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Closterovirus Encoded HSP70 Homolog and p61 in Addition to Both Coat Proteins Function in Efficient Virion Assembly

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Assembly of the viral genome into virions is a critical process of the virus life cycle often defining the ability of the virus to move within the plant and to be transmitted horizontally to other plants. *Closteroviridae* virions are polar helical rods assembled primarily by a major coat protein, but with a related minor coat protein at one end. The *Closteroviridae* is the only virus family that encodes a protein with similarity to cellular chaperones, a 70-kDa heat-shock protein homolog (HSP70h). We examined the involvement of gene products of *Citrus tristeza virus* (CTV) in virion formation and found that the chaperone-like protein plus the p61 and both coat proteins were required for efficient virion assembly. Competency of virion assembly of different CTV mutants was assayed by their ability to be serially passaged in *Nicotiana benthamiana* protoplasts using crude sap as inoculum, and complete and partial virus particles were analyzed by serologically specific electron microscopy. Deletion mutagenesis revealed that p33, p6, p18, p13, p20, and p23 genes were not needed for virion formation. However, deletion of either minor- or major-coat protein resulted in formation of short particles which failed to be serially transferred in protoplasts, suggesting that both coat proteins are required for efficient virion assembly. Deletion or mutation of HSP70h and/or p61 dramatically reduced passage and formation of full-length virions. Frameshift mutations suggested that the HSP70h and p61 proteins, not the RNA sequences, were needed for virion assembly. Substitution of the key amino acid residues in the ATPase domain of HSP70h, Asp^{7} to Lys or Glu^{180} to Arg, reduced assembly, suggesting that the chaperone-like ATPase activity is involved in assembly. Both HSP70h and p61 proteins appeared to contribute equally to assembly, consistent with coordinate functions of these proteins in closterovirus virion formation. The requirement of two accessory proteins in addition to both coat proteins for efficient assembly is uniquely complex for helical virions. © 2000 Academic Press

INTRODUCTION

The *Closteroviridae* family includes two definitive genera, the aphid-transmitted *Closterovirus* and the whitefly-transmitted bipartite *Crinivirus,* and it is probable that there will be at least one other genus consisting of mealybug-transmitted viruses (Bar-Joseph *et al.,* 1979; Karasev, 2000). The members of *Closteroviridae* contain two characteristic gene modules. The first is a "replication gene block," which includes one or two protease domains, methyltransferase- and helicase-like domains, and a +1 frameshift to an RNA-dependent RNA polymerase-like domain, with large intervening regions resulting in open reading frames (ORF) 1a and 1b of ~8–11 kilobases (kb). This gene block is involved in replication-associated functions (Klaassen *et al.,* 1996; Peremyslov *et al.,* 1998; Satyanarayana *et al.,* 1999). The second is the "signature gene module" of five genes in the 3′ region of monopartite or RNA 2 of bipartite viruses that encode a small (6 kDa) hydrophobic protein, proposed to be a membrane anchor, a protein closely related to the ubiquitous cellular heat shock induced chaperone, HSP70 (HSP70h), a protein of ~60 kDa, and minor- (CPm) and major- (CP) coat proteins. In addition to these gene modules, *Closteroviridae* members possess two to six other genes.

Citrus tristeza virus (CTV), a *Closterovirus*, is the largest plant RNA virus, causing the most economically damaging viral diseases of citrus throughout the world. It has a single-stranded, positive-sense RNA genome of ~19.3 kb that contains 12 ORFs potentially coding for at least 19 protein products (Pappu *et al.,* 1994; Karasev *et al.,* 1995; Karasev, 2000). The ten 3′ ORFs are expressed from 3′-coterminal subgenomic (sg) RNAs (Hilf *et al.,* 1995). Deletion mutagenesis of a CTV infectious cDNA clone demonstrated that none of the 3′ ORFs was necessary for replication (Satyanarayana *et al.,* 1999).

Closteroviridae virions are flexuous filamentous rods with an open helical symmetry with lengths of 700-2000 nm defined by the size of the genomic RNA. In contrast to other helical plant viruses, these virions contain two coat proteins. Most of the RNA is encapsidated by the CP, but about 5% is encapsidated by CPm, resulting in a polar conformation termed "rattlesnake-like virions" (Agranovsky *et al.,* 1995; Febres *et al.,* 1996; Tian *et al.,* 1999). The polar positioning of two coat proteins in a

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helical virion raises intriguing questions regarding assembly. Are both coat proteins necessary for producing viable particles? In addition to the coat proteins, virion preparations of *Lettuce infectious yellows virus* (LIYV), a *Crinivirus*, contained virus-encoded HSP70h and p59 (Tian *et al.,* 1999), and ~10 molecules of HSP70h were physically associated with each virion particle of the *Closterovirus Beet yellows virus* (BYV) (Napuli *et al.,* 2000). Are these additional virus-encoded proteins that are found in virion preparations involved in virion formation or are they only contaminants of the purification procedure?

