Expression of the genome of potato leafroll virus: readthrough of the coat protein termination codon \textit{in vivo}

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An antiserum was raised against a fusion protein containing part of the 56K polypeptide (P5) encoded by the open reading frame (ORF) at the 3' end of the genome of potato leafroll virus (PLRV). This antiserum reacted specifically with 80K and 90K polypeptides in PLRV-infected protoplasts, with a 90K polypeptide in infected potato tissue and with a 53K polypeptide in protein extracted from purified particles of PLRV. Monoclonal antibodies raised against purified PLRV particles also reacted with these polypeptides, as well as with the 23K coat protein. Virus particles partially purified from infected protoplasts contained some 90K polypeptide as well as the major 23K coat protein. The ORFs of the 23K coat protein and P5 are contiguous and in frame. The results suggest that the P5 polypeptide of PLRV occurs in infected cells as part of a readthrough protein comprising the 23K coat protein joined to the P5 amino acid sequence. Moreover the readthrough protein can be assembled into virus particles as a minor component together with the main 23K component. The P5 protein may thus contribute to properties of PLRV determined by its virus particle surface.

Introduction

Potato leafroll virus (PLRV; luteovirus group) causes a serious disease of potatoes that results in significant crop losses in various parts of the world. It is transmitted in a persistent manner by aphids both from infected plants and from preparations of purified virus (Harrison, 1984). Particles of PLRV contain a 6 kb RNA encapsidated in a protein coat made of a 23K polypeptide. Recently, the complete nucleotide sequences of cDNA copies of the genome RNA of two isolates of PLRV have been described (Mayo \textit{et al.}, 1989; van der Wilk \textit{et al.}, 1989). Sequences have also been reported for the RNA of beet western yellows (BWYV; Veidt \textit{et al.}, 1988) and barley yellow dwarf (BYDV; Miller \textit{et al.}, 1988) luteoviruses.

Although little is known about the replication of luteoviruses in infected cells, analysis of the nucleotide sequences suggests a number of strategies for gene expression. In particular, the sequence of PLRV RNA contains six large open reading frames (ORFs) arranged either side of a 200 nucleotide long non-coding region. The gene (ORF 4) for the 23K coat protein (P3) is located on the 3' side of the non-coding sequence; ORF 6, which is downstream from ORF 4 and is shown in Fig. 1 as between the first AUG codon and the first termination codon, could encode a 53K polypeptide (Mayo \textit{et al.}, 1989). The nucleotide sequences around the amber termination codon of the PLRV 23K protein (Mayo \textit{et al.}, 1989; van der Wilk \textit{et al.}, 1989), and those of BYDV and BWYV coat proteins (Miller \textit{et al.}, 1988; Veidt \textit{et al.}, 1988), resemble those around several other virus RNA termination codons that are misread by naturally occurring suppressor tRNAs to generate readthrough proteins. Readthrough of the termination codon of the gene for BWYV coat protein has been shown to occur during translation of \textit{in vitro} transcribed transcripts of BWYV cDNA (Veidt \textit{et al.}, 1988). It was therefore suggested (Mayo \textit{et al.}, 1989; van der Wilk \textit{et al.}, 1989) that in PLRV RNA readthrough of the termination codon of ORF 4 results in translation of the downstream ORF to give a readthrough protein of about 80K. In this paper we present serological evidence that the predicted 80K readthrough protein is produced in PLRV-infected cells. Although the major protein component of purified particles of luteoviruses is the 23K polypeptide, several authors have reported the presence of minor, higher Mr, components (Rochow & Duffus, 1981; Waterhouse, 1981; Murant \textit{et al.}, 1985). We demonstrate that in PLRV particles the 80K readthrough protein gives rise to a minor high Mr component, presumably by the loss of C-terminal sequences.
Methods

**Virus propagation and purification.** The PLRV isolate was the Scottish isolate of strain 1 (Tamada et al., 1984) and was purified as described by Mayo et al. (1989). Particles were partially purified from infected protoplasts by suspending a pellet of about 3 x 10^5 protoplasts in 2 ml of 0.01 M-sodium phosphate buffer pH 7, centrifuging the extract at about 10000 g for 5 min and pelleting virus particles from the supernatant fraction by centrifugation through a 1 ml cushion of 20% sucrose in 0.01 M-sodium phosphate pH 7 at 45000 r.p.m. in a Beckman SW50 rotor at 10 °C for 2.5 h.

