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The *Lettuce infectious yellows virus* (LIYV)-encoded P26 is associated with plasmalemma deposits within LIYV-infected cells

V. Medina[a], M.R. Sudarshana[b,1], T. Tian[b], K.S. Ralston[b,2], H.-H. Yeh[b,3], B.W. Falk[b,*]

[a]*Department de Producció Vegetal Ciència Forestal. Universitat de Lleida (UdL), Avda. A. Rovira Roure 177, 25198 Lleida, Spain*
[b]*Department of Plant Pathology, University of California, Davis, One Shields Avenue, Davis, CA 95616-8500, USA*

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Abstract

Cytological, immunological, and mutagenesis approaches were used to identify the viral factors associated with the formation of plasmalemma deposits (PLDs) in whole plants and protoplasts infected by *Lettuce infectious yellows virus* (LIYV). Transmission electron microscopy and immunogold labeling using polyclonal antibodies to four of the five LIYV RNA 2-encoded large proteins, capsid protein (CP), minor capsid protein (CPm), HSP70 homolog (HSP70h), and P59, showed specific labeling of LIYV virions or virion aggregates around the vesiculated membranous inclusions, but not PLDs in LIYV-infected *Nicotiana benthamiana*, *Nicotiana clevelandii*, *Lactuca sativa*, and *Chenopodium murale* plants, and *Nicotiana tabacum* protoplasts. In contrast, antibodies to the RNA 2-encoded P26 showed specific labeling of PLDs but not virions in both LIYV-infected plants and protoplasts. Virion-like particles (VLPs) were seen in protoplasts infected by all LIYV RNA 2 mutants except for the CP (major capsid protein) mutant. PLDs were more difficult to find in protoplasts, but were seen in protoplasts infected by the CP and CPm mutants, but not in protoplasts infected by the P26, HSP70h, or P59 mutants. Interestingly, although the CPm mutant showed VLPs and PLDs, the PLDs did not show associated virions/virion-like particles as was always observed for PLDs seen in protoplasts infected by wild-type LIYV. Immunoblot analyses performed on purified LIYV virions showed that P26 was not detected with purified virions, but was detected in the cell wall, 1000 g and 30,000 g pellet fractions of LIYV-infected plants. These data suggest that P26 is associated with the LIYV-induced PLDs, and in contrast to the other RNA 2-encoded large proteins, P26 is not a virion protein.
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Keywords: *Crinivirus*; Plasmodesmata; Immunogold labeling

Introduction

Lettuce infectious yellows virus (LIYV) is the type member of the genus *Crinivirus*, family *Closteroviridae* (Martelli et al., 2002). LIYV has a bipartite ssRNA genome, and is transmitted from plant-to-plant by the sweet potato whitefly, *Bemisia tabaci* Genn. (Duffus et al., 1986; Karasev, 2000; Klaassen et al., 1995; Martelli et al., 2002). LIYV RNA 1 (8,117 nucleotides) contains 3 ORFs (ORFs 1a, ORF 1b and ORF 2) and alone is competent for replication in protoplasts (Klaassen et al., 1995, 1996; Medina et al., 1998; Yeh et al., 2000). The LIYV ORF 1a protein contains motifs for a papain-like protease, a methyl transferase and helicase. ORF 1b overlaps ORF 1a and the encoded protein contains motifs characteristic for a putative RNA-dependent RNA polymerase. The LIYV RNA 1 ORF 2 encodes a protein, P34, which appears to be important for efficient replication in trans of LIYV RNA 2, and LIYV RNA 2 defective RNAs (D RNAs; Fang, 2003; Rubio et al., 2000; Yeh et al., 2000). LIYV RNA 2 contains seven ORFs, but none appear to encode for proteins that facilitate RNA replication (Klaassen et al., 1995, 1996; Yeh et al., 2000). However, RNA 2 contains the five-gene module closterovirus hallmark gene array common to all viruses of the family *Closteroviridae*

* Corresponding author. Fax: +1 530 752 5674.
E-mail address: bwfalk@ucdavis.edu (B.W. Falk).
[1] Current address: Western Institute of Food Safety and Security. One Shields Ave., University of California, Davis, CA 95616.
[2] Current address: Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095, USA.
[3] Current address: Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 106, Taiwan.