Closteroviridae members are the only viruses known to encode an HSP70 homolog of cellular molecular chaperone-like proteins (Karasev, 2000). The cellular HSP70 are ubiquitous proteins that are involved in a wide range of cellular processes, even in unstressed cells, including protein folding, assembly and disassembly of multi-subunit complexes, translocation into organelles, and intercellular transport (Hartl, 1996; Bukau and Horwich, 1998; Feder and Hofmann, 1999). The HSP70s have an *N*-terminal ATPase domain and a *C*-terminal domain (Flaherty *et al.,* 1990; Zhu *et al.,* 1996). The *N*-terminal ATPase domain displays a protein-binding property and the *C*-terminal domain plays a critical role in HSP70 interaction with target proteins (Boorstein *et al.,* 1994; James *et al.,* 1997). Computer analysis revealed that the motifs identified in the ATPase domain of cellular HSP70s are conserved in closteroviral HSP70h proteins, whereas the more variable *C*-terminal domain showed limited homology between cellular and closteroviral HSP70h proteins (Agranovsky *et al.,* 1991; Bork *et al.,* 1992). Do the closteroviral HSP70h function as molecular chaperones? It has been reported that BYV HSP70h has an ATPase activity, but does not appear to bind to denatured proteins (Agranovsky *et al.,* 1997).

The ~p60, which is conserved among the members of the *Closteroviridae*, also has been shown to be associated with virion preparations (Tian *et al.,* 1999). The corresponding genes of CTV (p61), BYV (p64), and *Grapevine leaf roll-associated virus*-2 (p63) have been reported to contain conserved motifs with sequence similarity with another cellular chaperone, HSP90 (Koonin *et al.,* 1991; Pappu *et al.,* 1994; Zhu *et al.,* 1998). However, the significance of that similarity has been questioned (V. V. Dolja, personal communication) and other members of the family do not possess this level of similarity with HSP90.

We examined the ten 3′ genes of CTV, which are not required for replication, for involvement in virion formation. We report that both coat proteins plus the HSP70h and p61 are needed for efficient virion formation based on serial passages of virions in protoplasts and serologically specific electron microscope (SSEM) analysis. Thus, all but the p6 of the conserved "signature gene module" of the *Closteroviridae* are involved in virion assembly of this closterovirus.

RESULTS

Assay for virion formation

The first requirement of this study was to develop an effective assay system to examine CTV virion formation. We reasoned that the most stringent assay would be a functional assay to examine the ability of the capsid proteins to protect the genomic RNA. Thus, we examined whether virions could withstand incubation in crude sap by assaying their ability to be passaged from protoplasts to protoplasts.

The CTV full-length RNA transcripts infect protoplasts with very low efficiency, since *in vitro* transcripts containing the green fluorescent protein ORF from jellyfish (Chalfie *et al.,* 1994), fused in-frame to the p20 gene (Gowda *et al.,* 2000), infected less than 0.1% of protoplasts (unpublished data). Yet, smaller CTV RNAs (~12–17 kb), mutants with large deletions, infected an exponentially greater proportion of protoplasts (Satyanarayana *et al.,* 1999), suggesting that the large size of the RNA decreases the efficiency of protoplasts infection. However, virions infect an even greater proportion of protoplasts (Navas-Castillo *et al.,* 1997), and remarkably, crude sap from infected bark tissue is several fold more infectious than purified virus preparations (Mawassi *et al.,* 2000). Inoculation with 1:10,000 diluted crude sap from infected bark tissue resulted in higher levels of infectivity than our most optimal inoculation with full-length RNA transcripts (data not shown). Using crude sap as an inoculum, up to 80% of the protoplasts became infected (unpublished data). Thus, wild-type virions in crude sap are very infectious.

