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Immunological analysis of potato leafroll luteovirus (PLRV) P1 expression identifies a 25 kDa RNA-binding protein derived via P1 processing

Dirk Prüfer*, Lawrence Kawchuk[1], Michael Monecke[2], Sabine Nowok, Rainer Fischer[2] and Wolfgang Rohde

Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné Weg 10, 50829 Köln, Germany, [1]Agriculture and Agri-Food Canada, PO BOX 3000, Lethbridge, AB TIJ 4B1, Canada and [2]Rheinisch-Westfälische Technische Hochschule Aachen, Worringer Weg 1, Aachen, Germany

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ABSTRACT

Mono- and polyclonal antibodies directed against different domains of the potato leafroll luteovirus (PLRV) P1 (ORF1) protein were applied to the analysis of P1 expression during PLRV replication *in planta*. Western analyses detected P1 and a protein of ~25 kDa (P1-C25) that accumulated to readily detectable amounts in PLRV-infected plants, but was not detected by *in vitro* cell-free translation of P1. P1-C25 represents the C-terminus of P1 and is a proteolytic cleavage product produced during P1 processing. On the basis of its molecular weight, the N-terminus of P1-C25 is either identical to or located adjacent to the previously identified PLRV genome-linked protein, VPg. P1-C25 is not associated with virus particles, and subcellular localization experiments detected P1-C25, but not P1, in the membrane and cytoplasmic fractions of PLRV-infected cells. In addition, P1-C25 exhibits nucleic acid-binding properties. On the basis of its biosynthesis, localization and biochemical properties, P1-C25 may facilitate the formation of P1/PLRV RNA complexes in which the spatial proximity allows for covalent bond formation between PLRV RNA and VPg.

INTRODUCTION

Processing of polyprotein precursors into intermediates and mature proteins is a well-known phenomenon with animal and plant viruses. One example from the animal virus field is poliovirus: the positive-sense RNA genome of this picornavirus is first translated into a single polyprotein precursor that requires three different virus-coded proteinases for the maturation of viral proteins and completion of the infection cycle (1). During maturation of the polioviral primary translation product, several proteolytic intermediates containing the VPg and replication-associated protein sequences were identified by immunological techniques. One of these intermediates, which leads to the release of VPg and its covalent linkage to RNA, is the P3-9 protein of 12 kDa that is abundantly associated with membrane structures of infected cells where replication of picornaviral RNA is known to occur (2).

In the plant virus field, potyviruses (3,4) are an example of positive-sense, single-stranded RNA viruses with a terminal RNA-linked VPg that share the translational strategy of poliovirus (processing of a primary polyprotein). In contrast, the expression of luteoviral genes occurs via subgenomic (sg)RNAs, but its VPg, as demonstrated recently for potato leafroll virus (PLRV; 5), is located within the C-proximal part of P1 (ORF1) from which it must be released through proteolytic cleavage. PLRV is a typical member of the luteovirus subgroup II (polerovirus), whose genome consists of a 5.8 kb single-stranded positive-sense genomic RNA (gRNA) with eight major open reading frames (ORFs) (6–9; Fig. 1A). The 5′ gene cluster (ORFs 0–2) is highly divergent among luteoviruses and is separated from the 3′-proximal group of relatively conserved genes (ORFs 3–7) by an untranslated region of ~200 nt located near the centre of the genome. The 3′ genes are expressed through the transcription of at least two sgRNAs, sgRNA1 (9,10) and sgRNA2 (11), and their translation involves non-canonical strategies (12) such as internal initiation and amber stop codon suppression. Both sgRNAs function as polycistronic mRNAs, and this also applies to PLRV gRNA: expression of the 5′ located genes P0 (ORF0) and P1 (ORF1) occurs from gRNA by initiation at their respective AUG codons. A –1 ribosomal frameshift within the 5′ end of the 582 nt overlap between P1 and P2 allows for the translation of P2 as a P1/P2 transframe protein (13,14). Functions attributed to these gene products are based on several lines of evidence, such as the development of disease symptoms in transgenic plants expressing P0 (15; D.Prüfer, unpublished), protein sequencing of the VPg N-terminus (5), or the presence of motifs characteristic of helicases (P1 and P2; 16), proteases (P1; 17), and RNA-dependent RNA polymerases (P2; 18).

