Mutational analysis of the proteinase function of Potato leafroll virus

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cDNA expression vectors of Potato leafroll virus (PLRV) were used to analyse specific mutations in the proteinase and replicase domains of the proteins encoded by ORF1 and ORF2. Agrobacterium-mediated DNA transfer was used to introduce a PLRV RNA expression unit, controlled by the 35S promoter of Cauliflower mosaic virus, into potato leaf cells. Expression of unmodified PLRV cDNA led to the replication of viral genomic and subgenomic RNAs and accumulation of the viral capsid protein, whereas alteration of amino acids GDD513–515 of the replicase to VHD abolished PLRV replication. Mutations in the presumed H-D-S catalytic triad of the viral proteinase abolished the formation of viral genomic and subgenomic RNAs as well as synthesis of the viral capsid protein. Co-agroinoculation of the GDD mutant along with any of the proteinase mutants restored virus replication in leaf discs, showing that these mutants are able to complement each other. Moreover, mutation of the postulated serine residue of the catalytic triad of the proteinase altered the pattern of proteins synthesized in vitro in comparison to wild-type, further supporting the relevance of the H-D-S motif.

Introduction

Potato leafroll virus (PLRV) is the type member of the genus Polerovirus of the family Luteoviridae (Pringle, 1998). Poleroviruses possess small (23 nm) isometric virions which encapsidate the single-stranded, positive-sense, monopartite RNA genome (Mayo et al., 1982). Similar to the other poleroviruses, PLRV is limited to the phloem tissue during infection of host plants and is transmitted by its aphid vector (Myzus persicae) in a circulative, nonpropagative manner (Harrison, 1984). The nucleotide sequences of several different geographical isolates of the ~ 6 kb PLRV RNA genome have been reported (Mayo et al., 1989; van der Wilk et al., 1989; Keese et al., 1990, Palucha et al., 1994). They all show a high degree of similarity, ranging from 93 to 97%. The viral genome (Fig. 1A) contains six main open reading frames (ORFs) located in two blocks separated by an intercistronic region.

PLRV and the other viruses in the family Luteoviridae show remarkably diverse mechanisms of gene expression, including overlapping reading frames, subgenomic RNA synthesis, ribosome leaky scanning, ribosomal frameshifting, stop codon read-through and protein self-processing (reviewed by Rohde et al., 1994; Miller et al., 1997). Proteins encoded by the first cluster (ORF0, ORF1, ORF2) are translated directly from the genomic RNA. The structural proteins, including the viral capsid protein (ORF3), the presumptive movement protein (ORF4) and the putative aphid transmission factor (ORF5), are expressed from a subgenomic RNA which is synthesized from the (−)-RNA template in infected cells (Smith & Harris, 1990). The function of the putative ORF0 protein is so far unknown. ORF1 encodes a polyprotein (calculated molecular mass 70 kDa) consisting of a putative proteinase and a VPg domain; it presumably undergoes (self) processing to liberate the VPg (van der Wilk et al., 1997; reviewed in Miller et al., 1997). It contains the sequence motif HX25D/EX70–85TR/KXGS (where X denotes any amino acid) characteristic of chymotrypsin-like serine proteinases where H, D/E and S constitute the putative catalytic triad (Gorbalenya et al., 1989). Mutagenesis of any of these residues abolishes processing of the ORF1 product expressed in a baculovirus system (Li et al., 2000). The VPg is located downstream of the proteinase domain, as determined by direct amino acid sequencing of the N-terminal region of the VPg.
A prerequisite for the application of recombinant DNA techniques to RNA viruses is the generation of infectious cDNA clones or of infectious transcripts from cloned viral cDNA (reviewed by Boyer & Haenni, 1994). The availability of an infectious clone or transcript from a viral RNA genome makes it possible to determine the function of particular ORFs and non-coding regions in the virus life-cycle by targeted mutagenesis. Infectious clones have been described for several members of the Luteoviridae (Young et al., 1990; Veidt et al., 1992; Demler et al., 1993; Prüfer et al., 1995, 1997). In addition, Leiser et al. (1992) used agroinfection as a system to study Beet western yellows virus (BWYV; Luteoviridae). However, only few
Table 1. Mutations introduced into the expression cassette of the PLRV genome

