Specific inclusion bodies are associated with replication of lettuce infectious yellows virus RNAs in *Nicotiana benthamiana* protoplasts

Vicente Medina,1 Tongyan Tian,2 Jacek Wierzchos3 and Bryce W. Falk2

1 Dept de Producció Vegetal I Ciència Forestal de la Universitat de Lleida (UdL), Avda. Alcalde Rovira Roure 177, 25198 Lleida, Spain
2 Department of Plant Pathology, University of California, 1 Shields Avenue, Davis, CA 95616, USA
3 SUIC-ME de la UdL, Avda. Alcalde Rovira Roure 44, 25006 Lleida, Spain

*Nicotiana benthamiana* mesophyll protoplasts, either mock-inoculated or inoculated using *in vitro* transcripts derived from lettuce infectious yellows virus (LIYV) RNA 1- and/or RNA 2-cloned cDNAs were analysed by transmission electron microscopy (TEM) and, in some cases, also by immunogold labelling. TEM revealed the main cytopathological effects of LIYV infections in *N. benthamiana* protoplasts infected with RNAs 1 and 2: (a) typical closterovirus-induced (beet yellows virus-type) accumulations of vesiculated cytoplasmic membranes as inclusion bodies, sometimes with associated virions; (b) scattered aggregations of virions within the cytoplasm; and (c) electron-dense plasmalemma deposits. These were not seen in mock-inoculated protoplasts. Protoplasts inoculated only with LIYV RNA 1 contained vesiculated cytoplasmic inclusion bodies, but not virions or plasmalemma deposits. Thus, infection by only LIYV RNA 1 is sufficient to induce characteristic closterovirus vesiculated cytoplasmic inclusion bodies. However, both LIYV RNAs 1 and 2 are needed for production of virions and plasmalemma deposits.

Lettuce infectious yellows virus (LIYV) is the type member of the genus *Crinivirus* of the family *Closteroviridae*. The LIYV genome is bipartite, divided among two ssRNAs of 8118 and 7193 nucleotides (RNAs 1 and 2 respectively), giving a total of 15311 nucleotides (Klaassen *et al.*, 1995). On LIYV RNA 2 is the ‘hallmark closterovirus gene array’ including five genes encoding: a small hydrophobic protein of as yet unknown function (ORF 1); an HSP70 homologue (ORF 2); a protein of unknown function (p59, ORF 3); the capsid protein (CP, ORF 5); and the duplicated capsid protein (dCP, ORF 6) (Agranovsky, 1996; Dolja *et al.*, 1994; Klaassen *et al.*, 1995). Many of these genes are unique to closteroviruses (i.e. the HSP70 homologue gene and the gene encoding the dCP), and the functions of the encoded proteins and their possible roles in LIYV biology are as yet unknown.

Besides being obligately transmitted by the whitefly, *Bemisia tabaci* (Gennadius), another characteristic property exhibited by LIYV is its absolute phloem tropism within the plant host. Previous studies have shown that closterovirus infections induce characteristic inclusion bodies within phloem-associated cells, including phloem parenchyma and companion cells (Lesemann, 1988). These inclusion bodies include cytoplasmic vesiculated membranous areas within infected cells, referred to as BYV-type vesicles (beet yellows virus; Francki *et al.*, 1985), surrounded by lipid droplets. Virions are often associated with these inclusion bodies, but virions can also occur as aggregated masses composing banded inclusion bodies (Matthews, 1991). Another type of inclusion body, so far unique to LIYV, is represented by the conical plasmalemma deposits found within phloem cells and occurring in high densities near pit fields (Hoefert *et al.*, 1988; Pinto *et al.*, 1988).

The virus and/or host factors which give rise to LIYV inclusion body formation are as yet unknown. We have used infectious transcripts generated from full-length cDNA clones of LIYV RNAs 1 and 2 (Klaassen *et al.*, 1996) to infect *Nicotiana benthamiana* protoplasts in order to determine if the aforementioned inclusion bodies could form within mesophyll protoplasts. We also inoculated protoplasts with only LIYV RNA 1 in order to determine whether replication of both LIYV RNAs was necessary for inclusion body formation. Immunogold labelling and transmission electron microscopy (TEM) were used to confirm the presence of LIYV CP in specific inclusion bodies.