**Preparation, inoculation and culture of protoplasts.** Protoplasts were isolated from the palisade mesophyll of leaves of *Nicotiana tabacum* cv. Xanthi using a two-step enzyme digestion as described by Kubo et al. (1975). Protoplasts were inoculated with phosphate-buffered mixtures of PLRV particles (0.2 pg/ml) and poly-L-ornithine (1 ktg/ml), as described by Mayo & Barker (1983); mock inoculation was identical except that virus particles were omitted. Protoplasts were then cultured as described by Kubo et al. (1975) until they were recovered by centrifugation and assayed either by extracting the pellets or by staining with fluorescent antibodies (Mayo & Barker, 1983). In most experiments 50% to 80% of morphologically intact protoplasts stained 2 days after inoculation were found to be infected.

**Construction of expression vectors.** DNA corresponding to nucleotides 4646 to 5280 of the sequence described by Mayo et al. (1989) was inserted into pRIT21 downstream from and in frame with the Protein A gene as follows. pRIT21 (a gift from Dr Nigel Stow) was derived from pRIT2T (Nilsson 1985) by cleavage with EcoRI, treatment with mung bean nuclease to remove 5' extensions and religation. pRIT21 was cleaved with PstI, treated with mung bean nuclease and recleaved with BamHI. Recombinant plasmid DNA containing the PLRV P5 gene was cleaved with EcoRI, treated with mung bean nuclease and recleaved with BamHI. The PLRV fragment corresponding to nucleotides 4646 to 5280 was isolated by electroelution from a polyacrylamide gel and inserted into the cleaved pRIT21 to yield the recombinant plasmid pRIT21-EP4.

**Preparation of fusion protein.** N4830 Escherichia coli cells containing pRIT21-EP4 were grown to a density of 0.6 at OD600 at 28 °C and recombinant plasmid pRIT21-EP4 were grown to a density of 0.6 at OD600 at 28 °C and protein extract was prepared from pellets of *Escherichia coli* cells expressing the PLRV P5 gene product. Cells were collected, treated with 2 mg/ml lysozyme, lysed in phosphate-buffered saline (PBS; 0.15 M-NaCl, 0.01 M-sodium phosphate pH 7.0) containing 0.05% Tween-20 and centrifuged. The resulting protein extract was applied to a 4 ml column of IgG-Fast Flow Sepharose (Pharmacia) and the column was washed with 50 bed volumes of PBS containing 0.05% Tween-20. Bound protein was eluted with 0.1 M-glycine–HCl pH 3.0, immediately neutralized with NaOH and then concentrated and desalted by ultrafiltration.

**Preparation of antisera.** Rabbits were injected with 250 μg of fusion protein emulsified in Freund's complete adjuvant. Three booster injections of the same amount of protein emulsified in incomplete adjuvant were given at 20-day intervals starting 1 month after the initial injection. The mouse monoclonal antibody (MAb) was SCR 1 (Massalski & Harrison, 1987) which was made following immunization with purified particles of PLRV.

**Electrophoresis.** Protein extracts were prepared from pellets of protoplasts or from fresh leaf tissue by suspension in 2% SDS, 0.15 M-dithiothreitol, 10% glycerol pH 6.8. Samples equivalent to about 3 x 10^5 protoplasts or 50 mg fresh weight of leaf tissue were separated by electrophoresis in 10% SDS-polyacrylamide gels essentially as described by Laemmli (1970). Polypeptide Mr values were estimated using as markers polypeptides from virions of adenovirus type 2 (Mr 113000), phosphorylase b (95000), bovine serum albumin (67000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000) and trypsin inhibitor (20000). Samples of PLRV particle protein contained about 100 ng for immunoblotting and several μg for staining with Coomassie blue.