<table>
<thead>
<tr>
<th>Site</th>
<th>Mutant</th>
<th>Original sequence</th>
<th>Mutated sequence</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S transcription initiation site–viral cDNA junction</td>
<td>Δ11</td>
<td>5’ GAGGaacgtagtaccACAAAAGAA</td>
<td>5′ GAGGACAAAAGAA</td>
<td>Transcription start point in bold; viral cDNA in italics; KpnI restriction site underlined; deleted nucleotides in lower case</td>
</tr>
<tr>
<td>DNA sequence encoding the GDD motif in ORF2 (nt 3076–3084)</td>
<td>GDD513–515VHD</td>
<td>5’ GGG GAC GAT</td>
<td>5’ GTC GAC GAT</td>
<td></td>
</tr>
<tr>
<td>DNA sequence encoding histidine-254 of the catalytic centre of the PLRV proteinase (nt 965–967)</td>
<td>H254L</td>
<td>CAC TGT CTA GAA</td>
<td>CTC TGC CTA GAA</td>
<td>Introduced AspL1 site underlined; amino acids altered by mutation in bold Destroyed XhoI site underlined; altered histidine residue marked by an asterisk</td>
</tr>
<tr>
<td>DNA sequence encoding aspartic acid-286 of the catalytic centre of the PLRV proteinase (nt 1058–1060)</td>
<td>D286A</td>
<td>GAT ATC</td>
<td>GCT ATC</td>
<td>Destroyed EcoRV site underlined; altered aspartic acid residue marked by an asterisk</td>
</tr>
<tr>
<td>DNA sequence encoding serine-353 of the catalytic centre of the PLRV proteinase (nt 1263–1265)</td>
<td>S353A</td>
<td>TCC GGA</td>
<td>GCC GGA</td>
<td>Destroyed MroI site underlined; altered serine residue marked by an asterisk</td>
</tr>
</tbody>
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data on the effect of site-directed mutants of any PLRV ORF are available. Recently, we reported that in vitro synthesized transcripts from cloned cDNA of PLRV replicated in protoplasts and produced viral capsid protein (Sadowsky et al., 1998). Here we report the construction of expression cassettes of the PLRV genome, consisting of a full-length copy of the viral genome, flanked by the 35S promoter and terminator sequences of Cauliflower mosaic virus (CaMV; Caulimoviridae), in a binary T-DNA vector of Agrobacterium tumefaciens. We show that transcripts derived from the 35S promoter replicate in agroinoculated potato leaf discs and we prove the functional importance of two protein sequence motifs for the biology of PLRV. We also demonstrate that mutants in the GDD and H-D-S motifs complement each other. Finally, we show that a mutation in the putative catalytic triad of the proteinase influences in vitro processing of the ORF1 product.

Methods

Construction of the PLRV expression cassette in a binary vector. Plasmid pJF (Sadowsky et al., 1998), containing the full-length cDNA of the PLRV genome (Polish isolate denoted PLRV-P; Pałucha et al., 1994) fused to the T7 RNA polymerase promoter, was used for further constructions (Fig. 1B). The sequence of the T7 promoter, present between the KpnI site and the viral cDNA, was removed from pJF. For this purpose the DNA of pJF was used as a template for PCR with oligonucleotides B1 (5’GCTTCTATGCTGAAGTCCAG) and K1 (5’GGTACCAGGAGAATACCCAGGAGG; non-viral nucleotides underlined). The product was digested with KpnI and Apal (position 369 of the viral genome) and inserted into pJF digested with the same enzymes, resulting in plasmid pPAK.

Plasmid pRT-X, a modified pRT104 (Töpfer et al., 1987) containing the CaMV 35S promoter (Guilley et al., 1982) and terminator, separated by a multiple cloning site, was used as acceptor of the PLRV cDNA. The modification of pRT-104 involved a deletion of 19 bp between the KpnI and XhoI sites. A KpnI–BamHI fragment of pPAK carrying 4166 bp of the PLRV genome was inserted into KpnI/BamHI-digested pRT-X resulting in plasmid pKB. Next, the PflMI–BamHI fragment of pKB was replaced by the PflMI–ScaI fragment of pPAK yielding expression plasmid p35PFX.