Passage of virions from protoplasts to protoplasts using crude sap exposes the virions to nucleases present in the sap. To examine whether this process discriminates between virions and free RNA, *in vitro* transcripts of wild-type CTV from pCTV9 and the free-RNA mutant CTV-ΔCla, which has all of the 3′ ORFs deleted including both coat proteins (Fig. 1A; Satyanarayana *et al.,* 1999), were used to inoculate *Nicotiana benthamiana* protoplasts. The primary protoplasts were lysed at 4 days postinoculation (dpi), and the resulting crude sap was used to inoculate another batch of protoplasts. Replication in each passage was examined by Northern blot hybridizations. In the primary protoplasts that were inoculated with RNA transcripts, the level of accumulation of CTV-ΔCla RNA was much higher than that of the full-length wild-type CTV9 (Fig. 1B). In the secondary protoplasts (1st passage), the level of accumulation of the wild-type virus was ~40 times more than that in the primary protoplasts. In contrast, there was no evidence that the secondary protoplasts were infected with CTV-ΔCla (Fig. 1B). These data suggested that the free RNA of

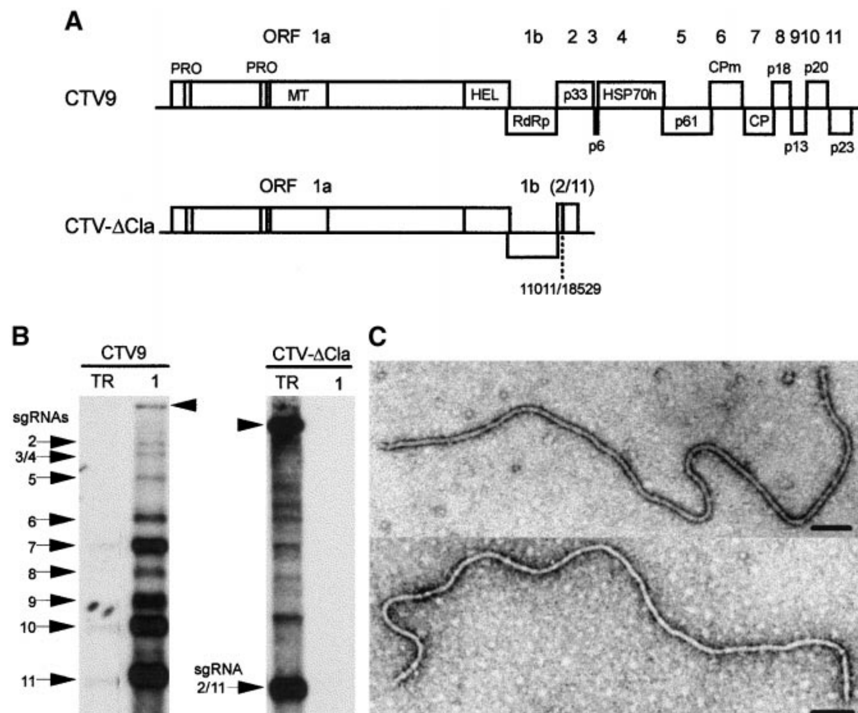

FIG. 1. (A) Genome organization of wild-type CTV (CTV9) and deletion mutant CTV-ΔCla showing putative domains of papain-like protease (PRO), methyltransferase (MT), helicase (HEL), RNA-dependent RNA polymerase (RdRp), and ORFs (open boxes) with respective numbers and translation products. HSP70h, HSP70 homolog; CPm, minor coat protein; CP, major coat protein; 2/11 represent fusion of ORFs 2 and 11. (B) Replication of CTV9 and CTV-ΔCla from *in vitro* transcripts (TR) and subsequent passage (1) in sap prepared from 4 dpi *Nicotiana benthamiana* protoplasts. Northern blot hybridizations were carried out using a 3′ positive-stranded RNA-specific probe. The positions of genomic and subgenomic RNAs of ORFs 2–11 are indicated by arrowheads and arrows, respectively. (C) Negative stained electron micrographs showing full-length virus particles from CTV9-transfected protoplasts at 4 dpi. Bar, 100 nm.

CTV-ΔCla was digested by endogenous ribonucleases in the sap while the RNA of CTV9 was protected and transferred efficiently to the next batch of protoplasts. To confirm that the protected viral RNA was encapsidated as virions, the sap extract from the primary protoplasts was examined by electron microscopy (EM). Complete virions were observed in the sap extract of CTV9-infected protoplasts (Fig. 1C), whereas no particles were observed in the sap extract of CTV-ΔCla-infected protoplasts. We also attempted to examine assembly by immunocapture RT-PCR, but were unable to eliminate false positives from mutants with no coat protein genes.

Both coat proteins are essential for efficient formation of viable virions

To examine whether viable virions were produced in the absence of either CP or CPm, coat protein mutations were generated in pCTV9 (Fig. 2A). CTV-ΔCPm had an in-frame deletion of 62 amino acid codons between nts 15606 and 15793. CTV-ΔCP contained an out-of-frame deletion between nts 16542 and 16661, translating only the *N*-terminal 129 amino acids of the CP (Fig. 2A). In general, both mutants failed to be transferred to the secondary protoplasts (Fig. 2B). However, in one experiment there was a weak transfer of CTV-ΔCPm to the secondary protoplasts, resulting in detectable accumulation of viral RNA, but the transfer to the third batch of protoplasts failed (data not shown). This result suggested that some virus particles were produced with CP alone to sufficiently protect the RNA to allow some infection of secondary protoplasts.