As deduced from the putative functions of the various proteins, P1 apparently plays an important role in the replication of PLRV RNA. It is not only translated in at least two forms (P1, P1/P2 transframe protein), but it additionally must serve as a precursor for the generation of VPg (and possibly other proteolytic cleavage products of yet unknown function). In this study, we demonstrate by immunological analyses that P1 is in fact proteolytically

*To whom correspondence should be addressed. Tel: +49 221 5062 423; Fax: +49 221 5062 413; Email: pruefer@mpiz-koeln.mpg.de

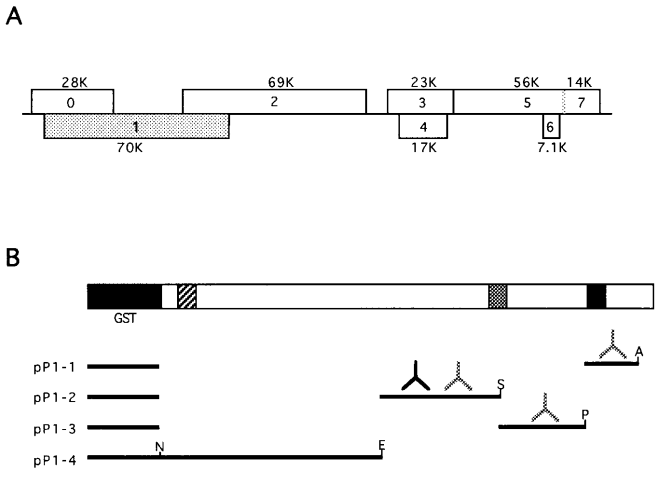

Figure 1. Schematic diagram of the PLRV genome and of P1 derivatives used in the production of antibodies. (**A**) Representation of PLRV gRNA. Rectangles represent ORFs. The position of P1 (ORF1) is indicated by shading and occurs in a different reading frame as compared with P0 (ORF0) and P2 (ORF2). (**B**) Deletion mutants of P1 expressed as pGEX fusion proteins used to prepare monoclonal and polyclonal antibodies. Termini of the fusion proteins are indicated by restriction sites within the coding sequence (A) *Alu*I (coordinate 2160; 8), (P) *Pst*I (coordinate 1950; 8), (S) *Swa*I (coordinate 1597; 8), (E) *Eco*RV (coordinate 1165; 8), and (N) *Nde*I (coordinate 326; 8). Polyclonal antiserum was raised to antigen labelled with a black inverted Y and a grey Y represents mAbs. For pP1-4, monoclonal antibody production was unsuccessful. P1 domains are indicated as follows: hydrophobic domain, hatched rectangle; VPg, grey rectangle; nucleic acid-binding domain, black rectangle (see also Fig. 5).

processed and that one of the products (P1-C25) accumulates to readily detectable amounts in PLRV-infected plants. P1-C25 originates from the P1 C-terminus, it is localized in membranes and cytoplasmic fractions and exhibits nucleic acid-binding activity. A possible function of P1-C25 during VPg maturation will be discussed.

MATERIALS AND METHODS

Preparation of monoclonal antibodies (mAbs)

Immunization, myeloma cells and culture media. GST fusion proteins were prepared by cloning deletion derivatives of the ORF1 coding sequence into the *Sma*I restriction site of the pGEX vector (19) as described in Figure 1B and purified from bacterial extracts by affinity chromatography on glutathione–Sepharose columns (Pharmacia). For the initial immunization, 100 µg of GST fusion proteins were emulsified 1:1 (v/v) with complete Freund's adjuvants (Gibco BRL, Eggenstein, Germany). One half each of this emulsion was applied to Balb/c mice by intraperitoneal and intramuscular injection, respectively. At 3 and 6 weeks after the first immunization, booster immunizations were administered using incomplete Freund's adjuvants. Three days before removing the spleen for cell fusion, an intravenous boost was given with 50 µg GST–ORF1 fusion proteins in 50 µl of physiological saline. The serum titer was determined by enzyme-linked immunosorbent assays (ELISA; 20) in microtitre plates (Dynatech M 129B) as described by Clark and Adams (21) using affinity-purified alkaline phosphatase (AP)-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) and a p-nitrophenyl-phosphate substrate (PNPP; Sigma, Deisenhofen, Germany). Only mice showing a GST–ORF1 fusion protein-specific antibody titer higher than 1:250 000 were used for the generation of hybridoma lines.