The PLRV expression cassette of p35PFX was transferred into the binary T-DNA vector pBin19 (Bevan, 1984; Frisch et al., 1995) by a three-fragment ligation. The HindIII–Apal and Apal–SphI restriction fragments of p35PFX were inserted into pBin19, blunt-ended with T4 DNA polymerase and cut with HindIII, thus yielding pBPF. This plasmid contains the full-length PLRV cDNA flanked by the CaMV 35S promoter and terminator (Fig. 1B). Finally, the 11 additional base pairs present at the 5’ end of the cDNA in pBPF were deleted by site-directed mutagenesis (QuikChange, Stratagene). For this purpose it was necessary to sub-clone the EcoRI–Apal fragment of pBPF into pBluescript KS(+) (Stratagene) digested with EcoRI/Apal. The resulting plasmid, pPGA, carrying the first 369 bp of the viral genome, was used for mutagenesis with oligonucleotides del-1 (nt 101–106) and del-2 (nt 330–335), thus introducing ApaI restriction sites. A transcript from cloned cDNA of PLRV replicated in proto-plasts and produced viral capsid protein (Sadowsky et al., 1994) fused to the T7 RNA polymerase promoter, was used for mutagenesis with oligonucleotides del-1 (nt 101–106) and del-2 (nt 330–335), thus introducing ApaI restriction sites. A transcript from cloned cDNA of PLRV replicated in proto-plasts and produced viral capsid protein (Sadowsky et al., 1994) fused to the T7 RNA polymerase promoter, was used for mutagenesis with oligonucleotides del-1 (nt 101–106) and del-2 (nt 330–335), thus introducing ApaI restriction sites.
the altered promoter fragment was used to replace the corresponding fragment of pBPF, resulting in pBOK. The schematic maps of the relevant plasmids and expression vectors are presented in Fig. 1.

**Construction of GDD and H-D-S mutants.** To introduce a mutation into the sequence encoding the GDD motif in plasmid pES (Sadowy et al., 1998) oligonucleotides gdd-1 (5' GGCGAATGGC-ATGGTGCAGGTGTCCG) and gdd-2 (5' GGCGTCTGAGACGTTCG) were used (mutated nucleotides in bold). As a result, the glycinine codon (GGG) at position 3076–3078 of the viral genome was changed to a valine codon (GTG) and the aspartic acid codon (GAC) at 3079–3081 to a histidine codon (CAC) at 3076–3078 of the viral genome was changed to a valine codon (GTG) and the aspartic acid codon (GAC) at 3079–3081 to a histidine codon (CAC) (Table 1). Concomitantly, this introduced an ApaI site at position 3076 facilitating screening. The resulting plasmid carrying the mutations was denoted pGDD513-515VHD.

The sequence of viral cDNA in the clone was verified and a SpeI-PmlI restriction fragment of pGDD513-515VHD (corresponding to nt 1070–3569 of the viral genome) was transferred to plasmid pBOK digested with the same enzymes. The full-length plasmid, pB-GDD513-515VHD, contains the expression cassette of the full-length cDNA of PLRV in which the amino acids GDD of ORF2 are replaced by VHD.

The H and D amino acids in the putative catalytic centre of the proteinase (ORF1) were altered by site-directed mutagenesis of plasmid pES (Sadowy et al., 1998) using oligonucleotides his-1 (5' GTGGAGCAGCTGAACGTGCTGATAGAAAGCGGTCTCTG) and his-2 (5' CGAAGGGCTTCTGAGTCAGGCTG) mutated nucleotides in bold) to change the histidine codon (CAC; nt 965–967) to a leucine codon (CTC), and oligonucleotides asp-1 (5' GTGCCCGTGAAGCTCTG) and asp-2 (5' GTGATGAGAAGCCTACCCG) for the replacement of the acidic aspartic acid codon (GAT; nt 1058–1060) by an alanine codon (GCA) (CT; Table 1). Next, the ApaI–SpeI fragments carrying either the mutation of the histidine or acidic acid codons were sequenced and inserted into ApaI–SpeI-digested pBOK resulting in plasmids pB-H254L and pB-D286A, respectively.