The accumulation of virus particles in the primary protoplasts infected with the RNA transcripts was too low to be effectively examined by electron microscopy directly because of the low percentage of protoplasts infected with large RNA transcripts and the poor assembly of the mutants. Therefore, we used SSEM which uses virion-specific antibodies attached to EM grids to concentrate virus particles from dilute samples (Derrick and Brlansky, 1976). In wild-type CTV9-infected protoplasts, full-length and small (partial or broken) particles were found at a frequency of 41.6 ± 10.3 and 6.7 ± 2.1 per mesh in 200 mesh copper grid, respectively. Using antibodies to CP to capture particles from CTV-ΔCPm-infected protoplasts, we failed to detect full-length particles encapsidated only by CP, but found small particles ranging from 200-1000 nm (6.2 ± 2.3 per mesh; Table 1; Fig. 2C). We used antibodies to CPm to capture CTV-ΔCP particles and found only small particles of 100–150 nm in length at a level of 6.9 ± 1.9 per mesh (Table 1; Fig. 2C).

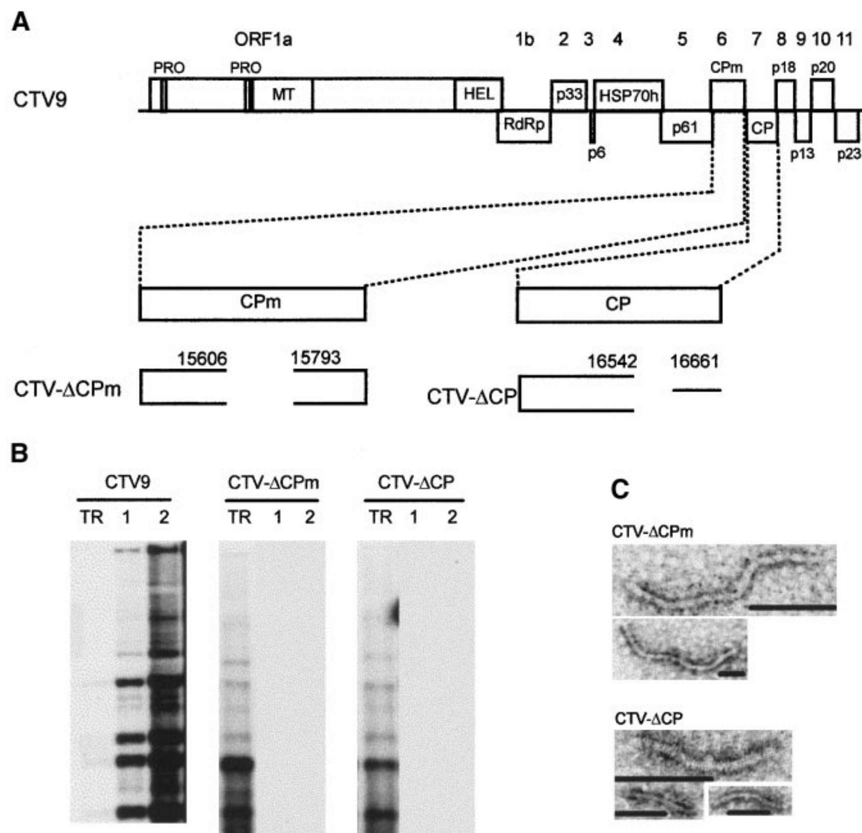

FIG. 2. (A) Diagrams of wild-type CTV (CTV9) and deletion mutants in CPm (CTV-ΔCPm) and CP (CTV-ΔCP); boxes, translatable sequences; lines, nontranslatable sequences; gaps, deletions. (B) Replication of CTV9, CTV-ΔCPm, and CTV-ΔCP from *in vitro* transcripts (TR), and serially passaged to 1st (1) and 2nd (2) transfers in 4-dpi protoplasts sap. Northern blot hybridizations were carried out using a 3′ positive-stranded RNA-specific probe. (C) Electron micrographs of virus particles from CTV-ΔCPm- and CTV-ΔCP-transfected protoplasts. Bar, 100 nm.

Although some mostly incomplete particles were produced in the absence of either coat protein, these results indicate that both coat proteins were needed for efficient production of viable virions.

