Electron-lucent inclusion bodies are structures specialized for aphid transmission of cauliflower mosaic virus

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Cauliflower mosaic virus (CaMV) is transmitted by aphids. For acquisition by the vector, a transmissible complex must form, composed of the virus particle, the viral coat-associated protein P3 and the helper protein P2. However, the components of the transmissible complex are largely separated in infected plant cells: most P3 virions are confined in electron-dense inclusion bodies, whereas P2 is sequestered in electron-lucent inclusion bodies (elIBs). This spatial separation controls virus acquisition by favouring the binding of virus-free P2 to the vector first, rendering the vector competent for later uptake of P3 virions. Consequently, sequential acquisition of virus from different cells or tissues is possible, with important implications for the biology of CaMV transmission. CaMV strains Campbell and CM1841 contain a single amino acid mutation (G94R) in the helper protein P2, rendering them non-transmissible from plant to plant. However, the mutant P2-94 protein supports aphid transmission when expressed heterologously and supplied to P3-CaMV complexes in vitro. The non-transmissibility of P2-94 was re-examined in vivo and it is shown here that the non-transmissibility of this P2 mutant is not due to low accumulation levels in infected plants, as suggested previously, but more specifically to the failure to form elIBs within infected plant cells. This demonstrates that elIBs are complex viral structures specialized for aphid transmission and suggests that viral inclusion bodies other than viral factories, most often considered as 'garbage cans', can in fact exhibit specific functions.

INTRODUCTION

Cauliflower mosaic virus (CaMV) infection results in formation of two different types of inclusion body in plant cells, the so-called electron-dense and electron-lucent inclusion bodies (edIBs and elIBs, respectively). Whereas edIBs are thought to be viral factories (reviewed by Hohn & Füttener, 1997), the function of elIBs seems to be related to vector transmission (Drucker et al., 2002; Espinoza et al., 1991).

This assumption is based on the fact that elIBs contain the totality of the viral protein P2 that is essential for viral transmission (Armour et al., 1983). CaMV is transmitted by aphids from one plant to another and employs the non-circulative transmission mode, meaning that only the cuticle lining of the aphids’ feeding apparatus (the stylets) specifically retains transmissible virus particles; internalization of the virus is not required (reviewed by Blanc et al., 2001; Gray & Banerjee, 1999). CaMV uses the helper strategy for transmission, i.e. a viral non-structural protein, the helper component, is needed for aphid transmission, which mediates (as a molecular bridge) binding of virus particles to the stylets. Correspondingly, the complete transmissible complex is composed of the virion and the helper component. In the case of CaMV, the virion is composed of the genome enclosed in an icosahedral shell, built of capsid protein (P4) and viral protein P3 associated with the capsid surface (Plisson et al., 2005). P2 is the helper component (Armour et al., 1983). P2 does not bind directly to the capsid, but to capsid-associated P3 (Leh et al., 1999, 2001). Accordingly, the CaMV transmissible complex consists of P2 and P3 virions.

CaMV has evolved a sophisticated mode of aphid transmission that takes into account plant morphology, vector behaviour and intracellular distribution of P2 and P3 virions in the two different types of inclusion body: edIBs contain approximately 95 % of a cell’s P3 virions enclosed in a matrix composed of viral protein P6, whereas elIBs are composed of viral P2, virion-free P3 and the remaining (approx. 5 %) P3 virions (Drucker et al., 2002; Espinoza et al., 1991). Thus, the components of the transmissible complex are essentially separated spatially in