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(Dolja et al., 1994a, 1994b; Martelli et al., 2002). For LIYV, and the related closteroviruses *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV), the genes within the hallmark closterovirus gene array have been shown to encode for proteins of various functions, but at least the four largest are virion proteins, or at least associated with efficient virion assembly (Alzhanova et al., 2001; Dolja, 2003; Napuli et al., 2003; Peremyslov et al., 2004a, 2004b; Satyanarayana et al., 2000, 2004; Tian et al., 1999). The LIYV RNA 2 3′ terminal ORF encodes a putative protein of MW 26,000 (P26; Klaassen et al., 1995), but its function and whether or not it also is a virion-associated protein are unknown. The LIYV-encoded P26 shows no significant similarity to other proteins in the existing databases, but similarly-positioned ORFs encoding proteins of similar size are found on the analogous genomic RNAs of other criniviruses (Aguilar et al., 2003; Hartono et al., 2003; Kreuze et al., 2002).

LIYV-infections show cytopathological effects similar to those seen for other viruses of the family *Closteroviridae* including scattered and aggregates of virions, and typical membranous vesicular inclusion bodies (*Beet yellows virus*-(BYV) type IBs) (Medina et al., 2003). However, LIYV infections also show a so far unique cytopathic effect: the conical plasmalemma deposits (PLDs) (Medina et al., 1998). The PLDs are usually found near plasmodesmata between companion cells and sieve tubes, and have associated large numbers of LIYV virions, often appearing to be oriented almost perpendicular to the plasmalemma (Hoefert et al., 1988; Medina et al., 2003; Pinto et al., 1988). Co-infection of protoplasts with both LIYV genomic RNAs also results in all of these cytopathological structures, but infection by RNA 1 alone only induces formation of vesiculated membranous inclusion bodies (Medina et al., 1998). As PLDs are formed in protoplasts only upon infection with both LIYV genomic RNAs, it seems likely that some LIYV RNA 2-encoded proteins might be associated with the LIYV-specific PLDs, or responsible for their formation.

We used transmission electron microscopy (TEM) and immunogold labeling analyses, subcellular fractionation, immunoblot analysis and mutagenesis to identify and localize specific LIYV-encoded proteins within LIYV-infected plants and protoplasts. Here, we show that antibodies to the LIYV RNA 2-encoded P26 specifically label the PLDs in LIYV-infected plants and protoplasts. Furthermore, P26 is not a virion protein, but is associated with the cell wall, 1000 g and 30,000 g subcellular fractions of LIYV-infected plants.

Results

LIYV-infected plants and protoplasts both show several types of specific cytopathological structures including vesiculated membranous inclusion bodies, virions or virion-like particles (VLPs) scattered and in large aggregated masses, and PLDs (Hoefert et al., 1988; Medina et al., 1998, 2003; Pinto et al., 1988). Therefore, we used both plants and protoplasts for our work here. When antibodies to the five large LIYV RNA 2-encoded proteins were used for immunogold labeling and TEM, specific labeling was seen with all antibodies for LIYV-infected but not healthy *N. benthamiana* plants and tobacco protoplasts. The four antisera corresponding to LIYV virion proteins (CP, CPm, HSP70h, and P59) showed labeling of masses of virions in the cytoplasm, but the labeling patterns were different depending on the antibody used. For example, virions were clearly labeled with the CP antisera (Fig. 1A). In contrast, antisera to the HSP70h, P59, and CPm did not give the same intensity or specificity of labeling, but clearly showed a greater density of gold particles associated with virion masses and sometimes with membranous vesiculated bodies (Figs. 1B, C, and D). None of these antibodies showed labeling of the PLDs. However, when we used the P26 antiserum virions were not labeled, vesiculated membranous bodies were only poorly labeled, but PLDs were specifically and heavily labeled (Fig. 1E). Examination of LIYV-infected leaves of *N. clevelandii*, *L. sativa*, and *C. murale*, and of