TABLE 1

The Number of Full-Length and Small CTV Particles in *Nicotiana benthamiana* Protoplasts Transfected with Different Mutants

	Number of virus particles[a]	
Construct/mutant	Full-length	Small
CTV9	41.6 ± 10.3	6.7 ± 2.1
CTV-Δp33	38.3 ± 13.3	5.7 ± 2.8
CTV-Δp33-p61	0.4 ± 0.5	9.5 ± 2.7
CTV-Δp33-HSP70h	0.5 ± 0.7	12.3 ± 3.8
CTV-ΔHSP70h-p61	0.2 ± 0.4	12.2 ± 4.3
CTV-Δp33ΔHSP70h (ΔNC-domains)	0.2 ± 0.4	2.5 ± 1.5
CTV-Δp33Δp61C	0.2 ± 0.4	2.2 ± 1.1
CTV-Δp33Δp61N	0	6.2 ± 2.3
CTV-ΔCPm	0	6.2 ± 2.3
CTV-ΔCP	0	6.9 ± 1.9

[a] Number of CTV particles per mesh in 200 mesh copper grid. The virus particles in 10-15 mesh areas (average number of particles per mesh ± standard deviation).

Examination of the role of other 3′ genes in virion formation

To examine the possible role of other proteins encoded by the 3′ genes in virion formation, we generated a series of mutants either with deletions within the ORFs or deletions in the intergenic regions upstream of the ORFs, which abolished the production of the respective sgRNAs.

p33. The requirement of p33 ORF in virion formation was examined by deleting ~80% (754 nts) of the gene to obtain CTV-Δp33 (Fig. 3A, b). The ability of this mutant to form virions was examined by serial passages in protoplasts using crude sap as inoculum. The high levels of accumulation of genomic and sgRNAs in serially passaged protoplasts indicated that CTV-Δp33 was passaged efficiently, similar to that of wild-type CTV9 (Fig. 3B, b). SSEM analysis also revealed that CTV-Δp33 produced approximately the same number and quality of virions as the wild-type virus (Fig. 3C; Table 1). These results demonstrated that p33 was not required for virion formation.

The size of the CTV genome and the lack of unique restriction endonuclease sites requires multiple steps of