To alter the serine residue in position 353, a 0.9 kb fragment was amplified on pJF by PCR using primers H8 (5' CGACTCCCGCCGTTGGAGCC) and ser-2 (5' CACTGGACCCGATATGCGGGAGGTTT; mutated nucleotide in bold) thus changing the serine to a glycine codon (TCC; nt 1262–1264) to an alanine codon (GCC). The PCR product was digested with NciI and PstI and the fragment obtained, corresponding to nt 1255–1846, was cloned together with the ApaI–NciI fragment of pJF (nt 369–1255) into ApaI–PstI-digested pBluescript SK (+). The SpeI–AatII fragment of the plasmid thus obtained (nt 1070–1725) was sequenced and exchanged with the corresponding fragment of pJF resulting in pS353A. The SpeI–PstI fragment of the plasmid thus obtained (nt 1069–3569) was transferred to SpeI–PstI-digested pBOK, resulting in pS353A.

**Agroinoculation of leaf discs.** Plasmids were introduced into A. tumefaciens strain LBA 4404 via electroporation (Mattanovich et al., 1988). The biological activity of the PLRV expression plasmids pBPF and pBOK (Fig. 1B) was checked in potato leaf discs. In contrast to pBOK, pBPF contains 11 non-viral nucleotides between the 35S promoter and PLRV cDNA (see Methods). Discs were inoculated with suspensions of A. tumefaciens harbouring plasmids pBin19, pBPF or pBOK; 3, 5, 7 and 10 days post-inoculation (p.i.) the presence of viral RNA and capsid protein was monitored to investigate the biological activity of the cloned cDNA.

**Detection of viral RNA and proteins.** RNA was isolated using TRI Reagent (Molecular Research Company, MRC), following the procedure supplied by the manufacturer. Electrophoretic fractionation of RNA (10 µg per lane or 2 µg per lane in the case of RNA preparations from PLRV-infected Physalis floridana) was performed in denaturing agarose–formaldehyde gels and RNA was blotted onto Hybond-N membranes (Amersham) by capillary transfer (Ausubel et al., 1987). The efficiency of the transfer and level of RNA loading were examined by methylene blue staining (MRC) as recommended by the manufacturer. Viral RNA was detected by non-radioactive hybridization with anti-PLRV antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions.

Protein isolation from plant tissue was as described by Wu & Wang (1984). Electrophoresis of proteins (amount per lane corresponding to 5 mg of tissue) was performed in denaturing 12.5% polyacrylamide gels (Laemmli, 1970). Proteins were electroblotted onto nitrocellulose membranes and revealed by polyclonal anti-PLRV antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) as described (Ausubel et al., 1987).

**RT–PCR analysis of viral RNA.** RNA isolated as described above was treated with RNase (Promega) and used for reverse transcription with Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) using primers 1604-2 (5' GTCTCTCTCTGAGTGGTTGACG) or 3725-2 (5' CTTCGTCTGGGGCCGTTTGACG) under the conditions recommended by the manufacturer. One-tenth of the reaction was used for PCR with primers 1604-2, 590-1 (5' CCTATGG-CATTATACACATTTGACG), and 3725-2, 1730-1 (5' CGCGTTGGCCTACAACGCCACACTACATTGACG) respectively, to amplify the regions of the genome encoding the active centres of the proteinase and replicase. PCR products were then digested with appropriate restriction enzymes (Table 1).

**In vitro transcription and translation.** Plasmids used as templates for in vitro transcription (pJF and pS353A) were linearized with Scal (nt position 5882). In vitro transcription was performed as described by Kujawa et al. (1993). The uncapped transcripts (200 ng) were used for in vitro translation in a rabbit reticulocyte lysate (Boehringer Mannheim) supplemented with L-[35S]methionine in conditions recommended by the manufacturer. Proteins synthesized after incubation for 1 or 24 h were analysed by electrophoresis in denaturing polyacrylamide gels followed by autoradiography.

**Results**

**PLRV RNA derived from a nuclear expression cassette replicates in potato leaf cells**

The biological activity of the PLRV expression plasmids pBPF and pBOK (Fig. 1B) was checked in potato leaf discs. In contrast to pBOK, pBPF contains 11 non-viral nucleotides between the 35S promoter and PLRV cDNA (see Methods). Discs were inoculated with suspensions of A. tumefaciens harbouring plasmids pBin19, pBPF or pBOK; 3, 5, 7 and 10 days post-inoculation (p.i.) the presence of viral RNA and capsid protein was monitored to investigate the biological activity of the cloned cDNA.