**Immunoblotting.** Polypeptides were transferred from gels to nitrocellulose (Randall & Young, 1988) by using a 'semi-dry blotter' (LKB) using a discontinuous buffer system according to the manufacturer's instructions. Alternatively, extracts of protoplasts were applied to nitrocellulose as 5 μl dots. Nitrocellulose sheets were blocked with 1/100 dilution of polyclonal anti-P5 or a 1/1000 dilution of MAb SCR 1 (anti-P3) in PBSMN. After the blots had been washed, antibodies were detected by incubation with either (i) 0.5 μCi/ml of 125I-labelled Protein A (Amersham) in PBSMN followed by autoradiography or (ii) a 1/1000 dilution of an alkaline phosphatase conjugate of goat anti-rabbit or goat anti-mouse serum (Sigma) in PBS containing 10% Marvel followed by reaction with NBT/BCIP substrate (Bio-Rad).

**Results**

**Antibodies directed against the P5 gene product**

To generate antisera directed against the protein encoded by ORF 6, cDNA to this region of the genome (Fig. 1) was inserted into the prokaryotic expression vector pRIT21 in such a way that an in-frame fusion was constructed with the protein A gene of *Staphylococcus aureus* (Nilsson et al., 1985). The antiserum raised in rabbits against this protein (anti-P5) reacted in dot blots at dilutions of 1/512 or less with extracts of PLRV-infected protoplasts but did not react at dilutions of more than 1/4 with extracts of mock-inoculated protoplasts (data not shown).

**Detection of P5 product in extracts of PLRV-infected protoplasts**

To detect polypeptides encoded by the P5 region of the PLRV genome, proteins present in PLRV-infected and mock-inoculated protoplasts were analysed by immunoblotting. Samples were taken 1, 2 and 3 days after inoculation with purified particles of PLRV.
inoculation and immunoblotted with anti-P5 or MAb SCR 1. Anti-P5 detected polypeptides of 66K, 80K and 90K in PLRV-infected protoplasts, particularly 2 and 3 days after inoculation (Fig. 2, lanes 12 and 14), but did not react with polypeptides present in mock-inoculated protoplasts (Fig. 2, lanes 8 to 10). MAb SCR 1 did not react with proteins from mock-inoculated protoplasts (Fig. 2, lanes 1 to 3) but reacted strongly with a 23K polypeptide which comigrated with the major polypeptide component of purified virus particles (CP; Fig. 2, lane 4) and also reacted with polypeptides of 90K and 80K which comigrated with products detected by anti-P5 (Fig. 2, lanes 5 to 7). In addition, SCR 1 detected a protein of 53K in purified preparations of virions (Fig. 2, lane 4) which was not detected in infected protoplasts (see below). A faint band corresponding to the 53K protein was visible in the original autoradiograph in lane 11. These data suggest that the 80K and 90K polypeptides represent the predicted readthrough protein which consists of the 23K polypeptide at the N terminus (detected by SCR 1) and polypeptide P5 at the C terminus (detected by anti-P5).

Similiar results were obtained when antisera conjugated to alkaline phosphatase were used to detect antibodies bound to blots (Fig. 3) although with this method several polypeptides present both in PLRV-infected and in mock-inoculated protoplasts bound anti-P5 (Fig. 3, lanes 4 and 5). Attempts to decrease this background reaction by using purified gamma globulin were unsuccessful.

**Immunoblots of protein from purified PLRV particles**

If, as predicted, the readthrough proteins of 80K and 90K detected in infected protoplasts contain the 23K protein it seems probable that they could be assembled with the 23K protein into virus particles. This was tested by immunoblotting proteins extracted from purified virus preparations. The major protein component detected by staining with Coomassie blue was the 23K protein but small amounts of a 53K protein were always present (Fig. 3, lane 7). Both proteins reacted with SCR 1 (Fig. 3, lane 3) but only the 53K protein reacted with anti-P5 antiserum (Fig. 3, lane 6). This result suggests...
that the 53K protein is a readthrough protein derived from the 80K and 90K polypeptides found in infected protoplasts. A 53K polypeptide has been detected in protein from particles of PLRV in preparations purified from potato tissue and Physalis floridana using either Celluclast (as in this work) or Driselase (as used by Waterhouse, 1981) to macerate the tissue prior to extracting the virus particles.