*N. benthamiana* mesophyll protoplasts were isolated and inoculated using transcripts derived from LIYV RNA 1, or both RNA 1 and RNA 2 cDNA clones, as previously described (Klaassen *et al.*, 1996). Protoplasts were harvested 48 or 72 h post-inoculation, collected by low speed centrifugation and...
Fig. 1. TEM analysis of LIYV RNAs 1 and 2-infected *N. benthamiana* mesophyll protoplasts prepared by standard double-fixation (A, B), or cold embedding and immunogold labelling (C, D). (A) Lower magnification image showing a portion of a cell containing the nucleus (N) and cytoplasm. Arrowheads indicate three inclusion bodies (IB) seen within the cytoplasm. P, plasmalemma. (B) High magnification of (A) showing the vesicles (VE) within the IB, and surrounding lipid droplets (LD). (C) An IB in the cytoplasm, and plasmalemma deposit (PD) in the lower left. (D) High magnification image of the PD from (C) which shows virions (V) plus gold label. Note the virions extending from the PD and in the cytoplasm, and gold label surrounding them. Bars: 4.60 µm (A); 0.73 µm (B); 2.3 µm (C); 0.91 µm (D); gold particle size, 30 nm.

processed for microscopic analysis using two methods. These were: (a) standard double-fixation using glutaraldehyde and osmium tetroxide (both in buffer phosphate 0·1 M pH 7·4) (with three 10 min washes after each fixation) as preliminary steps, a dehydrating ethanol series, embedding in LR White resin and polymerization at 60 °C for 12 h; or (b) cold
Interestingly, and despite our thorough analysis, protoplasts infected with only RNA 1 did not contain LIYV virions. However, a detailed examination showed that protoplasts readily observed within protoplasts infected with LIYV RNA inclusion bodies (Fig. 2A–D). These were abundant, and protoplasts contained the typical vesiculated cytoplasmic lication. TEM analysis showed that LIYV RNA 1-infected only with RNA 1 in order to determine if any of the above-et al (1988). Distinct and characteristic vesiculated inclusion bodies could be seen in the cytoplasm (Fig. 1A). Higher magnification analysis showed that these were the typical BYV-type vesiculated inclusion bodies, and were often surrounded by lipid droplets (Fig. 1B). In many sections, in addition to the typical BYV-type inclusion bodies, electron-dense plasmalemma deposits were also seen in protoplasts inoculated with both LIYV RNAs (Fig. 1C, D). These deposits were always associated with the plasmalemma and appeared identical to those described previously from LIYV-infected lettuce plants (Hoefert et al., 1988; Pinto et al., 1988). Long filamentous particles resembling LIYV virions were also seen scattered in the cytoplasm (Fig. 1B, C), and in many cases were seen to be associated with plasmalemma deposits (Fig. 1C, D). When cold-embedded samples were analysed by immunogold labelling for LIYV CP, abundant gold label was observed in the vicinity of the filamentous particles in the cytoplasm and at the plasmalemma deposits (Fig. 1D), providing further evidence that these particles are LIYV virions. Some background label was scattered, but the majority was concentrated in regions containing particles in the cytoplasm or at the plasmalemma deposits. Because the samples used for immunogold labelling (Fig. 1C, D) were cold-embedded to preserve antigenicity, some resolution and membrane integrity was lost. However, the characteristic features of the plasmalemma deposits were clear. No similar structures resembling the above-described inclusion bodies or LIYV virions were seen in mock-inoculated protoplasts.

Because LIYV RNA 1 alone is sufficient for replication (Klaassen et al., 1996), we also examined protoplasts inoculated only with RNA 1 in order to determine if any of the above-described inclusion bodies resulted only from RNA 1 replication. TEM analysis showed that LIYV RNA 1-infected protoplasts contained the typical vesiculated cytoplasmic inclusion bodies (Fig. 2A–D). These were abundant, and readily observed within protoplasts infected with LIYV RNA 1. However, a detailed examination showed that protoplasts infected with only RNA 1 did not contain LIYV virions. Interestingly, and despite our thorough analysis, protoplasts infected with only LIYV RNA 1 did not contain detectable plasmalemma deposits.