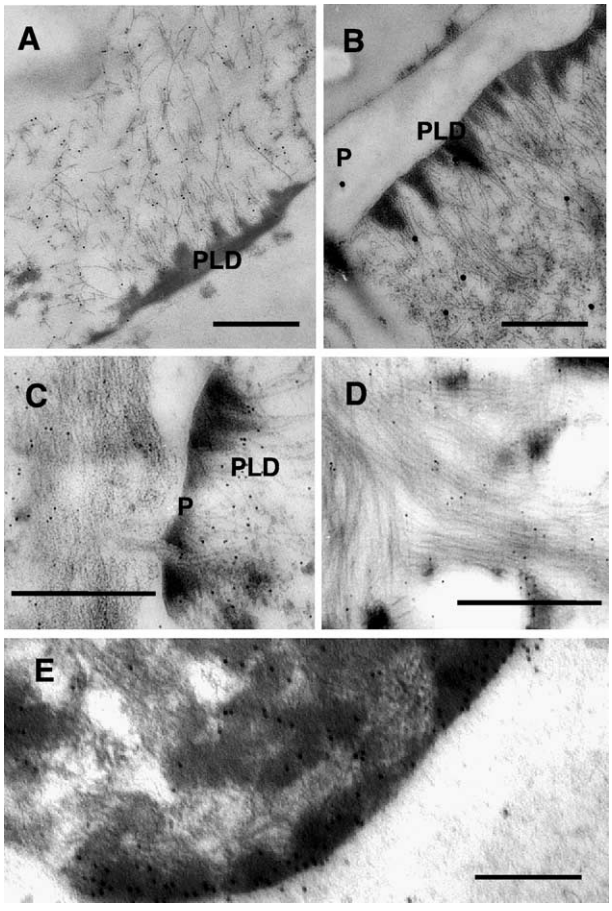

Fig. 1. Immunogold labeling of LIYV-infected *N. benthamiana* plants using antisera against the LIYV CP (A), HSP70h (B), CPm (C) P59 (D), and P26 (E). Dark conical structures in sections are plasmalemma deposits (PLD) and LIYV virions appear as filamentous particles. The plasmodesma in C is noted with a *P*. Scale bars: A = 0.58 μm; B = 0.48 μm; C = 0.79 μm; D = 0.91 μm; E = 0.25 μm. Gold particle size. A, B = 30 nm; C and D = 15 nm; E = 10 nm.

tobacco protoplasts also showed conical PLDs heavily labeled with P26 antiserum (Figs. 2A–E). High magnification analyses showed that PLDs always had associated LIYV virions which appeared to be oriented almost perpendicular to the plasmalemma (see Fig. 2E). PLDs in LIYV-infected *N. clevelandii* plants often were particularly large and numerous (Fig. 2A).

We next used replication competent, specific RNA 2 gene knock-out mutants (Yeh et al., 2000) in order to eliminate production of individual RNA 2-encoded large proteins and to assess resulting effects on LIYV virion and PLD formation/incidence in infected protoplasts. Transcripts of the corresponding mutants were inoculated to protoplasts and the resulting infected cells were examined for LIYV virions and PLDs. Because replication of RNA 1 induces formation of the large, BYV-type vesiculated membranous inclusion bodies (Medina et al., 1998), it was possible to easily identify protoplasts supporting LIYV RNA 1 replication, and these were then examined further for LIYV virions and/or PLDs. These analyses showed that many protoplasts supported RNA 1 replication, but evidence for RNA 2 replication (virions or PLDs) was not always detected in the same cells. Even for protoplasts inoculated using wild-type LIYV virion RNAs, vesiculated membranous inclusion bodies were readily seen, but virions were not always detected in the same cell sections (Table 1). For example, when LIYV virion RNAs were used as inocula, only 9.1% of protoplasts that showed vesiculated membranous inclusion bodies also showed virions. Similarly, when wild-type LIYV transcripts were used as inocula, 4% of protoplasts showing vesiculated membranous inclusion bodies also showed virions (Table 1). As a control when protoplasts were only inoculated with LIYV RNA 1 transcripts, vesiculated membranous inclusions were easily seen, but none showed particles resembling LIYV virions (Table 1). The above results could suggest that LIYV RNA 1 infected more cells than did LIYV RNAs 1 plus 2, or that RNA 1-induced structures (the vesiculated membranous inclusions) were more common and likely to be detected by TEM in a given section through an LIYV-infected cell than were viral products and PLDs associated with LIYV RNA 2 replication. In any case, these results suggested that the presence of the RNA 1-induced vesiculated membranous inclusion bodies, but lack of any RNA 2-associated structures (virions or PLDs), must be carefully interpreted.

