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Virus-like particles assemble in plants and bacteria expressing the coat protein gene of *Indian peanut clump virus*

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cDNA copies of the coat protein (CP) gene of *Indian peanut clump virus* (IPCV)-H were introduced into cells of *Nicotiana benthamiana* or *Escherichia coli* by transformation with vectors based on pROKII or pET respectively. In both plant and bacterial cells, IPCV CP was expressed and assembled to form virus-like particles (VLP). In plant extracts, the smallest preponderant particle length was about 50 nm. Other abundant lengths were about 85 and about 120 nm. The commonest VLP length in bacterial extracts was about 30 nm. Many of the longer VLP appeared to comprise aggregates of shorter particles. The lengths of the supposed 'monomer' VLP corresponded approximately to those expected for encapsidated CP gene transcript RNA. Immunocapture RT–PCR, using primers designed to amplify the CP gene, confirmed that the VLP contained RNA encoding IPCV-H CP. The results show that encapsidation does not require the presence of the 5′-terminal untranslated sequence of the virus RNA and suggest that if there is an 'origin of assembly' motif or sequence, it lies within the CP gene. When transgenic plants expressing IPCV-H CP were inoculated with IPCV-L, a strain that is serologically distinct from IPCV-H, the virus particles that accumulated contained both types of CP.

Introduction

Indian peanut clump virus (IPCV) affects several crops of the Indian subcontinent. It induces clump disease of groundnut (*Arachis hypogaea* L.) in India (Reddy, 1983). No source of natural resistance has been identified in groundnut (Reddy *et al.*, 1988) and pathogen-derived resistance is therefore thought to offer the most practical possibility for disease control (Mayo & Naidu, 1997). To evaluate the potential of a coat protein (CP)-mediated resistance strategy, transgenic *Nicotiana benthamiana* plants have been obtained that express sequence from the 5′ region of RNA-2 of IPCV-H containing the translatable sequence of the CP gene (Mayo & Naidu, 1997; Wesley, 1993).

Expression of CP genes in bacteria or in plants can give rise to virus-like particles, as shown for viruses as different as *Alfalfa mosaic virus* (Yusibov *et al.*, 1996), *Cowpea chlorotic mottle virus* (Zhao *et al.*, 1995), *Cowpea mosaic virus* (Wellink *et al.*, 1996) and the potyviruses, *Johnsongrass mosaic virus* and *Potato virus Y* (Jagadish *et al.*, 1991, 1993; Stram *et al.*, 1993). VLP of *Tobacco mosaic virus* (TMV) CP were detected in both transgenic plants (Sleat *et al.*, 1989) and *Escherichia coli* (Hwang *et al.*, 1994), if the encapsidated RNA contained an origin of assembly sequence (OAS). The assembly of VLP in either plants or bacteria has been proposed as a way to protect and accumulate specific mRNA (Sleat *et al.*, 1989), as a means to study the molecular assembly of the capsid (Jagadish *et al.*, 1991; Hwang *et al.*, 1994) or for the production of oral vaccines (Mason *et al.*, 1996). In this paper, we report the occurrence of VLP in transgenic plants expressing the 5′-most sequence of IPCV-H RNA-2 and in cells of *E. coli* expressing the IPCV-H CP gene.

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Methods

■ **Plants, viruses and their propagation.** All the plants were grown in environmentally controlled glasshouses at 22 °C. Transgenic lines AC14 and AC20 were selected from a collection of *Nicotiana benthamiana* transformed using a pROKII-derivative pPCV3 containing the 5′-most region of IPCV-H RNA-2 (nucleotides 13–1133) (Wesley,

