than wild-type. In fact, necrosis of vascular tissues induced rapid death of the whole plant. In all these cases, it was noticed that the concentration of 0.8 kb sg RNA was higher than in wild-type infections after 7–10 days. In the absence of encapsidation, progeny RNA from these clones may be available for transcription at all times during PoLV infection. This may lead to excess production of the necrosis-inducing 14 kDa protein which causes death of infected cells, including those of vascular tissues, and wilting of the tissues above the necrotic patches. In wild-type PoLV infections this may not occur, because genomic RNA is eventually encapsidated and made unavailable for transcription. Encapsidation of sg RNAs does not seem to have a major role in preventing the synthesis of the 14 kDa protein since these sg RNAs are rarely found in virus particles.

Since mutants DBg/Bs and DBg/Bs/GUS contain part of the R domain, it cannot be concluded that short- and long-distance movement of PoLV take place in the complete absence of CP. On the contrary, there is indirect evidence that the R domain is required for movement, since attempts to produce mutants unable to synthesize the N-terminal region of the CP failed, because of reversion to mutants with an AUG start codon in this region. It is likely, therefore, that a strong selection pressure operated to generate revertants able to form a CP containing at least part of the R domain. Interestingly, such CP can assemble to give rise to stable virions, which, at least in the case of All, are also infectious.

Our findings on the molecular biology of PoLV conform only in part to the molecular biology of tombusviruses. The main similarity resides in the fact that similarly located genes have identical functions (Rochon et al., 1991; Dalmay et al., 1993; Scholthof et al., 1991; Russo et al., 1994). A further point of similarity lies in the dispensability of encapsidation-competent CP for cell-to-cell and long-distance movement: It is not clear whether the R domain could play a role in this respect by binding to RNA, as suggested by Scholthof et al. (1993) for tomato bushy stunt tombusvirus (cherry).

A peculiar characteristic of PoLV is the high sensitivity of infected plants to the product of ORF 5, the 14 kDa protein. The amount of 0.8 kb sg RNA, from which this protein is translated, is low in wild-type infections, except at the initial stage of infection, when the 27 kDa movement protein product of the nested ORF 4 is necessary for completion of the virus life-cycle. On the other hand, in the absence of encapsidation-competent CP, transcription and translation may no longer be controlled and the 14 kDa protein reaches a level incompatible with host survival.

It is noteworthy that PoLV is unable to generate DI RNA(s) which reduce(s) the synthesis of tombusvirus sg RNAs and hence their products (Scholthof et al., 1995b). It was suggested that plants chronically infected with tombusviruses in the presence of DI RNA provide a reservoir for virus survival. Alternatively, the necrotic response in the absence of DI RNA provides high levels of inoculum in the soil (Scholthof et al., 1995b). PoLV may not utilize either mechanism, since it down-regulates the level of the 0.8 kb sg RNA in the absence of DI RNA, and accumulation of necrotic tissue in the environment may not take place.

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References


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