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circular B-JI genome. The latter oligonucleotide contained the mutation C→T at position 1629, changing G94 of P2 to R, and a silent A→T mutation at position 1625, introducing a DraI site into gene II to facilitate identification of recombinants (mutations are shown in lower case; Xhol and DraI sites are underlined). The PCR product was digested with BstEII and Xhol and ligated into pCa24 cut with the same enzymes to yield plasmid pCa24-94. The resulting CaMV mutant was named B-J94. To create a CaMV mutant expressing HP2 (P2 with an N-terminal His tag), the P2-coding sequence of plasmid p119-HP2 (Hebrard et al., 2001) was amplified with oligonucleotides GGACTACGGCTAGGGAACCCAGCCACCACCAACCCAG and GGACTACGGCTAGCAAAATTTATCTTTATCC, containing SpeI sites (underlined), and introduced into the SpeI site of pCa37AP2, which encodes the genome of CaMV strain Cabb S, where ORF II is replaced by a SpeI site (Froissart et al., 2004). The resulting P2 mutant contained the additional 14 aa GHHHHHHHATAEGR, comprising a His tag and a factor Xa cleavage site at the N terminus of P2.

To obtain a recombinant baculovirus coding for P2-94 His-tagged at the N terminus (HP2-94), ORF II was amplified by PCR using pCa24-94 as template and introduced into the p119His transfer plasmid as described by Hebrard et al. (2001). To obtain a baculovirus encoding P2-94, ORF II from pCa24-94 was PCR-amplified by using the primers CCACATGTCCATTACGGGTCAACCGCATG, containing an AgeI site, and AAAACTGGTAGTTAGGAAATATTCTGT- TTAATCC, containing a PstI site (restriction sites are underlined). After digestion with AgeI and PstI, the PCR fragment was inserted into p119His cut with Ncol (this site is 5′ of the His tag in p119His) and PstI. Recombinant baculoviruses were obtained by cotransfecting S9 cells with the respective P2-coding plasmids and AcSLP10 DNA as described by Chabibi et al. (1993). All recombinants were verified by sequencing. The baculoviruses coding for HP2 and HP2 were described previously (Blanc et al., 1993b; Hebrard et al., 2001).

**Aphid-transmission assays.** Aphid-transmission assays were carried out as described previously (Blanc et al., 1993b). Briefly, groups of starved Myzus persicae (Sulz.) were allowed to probe different CaMV protein-containing solutions in SES buffer (200 mM Tris, pH 7.6, 100 mM EGTA, 50 mM MgCl2)/10% sucrose through stretched Parafilm membranes (American National Can) for 30 min or were placed on an infected turnip leaf for acquisition. Groups of 10 aphids were then placed on young turnip test plants for inoculation and allowed to feed overnight before treatment with Confidor insecticide (350 μl l−1). Symptoms were recorded 3 weeks later by visual inspection.

**Protein preparation.** Crude extracts of S9 cells expressing wild-type P2 and mutants were prepared by resuspending cells, harvested after infection with a recombinant baculovirus, in SES buffer, followed by ultrasonication to lyse cells. Crude extracts of P3-expressing S9 cells were prepared as described by Drucker et al. (2002). Extracts were either used directly in aphid-transmission assays or stored at −80 °C. His-tagged proteins were purified by Ni–NTA affinity chromatography as described previously (Hebrard et al., 2001), except that proteins were eluted with SES buffer containing 400 mM EGTA.

**Purification of viral inclusion bodies.** Inclusion bodies were purified from infected turnip leaves essentially as described by Al Ani et al. (1980). Denervated leaves were homogenized in 4 vols IB buffer [10 mM MES (pH 6.5), 1 mM CaCl2, 1 mM dithiothreitol] and passed through one layer of cheesecloth and one layer of Miracloth (Calbiochem). Triton X-100 was added to a final concentration of 1% from a 10% Triton X-100 solution in IB buffer and the homogenate was shaken for 10 min on ice. Then, the homogenate was centrifuged for 15 min at 800 g in a swing-out rotor through a
cushion of 1.2 M sucrose in IB buffer, and the pellet was resuspended in 2 vols IB buffer (g starting material) \(^{-1}\). Triton X-100 was added to a final concentration of 1% and the incubation and centrifugation steps described above were repeated twice. Finally, the pellet was washed once with IB buffer and stored at -80 °C.