P3-X63-Ag8.653 myeloma cells (22) were grown in RPMI 1640 (Cytogene, Berlin, Germany) containing 15% fetal calf serum (Cytogene), 25 mM $NaHC0_3$, 1 mM L-glutamine, 50 µM 2-mercaptoethanol, 24 mM sodium bicarbonate, 50 IU penicillin and 50 µg streptomycin/ml (Gibco BRL). For selection of hybridoma cells, 100 µM hypoxanthine, 10 µM aminopterine and 16 µM thymidine (Sigma) were added to complete RPMI medium (HAT medium). After two limiting dilution cloning steps in 96-well microculture plates (Falcon), the cells were grown in suspension mass cultures at 37°C in a humidified incubator at 5% CO_2.

Production and purification of mAbs. The fusion of myeloma and spleen cells was carried out according to Westerwoudt (23). For *in vitro* production of mAbs, hybridoma cells were cultured in ten 250 ml tissue culture flasks each. The presence of ORF1-specific mAbs was determined by ELISA, and affinity-purified GST was used to identify and eliminate GST-specific mAbs during screening. Determination of mAb reactivity, affinity, isotypes and specificity was performed as described (24). MAbs from hybridoma culture supernatants were concentrated by affinity chromatography on Prosep-A HC (Bioprocessing, Consett, UK). The purity of the mAb preparation was determined by SDS–PAGE (25).

Polyclonal antiserum. Polyclonal antiserum was prepared by emulsifying 1:1 (v:v) pP1-2 fusion protein with complete Freund's adjuvant and introduced into chickens by intramuscular injection. At 3 and 6 weeks after the primary immunization, booster injections were administered using incomplete Freund's adjuvant. Serum was extracted from eggs and titred as described for mAbs used in this study.

Immunological examination of PLRV P1 and proteolytic products *in planta*

Total protein was isolated under denaturing conditions from the leaves of healthy and systemically PLRV-infected *Solanum tuberosum* and *Physalis floridana* plants according to Baunoch *et al.* (26). Aliquots of 10 µg were separated by 10% SDS–PAGE and electroblotted to nitrocellulose membranes as described by Niesbach-Klösgen *et al.* (27). Membranes were blocked overnight at 4°C in 1× phosphate buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) containing 5% skimmed milk powder and 0.5% Tween 20. Immunodetection experiments were carried out according to established protocols (27).

To study the association of P1-C25 with subcellular structures, plant extracts were isolated under non-denaturating conditions and fractionated on discontinuous sucrose gradients as described by Niesbach-Klösgen *et al.* (27).

Purification and examination of PLRV

Isolation of PLRV virus particles from *S.tuberosum* was performed as described by D'Arcy *et al.* (28) with the following modifications. Cell walls were digested with 1% (w/v) cellulase (Sigma) and 8% (w/v) macerozyme (Serva) for 12 h prior to adding 2-mercapto-ethanol to a final concentration of 100 mM. Virus collected from rate zonal centrifugation on sucrose gradients was further purified by $CsSO_4$ density gradient centrifugation and quantified by absorption at 260 nm and ELISA assay. Aliquots of 1 µg of

purified PLRV and phenol/chloroform extracted viral RNA were treated with ribonuclease A and separated on 18% SDS–polyacrylamide gels.

Nucleic acid-binding assays

For *in vitro* nucleic acid-binding studies, bacterial extracts expressing the P1 (ORF1) deletion derivatives described in Figure 1B were separated by PAGE and blotted onto nitrocellulose membranes. Filter binding experiments were performed as described by Gramstat *et al*. (29) using a ^{32}P-labelled PLRV probe obtained by *in vitro* transcription (30).

RESULTS AND DISCUSSION

Immunological analysis of the PLRV P1 protein *in planta*

A set of mono- and polyclonal antibodies directed against different domains of PLRV P1 (Fig. 1B) were raised and their activity characterized by ELISA and western blot analysis. Generation of antibodies against pP1-4 has not been successful to date, while mono- (pP1-1, pP1-2, pP1-3) and polyclonal (pP1-2*) antibodies showed a strong and significant reactivity to the corresponding antigens.