This work demonstrated that N. benthamiana mesophyll protoplasts can be used for studies on the cytopathology of LIYV infections, and that the three characteristic LIYV cytopathological structures, previously identified in LIYV-infected lettuce plants (virions, BYV-type inclusion bodies and plasmalemma deposits), also can be found in LIYV-infected protoplasts (Hoefert et al., 1988; Pinto et al., 1988). However, by comparing protoplasts infected with LIYV RNA 1 with those infected with LIYV RNAs 1 and 2, we found that infection by only LIYV RNA 1 is sufficient to induce formation of the typical BYV-type vesiculated inclusion bodies. These vesiculated inclusion bodies are recognized as characteristic of plant infections by closteroviruses (viruses in the family Closteroviridae) and have been identified in plants infected with aphid-, mealybug- and whitefly-transmitted closteroviruses (for a review see Lesemann, 1988). Their origins, whether derived from specific cellular organelles or generated de novo, are not yet clear but previous authors have speculated that they likely contain viral RNA, and thus have a role(s) in replication (Esau & Hoefert, 1971). Vesicular bodies of various sizes and shapes are cytopathological structures associated with infections of many different types of plant viruses, and in some cases these have been shown to contain viral dsRNAs and/or RNA-dependent RNA polymerases (Burgyan et al., 1996; Martelli et al., 1984; De Graaff & Jaspars, 1994; Lesemann, 1988). These observations and the fact that LIYV RNA 1 is alone competent for replication and primarily encodes proteins predicted to be involved in replication (Klaassen et al., 1995), further support the idea that the vesicles induced by LIYV are associated with LIYV RNA replication.

Although transcripts derived from cloned cDNAs for LIYV RNAs 1 and 2 are infectious in protoplasts, we have as yet been unable to infect whole plants using transcripts. This is likely due at least in part to the phloem-limited nature of LIYV infections in whole plants (Hoefert et al., 1988). However, as shown here, protoplasts offer a good alternative approach to studying the cytopathology of LIYV infections. In addition to the BYV-type inclusion bodies described above, we also identified LIYV virion aggregates and the LIYV-specific plasmalemma deposits, but only in protoplasts infected with both LIYV RNAs 1 and 2. As the LIYV CP gene is encoded on RNA 2 (Klaassen et al., 1995), the lack of virion formation when cells were infected with only RNA 1 is to be expected. However, the lack of plasmalemma deposits in cells infected with only LIYV RNA 1, in contrast to their presence in cells infected with both LIYV RNAs 1 and 2, is intriguing. LIYV RNA 2 contains seven ORFs, including the five-ORF hallmark closterovirus gene array (Klaassen et al., 1995; Dolja et al., 1994). Whether any of the LIYV RNA 2-encoded gene products have roles in plasmalemma deposit formation is not yet known. Also, what function(s) the plasmalemma deposits have in LIYV infections are not yet known. Hoefert et al. (1988)
Fig. 2. TEM analysis of LIYV RNA 1-infected *N. benthamiana* mesophyll protoplasts prepared by standard double-fixation. (A) Typical vesiculated closterovirus-specific inclusion body (IB) surrounded by lipid droplets (LD). (B) High magnification image of the IB in (A) which shows greater detail. (C) Distorted chloroplasts (ChP) around a typical vesiculated IB. (D) High magnification close-up image of a typical vesiculated IB from (A) showing vesicles (VE). Note that no typical LIYV virions are present. Bars: 3.63 µm (A, C); 1.81 µm (B); 0.91 µm (D).

and Pinto *et al.* (1988) first described the LIYV-induced plasmalemma deposits, and noticed their association with the plasmalemma, particularly in regions such as pit fields on the parenchyma side of plasmodesmatal connections between phloem parenchyma and sieve tube elements. They also noted that what appeared to be LIYV virions were sometimes associated with plasmalemma deposits, and that when associated with plasmalemma deposits, these particles were oriented perpendicular to the plasmalemma towards the plasmodesmata. We also observed similar particles and by immunogold labelling showed them to be LIYV virions. Interestingly, and as noted by Pinto *et al.* (1988), Raine *et al.* (1979) described similar plasmalemma deposits and oriented virus-like particles in tissues of little cherry-diseased (LChD) cherry trees. LChD has recently been shown to be caused by little cherry disease virus (LChV), and nucleotide sequence
analysis has shown that LChV is a member of the Closteroviridae and related to LIYV (Jelkmann et al., 1997). Whether plasmalemma deposits are characteristic inclusion bodies associated with plant infections caused by clostero-viruses related to LIYV (i.e., those in the genus Crinivirus) is as yet not known, but whether specific LIYV genes or gene products facilitate their development can now be addressed by using approaches such as we have employed here.

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References

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