**SDS-PAGE and Western blotting.** SDS-PAGE was carried out with 13.5% gels using the Tris/glycine system (Laemmli, 1970). Proteins were either stained with Coomassie blue or transferred to nitrocellulose membranes and processed as described by Drucker et al. (2002). Primary antisera were used at a dilution of 1:1000 (rabbit anti-P2, rabbit anti-P3, rabbit anti-P6) or 1:2000 (rabbit anti-CaMV). Bound secondary antibodies were revealed by using the NBT/BCIP colour reaction.

**Limited proteolysis by trypsin.** Affinity-purified HP2 and HP2-94 protein in DB5 buffer (Hebrard et al., 2001) was incubated with 0.1 vol. 2.5% trypsin solution (Invitrogen) at 37 °C for the times indicated. The reaction was stopped by addition of 0.33 vol. 4× Laemmli buffer and boiling for 5 min. Then, the digests were separated by SDS-PAGE and gels were stained with Coomassie blue for analysis.

**Microscopy.** Samples for electron microscopy were processed essentially as described by Drucker et al. (2002). For transmission electron microscopy, infected turnip leaves were fixed with 4% glutaraldehyde, post-fixed with 2% OsO\(_4\) and embedded in Epon resin. For immunoelectron microscopy, leaves were fixed with 0.5% glutaraldehyde and 2% paraformaldehyde and embedded in LR Gold resin (London Resin Co.). All primary antisera and 10 nm gold-conjugated secondary antibodies were used at a 1:25 dilution. The grids were observed under a JEOL JEM 100CX II electron microscope operated at 60–80 kV.

For immunofluorescence, protoplasts were prepared from infected turnip leaves by overnight digestion with 0.5% Cellulase R10 and 0.05% Macerozyme (Yakult) in 0.5 M mannitol and 5 mM MES (pH 5.5), followed by filtration on Miraloch and one wash with 0.5 M mannitol. Protoplasts were fixed for 20 min at room temperature with 3% paraformaldehyde. Fixation was stopped by two washes with TBS; then, protoplasts were incubated for 20 min in methanol. After another wash with water, protoplasts were immobilized on polylysine-treated slides and permeabilized by 10 min incubation in 0.2% Triton X-100. Slides were blocked for 30 min with 5% milk powder in TBS before incubation for 1 h with P2 antiserum diluted 1:100 in the same buffer. After three washes with TBS, slides were incubated for 1 h with anti-rabbit Alexa Fluor 488 (Invitrogen), again rinsed three times with TBS and mounted in 90% glycerol in TBS/1% propylgallate for observation under an Olympus BX60 epifluorescence microscope equipped with a narrow-band filter set (excitation, 470–490 nm; emission, 515–550 nm) and ×40 air and ×100 oil objectives. Images were recorded with a Canon PowerShot S50 camera using Canon Remote Capture 2.7 software; final images were mounted by using GIMP 2.2.13 software (http://www.gimp.org).

**RESULTS**

**Aphid-transmission activity of P2-94**

Non-transmissibility of the CaMV Campbell and CM1841 strains has been attributed to the G94R mutation in P2 (Woolston et al., 1987). We introduced this mutation into the transmissible reference strain B-JI, obtaining the CaMV mutant B-JI94.

We inoculated turnip plants with B-JI and B-JI94 plasmid DNA. Plants inoculated with B-JI94 developed systemic infection, with kinetics and symptoms similar to those of wild-type B-JI. We then compared plant-to-plant aphid transmission of the two viruses. Table 1(a) shows that this single amino acid change was indeed sufficient to abolish aphid transmission of B-JI.