The results of immunodetection experiments with protein extracts from healthy and PLRV-infected *P.floridana* plants are shown in Figure 2. Only mAbs directed to the C-terminus of P1 (pP1-1, pP1-3; lanes b and d) detected two immunoreactive proteins that were absent in the healthy control (lane a). The larger protein corresponded in size to P1, while the smaller product displayed a molecular weight of ~25 kDa (P1-C25). MAbs raised against epitopes within the central domain of P1 (pP1-2) and reacting with the bacterially expressed P1 fusion proteins, were unable to detect the denatured P1 and P1-C25 proteins (Fig. 2, lane c) from PLRV-infected plants. To further verify the *in planta* presence of P1 and define the location of the P1-C25 N-terminus, a polyclonal antiserum was prepared against pP1-2. As shown in Figure 2 (lanes e and f) the polyclonal antibody detected P1, but not P1-C25 confirming the origin of P1-C25 from the ORF1 C-terminus. *In vitro* cell-free translation of PLRV gRNA resulted in P1 as the only immunoreactive product (L.Kawchuk, unpublished), and additional host and/or viral factors may be required for P1 processing.

By direct VPg sequencing, van der Wilk and co-workers (5) localized the PLRV VPg within the P1 coding sequence and postulated its release by P1 proteolytic cleavage. As deduced from the apparent molecular weight of P1-C25, the N-termini of P1-C25 and the VPg are located at approximately comparable positions within the P1 protein, indicating that P1-C25 may function as a possible precursor during VPg maturation or represent a product following VPg cleavage. The pP1-2 fusion protein (Fig. 1B) contains the entire VPg sequence as determined by van der Wilk *et al*. (5), but polyclonal antibodies directed against pP1-2 did not react with P1-C25, suggesting that VPg is either not present on P1-C25 or that it is not an efficient epitope in the fusion protein pP1-2. Only the availability of antibodies directed against the sequenced PLRV VPg will allow us to distinguish between these two possibilities. With poliovirus, the VPg is stable in the infected cell and cell extracts, as long as it is part of the P3-9 precursor (2), whereas it is rapidly degraded in its free form. Therefore, it has been suggested that P3-9 may protect the VPg from cellular proteases through protein folding (31,32), and P1-C25 may serve the same purpose for PLRV VPg.

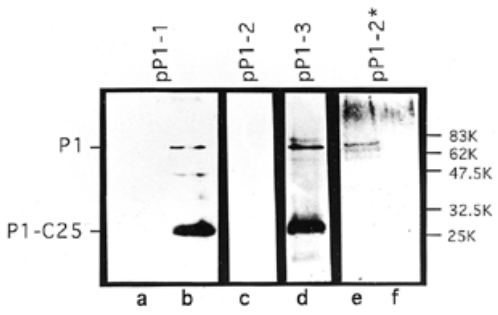

Figure 2. Immunological analyses of P1 and its derivatives *in planta*. Detection of P1 and P1-C25 in total protein extracts from PLRV-infected *P.floridana* plants using monoclonal (lane b, pP1-1; lane c, pP1-2; and lane d, pP1-3) and polyclonal (pP1-2*) antibodies (lane e). Similar proteins were not detected in extracts from healthy *P.floridana* plants (lanes a and f).

Immunological analysis of the PLRV P1 proteins in subcellular fractions

In addition to the mature poliovirus VPg—either bound to virion RNA or in its free form (33)—six precursor proteins containing the VPg amino acid sequence were detected in poliovirus-infected cells (34). Only one of these, a 12 kDa protein, was found in membrane structures of infected cells where picornaviral replication is known to occur (2). Thus in an effort to study the subcellular localisation of PLRV P1 and P1-C25, immunological studies were performed with isolated fractions of nucleus/chloroplast, membranes/mitochondria, cytoplasmic proteins and cell wall proteins. As depicted in Figure 3, the P1-C25 protein was detected in the P30 (membrane), S30 (cytoplasmatic) and CW (cell wall) fractions. In addition, minor immunoreactive proteins of ~18 (P30) and ~6 kDa (S30) were detected which were also present in total protein extracts from PLRV-infected plants after incubation for 30 min at room temperature prior to loading on a gel. The high amounts of P1-C25 associated with membrane structures is surprising in view of the highly hydrophilic character of the P1-C25 protein. In contrast, P1 contains the hydrophobic sequence SIEAFCLILLGCITSLI (P1 amino acids 152–168) at its N-terminus which is characteristic for membrane-associated proteins. Thus P1 may associate via this domain with cellular membranes, and in this complex P1-C25 may be produced by proteolytic cleavage and remain associated with this complex by virtue of its nucleic acid-binding capacity (see below).