To determine the relation between the 53K polypeptide and the 90K/80K polypeptides detected in infected protoplasts, protein was extracted from PLRV particles that had been isolated rapidly without enzyme treatment from protoplasts 2 days after infection and immunoblotted using SCR 1. The main polypeptides detected by SCR 1 in these partially purified virus particles were the same species (CP, 80K and 90K; Fig. 4, lane 3) as were detected in unfractionated protoplasts (Fig. 4, lane 4) and little reactive polypeptide comigrated with the 53K component present in protein from virus particles purified from whole plants by the standard enzyme procedure (Fig. 4, lane 1). Polypeptides in a similar preparation from mock-inoculated protoplasts did not react with SCR 1 (Fig. 4, lane 2). In a second experiment, in which samples were immunoblotted with anti-P5 or SCR 1 (Fig. 4, lanes 5 to 7), anti-P5 reacted with several polypeptides in extracts of mock-inoculated protoplasts (Fig. 4, lane 7; as in the experiment shown in Fig. 3, lane 4). However both antisera reacted with several polypeptides in virus purified from PLRV-infected protoplasts including 80K and 90K polypeptides, although the strongest reaction of SCR 1 (although not anti-P5) with polypeptides larger than 23K was with a 53K species (Fig. 4, lanes 5 and 6). The doublet band in lane 6 that reacted weakly with anti-P5 but not SCR 1 may be some of the C-terminal part of P5 removed by the cleavage to form the 53K protein, but further experiments are necessary to test this hypothesis.

When protein that had been extracted from leaves of
potato or *N. clevelandii* plants infected with PLRV was immunoblotted, infection-specific polypeptides were less readily detected than in protoplast samples but those detected were almost exclusively 23K and 90K species (Fig. 5, lanes 1 to 3). In this experiment a sample from PLRV-infected protoplasts contained a 90K polypeptide that reacted with SCR 1 but the 80K species detected in other experiments was absent (Fig. 5, lane 4). SCR 1 also detected a small amount of a 53K polypeptide which comigrated with the 53K species found in purified preparations of PLRV. No reaction was detected when protein from *N. clevelandii* tissue infected with BWYV was immunoblotted with SCR 1 or anti-P5 (data not shown).

**Discussion**

The results show that although most of the protein made in infected cells that reacts with SCR 1 is found as a 23K product, some is linked to the polypeptide encoded by the next ORF downstream (ORF 6). No polypeptide corresponding to the primary translation product of ORF 6 (Mayo *et al.*, 1989) was detected in infected protoplasts or, in a more limited examination, in infected potato tissue. It appears that this ORF is expressed only as a result of suppression of the amber termination codon of ORF 4, the gene for the 23K coat protein (P3). Presumably this suppression is mediated by a rare, naturally occurring tRNA. This type of readthrough is known to occur during the translation of several other plant virus RNA species, for example suppression of the amber termination codon of the tobacco mosaic virus 126K gene by tyrosine-accepting suppressor tRNA to yield a 183K protein (Morch & Haenni, 1987) and during translation of animal virus RNA in the synthesis of the gag-pol precursor of murine leukemia virus (Philipson *et al.*, 1978; Shinnick *et al.*, 1981); in this case the amber codon of the gag gene is read as glutamine (Yoshinaka *et al.*, 1985).