The G94R mutation was also introduced into the B-JI P2 protein sequence for expression with the baculovirus/S9 system, either in its native state or fused with an N-terminal His tag. We first verified that P2-94 and its His-tagged variant (HP2-94) retained aphid-transmission activity. For this, crude extracts from S9 cells, together with recombinant P3 and purified B-JI virions, were used for aphid feeding across stretched Parafilm membranes. After this acquisition period, the aphids were placed on healthy turnip plants for inoculation. Table 1(b) shows that P3 and virions alone did not support aphid transmission. Addition of recombinant wild-type or His-tagged P2 or P2-94 restored aphid transmission to comparable levels.

**P2 and P2-94 show the same biochemical properties**

As it has been suggested that P2-94’s lack of aphid-transmission activity might be due to protein instability, we compared accumulation kinetics of P2 and P2-94 in insect S9 cells infected with corresponding recombinant baculoviruses. Fig. 1(a) shows that the two proteins accumulated to comparable levels in the first 48 h after infection. Thereafter, at a time when the S9 cells were killed by the baculovirus, the quantities of P2 and P2-94 remained constant throughout the observation period. Thus, it seemed that the two proteins were equally stable.

To examine the proteolytic sensitivity of P2 and P2-94 more precisely, their His-tagged variants were purified from recombinant baculovirus-infected S9 cells by Ni-NTA affinity chromatography and subjected to in vitro proteolysis by trypsin, a method to identify domains resistant to proteolysis. Again, no major difference was found between wild-type and mutant P2, as illustrated in Fig. 1(b). The two proteins were degraded with comparable kinetics, concomitant with the appearance of a similar processing product migrating slightly faster than undigested protein.

**Accumulation of P2 and P2-94 in infected plants**

Failure of B-JI94-infected plants in aphid transmission might be due to insufficient accumulation of components of the transmissible complex during infection. We therefore analysed accumulation of P2, P3, the viral capsid protein P4 and, as a marker of edIBs, accumulation of P6 in fractions enriched in inclusion bodies prepared from infected leaves. Fig. 2 shows that early in infection, at day 2 after emergence of systemic symptoms [post-emergence (p.e.)], P2 and P2-94 displayed similar accumulation...
kinetics. Thereafter, however, wild-type P2 levels remained essentially stable between 15 days p.e. and the end of the observation period at 36 days p.e., when leaves became senescent, whereas P2-94 levels were maximal at 15 days p.e., then decreased rapidly and became undetectable at 36 days p.e. This result shows that, early in infection, P2 and P2-94 levels were comparable.

P3 accumulation (Fig. 2) was identical for the two strains except that P3 was already detected at 2 days p.e. in wild-type-infected leaves. Also, kinetics of virion accumulation (Fig. 2), as judged by P4 levels, were similar for the two strains, with a stable and approximately equivalent level after 15 days p.e.

Accumulation kinetics of the edIB marker P6, on the other hand, were different in the two isolates, with P6 accumulating to higher amounts at 15–22 days p.e. in B-JI94-infected plants (Fig. 2). Thereafter, P6 levels dropped to comparable levels. Similar results were obtained when whole-leaf extracts were probed (data not shown).

Densitometry analysis of blots from different independent inclusion-body or whole-leaf preparations indicated an about 1.1–2.0-fold increase of P6 protein in B-JI94-infected plants at roughly 10–20 days p.e. (data not shown). Thus, the mutation in B-JI-P2-94 affected P6 levels, as well as accumulation of P2.