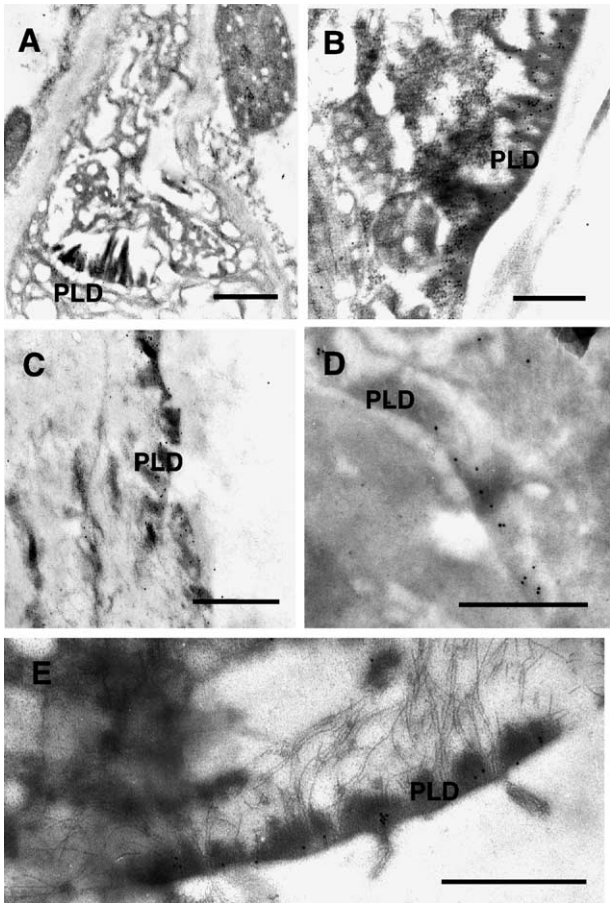

Fig. 2. Immunogold labeling with antisera against the LIYV-encoded P26 in different LIYV-infected plant hosts and protoplasts. A and B are *N. clevelandii*; C is *L. sativa*; D is *C. murale*; and E is a *N. tabacum* protoplast. PLD denotes plasmalemma deposits. Scale bars represent A = 1.16 μm; B, C, D and E = 0.58 μm. Gold particle size = 15 nm.

When protoplasts inoculated with the specific LIYV RNA 2 mutants were examined, typical RNA 1-induced vesiculated membranous inclusion bodies were seen as for wild-type infections. Protoplasts inoculated with LIYV wild-type RNA 1 plus each of the LIYV RNA 2 mutants also showed at least one of the RNA 2-associated cytopathological structures. Because of the low percentage of LIYV-infected protoplasts observed by us, and because immunogold labeling required a separate fixation process and cold embedding which resulted in poor membrane integrity thereby making it more difficult to identify the vesiculated membranous inclusion bodies, protoplasts inoculated with the LIYV RNA 2 mutants were prepared by conventional fixation and thus were not analyzed with specific antibodies. Therefore, particles that appeared indistinguishable from those confirmed to be LIYV virions in previous analyses are referred to here as virion-like particles (VLPs). VLPs were seen in protoplasts inoculated using all LIYV RNA 2 mutants except for the CP-mutant (Fig. 3 and Table 1). Also, PLDs were seen in cells infected by the CP- and CPm-mutants, but not in cells infected by P26-, HSP70h-, or the P59-mutants (not shown). As noted above, because so few cells showed LIYV RNA 2-associated inclusion bodies, lack of detection of specific structures was not considered as significant as was their presence. Still, two observations here are of interest. One is that for the P26-mutant, we examined more RNA 1-infected protoplasts (86) than for any other construct and four of these also contained VLPs but none contained PLDs. Second is that in all cells examined for all constructs, whenever PLDs and VLPs were seen in the same cell, except for one instance, the PLDs always had VLPs associated with them and oriented ~perpendicular to the