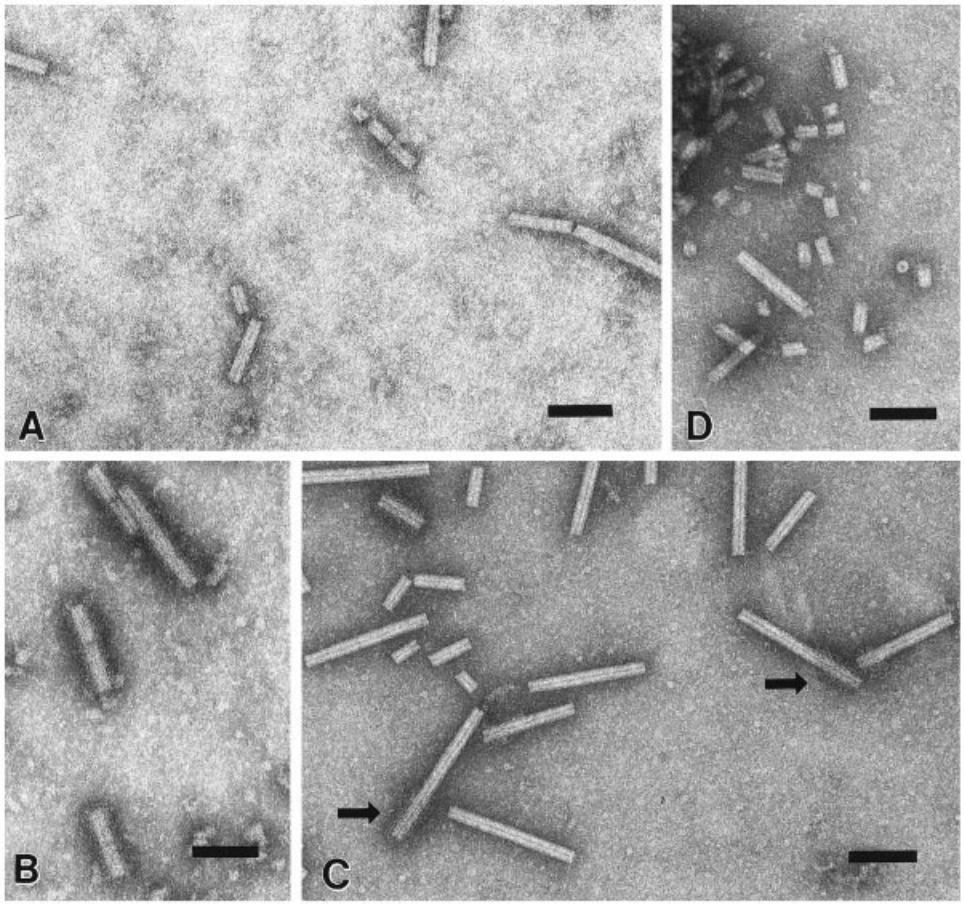

Fig. 1. Electron micrographs of particles trapped on grids coated with antibodies to IPCV-H. (A) VLP from extracts of plants of line AC20. (B) As (A) but grids treated after trapping VLP with antibodies to IPCV-H; the VLP show specific decoration. (C) Particles trapped from transgenic *N. benthamiana* AC20 plants inoculated with IPCV-L and kept for 2 weeks in a glasshouse. Grids were treated with antibodies to IPCV-H after trapping. Some IPCV-L particles were bound non-specifically, some became decorated in patches with ant-IPCV-H. (D) VLP trapped from extracts of *E. coli* expressing pET containing the IPCV-H CP gene. Bar indicates 100 nm.

1993). The expression levels in F_2 plants were assessed by immunoblotting using IgG (1 μg/ml) from a rabbit antiserum directed against IPCV-H. The IPCV strains used were IPCV-H and IPCV-L (Nolt *et al.*, 1988).

■ **Purification of IPCV particles and IPCV-like particles.** Virus particles were purified from *N. benthamiana* plants infected by mechanical inoculation as described by Reddy *et al.* (1985) and were stored at −20 °C in 0·06 M NaCl, 0·01 M Tris–HCl, 0·003 M EDTA. Infectivity was assessed by mechanical inoculation on *N. benthamiana*, *Chenopodium amaranticolor* and *Phaseolus vulgaris* cv. Topcrop. The same protocol was used in attempts to purify VLP from ca. 200 g batches of F_2 transgenic plants. Fractions obtained after centrifugation through a sucrose cushion, as well as after a sucrose gradient step were assessed by ELISA and immunosorbent electron microscopy (ISEM).