**Table 1. Aphid-transmission assays**

<table>
<thead>
<tr>
<th>CaMV strain/recombinant P2 variant</th>
<th>Aphid transmission (%) (± SEM)*</th>
<th>No. plants inoculated</th>
<th>No. experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) CaMV mutant B-JI94 is not aphid-transmitted from plant to plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-JI</td>
<td>57 (±9)</td>
<td>106</td>
<td>3</td>
</tr>
<tr>
<td>B-JI94</td>
<td>0</td>
<td>200</td>
<td>5</td>
</tr>
<tr>
<td>(b) Recombinant P2-94 supports aphid transmission after <em>in vitro</em> acquisition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no P2)</td>
<td>0</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>P2</td>
<td>33 (±24)</td>
<td>57</td>
<td>3</td>
</tr>
<tr>
<td>P2-94</td>
<td>31 (±13)</td>
<td>257</td>
<td>4</td>
</tr>
<tr>
<td>HP2</td>
<td>40 (±28)</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>HP2-94</td>
<td>33 (±11)</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>(c) Inclusion-body fractions purified from B-JI94-infected plants support aphid transmission after <em>in vitro</em> acquisition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-JI</td>
<td>31 (±19)</td>
<td>148</td>
<td>4</td>
</tr>
<tr>
<td>B-JI94</td>
<td>5 (±4)</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>(d) Cabb S-HP2 is not aphid-transmitted from plant to plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabb S</td>
<td>49 (±11)</td>
<td>92</td>
<td>2</td>
</tr>
<tr>
<td>Cabb S-HP2</td>
<td>0</td>
<td>180</td>
<td>4</td>
</tr>
</tbody>
</table>

*Aphids were starved for 30 min before being allowed a 30 min acquisition feed on: (a, d) a leaf infected systemically with the indicated CaMV strain; (b) a solution containing purified virus particles, recombinant P3 and the indicated recombinant P2 variant; or (c) a solution containing inclusion bodies purified from plants infected with the indicated CaMV strain. The aphids were then placed on healthy test plants for overnight inoculation. For (a) and (d), leaves from plants at 5–15 days p.e. were used when mutant P2 levels were maximal; for (c), inclusion bodies were purified at 8 days p.e. from the same leaves as in Fig. 2, when the P2 content was comparable in B-JI- and B-JI94-infected plants.

**Fig. 1.** P2-94 is as stable as P2. (a) Sf9 cells were infected with baculoviruses encoding P2 (left panel) or P2-94 (right panel). Equal aliquots of total cell extracts were taken at the indicated time points and analysed for the presence of P2 by Western blot. (b) His-tagged variants of P2 and P2-94 (HP2 and HP2-94) expressed in Sf9 cells were affinity-purified and incubated with trypsin for the time spans indicated, and analysed by SDS-PAGE and Coomassie blue staining. The arrows point to the protein bands corresponding to non-degraded HP2 and HP2-94. Both proteins were degraded with comparable kinetics.

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transmissible complex) were present in wild-type- and B-JI94-infected plants. So – bearing in mind that baculovirus-expressed P2-94 supported aphid transmission – aphid transmission of B-JI94 should theoretically be possible. To test this, we used inclusion body-enriched fractions from the pooled five leaves and aliquots normalized to equal fresh weight starting material were used for Western blotting. The blots were probed with antisera as indicated.

Fig. 2. Accumulation kinetics of P2, P3, P4, and P6. The first systemically infected leaf from five plants was harvested at the time after systemic symptom emergence (days p.e.) indicated. Fractions enriched in inclusion bodies were prepared from the pooled five leaves and aliquots normalized to equal fresh weight starting material were used for Western blotting. The blots were probed with antisera as indicated.

Fig. 3. B-JI94-infected plants contain paracrystals instead of eILBs. (a, b) Immunofluorescence microscopy of protoplasts purified from infected turnip leaves shows that (a) in B-JI-infected cells, most P2 accumulates in rounded, single, large, cytoplasmic inclusion bodies, whereas (b) in B-JI94-infected cells, P2 forms fibrous, star-like structures. (c, d) Transmission electron microscopy of (c) a B-JI-infected and (d) a B-JI94-infected plant cell reveals that B-JI infection results in formation of both edILBs and paracrystals (arrows point to enclosed virions), but not of paracrystals, whereas in B-JI94-infected cells, only edILBs and paracrystals (arrows), but no eILBs, are detectable. (e) Close-up of the paracrystals detected in (d). Bars, 10 μm (a, b); 200 nm (c, d); 100 nm (e).