Table 1
Detection of LIYV-specific vesiculated, membranous inclusion bodies (IBs), virions or virus-like particles (VLPs), and plasmalemma deposits (PDs) in protoplasts inoculated with wild-type (WT) LIYV and specific RNA 2 mutants

Sample	IBs	VLPs	PDs
Virion RNA	76	7 (9.2%)	6 (7.9%)
WT transcripts	47	2 (4.2%)	2 (4.2%)
CP-	30	0 (0%)	1 (3.3%)
CPm-	27	2 (7.4%)	1 (3.7%)
HSP70h-	69	1 (1.4%)	0 (0%)
P59-	41	1 (2.4%)	0 (0%)
P26-[a]	86	4 (4.6%)	0 (0%)
RNA 1	23	0 (0%)	0 (0%)

Others include only protoplasts from cultured cells.
[a] These include protoplasts made from cultured *N. tabacum* cells (lacking chloroplasts), and mesophyll protoplasts from *N. benthamiana* leaves.

plasmalemma. The exception here was cells infected by the CPm-mutant where VLPs and PLDs were seen in the same cell, but PLDs did not have associated VLPs.

Because PLDs were readily and consistently observed in all LIYV-infected plants tested here, and at least in wild-type LIYV-infected protoplasts, and because they showed good labeling with the P26 antibody, this suggested that P26 was relatively abundant in LIYV-infected cells. Furthermore, because PLDs and P26 labeling were distinctly associated with the cell periphery, but not with virion aggregates in the cytoplasm, it seemed likely that P26 is not a virion structural protein. Therefore, we fractionated extracts from healthy and LIYV-infected *C. murale* plants and used immunoblotting to compare specific subcellular fractions and LIYV virions for P26 and the four LIYV virion proteins. Subcellular fractions included the cell wall (CW), membrane (1000 g and 30,000 g) and soluble fractions (30,000 g supernatant; Donald et al., 1993) and were analyzed for specific LIYV proteins using antibodies to five of the seven LIYV RNA 2-encoded proteins (CP, CPm, HSP70h, P59, and P26). As expected, antibodies to the LIYV CP, CPm, P59, and HSP70h gave clear, positive reactions with purified LIYV virion proteins for proteins of the correct size (Fig. 4, lane V). In contrast, antibodies to P26 did not react with the LIYV virions. However, all antibodies used here, including those to P26, reacted with specific proteins of expected size in subcellular fractions from LIYV-infected plants, and each of these LIYV proteins was found in more than one of the fractions. The LIYV ~29 kDa CP was detected in all fractions from LIYV-infected, but not healthy plants (Fig. 4, denoted by the arrow in panel CP). Some smaller (~22 kDa), likely degradation products, were detected only in extracts from LIYV-infected and not healthy plants, particularly in the CW and 1000 g fractions (Lanes CW-I and P1-I, Fig. 4). The LIYV-encoded P26, CPm, HSP70h, and P59 proteins were detected in the CW, 1,000 g (P1) and 30,000 g pellet (P30) fractions, and the HSP70h was sometimes also detected in the 30,000 g supernatant fraction (S30-I, Fig. 4). Antibodies to P59 and