■ **Extraction of virus-like particles from *E. coli*.** Cells of *E. coli* BL21(DE3)pLysS were transformed with the plasmid pET-15b (Novagen, AMS biotechnology) containing the IPCV-H coat protein gene (nucleotides 503–1129, i.e. lacking non-coding sequences). After an overnight preculture, cells were grown in 20 ml LB + ampicillin at 37 °C for 4 h, before induction with 1 mM IPTG. Cells were then collected by centrifugation (6000 ***g***, 4 °C, 10 min), resuspended in 1 ml 10 mM Tris–HCl, 1 mM EDTA, pH 8 (TE) and stored at −20 °C for further use. Samples of the suspension were denatured and subjected to SDS–PAGE [15% (w/v) gel] as described by Reddy & Mayo (1985). After electrophoresis and staining in Ponceau S (1%), proteins were transferred to nitrocellulose and probed with IgG from a rabbit antiserum specific for IPCV-H at 1 μg/ml followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) and colorimetric reaction. Controls included a purified preparation of IPCV-H and an extract from an IPCV-H-infected *N. benthamiana*.

■ **Electron microscopy.** For electron microscopy, frozen suspensions of partially purified VLP were thawed on ice and centrifuged at 4 °C at 6000 ***g*** for 10 min to remove insoluble material. The remaining solution was kept on ice and sonicated. The resulting mixture was used for ISEM.

ISEM and antibody coating (AC) of antibody-trapped VLP were done as described by Roberts (1986). Antibodies to IPCV-H were diluted in 0·06 M phosphate buffer, pH 6·5. Grids were negatively stained with 2% sodium phosphotungstate, pH 6 and were examined in a Philips CM-10 or a JEOL 1200 Ex electron microscope.

■ **Immunocapture (IC)–RT–PCR.** Plants were selected as being transgenic by PCR using primers designed to amplify the IPCV-H CP gene (nucleotide position 1141, 5′ AGACGTCGACATTAATTCACCC 3′, and nucleotide position 496, 5′ ACGGACATGTCTAATTTGTCTG 3′).

IC–RT–PCR experiments were done on transgenic *N. benthamiana* plant extracts as described by Brandt & Himmler (1995). PCR tubes were coated with 100 µl anti-IPCV-H IgG (1 µg/ml) in 0·1 M carbonate buffer, pH 9·6. After 2 h incubation, tubes were washed twice in PBS–0·05% Tween and twice in PBS. Plant extracts were made in phosphate buffer (0·01 M, pH 6·5) using a 1:10 leaf/buffer ratio (w/v). Extracts were centrifuged to remove plant debris and 100 µl samples were incubated overnight at 4 °C in the coated tubes. Tubes were then washed three times with 200 µl PBS–Tween and three times with 200 µl PBS. For assays, tubes were washed by vortexing with 10 µl Triton X-100 in TE and eight samples were subjected to the two-step RT–PCR done according to manufacturer's instructions (Expand reverse transcriptase, Expand high fidelity, Boehringer Mannheim). The primers already described for PCR were also used for the IC–RT–PCR. As a control, the same plants were submitted to an IC–RT–PCR using primers designed to amplify the 250 bp from the 3′ end of both RNA-1 and RNA-2 of IPCV.

■ **Inoculation of IPCV-L virus in transgenic *N. benthamiana* expressing the IPCV-H CP gene.** To increase the number of particles available for analysis, the transgenic *N. benthamiana* line AC20 was inoculated with plant sap of a freezer-stored isolate of IPCV-L serotype (Nolt *et al.*, 1988). Plants were then tested for the presence of particles by ISEM and immunodecoration using an anti-IPCV-H antiserum.

