Lettuce infectious yellows virus: \textit{in vitro} acquisition analysis using partially purified virions and the whitefly \textit{Bemisia tabaci}

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Virions of lettuce infectious yellows virus (LIYV; genus \textit{Crinivirus}) were purified from LIYV-infected plants and their protein composition was analysed by SDS–PAGE and immunoblotting. Virion preparations contained the major capsid protein (CP), but the minor capsid protein (CPm), p59 and the HSP70 homologue were also identified by immunoblot analysis. Immunogold labelling analysis showed that CP constitutes the majority of the LIYV virion capsid, but CPm was also part of the capsid and localized to one end of the virion, similar to the polar morphology seen for viruses in the genus \textit{Closterovirus}. p59 and the HSP70 homologue were not detected on virions by immunogold labelling, but were always detected in virion preparations by immunoblot analysis. Purified LIYV virions were used for \textit{in vitro} acquisition analysis with \textit{Bemisia tabaci} whiteflies and were efficiently transmitted to plants. Infectivity neutralization analyses were done using antisera to the LIYV-encoded CP, CPm, p59 and HSP70 homologue. Only antiserum to the CPm effectively neutralized LIYV transmission by \textit{B. tabaci}. These data suggest that the LIYV–\textit{B. tabaci} transmission determinants are associated with purified virions, and that the LIYV virion structural protein CPm is involved in transmission by \textit{B. tabaci}.

Introduction

Lettuce infectious yellows virus (LIYV) is the type member of the genus \textit{Crinivirus} in the family \textit{Closteroviridae} (G. P. Martelli and others; to be published in the \textit{Seventh Report of the International Committee on Taxonomy of Viruses}). The LIYV genome organization and composition are similar to other members of the family \textit{Closteroviridae} including beet yellows virus (BYV) and citrus tristeza virus (CTV) (Klaassen \textit{et al.}, 1995; Agranovsky \textit{et al.}, 1994; Karasev \textit{et al.}, 1995). However, in contrast to BYV and CTV, the LIYV genome is bipartite. LIYV RNA1 (8118 nt) encodes proteins associated with RNA replication while LIYV RNA2 (7193 nt) potentially encodes seven proteins and contains the closterovirus hallmark gene array (Klaassen \textit{et al.}, 1995, 1996). Genes in this array code for p5, a small hydrophobic protein, a heat shock protein 70 homologue (HSP70), p59 (a protein of unknown function), the LIYV major capsid protein (CP) and the minor capsid protein (CPm) (Klaassen \textit{et al.}, 1995; Dolja \textit{et al.}, 1994; Agranovsky 1996; and see Fig. 1). LIYV RNA1 is capable of replication in protoplasts in the absence of LIYV RNA2, but LIYV RNA2 replicates only when it is co-inoculated with LIYV RNA1 (Klaassen \textit{et al.}, 1996). However, LIYV virions are formed only in protoplasts inoculated with both LIYV RNA1 and RNA2 (Medina \textit{et al.}, 1998).

The virions of both BYV and CTV are composed of two capsid proteins: CP constitutes the majority of the capsid and CPm encapsidates only the terminal region of the RNA at one end of the virion (Agranovsky \textit{et al.}, 1995; Febres \textit{et al.}, 1996). Thus, the filamentous virions are morphologically polar. This type of virion structure is unique among plant viruses but the functions of the two capsid proteins, other than encapsidation of the genomic RNA, are not known. It was not known if LIYV virions, or those of other viruses in the genus \textit{Crinivirus}, have a similar polar morphology.