RNA preparations isolated from agroinoculated leaf discs 3, 5, 7 or 10 days p.i. were analysed by Northern blot hybridization. RNA fractionated by electrophoresis in denaturing agarose gels was transferred to a Hybond-N membrane and probed with a DIG-labelled riboprobe. Two
Fig. 2. Accumulation of RNAs and proteins synthesized by cDNA expression plasmids of the PLRV genome. (A) Northern blot analysis of the RNA isolated from potato leaf discs inoculated with agrobacteria containing pBPF, pBOK or pBin19. The same amount of RNA (10 µg) was loaded onto each lane as revealed by methylene blue staining of 28S rRNA. Lane 1, RNA of Physalis floridana naturally infected by PLRV (2 µg RNA); lane 2, RNA of leaf discs agroinoculated with pBin19, 10 days p.i.; lanes 3–6, RNA of leaf discs agroinoculated with pBPF, 3, 5, 7 and 10 days p.i., respectively; lanes 7–10, RNA of leaf discs agroinoculated with pBOK, 3, 5, 7 and 10 days p.i., respectively; lane 11, RNA of leaf discs inoculated with pB-GDD513-515VHD. gRNA, genomic RNA; sgRNA, subgenomic RNA. (B) Western blot analysis of proteins isolated from potato leaf discs inoculated with agrobacteria containing pBPF or pBOK. Lane 1, virions of PLRV; lane 2, protein extracts of leaf discs agroinoculated with pBin19, 10 days p.i.; lanes 3–6, protein extracts of leaf discs agroinoculated with pBPF, 3, 5, 7 and 10 days p.i., respectively; lanes 7–10, protein extracts of leaf discs agroinoculated with pBOK, 3, 5, 7 and 10 days p.i., respectively; lane 11, protein extracts from leaf discs inoculated with pB-GDD513-515VHD. CP, capsid protein.

These results demonstrate the ability of the 35S-derived PLRV transcripts to replicate in plant cells and to produce subgenomic RNA. The RNA thus synthesized was also able to direct in vivo synthesis of the viral capsid protein. Eleven additional nucleotides, present at the 5′ end of the pBPF-derived transcript, diminished but did not completely inhibit virus replication. In all subsequent experiments, pBOK was used as a positive control and samples were collected 7 days p.i. This plasmid also served for construction of the replicate and proteinase mutants used in further studies.

To ascertain that the PLRV RNAs detected in the leaf discs were products of replication and not merely transcripts of the 35S promoter and potential processing products thereof, a mutant in the GDD513–515 motif of the ORF2 product was constructed (pB-GDD513–515VHD; Table 1). The amino acid motif GDD encoded by ORF2 at nt 3076–3084 is characteristic of all viral RNA-dependent RNA polymerases (Kamer & Argos, 1984). Molecularly, the two active site aspartates are implicated in the co-ordination of divalent cations (Mg$^{2+}$, Mn$^{2+}$) required for catalysis in several polymerases (for a review see Joyce & Steitz, 1994). Neither viral genomic RNA and subgenomic RNA nor the viral coat protein were detectable in leaf discs agroinoculated with pB-GDD513-515VHD (Fig. 2A, lane 11 and Fig. 2B, lane 11).

Amino acids H254-D286-S353 are essential for the PLRV proteinase function

Alignment of the ORF1 protein sequence with those of the serine proteinase family showed the presence of the characteristic amino acid motif H-D-S in the ORF1 product (Bazan & Fletterick, 1990; Koonin & Dolja, 1993), thought to constitute the catalytic centre of the proteinase. In the case of PLRV, this centre would consist of histidine-254, aspartic acid-286 and serine-353. Although suggestive, experimental evidence of the importance of these amino acids is missing for PLRV and all other poleroviruses. To confirm the importance of these amino acids for virus amplification, each codon specifying an amino acid of the putative catalytic triad was mutated individually (Table 1), and the effect of the respective mutation on virus replication was examined.

Three plasmids carrying the mutations H254L (pB-H254L), D286A (pB-D286A) or S353A (pB-S353A) were used for agroinoculation of potato leaf discs. Northern blot hybridization of total plant RNA isolated from leaf discs agroinoculated with each of the three mutants failed to produce any signal of viral genomic or subgenomic RNA (Fig. 3A, lanes 3–5). The genomic and subgenomic RNAs were readily detected in RNA preparations from leaf discs agroinoculated with pBOK (Fig. 3A, lane 1).