While monoclonal and/or polyclonal antibodies detected P1 in total protein extracts of PLRV-infected plants (Fig. 2), it was not detectable in subcellular fractions (Fig. 3; data not shown). Since these fractions were prepared under native conditions, it is possible that P1 is rapidly degraded and may, therefore, be only transiently stable as is often observed with autocatalytic polypeptides as in the case of the picornaviruses VPg precursors (2). Also, western analysis of purified PLRV particles with mAbs to the C-terminus of P1 did not detect P1 or P1-C25, indicating that they are not associated with the virion (data not shown).

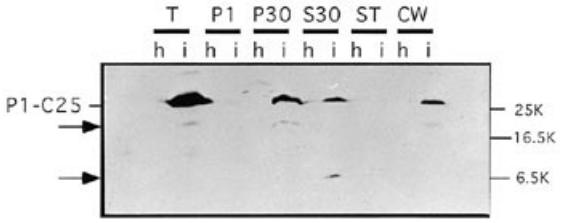

Figure 3. Subcellular localization of P1 and proteolytic P1 products. A western blot was performed with protein extracts from healthy (h) and PLRV-infected *P.floridana* plants (i) and assayed with a mixture of mAbs prepared against pP1-1 and pP1-3. P1-C25 is associated with the membrane fraction (P30), soluble fraction (S30) and cell wall fraction (CW). Immunoreactive proteins of ~18 and 6 kDa, respectively, are indicated by arrows.

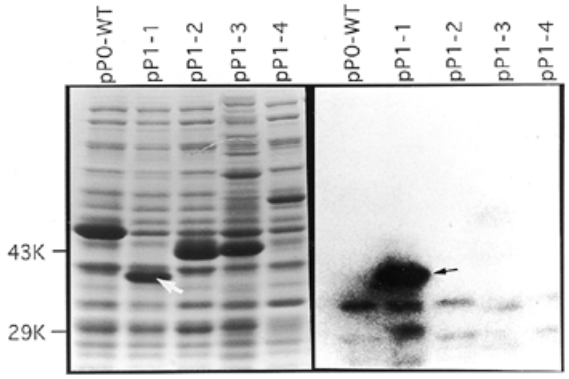

Figure 4. Nucleic acid-binding activity of P1 derivatives. Total protein extracts from bacterial cultures expressing the respective proteins were separated by PAGE on 10% SDS-containing gels and stained with Coomassie Blue (left panel). After electroblotting to nitrocellulose, the membrane was incubated with a ^{32}P-labelled PLRV sense RNA. Binding of RNA to pP1-1 protein (indicated by arrows) was visualized by autoradiography.

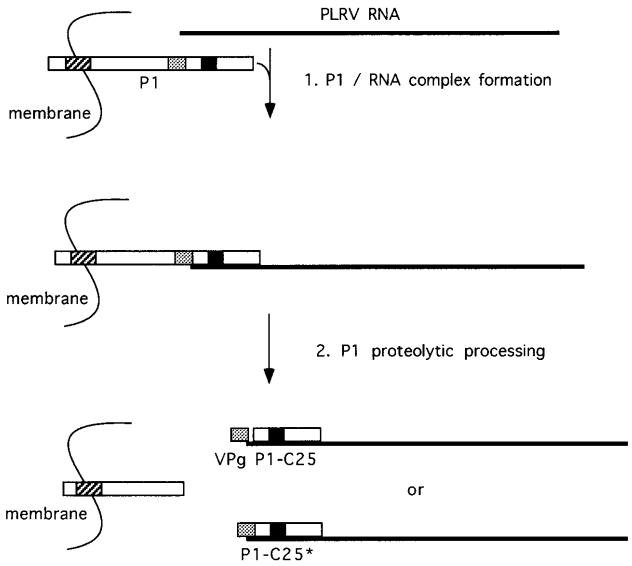

Figure 5. Schematic model of P1 involvement in VPg maturation. A hydrophobic sequence located at the N-terminus of P1 targets the protein to cellular membranes, while a hydrophilic basic nucleic acid-binding domain, located towards the C-terminus, binds to PLRV RNA. This membrane-bound complex serves as a site for VPg maturation with proteolytic processing occurring to release either both P1-C25 and VPg with concomitant covalent VPg linkage to the 5′ end of PLRV RNA or VPg as part of P1-C25 (P1-C25*). See legend to Figure 1 for explanation of domains.