Like the aphid-transmitted BYV and CTV, LIYV and other viruses in the genus \textit{Crinivirus} are transmitted from plant to plant by whitfly vectors in a non-circulative, semi-persistent manner (Duffus \textit{et al.}, 1986; Nault, 1997; Manjunath, 1985). Within the plant host, infections are phloem-limited and the viruses are acquired and transmitted by their respective vectors only upon phloem feeding. Recent studies have provided important information regarding specific virus–vector interactions which facilitate the transmission of several plant viruses, including those having non-circulative non-persistent,
circulative non-propagative and circulative propagative transmission relationships (Schmidt et al., 1994; Bruyere et al., 1997; Hofer et al., 1997; Bandla et al., 1998; Wang et al., 1998; Kikkert et al., 1998). In all cases, virion capsid or membrane proteins are associated with vector-mediated virus transmission and for potyviruses and caulimoviruses, a second protein (the HC-Pro or ATF, respectively) is essential for acquisition and transmission of LIYV and other viruses in the family Closteroviridae has been the lack of a suitable in vitro acquisition system which can be used to identify virus-encoded proteins associated with virus acquisition and transmission. Herein we report the development of an in vitro acquisition system for LIYV. We also show that specific LIYV-encoded proteins are associated with LIYV virions and that specific antibodies to LIYV-encoded proteins can neutralize LIYV transmission by *Bemisia tabaci* (Genn.).

**Methods**

**LIYV and *B. tabaci* maintenance.** LIYV was maintained in lettuce plants (*Lactuca sativa* L. cv. Summer Bibb) by continuous transmission using the whitefly *B. tabaci* as described previously (Klaassen et al., 1994). *B. tabaci* was maintained on lima bean plants (*Phaseolus lunensis* (Macf.) in a growth chamber as described (Perring et al., 1993). LIYV was also transmitted to *Chenopodium murale* (L.) and *Nicotiana clevelandii* (Gray) plants for virion purification.

**LIYV virion purification.** LIYV virions were purified from 25 g of LIYV-infected *C. murale* or *N. clevelandii* plants using the protocol described by Klaassen et al. (1994). Virions were resuspended in 1 ml of TE (10 mM Tris–HCl pH 7.4, 1 mM EDTA) following differential centrifugation. In some instances, virions were further purified using cesium sulphate (Cs2SO4)–sucrose density gradients (Klaassen et al., 1994). The fractions containing LIYV virions were dialysed overnight in TE. Purified virions were stored at 4 °C in TE or used immediately for further analyses.

**Antisera to LIYV-encoded proteins.** Polyclonal antiserum to LIYV CP was prepared by immunization of a New Zealand White rabbit with bacteriophage gene 10–LIYV CP fusion protein expressed in *Escherichia coli* (Klaassen et al., 1994). Polyclonal antisera was also produced to the LIYV HSP70 homologue, p59 and CPM. The CPM and p59 coding regions were amplified by PCR using the LIYV RNA 2 full-length cDNA clone (pSP6) as the template (Klaassen et al., 1996). The upstream primer for the p59 coding region (5′ ATCGGATTCGACCTCATGGAATGACAGA 3′) contained a BamHI site (italics) and the downstream primer (5′ ACTGGATCCGAATTCGTATGATTCCATGAA 3′) contained an EcoRI site (italics). The CPM coding region upstream primer (5′ CAATCTCGAGGATCCATGGAATGACAGA 3′) and downstream primers (5′ TACGCTCGAGCTTTCCATGGAATGACAGA 3′) contained BamHI sites (italics). The amplified DNAs were digested using the corresponding restriction enzymes and cloned into pRSET A (Invitrogen). The HSP70 coding region was obtained using a similar strategy and primers (5′ GTTGGATCCGGACTCATGACAGCATCA 3′) and downstream primer (5′ TACGCTCGAGCTTTCCATGGAATGACAGA 3′) contained BamHI sites (italics). The amplified DNAs were digested using the corresponding restriction enzymes and cloned into pRSET A (Invitrogen). The corresponding histidine-tagged proteins were expressed in *E. coli* followed by protein purification by Ni–NTA column chromatography (QIAGEN) and SDS–PAGE. Purified proteins were used for immunization of New Zealand White rabbits (Tian et al., 1995).