In parallel to the viral RNAs, the presence of the capsid protein in extracts from agroinoculated leaf discs was analysed by Western blotting. If replication [i.e. production of (−)-RNA] is a prerequisite for the synthesis of subgenomic RNA,
the messenger RNA for the capsid protein, no capsid protein synthesis would be expected in the mutants analysed. Indeed, no capsid protein was detected in protein preparations obtained from leaf discs agroinoculated with the PLRV expression plasmids mutated in any of the codons specifying an amino acid of the putative catalytic triad of the viral proteinase (Fig. 3B, lanes 3–5).

**Complementation between the replicase and proteinase mutants**

Mutations that change amino acid residues constituting the putative catalytic triad of the proteinase and a mutation in the GDD motif might also influence the secondary structure of the viral RNA and/or cis-acting signals involved in PLRV replication. Therefore, we tested whether these mutants can complement each other. Several independent assays of co-agroinoculation of pB-GGD513-515VHD with one of the proteinase mutants (pBS353A, pB-H254L or pH-D286A) resulted in wild-type or close to wild-type levels of synthesis of viral genomic and subgenomic RNA (Fig. 4A, lanes 4–6). Similarly, the capsid protein was easily detected in protein preparations from discs agroinoculated with pB-GDD513-515VHD and either pB-H254L, pB-D286A or pB-S353A (Fig. 4B, lanes 4–6, respectively). In contrast, no viral products were detected in discs co-agroinoculated with two constructs containing different mutations in the proteinase motif (Fig. 4A, lanes 7–9 and Fig. 4B, lanes 7–9).

Progeny viral RNA resulting from complementation between the proteinase and replicase mutants was analysed by RT–PCR followed by restriction enzyme digestion to determine whether the mutations introduced had been maintained. The amplified region, nt 576–1617, harbouring the sequence encoding the catalytic centre of the proteinase, was digested with MroI, XbaI and EcoRV in the case of the RT–PCR products synthesized on RNA templates from pB-GDD513-515VHD and either pB-H254L, pB-D286A or pB-S353A (Fig. 4B, lanes 4–6, respectively). In contrast, no viral products were detected in discs co-agroinoculated with two constructs containing different mutations in the proteinase motif (Fig. 4A, lanes 7–9 and Fig. 4B, lanes 7–9).
inoculations, respectively. In each case only partial digestion was obtained (Fig. 5A, lanes 7–9). Under the same conditions, the wild-type PCR products obtained on the pBOK template were digested to completion (Fig. 5A, lanes 1–3). The amplified region, nt 1716–3736, resulting from RT–PCR on the template of RNA preparations from the same mixed agroinoculations, contains the sequence encoding the catalytic centre of the replicase. The fragment was digested with ApoLI and yielded partially cleaved DNA (Fig. 5B, lanes 3–5). The PCR product obtained with the pB-GDDS513-515VHD template was completely digested by the enzyme (Fig. 5B, lane 2). The RT–PCR results indicate that the progeny viral RNAs consist of a mixture of both wild-type and mutant RNAs, and hence that replication does not result from reversion of a mutation or recombination between the RNAs produced.

**In vitro studies on processing of the ORF1 product**

Proteolytic activity of the wild-type and mutated ORF1 product was studied in an *in vitro* translation system. For this purpose plasmids pJF and pS353A were linearized by Scal and subsequently used as templates for *in vitro* transcription. Both uncapped transcripts, whose lengths corresponded to the total length of the PLRV genome, were translated *in vitro* in a rabbit reticulocyte lysate in the presence of \[^{35}\text{S}\]methionine. The translation products obtained after incubation for 1 or 24 h were separated by SDS–PAGE and visualized by autoradiography. The translation of both wild-type and mutant transcripts resulted in synthesis of three proteins of 118, 66 and 28 kDa after 1 h of incubation (data not shown). Prolonging incubation to 24 h (Fig. 6) resulted in the appearance of an additional band of 25 kDa in translations of pJF-derived transcripts but absent when the pS353A transcript was used. This protein probably corresponds to protein C25 detected *in planta* by Prufer et al. (1999).