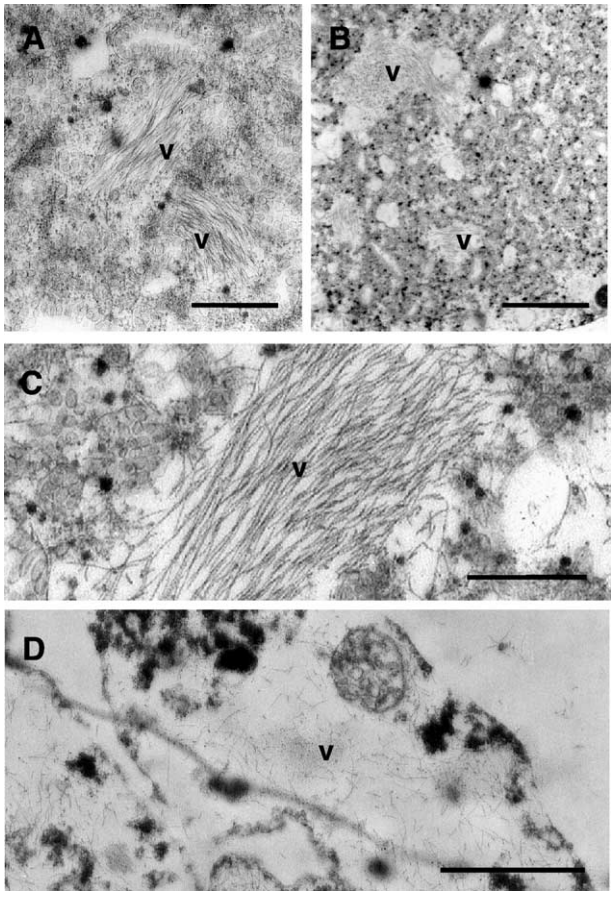

Fig. 3. Virus-like particles in *N. tabacum* protoplasts infected by specific LIYV mutants. A = HSP70h-; B = P59-; C = CPm-; D = P26-. V= virus-like particles. Scale bars in A = 0.91 μm; B = 1.81 μm; C = 0.72 μm; and D = 1.25 μm.

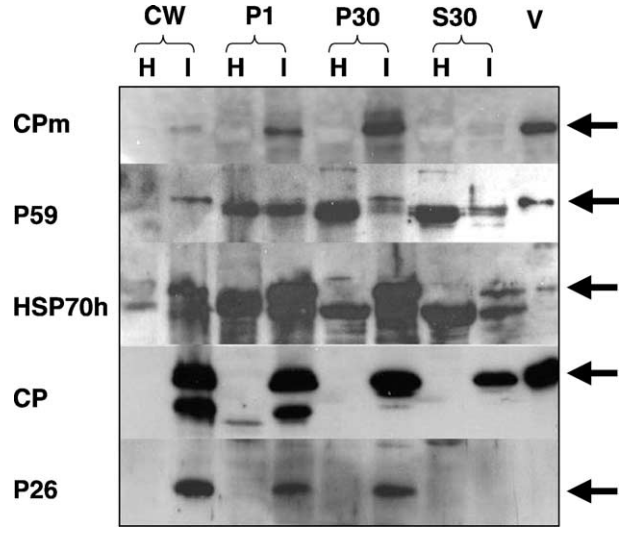

Fig. 4. Immunoblot analysis of fractionated proteins from LIYV-infected *C. murale* plants and purified virions. Lane V contains purified LIYV virions. Other lanes are subcellular fractions from healthy plants (H) and from LIYV-infected plants (L). CW represents the cell wall fraction; P1 is the 1000 g pellet fraction; P30 is the 30,000 g pellet fraction and S30 is the 30,000 g supernatant fraction. Arrows at the right indicate the positions of the respective proteins. Labels at left indicate the antiserum used to probe proteins in that panel.

HSP70h also showed reactions with proteins in healthy plant extracts making interpretation of these analyses more difficult. But by comparing the position of the respective protein in the lane containing LIYV virions (see arrow and lane V), reactions for the corresponding proteins in LIYV-infected, but not healthy plant extracts can be seen (for example, see the HSP70h-specific reaction in lanes designated I, just above the reaction with the plant protein which is present in all lanes). The P26 antibodies showed strong, specific detection of P26 in the CW, P1, and P30 subcellular fractions of LIYV-infected plants, no similar-sized proteins were detected in extracts of healthy plants (Fig. 4, panel P26).

Discussion

Viruses of the family *Closteroviridae* have the largest and most complex genomes of the ssRNA plant viruses (Karasev, 2000; Martelli et al., 2002). Their large genomes appear to encode >10 proteins, and for many of the proteins the functions are as yet unknown (Karasev, 2000; Martelli et al., 2002). In contrast to the monopartite members of the *Closteroviridae*, viruses of the genus *Crinivirus* have bipartite genomes and appear to encode proteins for replication on genome segment RNA 1, while proteins needed for other aspects of the virus life cycle are encoded by RNA 2 (Yeh et al., 2000). Recent evidence has also shown that the filamentous virions of viruses in the *Closteroviridae* are more structurally complex than are those of most filamentous plant viruses. *Beet yellows virus* (BYV), *Citrus tristeza virus* (CTV) and LIYV have morphologically polar virions with capsids composed of at least four to five virus-encoded proteins (Alzhanova et al., 2001; Dolja, 2003; Napuli et al., 2003; Peremyslov et al., 2004a; Satyanarayana et al., 2000, 2004; Tian et al., 1999). For LIYV, all of these virion proteins are encoded by four of the five RNA 2 ORFs capable of encoding large proteins (proteins >20 kDa; Tian et al., 1999).