**LIYV virion analysis.** Purified LIYV virions were analysed by SDS–PAGE (Laemmli, 1970) and immunoblotting as described previously (Klaassen et al., 1996). Immunogold labelling and transmission electron microscopy (TEM) were also used to identify virion structural proteins. Formvar/carbon-coated grids were allowed to float on drops of purified virions (in TE containing 100 µg/ml bacitracin). Grids were incubated with blocking buffer (10 mM Tris–HCL, 100 mM NaCl, 1% BSA, 0.1% Tween 20) for 10 min and transferred to drops of primary antiserum (1:200 in blocking buffer) for 1 h. Grids were then rinsed in TE containing 100 µg bacitracin/ml and placed in blocking buffer for 30 min. Grids were incubated with goat anti-rabbit antiserum conjugated with 10 nm gold (1:30 in blocking buffer) for 1 h. Grids were incubated in blocking

![Fig. 1. Upper panel, schematic representation of LIYV genomic RNAs 1 and 2 (Klaassen et al., 1995). Open boxes indicate open reading frames. P-Pro, papain-like protease; MTR, methyltransferase; HEL, RNA helicase; RDRP, RNA-dependent RNA polymerase; HSP70, HSP70 homologue; CP, major capsid protein; CPM, minor capsid protein. Lower panel, SDS–PAGE and immunoblot analysis of LIYV virions. Numbers at left indicate positions of protein standards (10−3 × M0). Panels (A)–(E) contain identical protein samples. H, proteins purified from healthy plants; V, proteins present in partially purified preparations of LIYV virions. Panel (A), proteins detected by staining with Coomassie blue; arrows indicate the ca. M0, 60,000, M1, 52,000, and M2, 28,000 proteins from top to bottom, respectively. Panels (B)–(E) are identical blots probed with polyclonal antisera to the LIYV CP (B), CPM (C), HSP70 homologue (D) and p59 (E). Arrows indicate positive serological reactions for each blot.](image-url)
Fig. 2. TEM and immunogold labelling analysis of partially purified LIYV virions. The virion in (A) was labelled using antiserum to the LIYV CP. Virions in (B) and (C) were labelled using antiserum to the LIYV CPm. The virion in (D) was labelled using pre-immune antiserum. Labelling was detected by using goat anti-rabbit antibodies conjugated with 10 nm gold particles. Bars represent 224 nm. Arrows in (B) and (C) indicate LIYV virion termini labelled using CPm antiserum. Arrow in (A) indicates a virion terminal region unlabelled with LIYV CP antiserum.

buffer for 30 min, rinsed with TE containing 100 µg/ml bacitracin and stained with 2% uranyl acetate. Grids were viewed with a Philips-400 transmission electron microscope.

In vitro acquisition assay. Approximately 100 adult B. tabaci were placed inside an acquisition cage with one side sealed with a single sheet of stretched Parafilm membrane (Duffus, 1990). After 18 h, 80–90 µl of LIYV virions (ca. 25–50 µg/ml) in 15% sucrose–TE was placed on the top of the Parafilm and covered with a second sheet of stretched Parafilm. The B. tabaci were allowed acquisition access for 6 h and transferred to lettuce plants (ca. 100 per plant) for a 24 h inoculation access period. Additional B. tabaci were given access to 15% sucrose in TE buffer as negative controls. Inoculated lettuce plants were sprayed with insecticide and kept in a greenhouse for 4–5 weeks. LIYV infections were determined by scoring symptoms and confirmed by dot blot hybridization analysis (Tian et al., 1996).