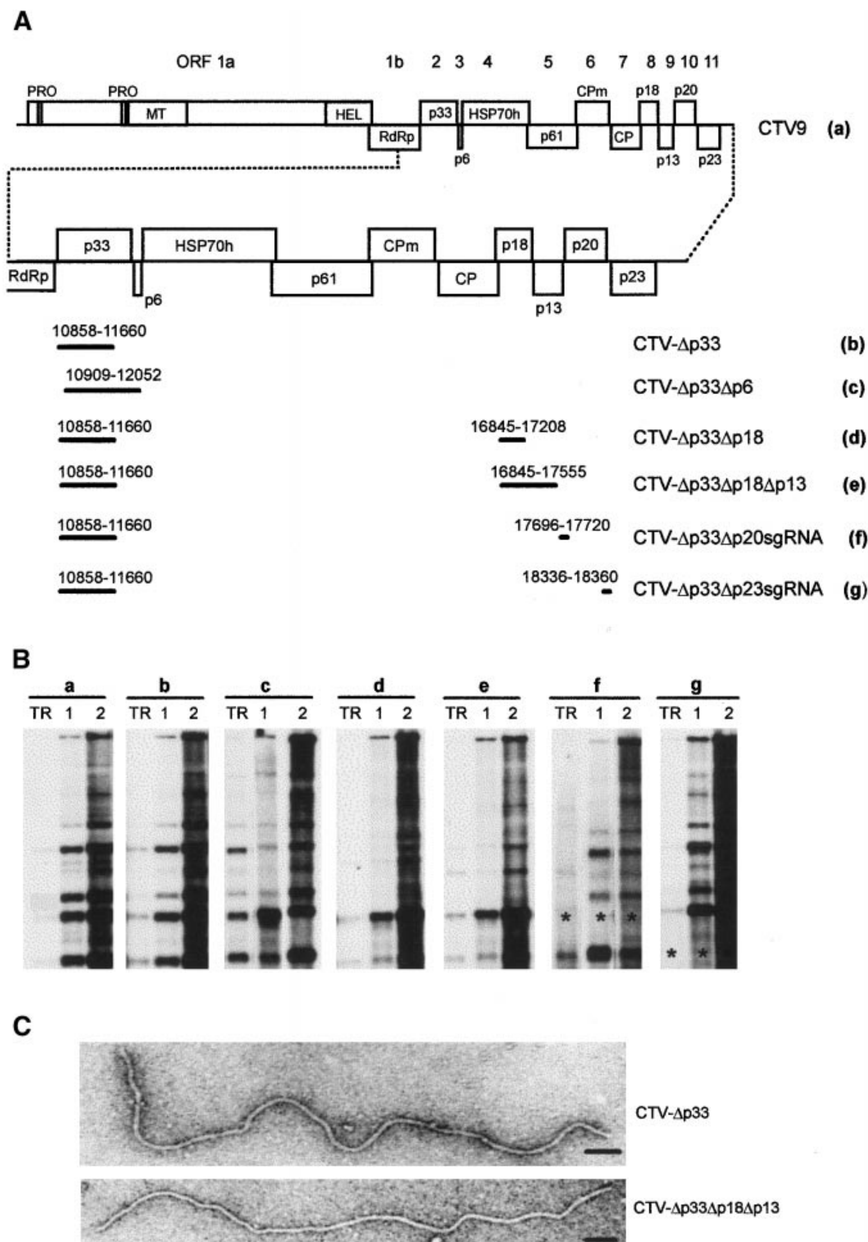

FIG. 3. (A) Schematic diagrams of wild-type CTV (CTV9) (a) and deletion mutants (b–g). The deleted regions are shown as thick horizontal lines with corresponding nucleotide numbers. (B) Replication of CTV9 (a) and deletion mutants (b–g) from *in vitro* transcripts (TR) or crude sap from 4-dpi protoplasts as inoculum in 1st (1) and 2nd (2) passages. Northern blot hybridizations were probed using a 3′ positive-stranded RNA-specific probe. Absence of p20 and p23 subgenomic (sg) RNAs from CTV-Δp33Δp20sgRNA- and CTV-Δp33Δp23sgRNA-transfected protoplasts, respectively, indicated by asterisks. (C) Electron micrographs of negative-stained virus particles from CTV-Δp33- and CTV-Δp33Δp18Δp13-transfected protoplasts at 4 dpi. Bar, 100 nm.

ligations and transformations to create CTV mutants from pCTV9. However, CTV-Δp33 provides convenient restriction sites that greatly simplifies mutations within the 3′ ORFs. Mutations in 3′ genes could be introduced with one simple ligation procedure into CTV-Δp33 compared to two to four ligation and transformation steps required to introduce the same mutations into pCTV9. Since CTV-Δp33 appeared to be identical to wild-type CTV in virion formation, we created a series of mutants from this construct to examine the requirement of other 3′ genes for virion formation.

p6. We examined the requirement of p6 in virion formation by fusing the p33 start codon to the second amino acid codon of HSP70h ORF to obtain CTV-Δp33Δp6, thus deleting both p33 and p6 ORFs completely (Fig. 3A, c). This manipulation probably destroyed the HSP70h sgRNA promoter, but placed the HSP70h ORF under control of the p33 sgRNA promoter. The virions produced

by CTV-Δp33Δp6 were passaged efficiently in sap to the next batch of protoplasts, with approximately the same level of accumulation of RNAs as CTV9 and CTV-Δp33 (Fig. 3B, c), demonstrating that neither p33 nor p6 was involved in virion formation.

p18 and p13. Deletions within the p18 or p13 ORFs were made in CTV-Δp33, retaining the 3′ nts of each ORF enough to provide promotion of the sgRNA of the corresponding downstream ORF. Thus, sequences between nts 16845 and 17208 comprising 73% of p18 ORF were deleted in CTV-Δp33Δp18 (Fig. 3A, d). The ORFs encoding CP and p18 overlap by 35 nts. To avoid deletion of CP ORF sequences, the deletion began after the end of CP through most of p18 ORF leaving 150 nts upstream of the start site of p13 ORF, thus retaining the p13 sgRNA promoter (Fig. 3A, d). Crude sap from CTV-Δp33Δp18-infected protoplasts was serially passaged with only slightly lower efficiency than that of CTV-Δp33 (Fig. 3B, d). The accumulation of the RNAs in the third set (2nd passage) of protoplasts was ~60-fold higher than that in primary protoplasts inoculated with *in vitro* RNA transcripts, suggesting that p18 was not required for virion formation.