Differential intracellular localization of P2 and P2-94 conditions aphid transmission

We examined P2 distribution patterns in infected leaves by immunofluorescence and electron microscopy. Immunofluorescence of protoplasts isolated from B-JI-infected turnip leaves at 10–20 days p.e. showed that, in about 70% of the mesophyll cells (mean of four experiments), P2 label was concentrated in large (up to 5 μm in diameter), rounded, cytoplasmic inclusion bodies (Fig. 3a). Astonishingly, in most cells, only one such inclusion body was detected. P2 labelling of mesophyll protoplasts prepared from B-JI94-infected plants at 10–20 days p.e. showed a different pattern of P2-94 distribution: in approximately 70% of the cells (mean of four experiments), P2-94 was found in star-like, fibrous structures, frequently seeming to emerge from a common centre; the rounded, P2-containing inclusion bodies, characteristic of B-JI-infected cells, were never observed (Table 1a), the difference was statistically significant (P ≤ 0.05, Fisher’s exact test using the combined median of aphid-transmission rates after B-JI94 acquisition from plants and inclusion bodies). Thus, B-JI94 acquisition by aphids could be restored under *in vitro* conditions, indicating that disruption of cells and inclusion bodies by the purification process and solubilization of P2-94 by SES buffer somehow enabled aphid transmission. This result shows clearly that P2-94 expressed during viral infection in plants is potentially functional in aphid transmission. So why is B-JI94, despite encoding and expressing a functional helper component, not transmitted from plant to plant by aphids?
These results suggested that the rounded inclusion bodies labelled by P2 in B-JI-infected cells correspond to eIIBs, and that the fibrous form of P2 detected in B-JI94-infected plants matches a hitherto-unknown form of P2 in infected plants.

To examine P2 distribution in wild-type- and B-JI94-infected plant cells more closely, we performed electron microscopy. Fig. 3(c) shows that B-JI-infected cells contained typical eIIBs and eIIBs. In contrast, despite extensive searching, no eIIBs were found in B-JI94-infected cells, although eIIBs were detected easily (Fig. 3d). Instead, we observed paracrystalline structures that were never observed in cells infected with wild-type CaMV (Fig. 3d, e). Paracrystals have been described previously to be the prevalent form of P2 in recombinant baculovirus-infected Sf9 cells and in plant-cell extracts (Blanc et al., 1993c), but never in intact plant cells. Immunogold labelling verified that the paracrystals indeed contained P2 (Fig. 4a). Besides paracrystals, no other cell structures were P2-labelled. This suggests that all P2 in B-JI94-infected cells is incorporated in paracrystals. Paracrystals often formed parallel bundles and were always found close to eIIBs, and sometimes seemed to emerge from or condense into them (see below). The electron microscopic data thus confirmed our observation of P2 by immunofluorescence in B-JI- and B-JI94-infected cells: the huge, singular P2-labelled inclusion bodies identified in B-JI-infected plants by immunofluorescence correspond to eIIBs and the fibres found in B-JI94-infected cells to paracrystals.

Also, the distribution of P3, P6 and virus particles in B-JI94-infected cells was examined by immunoelectron microscopy. Fig. 4(b) shows that P3 antiserum labelled eIIBs, whereas labelling of paracrystals was not observed. P6 label (Fig. 4c, d) was observed mainly in eIIBs; occasionally, some P6 label was detected in the cytoplasm around eIIBs and on paracrystals close to eIIBs. Paracrystals seemed to be more prominent around small, presumably young eIIBs containing only a few virus particles than around bigger eIIBs (compare Fig. 4c and d). Virions were found in eIIBs (Fig. 4a–d) and were sometimes aligned parallel to paracrystals, but never in their interior.