**Discussion**

Infectious transcripts of viral genomes have proven invaluable for the molecular analysis of plant viruses (reviewed in Boyer & Haenni, 1994). They have, however, some
limitations, as for instance the requirement for protoplasts and in vitro transcription. An alternative to infectious transcripts is represented by agroinoculation (Grimsley et al., 1987), first applied to a polerovirus by Leiser et al. (1992) who demonstrated that plants could thus be infected with appropriate constructs of full-length BWYV cDNA expressed under control of the CaMV 35S promoter. Similarly, Prüfer et al. (1997) described the construction of full-length cDNA clones of PLRV under the control of the 35S promoter for in vivo studies in protoplasts and stably transformed potato plants by Agrobacterium-mediated leaf disc transformation.

Here we have used the full-length cDNA of a Polish isolate of PLRV (Sadowy et al., 1998) to construct a nuclear expression cassette and show that the derived RNA replicates in leaf discs. In addition, we prove that PLRV-specific RNAs detected in leaf discs agroinoculated with such T-DNA-based expression vectors are generated by the action of the viral replicase, since a mutation in the GDD motif of the replicase abolishes RNA-dependent replication (Fig. 2).

In the case of certain comosp-, cucumo- and alfamoviruses, the presence of non-viral nucleotides at the 5' end of their in vitro-derived transcripts had an inhibitory effect on their biological activity (Eggen et al., 1989; Rizzo & Palukaitis, 1990; Dore et al., 1990). Therefore, the 11 additional nucleotides located between the transcript initiation site of the 35S promoter and the 5' end of the PLRV cDNA were removed from plasmid pBP, resulting in plasmid pBOK. Thus, the pBP- and pBOK-derived transcripts differ only by the presence or absence of the 11 nucleotides at the 5' end of the former, whereas the pBOK-encoded transcript starts precisely at the authentic viral sequence. The presence of the 11 additional nucleotides in the pBPF-derived transcript decreased virus replication (Fig. 2).

The 3' ends of pBP and pBOK transcripts are identical; both presumably contain an extension of about 200 nt and a poly(A) tail. The tolerance of additional sequences at the 3' end of the pBPF- and pBOK-derived transcripts is consistent with the results of similar experiments with other viruses, such as White clover mosaic virus (Potexviridae; Beck et al., 1990) and BWYV (Leiser et al., 1992). In the latter case, a 35S-derived transcript with a 3' extension was as infectious as one containing a 3' ribozyme to precisely restore the viral 3' end.

The importance of the amino acid motif GDD, found in the replicases of all (+)-strand RNA viruses (Koonin, 1991), is well established and has been investigated by site-directed mutagenesis of the infectious clones of numerous bacterial, animal and plant viruses (Inokuchi & Hirashima, 1987; Taschner et al., 1991; Ribas & Wickner, 1992; Longstaff et al., 1993; Jablonski & Morrow, 1995; Li & Carrington 1995; Molinari et al., 1998). In each case, the alteration of amino acids of this motif completely abolished infectivity of the viral RNA. Moreover, mutated forms of the viral replicases were inactive when tested by in vitro assays (Jablonski & Morrow, 1995; Hong & Hunt, 1996). Therefore, a mutant containing the sequence VHD instead of GDD (pB-GDD513-515VHD) was constructed to prove whether the RNA species observed following agroinoculation were products of the viral replicase. The absence of these RNAs in agroinoculation experiments using pB-VHD confirms that the viral RNAs observed following inoculation with pBPF and pBOK indeed result from viral replicase activity.

Proteinases are required for the processing of the polyproteins expressed by many viruses infecting eukaryotic hosts (reviewed in Dougherty & Semler, 1993). Often, the rate of proteolysis has a regulatory role during the virus life-cycle (Babe & Craik, 1997). In chymotrypsin-like serine proteinases, a triad composed of a histidine, an aspartic or glutamic acid and a serine residue constitute the active centre of the enzyme. In ORF1 of PLRV a sequence motif typical of serine proteinases, H(X23)D/E[K/R]GXX, was detected (triad in bold; Koonin & Dolja, 1993). Moreover, a VPg domain has been identified within ORF1 of PLRV (van der Wilk et al., 1997) and a 25 kDa cleavage product of the ORF1-encoded protein has been detected in infected plants (Prüfer et al., 1999). While these data suggest that a proteinase might be involved in the PLRV life-cycle, the present work provides the first genetic evidence that the serine residue of the putative triad encoded by ORF1 is indispensable for PLRV replication. Alteration of any of the three amino acids constituting the catalytic triad likewise abolished replication (Fig. 3). Consistent with these results, the viral capsid protein, expressed from the subgenomic RNA, was not detected in cells infected with any of the three mutants in the catalytic triad.