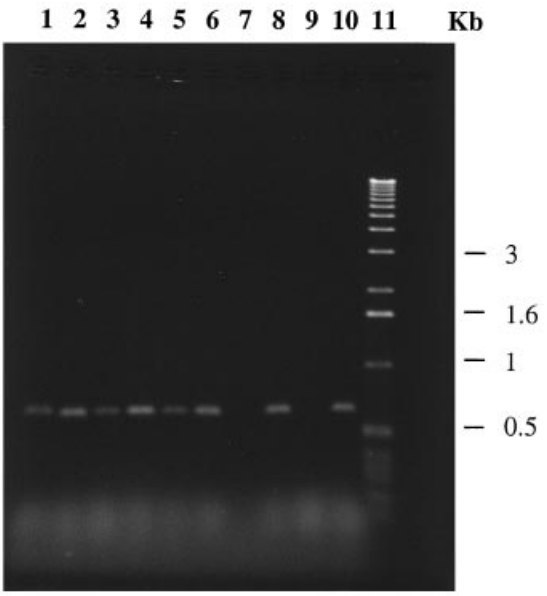

Fig. 2. IC–RT–PCR detection of the IPCV-H CP gene in transgenic *N. benthamiana* AC20. Plant extracts in phosphate buffer (0·01 M, pH 6·5), from eight selected plants, amplified by IC–RT–PCR (Brandt & Himmler, 1995) using primers designed to amplify the IPCV-H CP gene. Extracts from an IPCV-H infected and a non-infected *N. benthamiana* plant were used as negative and positive controls (lanes 9 and 10); lane 11 is a 1 kb ladder (Gibco).

Results

Expression of CP in transgenic plants

Although ELISA of leaf extracts of *N. benthamiana* lines AC14 and AC20 that had been transformed with the pROKII-derivative plasmid pPCV3 gave no evidence of accumulation of CP, CP was readily detectable when samples were analysed by immunoblotting. Comparisons with blots of protein from known amounts of IPCV suggested that about 50–100 pg/g fresh weight were produced in these transgenic plants. To assess the form in which the CP was present in the tissue, extracts were examined by ISEM.

VLP were detected in some extracts of AC20 plants, but not those of AC14. Changing the pH or composition of the extraction buffer did not result in better detection of VLP. The VLPs resembled particles of IPCV in diameter and in having a central hole, but were markedly shorter (Fig. 1A).

In order to obtain sufficient VLP to make reliable size estimates, leaves from transgenic plants were subjected to the purification protocol used for IPCV. Some preparations contained no detectable particles whereas a few yielded some VLP. In preparations from AC14 and AC20, the length of the particles detected ranged from 10 to 330 nm with about 80% of particles (in a sample of 542 particles) being between 40 and 150 nm long. In a particle length distribution histogram, there was a peak of length about 50 nm and other peaks of about 85 and 120 nm. Some particles had clearly been formed by end-to-end aggregation, although this was not obvious for some other long particles. The trapped VLPs were decorated with IPCV-H antiserum (Fig. 1B). A particle formed by encapsidation of the predicted transcript RNA from pPCV3 would, assuming it had the same quaternary structure as IPCV particles, be about 45 nm in length.

To test for the presence of the CP gene in the VLPs, plant extracts were assayed by IC–RT–PCR using primers chosen to amplify the CP gene. Of the eight AC20 transgenic plants tested, seven yielded a ca. 600 bp fragment that corresponded in size to the CP gene (Fig. 2). To exclude IPCV particles as the source of the PCR product, IC–RT–PCR was done using primers designed to amplify the conserved 3′ end of RNA-1 and RNA-2 of IPCV. Extracts of IPCV-H-infected *N. benthamiana* yielded the predicted product, but no amplification product was obtained from extracts of the transgenic plants.