A second P2 mutant defective in plant-to-plant aphid transmission

We looked for another CaMV mutant that expresses a P2 variant potentially active in in vitro acquisition assays, but
that might be impaired in plant-to-plant aphid transmission. To this aim, we constructed Cabb S-HP2, a CaMV mutant expressing N-terminally His-tagged P2 (HP2). HP2 has previously been shown to possess all known P2 properties, including aphid transmission (Table 1b; Hebrard et al., 2001). Cabb S-HP2 was infectious in plants, which produced symptoms comparable to those of wild-type infection (data not shown). When tested in plant-to-plant aphid-transmission assays, no transmission was recorded (Table 1d). HP2, P3 and viral coat protein were, however, detected by Western blot analyses using extracts from infected leaves (Fig. 5a), although HP2 accumulated less than wild-type P2 and also less than P2-94 (data not shown). This situation resembled the case of B-JI94 and we examined infected leaves by microscopy. In immunofluorescence, only about 30% of the cells were positive for HP2 (mean of three experiments). In these cells, most HP2 did not localize in big inclusion bodies, like P2, or in fibres, like P2-94, but formed a network extending throughout the cell (Fig. 5b). Double immunolabelling showed that the network colocalized with microtubules (data not shown). In electron microscopy, typical edIBs were found, but we never observed any elIBs, paracrystals or other prominent structures (Fig. 5c).

**DISCUSSION**

**P2 instability does not explain the non-transmissibility of B-JI94**

In this report, we re-examined the non-transmissibility by aphids of CaMV strains encoding the G94R mutation of P2. To rule out any other amino acid changes as a possible cause for the mutant P2-94’s failure in aphid transmission and for any change of biochemical properties, we introduced this mutation into the transmissible P2 protein variant B-JI for heterologous expression in insect Sf9 cells and into the genome of CaMV B-JI for analysis of the mutant protein in the context of a viral infection. Biochemical comparison of P2 and P2-94 stability in vitro in Sf9 cells and in vitro by limited trypsin proteolysis revealed no differences between the two proteins. Together with the fact that P2-synthesis rates are comparable in wild-type- and CM1841-transfected protoplasts (Nakayashiki et al., 1993), low P2-94 levels in infected plant cells are due neither to reduced expression nor to intrinsic protein instability, but to another reason.

As earlier reports demonstrated that recombinant P2 produced in Sf9 cells from isolate CM1841 supports aphid transmission after in vitro acquisition, the non-transmissibility of this strain has been suggested to result from insufficient concentration of P2-94 in infected plant cells (Blanc et al., 1993a; Nakayashiki et al., 1993). However, we show here that, although B-JI94 was not transmitted in plant-to-plant assays, extracts prepared from the same plants and acquired by aphids in vitro did support aphid transmission. This result demonstrates clearly that B-JI94-infected plants contain, at least temporarily, a sufficient amount of P2 that should, in principle, allow aphid transmission. However, we do not rule out the possibility that, later in infection, a low P2-94 level contributes to non-transmissibility.