The predicted size for the PLRV readthrough protein is therefore the sum of 23K, 53K (ORF 6) and the 4K encoded by the sequence between ORF 4 and ORF 6, a total of 80K. In most immunoblots of infected protoplasts, antisera to the 23K and 53K components reacted with two polypeptides of 80K and 90K. In one protoplast sample (Fig. 5, lane 4) and with several samples from infected potato and *N. clevelandii* leaves (Fig. 5, lanes 1 to 3), only the 90K polypeptide was detected. Although the predicted size of the readthrough protein is 80K, it is not possible, with the available evidence, to determine whether the 80K or the 90K polypeptide is the primary translation product. Possibly the 80K polypeptide is the primary product and is modified, for example by glycosylation (there are three potential glycosylation sites in P5: QNY, YNY and KNK which are 64, 201 and 394 amino acid residues downstream respectively of the 23K termination site), so that it migrates as if it were 90K rather than 80K. Alternatively the 90K polypeptide may be the primary product and may migrate unusually slowly, the 80K polypeptide arising from it by degradation.

The 53K polypeptide present in protein extracted from purified particles of PLRV resembled the 80K polypeptide in that it also reacted with SCR 1 and anti-P5. It is probable that the 53K protein in virus particles is derived by the loss of about 26K of the 80K protein, presumably from its C-terminal end. When partially purified particles were examined in one experiment the proteins resembled those in unfractionated protoplasts but in the other experiment the results suggested that some of the 80K/90K polypeptides had been degraded to form polypeptides intermediate between 80K and 53K but mainly 53K. This suggests that the proteolysis occurs relatively rapidly after infected cells are disrupted and that the 80K protein is cleaved at a limited number of sites.

The results also suggest that the readthrough protein can be assembled into virus particles. One feature of the amino acid sequence encoded by the nucleotide sequence immediately downstream of the 23K termination codon is a succession of proline residues alternating mainly with serine or threonine residues (Mayo *et al.*, 1989). This feature is shared with other luteoviruses (Miller *et al.*, 1988; Veidt *et al.*, 1988). Perhaps a function of this unusual sequence is to separate the 23K domain of the readthrough protein, which must assemble with other 23K polypeptides to form the capsid, from the 56K domain which would thus protrude from the virus particle. Indeed a small number of protrusions have been detected on the surfaces of PLRV particles in favourably stained preparations (Harrison, 1984). A similar readthrough protein composed of coat protein and part of the downstream ORF has been detected in particles of BYDV (P. M. Waterhouse, personal communication) and Veidt *et al.* (1988) have demonstrated suppression *in vitro* of the amber termination codon of the 23K protein ORF for BWYV. Therefore this means of expression of the ORF for P5 may be characteristic of luteoviruses.

A possible role of the P5 domain of the 80K protein is suggested by the results of comparisons (M. A. Mayo & C. A. Jolly, unpublished results) between the 23K proteins of aphid-transmissible isolates of PLRV and an isolate that is very poorly aphid-transmissible (strain V; Massalski & Harrison, 1987). Although some monoclonal antibodies that react with particles of transmissible isolates failed to react with particles of PLRV-V, the only amino acid changes between the
sequences of the 23K coat proteins were exchanges of very similar amino acids (arginine to lysine and isoleucine to valine). Thus it seems likely that at least one determinant for aphid transmissibility is located in the P5 domain. A 90K polypeptide that reacted with SCR-1 was detected in potato tissue infected with PLRV-V (data not shown) and thus it is likely that the determinant is altered rather than deleted in PLRV-V P5. The amino acid sequences of the P5 proteins of PLRV, BWYV and BYDV are appreciably similar in their N-terminal halves but are largely (PLRV and BWYV) or completely (BYDV and either PLRV or BWYV) distinct in their C-terminal halves (Mayo et al., 1989). The similarities among the N-terminal halves may be related to the location of a determinant for aphid transmission in this part of P5.

The results therefore suggest that P5 of PLRV is a part of the virus particle that possibly contributes biological properties to the virus. In evolutionary terms this strategy has the advantage of separating distinct roles of the virus particle so that changes in one do not cause disadvantageous changes in another. Indeed, whereas the sequences of the three known luteovirus 23K proteins are strikingly similar (Mayo et al., 1989), the P5 polypeptides are less similar, particularly at their C-terminal ends. However, even if correct for luteoviruses, this strategy is not shared by many other plant viruses, the only other examples of readthrough of the coat protein cistron.


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