In attempts to identify LIYV-encoded proteins which may be associated with LIYV transmission by B. tabaci, antiserum neutralization experiments were done by mixing partially purified LIYV virions with dilutions of specific LIYV antisera (Duffus, 1990). In Expt 1, antisera were mixed with virion preparations and 60% sucrose to achieve a final sucrose concentration of 15% and the solution was immediately fed to B. tabaci. For subsequent experiments, antisera and virion preparations were mixed and incubated for 30 min at room temperature before adding sucrose and feeding to B. tabaci. Pre-immune sera and a polyclonal antiserum against the beet western yellows virus p19 (Tian et al., 1995) were used as controls.

Results

LIYV virion analysis

Previously, we reported that LIYV virions have a capsid protein of ca. M, 28 000 (Klaassen et al., 1994), but lesser amounts of a ca. M, 52 000 protein were also seen in these preparations. Therefore, we further analysed LIYV virions by SDS–PAGE and immunoblotting using antisera specific to four LIYV-encoded proteins in attempts to identify other proteins associated with LIYV virions. SDS–PAGE confirmed previous results and showed that the most abundant protein in LIYV virion preparations was ca. M, 28 000, the LIYV CP (Fig. 1A). However, lesser amounts of proteins of ca. M, 52 000, and M, 60 000 were consistently observed in these same preparations (Fig. 1A). No similar proteins were detected in preparations extracted from healthy plants. When we analysed the LIYV virion-associated proteins by immunoblotting, the ca. M, 28 000 protein specifically reacted with LIYV CP antiserum, confirming it as the LIYV CP (Fig. 1B). However, the ca. M,
Table 1. LIYV–*B. tabaci*, in vitro acquisition and transmission analysis

<table>
<thead>
<tr>
<th>Expt 1</th>
<th>Partially purified LIYV virions*</th>
<th>Cs₂SO₄-purified LIYV virions†</th>
<th>TE buffer control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/10§</td>
<td>–</td>
<td>0/10</td>
<td>0/10</td>
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<tr>
<td>Expt 2</td>
<td>4/6</td>
<td>–</td>
<td>0/6</td>
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<tr>
<td>Expt 3</td>
<td>20/20</td>
<td>10/10</td>
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* LIYV virions were purified using differential centrifugation and resuspended in TE buffer. Virion preparations were mixed with 60% sucrose to a final sucrose concentration of 15% before acquisition.
† The Cs₂SO₄–sucrose gradient fraction containing LIYV virions was dialysed in TE buffer and used as described for (*).
‡ TE buffer was mixed with 60% sucrose (final sucrose concn 15%) and used as a negative control.
§ *B. tabaci* was used to inoculate lettuce plants after *in vitro* acquisition. Numbers indicate LIYV-infected lettuce plants over the total number inoculated.

TEM and immunogold labelling

To further assess the composition of LIYV virions, immunogold labelling analysis was performed with the same four antisera used in immunoblot analysis. When LIYV virions were analysed using the CP antiserum, gold particles were found concentrated along almost the entire length of the virions (Fig. 2 A). In contrast, immunogold labelling using the CPm antiserum gave labelling that was concentrated at one end of the virions only (Fig. 2 B, C). Thus, LIYV virions exhibit the same morphologically polar rattlesnake structure as described for BYV and CTV virions (Agranovsky *et al.*, 1995; Febres *et al.*, 1996). The LIYV virion length was ca. 800–900 nm, as previously reported (Duffus *et al.*, 1996). However, only the terminal ca. 75–85 nm was labelled by the CPm antiserum, similar to the situation described for BYV and CTV virions. No gold label was observed on virions analysed with pre-immune serum (Fig. 2 D). As immunoblot analyses indicated that the HSP70 homologue and p59 were associated with LIYV virions, we also used immunogold labelling to determine if these were virion structural proteins. Virions did not show distinct labelling using the HSP70 homologue and p59 antisera, that is labelling was no different than for the pre-immune control.