**elIBs are inclusion bodies specialized for aphid transmission**

Electron and immunofluorescence microscopy provided an answer to why P2-94-encoding CaMV isolates are non-transmissible. In contrast to wild-type-infected cells, B-JI94-infected cells contained no elIBs, a result also reported for the natural P2-94-encoding Campbell isolate (Espinoza et al., 1991). However, unlike these authors, we detected P2-labelled paracrystals in B-JI94-infected cells. The reason that Espinoza et al. (1991) did not detect paracrystals in cells infected by Campbell is probably explained by the fact that they used plant material for analysis at a time point in infection when the mutant P2 was already degraded.
P2 paracrystals are an inactive, polymerized form of P2 that is acquirable by aphid vectors and active in transmission assays only after solubilization (Blanc et al., 1993c). The incorporation of P2-94 into paracrystals instead of elIBs explains all features of B-JI94: reduced accumulation and apparent instability of P2-94 are probably due to enhanced degradation, caused by unfavourable exposure of P2-94 paracrystals to cellular proteases or proteasomes as opposed to P2 stocked in elIBs. Further, the non-transmissibility of B-JI94 is dictated primarily by ineffectiveness of P2-94 paracrystals in binding to the aphid vector. The mere presence of P2 and P3 virions in infected cells is insufficient for effective virus acquisition; in addition, P2 must be available to the vector in a form compatible with attachment to the aphids’ styles. Moreover, the only form of P2 compatible with plant-to-plant aphid transmission is that found in elIBs, where P2 colocalizes with P3 and some virions in a loose, non-crystalline matrix (Drucker et al., 2002). Thus, elIBs are specialized inclusion bodies that are entirely dedicated to permit aphid transmission. This view is strengthened by analysis of the Cabb S-HP2 mutant. Also, this virus encodes and expresses a potentially functional P2 protein, but was not transmitted from plant to plant. Also, this mutant did not form elIBs in infected cells; instead, most HP2 was found in reticulate structures. This further strengthens the hypothesis that P2 must be presented in a highly specific form, which appears to be elIBs rather than paracrystals (B-JI94) or a network (Cabb S-HP2), to assist in CaMV aphid transmission.

How do the P2-94 and HP2 mutations prevent formation of elIBs? The current hypothesis proposes that all viral proteins are translated in or close to elIBs. However, P2 has never been detected in elIBs, indicating that it is exported rapidly, together with P3 and some virions, to elIBs. P2-94 paracrystals, on the other hand, were always found close to elIBs and sometimes seemed to emerge from them. Thus, it is probable that transport of P2-94 and/or its in vivo interaction with P3 is affected by the mutation, resulting in the formation of P2-94 paracrystals. Faulty interaction of P2-94 with P3 and consequent failure to form elIBs might be indicative of a novel P2–P3 interaction, independent of the known one involved in binding to P3 on the capsid surface, which requires the C terminus of P2 (Leh et al., 1999) and is not affected by the P2-94 mutation, as shown by the aphid-transmission activity of solubilized P2-94. Evidence for such a (probably indirect) interaction is supported by the fact that native P2 and P3 do not interact in vitro, whereas they colocalize in baculovirus-infected Sf9 cells and in elIBs (Drucker et al., 2002). HP2 in Cabb S-HP2-infected cells was primarily found as a network-like structure and colocalized with microtubules. This indicates that P2, known to interact with microtubules (Blanc et al., 1996), in this mutant either interacts aberrantly with the cytoskeleton or is blocked in a step necessitating microtubule interaction for elIB formation.

As well as a lower amount of P2-94, we detected a transient increase of P6 in B-JI94-infected cells. This might be indicative of a direct or indirect interaction of P6 with P2, an interaction that was also suggested by Qiu et al. (1997). The biological relevance of this putative P2–P6 interaction remains, however, for the moment unknown. We suggest that it may be involved in the correct export of P2 from elIBs.

Hitherto, most inclusion bodies have either been implicated in viral replication or have been considered as functionless, dead-end structures or merely serving as ‘garbage containers’ stocking superfluous viral material (reviewed by Novoa et al., 2005). Only very few data are available describing alternative functions for viral inclusion bodies, e.g. baculovirus occlusion bodies shielding virus particles from exposure to the environment (Miller, 1997) or potyvirus CI protein in the form of cylindrical inclusion bodies presumed to facilitate viral cell-to-cell movement (Roberts et al., 1998). By describing a highly specialized role in aphid transmission for viral inclusion bodies, here we add to the scarce information available on alternative functions. It will be interesting to determine whether inclusion bodies from other viruses also possess specific functions, distinct from viral factories. Altogether, our findings support a new view of the role of inclusion bodies: rather than being just dead-end structures without any function, they fulfil distinct and important biological roles in the viral infection process.

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