Here, we show that the other LIYV RNA 2-encoded large protein, P26, is not a virion protein. However, P26 is abundant in LIYV-infected plants and was detected by immunoblot analysis in the cell wall, the 1000 g and 30,000 g subcellular fractions from LIYV-infected, but not healthy plants. This was reproducible from more than 10 separate experiments using plants at different times post-inoculation. Although we did not rigorously analyze the other contents of the subcellular fractions used here for immunoblotting, the cell wall, 1000 g and 30,000 g fractions can be expected to contain membrane and cell wall-associated proteins (Donald et al., 1993). It is interesting to note that the distribution of P26 in the cell wall and membrane fractions is similar to that shown by many typical plant virus-encoded proteins shown to have roles in cell-to-cell movement (Cooper and Dodds, 1995; Davies et al., 1993; Deom et al., 1990; Donald et al., 1993; Peremyslov et al., 2004b; Turina et al., 2000).

P26 was also seen by immunogold labeling and TEM to be localized with PLDs, distinct cytopathological structures, in cells of LIYV-infected plants and protoplasts. The PLDs are characteristic in their appearance and location within cells. In LIYV-infected plants, PLDs are found primarily within companion cells (sometimes in phloem parenchyma) at the cell periphery adjacent to sieve tube cells, and often plasmodesmata can be seen adjacent to the PLDs and traversing the cell wall at this location (e.g., see Fig. 1C here, and Fig 1b and 1D of Hoefert et al., 1988; Medina et al., 2003; Pinto et al., 1988). Hoefert et al. (1988) first described LIYV PLDs as a "new feature as yet unreported among other Closterovirus infections". They further analyzed PLDs and suggested them to be membrane lamellae deposits that likely functioned in intercellular trafficking of LIYV (Pinto et al., 1988). Our work here suggests that PLDs are not composed of membrane lamellae deposits as our immunogold labeling experiments used non-osmium fixed, cold-embedded cells showed that PLDs (and LIYV virions) retained their characteristic morphology (see Fig. 2), while the LIYV-induced vesiculated membranous inclusion bodies did not under these same conditions (not shown). However, the suggestion by Hoefert et al. (1988) that PLDs might have a role in intercellular trafficking of LIYV seems plausible based on data so far available. PLDs almost always showed LIYV virions associated with them and oriented nearly perpendicular to the plasmalemma and near plasmodesmata (except for those seen here in protoplasts infected with the LIYV CPm-mutant).

PLDs, or similar structures have not been observed in infected cells for other well studied viruses of the *Closteroviridae* such as BYV and CTV, or even for other viruses of the genus *Crinivirus* (Medina et al., 2003). Raine et al. (1979) did report structures resembling PLDs for *Prunus* spp. plants infected by *Little cherry virus* (LChV). LChV-1 and LChV-2 are members of the *Closteroviridae*, LChV-1 an unassigned member and LChV-2 a member of the genus *Ampelovirus* (Martelli et al., 2002). Despite the lack of PLDs associated with other criniviruses, it is interesting to note that comparison of the limited nucleotide sequence information available so far for other viruses of the genus *Crinivirus* shows that they also encode a similar-sized protein from an ORF also located at the 3′ terminus of RNA 2, but the encoded proteins do not exhibit similarity to LIYV P26 (Aguilar et al., 2003; Hartono et al., 2003; Kreuze et al., 2002). Thus, whether the LIYV-encoded P26, and the proteins encoded by similarly-positioned genes of other criniviruses have similar role(s) in their respective virus life cycles is as yet unknown.