CTV-Δp33Δp18Δp13 lacked p33, almost all p18, and 60% of p13 ORFs, but retained the 3′ 133 nts of p13 ORF, which contains the p20 ORF sgRNA promoter (Fig. 3A, e). Based on the accumulation of the RNAs in serially passaged protoplasts, this mutant also was readily transferred in crude sap, but apparently with less efficiency compared to the parental mutant CTV-Δp33 (Fig. 3B, e). Further passage to a fourth set of protoplasts resulted in high accumulation of RNA (data not shown), indicating that also p13 was not needed for virion formation. Corresponding full-length virus particles were observed by SSEM from CTV-Δp33Δp18Δp13-infected protoplasts similar to CTV-Δp33 (Fig. 3C).

p20 and p23. Parallel experiments have shown that deletions within the region of the extreme 3′ ORFs of CTV RNA, particularly the p20 and p23 ORFs, affect the amplification of free positive strands of genomic and subgenomic RNAs. Mutants with large deletions in this region tend to produce a ratio of positive-to-negative strands approaching 1:1 (unpublished data). Thus, to produce mutants with sufficient positive-sense single-stranded genomic RNA as a substrate for assembly, we attempted to create mutants unable to express these genes without making large deletions. CTV-Δp33Δp20sgRNA and CTV-Δp33Δp23sgRNA were generated by deleting nts −20 to +5 with respect to the sgRNA transcription start sites of p20 and p23 ORFs, respectively (Karasev *et al.,* 1997; Fig. 3A, f, g). This manipulation destroyed the ability of the mutants to synthesize the corresponding sgRNA (Fig. 3B, f, g). Following the transfections with transcript RNAs, these mutants were efficiently passaged via sap to the next batch of protoplasts resulting in levels of viral RNA accumulation approximately the same as that of CTV-Δp33 (Fig. 3B, f, g). These data suggested that neither p20 nor p23 was involved in virion formation.

HSP70h and p61. We next examined the involvement of HSP70h and p61 in virion formation by generating a series of mutations in these genes. CTV-Δp33-p61 lacked p33, p6, HSP70h, and p61 ORFs. In this mutant, the ORF 1b stop codon at nt 10858 was fused to nt 15118, leaving 245 nts of the 3′ end of p61 ORF, to maintain the sgRNA promoter of CPm (Fig. 4A, b). In CTV-Δp33-HSP70h, the ORF 1b stop codon was fused to nt 13540 in the 3′ end of the HSP70h ORF which deleted the entire p33 and p6 ORFs and most of HSP70h ORF, but retained 293 nts at the 3′ end to maintain the sgRNA promoter of p61 ORF (Fig. 4A, c). To exclude the possible effects of p33 and p6 deletions on function of the HSP70h and p61 proteins in virion formation, the HSP70h and p61 ORFs also were deleted in CTV9 to obtain CTV-ΔHSP70h-p61. The region between *Sph*I (nt 12168) and *Bst*EII (nt 15072) were deleted in pCTV9, resulting in a +1 frameshift at the ligation site (Fig. 4A, d). This mutant is predicted to translate only the *N*-terminal 40 amino acids of HSP70h and none of the p61 protein, while having functional p33 and p6 genes. The accumulation of CP from the mutant-transfected protoplasts was examined by SDS–PAGE and Western immunoblot analysis and revealed that mutations in HSP70h and/or p61 ORFs did not affect the expression of CP (data not shown).

Apparently because of the reduced size of these transfecting RNAs resulted in higher percentage of infected protoplasts, these mutants accumulated ~20–40 times more than that of the wild-type CTV9 in the primary protoplasts (Fig. 4B). Most of the time, these mutants were passaged by sap from primary protoplasts to the next set of protoplasts. However, the levels of accumulation of viral RNAs in the secondary protoplasts (1st passage) were ~30–40 times less than that of the wild-type virus (Fig. 4B). When transferred by sap to a third set of protoplasts (2nd passage), these mutants either failed to be transferred or the levels of accumulation were reduced further, estimated to be >100-fold lower than that of the wild type (Fig. 4B).

The ability of these mutants to be transferred in sap, albeit at reduced levels, suggested that some full-length virions were formed. Therefore, we examined by SSEM the nature of virus particles formed by these mutants (Fig. 4C). Few full-length particles and many small particles were found in extracts of the protoplasts infected with the HSP70h and/or p61 deficient mutants (Fig. 4C; Table 1). CTV-Δp33-p61, CTV-Δp33-HSP70h, and CTV-ΔHSP70h-p61 formed 0.4 ± 0.5, 0.5 ± 0.7, and 0.2 ± 0.4 complete/large particles per grid, respectively, compared to 41.6 ± 10.3 for CTV9, but many more short particles were found (Table 1). Immunogold labeling with CP-specific antibodies revealed that short particles produced from HSP70h and/or p61 deficient mutants were