In vitro acquisition assays

We used both partially purified LIYV virions and those purified on Cs₂SO₄–sucrose density gradients in attempts to

Table 2. LIYV serological infectivity neutralization analysis

<table>
<thead>
<tr>
<th>Expt</th>
<th>CP*</th>
<th>CPm</th>
<th>HSP70</th>
<th>p59</th>
<th>Pre-immune†</th>
<th>No serum</th>
<th>TE Control</th>
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<tbody>
<tr>
<td>1‡</td>
<td>5/5§</td>
<td>0/5</td>
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<td>2 2§</td>
<td>3/10</td>
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<td>6/10</td>
<td>9/10</td>
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* Indicates the LIYV antiserum used for neutralization studies.
† In Expt 1, antiserum against BWYV p19 was used as a control (see Tian *et al.*, 1995). In Expts 2–5, pre-immune antisera were used as controls.
‡ In Expt 1, LIYV virions were mixed at a 1:20 ratio of antiserum:virion preparation without additional incubation before *B. tabaci*-acquisition.
§ *B. tabaci* was used to inoculate lettuce plants after *in vitro* acquisition. Numbers show LIYV-infected lettuce plants over the total number inoculated.
₃ In Expts 2 and 4, LIYV virions were mixed at a 1:20 ratio of antiserum:virion preparations and in Expts 3 and 5 at a 1:100 ratio. Virion:antiserum mixtures were incubated for 30 min before *B. tabaci*-acquisition.
develop an in vitro acquisition assay. When lettuce plants were inoculated using B. tabaci whiteflies which were first given acquisition access to the LIYV virion preparations, typical LIYV symptoms developed on inoculated plants 2–3 weeks after inoculation. Similar transmission efficiencies were obtained using either the partially purified or Cs$_2$SO$_4$–sucrose-purified virion preparations (Table 1). Transmission efficiency was 100% for most experiments and no LIYV symptoms were observed on any lettuce plants inoculated using whiteflies which were given acquisition access to solutions of sucrose buffer only. Dot blot hybridization analysis confirmed that the symptomatic plants were LIYV-infected and that asymptomatic plants were not. Furthermore, B. tabaci were able to acquire and subsequently transmit LIYV from symptomatic plants inoculated via membrane feeding.

Because we observed four different LIYV-encoded proteins in LIYV virion preparations, we tested whether any of our LIYV antiserum could interfere with, or neutralize, in vitro acquisition and subsequent transmission of LIYV by B. tabaci. Results from several experiments show that LIYV transmission efficiency was not affected by pre-immune serum, antiserum to BWVV p19 or antiserum to LIYV p59 (Table 2). In only one experiment did the LIYV CP antibody reduce transmission (3/10) compared to pre-immune treatments. Similarly, the HSP70 homologue antiserum slightly reduced transmission efficiency (6/10) in one experiment only. Treatments with the CPm antiserum showed the greatest effects. In all but one experiment (Expt 5), the CPm antiserum completely eliminated LIYV transmission by B. tabaci. Only when the CPm antiserum was diluted to 1% final concentration was there incomplete neutralization of LIYV transmission by B. tabaci (Table 2, Expt 5).

Discussion

Our data show that partially purified virions of LIYV can be acquired in vitro by B. tabaci and subsequently transmitted to plants. We have been able to consistently achieve near 100% transmission efficiency using in vitro acquisition from partially purified LIYV virions. In addition to lettuce plants, we have transmitted LIYV to N. clevelandii plants after in vitro acquisition, and we have repeatedly transferred LIYV from the initially inoculated plants to additional test plants. LIYV in vitro acquisition and transmission have been attempted previously, but without success (Duffus et al., 1986). In vitro acquisition has been successful for the semi-persistent aphid-transmitted, helper-dependent parsnip yellow fleck virus (PYVV; Elnagar & Murant, 1976). However, successful PYVV transmission required that aphids had previously acquired the helper virus, anthriscus yellows virus (AYV), from AYV-infected plants. We are not aware of any reports of in vitro membrane feeding systems for other aphid- or whitefly-transmitted viruses which have semi-persistent non-circulative relationships with their vectors. This has led to some speculation that perhaps unlike the aphid-transmitted luteoviruses, clustroviruses may require additional proteins other than those on the virion for vector transmission (Nault, 1997; Pirone & Blanc, 1996). This requirement could be similar in function to the potyvirus HC-Pro or the caulimovirus ATF (Schmidt et al., 1994; Wang et al., 1998). However, our results show that the LIYV–B. tabaci transmission determinant(s) is within the LIYV virion preparation.