Conical/cylindrical-shaped structures associated with the plasmalemma and even with plasmodesmata are common in plants infected by viruses of the genus *Potyvirus*. The major component of these structures is the virus-encoded CI protein (Leseman, 1988), but careful time-course analysis

for *Tobacco vein mottling virus* (TVMV)-infected plants showed that the TVMV CP and RNA also accumulate within these, presumably as virions, and suggests a role for these structures in cell-to-cell spread of TVMV (Rodríguez-Cerezo et al., 1997). Functional analyses also have shown both the CI and CP proteins to be determinants for potyvirus cell-to-cell movement (Carrington et al., 1998; Dolja et al., 1994a, 1994b; Rojas et al., 1997). Analyses for the closterovirus, BYV, have demonstrated that BYV virion and non-virion proteins are required for cell-to-cell movement (Alzhanova et al., 2001; Dolja, 2003; Peremyslov et al., 2004a, 2004b) and it seems reasonable that this could apply for other viruses of the family *Closteroviridae*. Our present study is the first step in gaining insight on the role of the LIYV-encoded P26 protein in the LIYV life cycle and LIYV-induced cytopathology.

Materials and methods

LIYV and B. tabaci maintenance

LIYV was maintained in lettuce plants (*L. sativa* L. cv. Summer Bibb) by continuous transmission using the whitefly, *B. tabaci* as described previously (Klaassen et al., 1994; Tian et al., 1999). LIYV was also transmitted to *C. murale* (L.), *N. benthamiana* (Domin.) and *N. clevelandii* (Gray) plants for specific analyses. Virions were purified from LIYV-infected plants and virion RNAs extracted as done previously (Tian et al., 1999). LIYV virion RNAs, and specific LIYV transcripts, including those for RNA 2 mutants, were also used to inoculate *N. tabacum* protoplasts as previously described (Yeh et al., 2000).

LIYV P26 antiserum production and serological analyses

The coding region for the LIYV RNA 2 ORF encoding P26 was amplified by PCR and subcloned for protein expression in *Escherichia coli*. The LIYV RNA 2 P26 ORF was amplified by PCR from the LIYV RNA 2 infectious cDNA clone, pSP6 (Klaassen et al., 1996), using oligonucleotides TTCACA*GGATCC*ATGAATAATTTTCCTGAAATT (Italicized letters indicate a *Bam*HI site) and CCCGCT*AAGCTT*TTAAATTTTTATATCATTTAA (Italicized letters indicate a *Hin*dIII site). The 3′ terminal 21 nucleotides of the primers correspond to nucleotide positions 6319–6340 (identical sequence) and 7002–6981 (complementary sequence) of LIYV RNA 2, respectively. The resulting PCR product was digested using *Bam*HI and *Hin*dIII, ligated into *Bam*HI and *Hin*dIII digested pRSET A (Invitrogen) and transformed into competent *E. coli* DH5a. Plasmids were purified and nucleotide sequence analyses were performed to confirm the orientation and sequence of the cloned DNA fragments. The pRSET-P26 plasmid was transformed into *E. coli* BL21:DE3-CodonPlus (Stratagene), and protein expression was induced by using IPTG (Tian et al., 1999). The histidine-tagged P26 was purified using Ni-agarose column, followed by SDS-PAGE and used for polyclonal antibody production as we have done previously (Tian et al., 1999).

Transmission electron microscopy (TEM) and immunogold-labeling analyses

Samples of healthy and LIYV-infected plants and protoplasts were prepared for TEM using standard and cold-embedding procedures (Medina et al., 1998). Immunogold-labeling using specific antisera to the LIYV-encoded CP, CPm, HSP70h, P59, and P26 was done as described (Medina et al., 1998; Tian et al., 1999).

Subcellular fractionation and immunological analysis

Total proteins were extracted from healthy and LIYV-infected *N. clevelandii*, *Capsella bursa-pastoris* and *C. murale* plants and fractionated by filtration and centrifugation (Donald et al., 1993). Proteins from the different subcellular fractions were separated by SDS-PAGE, transferred to nictrocellulose membranes and immunoblot analyses were performed using antisera to the LIYV CP, CPm, HSP70h, P59, and P26 as previously (Klaassen et al., 1994; Tian et al., 1999).

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