In another attempt to increase the number of VLP available for analysis, transgenic plants were inoculated with IPCV-L and virus particles were purified 14 days later. Semi-purified virus preparations were examined by ISEM using an anti-IPCV-H rabbit antiserum. When grids carrying trapped VLP were treated with antiserum in decoration tests, a proportion of the particles became partially or wholly coated with antibodies (Fig. 1C). The particles that were coated with antibodies were similar in length to uncoated particles. The modal lengths corresponded to those observed in mechanically infected plants (ca. 160 and 220 nm), although very few such characteristic length particles were completely coated. The size of the coated regions (Fig. 1C, arrows) ranged from 10 to

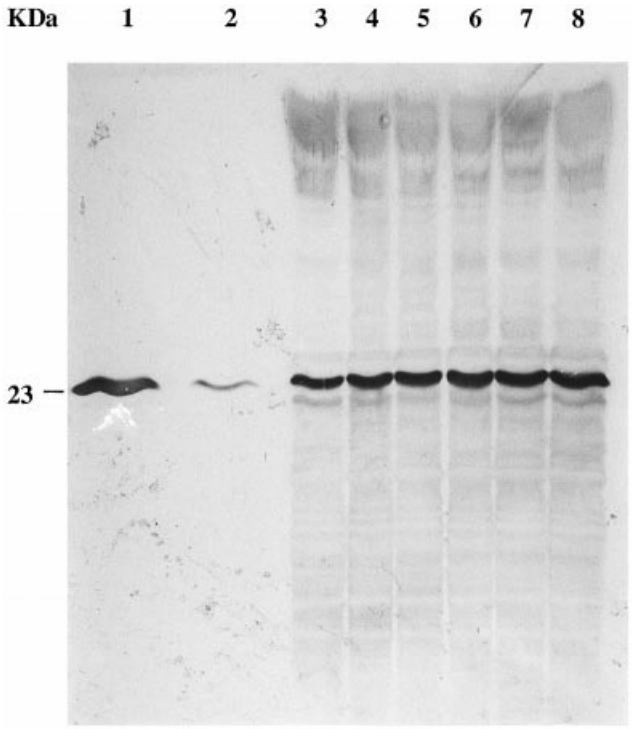

Fig. 3. Detection by immunoblotting of the IPCV CP synthesized in bacteria. Bacterial cells were grown for 4 h before induction. Fractions collected after 0·5, 1, 2, 3, 4 and 8 h (lanes 3–8, respectively) were analysed by SDS–PAGE. Samples were transferred onto a nitrocellulose membrane and an IPCV-H rabbit polyclonal antiserum was used to detect the CP. Purified IPCV-H (ca. 5 or 0·5 µg) was used as a positive control (lanes 1 and 2 respectively). The position of the IPCV-CP is indicated (23 kDa).

210 nm. Some coated particles appeared to consist of end-to-end aggregated particles. Although there is little cross-reaction between IPCV serotypes L and H, some particles of IPCV-L were trapped non-specifically on grids coated with the anti-IPCV-H antiserum.

Expression of CP in bacteria

E. coli BL21(pLysS) cells were transformed with pET15b containing IPCV-H CP. After induction of expression the levels of the CP were monitored over a 20 h period by immunoblotting. The CP was readily detectable in samples taken after 1 h and did not change much after longer induction periods (Fig. 3).

Extracts in 0·1 M citrate–phosphate buffer (pH 6·5) of bacterial cells that expressed the CP were examined by ISEM using antibodies to IPCV-H. Rod-shaped VLP (Fig. 1D) were observed in samples from cells that had been treated with IPTG for 8 or 20 h, but not in extracts of cells induced for shorter periods. The lengths of the measured VLP were between 10 and 140 nm, with a mode of ca. 30 nm. The length of a particle containing the IPCV-specific transcript RNA would be about 25 nm.