Based on what is known for other viruses in the family Crinivirus genus Crinivirus, it seemed possible that LIYV virions may also be structurally complex and composed of more than one type of capsid protein. BYV and CTV have morphologically polar virions composed of CP and CPm (Agranovsky et al., 1995; Febres et al., 1996). However, this had not previously been demonstrated for viruses in the genus Crinivirus such as LIYV. Furthermore, the LIYV CPm (ca. M$_r$ 52000) is twice as large as the LIYV CP (ca. M$_r$ 28000). In contrast the BYV CP is ca. M$_r$ 22000 and the CPm is similar in size, ca. M$_r$ 24000. Thus it was reasonable to question if proteins (the LIYV CPm and CP) of such different sizes could assemble to yield morphologically polar virions similar to those seen for BYV and CTV. Our immunoblot and immunogold-labelling data show that they do. Thus it seems reasonable to speculate that CPm may have a common function for viruses in the genera Crinivirus and Clustrovirus.

It was unexpected to find that the LIYV-encoded HSP70 homologue and p59 were also associated with LIYV virion preparations. We do not yet know whether the HSP70 homologue and p59 are LIYV virion structural proteins, although our immunogold labelling failed to identify either protein on purified virions. However, this failure could be due to the relatively small amounts of those proteins in the virion preparation. In any case, both proteins were consistently found to be associated with LIYV virions, even after Cs$_2$SO$_4$–sucrose gradient centrifugation.

It has been demonstrated for a number of plant viruses that virus-encoded proteins are required for vector transmission of the respective virus (Harrison & Murant, 1984; Pirone, 1991; Pirone & Blanc, 1996). This includes viruses from several taxonomically different genera including those in the families Potyviridae (Wang et al., 1998; Peng et al., 1998), Geminiviridae (Hofer et al., 1997), Bunyaviridae (Bandla et al., 1998; Kikkert et al., 1998), Luteoviridae (Bruyere et al., 1997), Caulimoviridae (Schmidt et al., 1994) and Bromoviridae (Perry et al., 1998). We have used serological blocking (infectivity neutralization) of LIYV acquisition and transmission from partially purified LIYV virions as a means to identify potential LIYV–B. tabaci transmission determinants. We expected that LIYV CP antibodies would block transmission, as has been definitively demonstrated in similar studies for luteovirus aphid transmission (Rochow & Duffus, 1978). Surprisingly, the LIYV CP antiserum did not block LIYV transmission. However, the LIYV CPm antiserum completely abolished LIYV transmissibility in all experiments except one. Our results suggest
that the LIYV CPm is important for B. tabaci transmission. Somewhat similar findings have revealed that aphid transmission of BYV was partially inhibited by using BYV CPm antibodies, while BYV CP antibodies were much less effective in preventing aphid transmission (He et al., 1998). In these latter experiments, aphids were first fed on BYV-infected plants and then allowed to feed on antiserum preparations before the inoculation access period. Our experiments were different in that the viroins and antiseras were mixed and incubated before in vitro acquisition. Thus, for the BYV experiments, the CPm antibody decreased transmission efficiency by viruliferous aphids, while in our experiments the LIYV CPm antibody probably affected LIYV acquisition, and possibly transmission. Definitive proof for the roles of specific LIYV-encoded proteins in B. tabaci-mediated transmission of LIYV will require further work, but the in vitro acquisition system described here provides a method to answer these questions.

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References


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