Characterization of nucleic acid-binding polypeptides derived from the P1 (ORF1) protein

Proteins of PLRV displaying nucleic acid-binding capacity such as P2 (ORF2; 13), P4 (ORF4; 35), and P7 (ORF7; 11) contain a cluster of basic amino acids. The basic motif identified for example in the ORF2 frame of the ORF1/2 transframe protein is responsible for its RNA-binding activity and could possibly represent the RNA template-binding site of the PLRV replicase (13). Computer analyses of P1 identified a similar stretch of basic amino acid residues (KxKxKKRxRRxxRxK) in the P1 C-terminus (coordinates 1848–1892 in the PLRV-G isolate; D.Prüfer, unpublished). This domain is not part of the VPg coding region or P1/2 (ORF1/2) transframe protein, but nested within the 25 kDa P1-C25 protein.

To test whether the VPg coding region as well as the C-terminal basic domain exhibit the capacity to bind to PLRV RNA, the ORF1 deletion derivatives used for the antibody production (Fig. 1B) were subjected to north-western analysis. After separation of bacterial protein extracts by PAGE (Fig. 4A) and electroblotting to nitrocellulose membranes, RNA-binding experiments were performed with radiolabeled single-stranded positive PLRV RNA as described previously (13). Only the fusion protein containing the basic C-terminus (pP1-1; Fig. 4B) exhibited the capacity to bind to PLRV RNA. In a control experiment the ORF0 protein did not display this capacity, as the GST/ORF0 fusion protein (pP0-WT; Fig. 4B) failed to show any binding activity.

This strongly basic region KxKxKKRxRRxxRxK present in P1 and P1-C25 is similar to the cluster of 8–10 amino acids high in lysine and arginine that are present in other nucleic acid-binding proteins (36). Viral proteins with nucleic acid-binding capabilities are often involved in replication and this would suggest a role for P1 and P1-C25 in PLRV multiplication. In fact, for the luteoviruses beet western yellows virus and barley yellow dwarf virus, the luteoviral sequence spanning P1 and P2 has been identified as sufficient for replication in protoplasts (37,38). Furthermore, earlier studies (13) have shown that the PLRV transframe protein P1/2 as part of the PLRV replicase complex contains both the GDD motif for polymerases as well as a basic nucleic acid-binding domain which resides on the P2 part. Thus P1/2 and P1 (as well as P1-C25) have sequence-unrelated binding domains and probably bind to different PLRV RNA sequences.

By analogy to the sequence of events described for picornaviruses VPg maturation, a model is proposed which involves PLRV P1-C25 and its nucleic acid-binding domain in VPg maturation (Fig. 5). Upon P1 translation from gRNA, the hydrophobic N-proximal P1 domain SIEAFCLILLGCITSLI targets P1 to membrane structures. The basic, hydrophilic C-terminus of P1 binds to PLRV RNA via its nucleic acid-binding domain such that the VPg sequence and the 5′ end of PLRV RNA are juxtaposed. In this membrane-bound complex, proteolytic cleavage of P1 occurs to generate either P1-C25* (containing the VPg) or P1-C25 plus proteolytically released VPg with concomitant covalent linkage of VPg to PLRV

RNA. In both cases, P1-C25 (or P1-C25*) would remain adjacent to VPg at the 5′ end and may play a protective role to prevent VPg proteolysis.

SUMMARY

We have shown by immunological analyses with a set of P1-specific mono- and polyclonal antibodies the presence of this 70 kDa protein in total extracts of PLRV-infected plants. In addition, a 25 kDa protein (P1-C25) that represents the C-terminus of P1 and exhibits nucleic acid-binding capacity, reacts with corresponding antibodies. As deduced from western and computer analyses, the P1-C25 N-terminus either contains the previously identified PLRV VPg or is located in its proximity. A possible role of P1-C25 during VPg maturation is discussed.

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