Discussion

We have shown that when a cDNA encoding the full-length CP of IPCV-H is expressed in *N. benthamiana* or *E. coli*, the resulting CP monomers assembled to form VLP. Apart from their shorter length, the VLP were similar in appearance to particles of IPCV, with a narrow stain-penetrable lumen and a diameter of about 20 nm. The VLP were shown by antibody binding and subsequent antibody decoration to consist of IPCV CP.

Little is known about the assembly of particles of IPCV and similar viruses such as *Beet necrotic yellow vein virus* (BNYVV) or *Potato mop-top virus*. Similarities in amino acid sequence among CPs of viruses with rod-shaped particles suggests that their assemblies share a common mechanism (Goulden *et al*., 1992). Thus the assembly mechanism for TMV CP could suggest a mechanism for IPCV. However, TMV particle assembly is initiated by the interaction between an origin of assembly sequence (OAS), a 75 nucleotide sequence that can form a hairpin loop, which interacts with a two-layer disk-like structure (reviewed in Butler & Mayo, 1987). The results of IC–RT–PCR and particle length measurements indicate that IPCV CP encapsidates the transcript RNA that encodes the CP in both prokaryotic and eukaryotic cells. Thus if IPCV RNA has an OAS, it lies within the CP gene. However, as with other viruses with more than one genome RNA, the OAS does not seem to be a particular nucleotide sequence as no common sequence was detected between RNA-1 (Miller *et al*., 1996) and the CP gene (Wesley *et al*., 1994) of IPCV-H. For example, with BNYVV Gilmer *et al*. (1992) have shown that a sequence of the non-coding 5′ end of BNYVV RNA-3 is required for RNA packaging *in vivo* although there is no similarity between this sequence and that of the other BNYVV RNAs. An alternative explanation is suggested by the work of Guilley *et al*. (1975), who showed that TMV CP could encapsidate sequences other than the OAS. A specifically encapsidated fragment (SERF) was found in the CP gene that was thought probably to be responsible for the encapsidation of RNA of *Sunn-hemp mosaic virus* (Hirth & Richards, 1981).

VLP were difficult to detect in transgenic plants, especially of line AC14. VLP were more readily detected in bacterial extracts, which indicates that assembly does not involve post-translational modification. Conditions for *in vitro* assembly of VLPs differ greatly from one virus to another, as for example for aggregation of CPs of TMV and *Tobacco rattle virus* (Mayo *et al*., 1993). Nevertheless, it is not yet clear at this stage how frequent is the assembly phenomenon, and under what conditions VLP are assembled inside the cells or during the processing of the cells extracts.

To our knowledge no VLP formation has been reported for any plant viruses transmitted by fungus. When extracts of transgenic plants that were expressing large amounts of PMTV CP (Barker *et al*., 1998) were searched for VLP, none was found (unpublished results).

The occurrence of VLP in transgenic plants raise questions about risks linked to heteroencapsidation, a phenomenon deduced to occur with other viruses for which VLP have been observed, such as potyviruses, from the results of challenge inoculation of transgenic plants (Jacquet *et al*., 1998; Hammond & Dienelt, 1997). We have observed a type of heteroencapsidation in the experiments reported here. Particles were formed that corresponded in length to particles containing RNA of IPCV-L, but patches of the protein coats of these particles consisted of CP of IPCV-H. This shows that IPCV-H CP is able to interact with IPCV-L RNA, and the pattern of patches of heterologous CP suggests that once IPCV-H CP had started to encapsidate the RNA, it was more likely to interact with itself than with the more abundant IPCV-L CP, and vice versa.

These results are another example of genomic masking as demonstrated by Rochow (1977) for barley yellow dwarf viruses. If, as with luteoviruses, vector transmission of IPCV depends on surface features of the virus particles, such masking could result in a change in vector specificity for the masked virus genome, and hence altered pathogenicity for this virus. It is not known if the IPCV–*Polymyxa graminis* interaction does involve the CP, but our results suggest a potential problem that should be considered if and when transgenes based on the CP gene of IPCV are used in attempts to obtain transgenic resistance.

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