Complementation is an effective way to rescue mutant viral genomes as studied extensively for Poliovirus (e.g. Bernstein et al., 1986; Charini et al., 1991; Teterina et al., 1995) and demonstrated for plant viruses such as Turnip yellow mosaic virus (Tymoviridae; Weiland & Dreher, 1993), Turnip crinkle virus (Tombusviridae; White et al., 1995), Artichoke mottled crinkle virus (Tombusviridae; Molinari et al., 1998) and Tomato bushy stunt virus (Tombusviridae; Oster et al., 1998). A complementation phenomenon also occurs in the natural viral population of Tomato aspermy virus (Bromoviridae; Moreno et al., 1997). Here we show that mixed agroinoculations of a mutant in the GDD motif of the viral replicase with any of the three mutants in the putative catalytic triad of the proteinase restores virus replication in leaf discs. The pattern of viral RNA did not differ from that produced by the wild-type clone (Fig. 4). RT–PCR analysis followed by digestion with diagnostic restriction enzymes revealed that the original mutations were still present in the progeny viral RNAs resulting from complementation (Fig. 5). It can therefore be concluded that both replication components, the proteinase and replicase, can act in trans on viral RNA templates. Such a result is consistent with the finding that the PLRV proteinase cleaves in trans (Li et al., 2000). On the other hand, the proteinase mutants themselves never complemented each other in co-agroinoculation experiments (Fig. 4). These observations indicate that the observed
replication was indeed basically due to complementation and not reversion of the mutants or recombination between the viral RNAs. Although we cannot exclude the possibility that some recombination occurs, if only the hypothetical recombinants were viable, one would expect them to rapidly become the predominant or exclusive form present in the discs, which is not the case.

The in vitro translation of wild-type and mutated full-length transcripts of PLRV resulted in the synthesis of the 118, 66 and 28 kDa proteins, corresponding to the ORF1–ORF2 frameshift protein, ORF1 and ORF0 products, respectively (Fig. 6). Translation for 24 h using wild-type transcripts produced an additional 25 kDa band, which probably results from slow autocatalytic processing of the ORF1 product. It is also known that in the case of some viral proteinases efficient cleavage requires the presence of membranes (Amberg et al., 1994; Pinon et al., 1997; Rubinio & Russo, 1998), metal cations (De Francesco et al., 1996; Petersen et al., 1999), polypeptide cofactors (Ding et al., 1996; Kim et al., 1996) or RNA (Matthews et al., 1994; Daros & Carrington, 1997). Lack of one or more such cofactors in the in vitro incubation conditions may limit autoproteolysis of the PLRV ORF1 product. Low cleavage efficiency may be also due to the fact that the PLRV proteinase presumably acts in trans (Li et al., 2000) and a low concentration of the enzyme might represent a limiting factor for processing. The 25 kDa protein produced in vitro presumably corresponds to the 25 kDa protein detected in PLRV-infected plants with antibodies specific to the C-terminal part of the ORF1 product (Prüfer et al., 1999). Moreover, mutagenesis of the putative catalytic serine prevented the appearance of the 25 kDa band by in vitro translation. These data strongly support the hypothesis that the ORF1 product is a serine proteinase. However, our attempts to further characterize the proteinase by the use of specific inhibitors such as phenylmethylsulfonylfluoride, aprotinin and diisopropylfluorophosphate to prevent self-cleavage were unsuccessful (data not shown). However, it is known that some proteinases, e.g. the NS2B-3 of Yellow fever virus (Flaviviridae), the epidermolysis toxin of Staphylococcus aureus or Npro of Sindbis virus (Togaviridae), do not react with such proteinase inhibitors (Amberg et al., 1994; Bailey et al., 1995; Rumenapf et al., 1998). Further experiments, in particular expression of ORF1 in vivo and structural studies, are required to elucidate the mechanism of the action of the PLRV proteinase.

The following paper (Sadowy et al., 2001) reports experiments to prove that the ORF0 product is indispensable for virus accumulation.

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