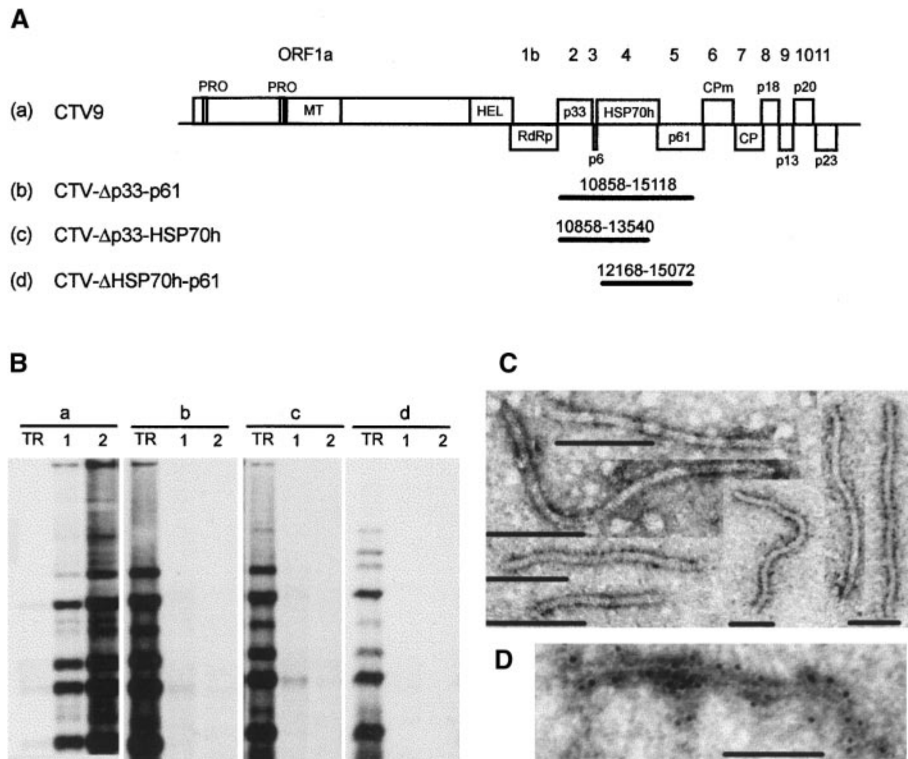

FIG. 4. Deletion mutational analysis of HSP70h and/or p61. (A) Genome organization of wild-type CTV (CTV9) (a) and the deletion mutants (b–d). The deleted regions are shown as thick horizontal lines with corresponding nucleotide numbers. (B) Replication of CTV9 (a) and the deletion mutants (b–d) from *in vitro* transcripts (TR), and subsequent 1st (1) and 2nd (2) passage of virions in crude sap from 4-dpi protoplasts. Northern blot hybridizations were carried out using a 3′ positive-stranded RNA-specific probe. (C) Electron micrographs of negative-stained virus particles from the deletion mutants (b–d) and (D) decoration with 10-nm gold particles locating the CP antibodies from the deletion mutant (b). Bar, 100 nm.

decorated with gold particles and are indeed CTV specific (Figs. 4D, 5D, and 6D). The greatly reduced ability of serial passage of these mutants in sap, coupled with the formation of mostly small/incomplete particles, suggested that both HSP70h and p61 were required for efficient assembly of virions.

Further examination of the HSP70h

The role of the computer-predicted *N*-terminal ATPase domain and *C*-terminal domain of CTV HSP70h (Agranovsky *et al.,* 1991; Pappu *et al.,* 1994) in virion formation was examined with mutants with a deletion in either of these domains and a mutant with deletion of both domains (Fig. 5A). The ΔATPase mutant contained an in-frame deletion of 153 amino acid codons between nts 12168 and 12628 in the *N*-terminal domain. Mutant Δ*C*-domain had a deletion of 71 amino acid codons between nts 13233 and 13446, which resulted in a +1 frameshift at the ligation site, thus deleting the *C*-terminal domain. The third mutant, Δ*NC* domains, had an internal in-frame deletion of parts of the *N*-terminal ATPase and *C*-terminal domains between nts 12168 and 13570, but retained the sequences encoding 40 amino acid residues of the *N*-terminal domain and 90 amino acid residues of the *C*-terminal domain. All three mutants failed to be transferred efficiently through protoplasts using sap, suggesting that both HSP70h domains were essential for efficient virion formation (Fig. 5B). SSEM analysis of the particles produced by Δ*NC* domains revealed a few large particles (0.2 ± 0.4 per mesh) and small particles (2.5 ± 1.5 per mesh) compared to CTV-Δp33 (38.3 ± 13.3 large particles and 5.7 ± 2.8 small particles per mesh; Fig. 5C; Table 1).

To exclude the possibility that these mutations resulted in loss of a *cis*-acting sequence in the RNA or an origin of assembly that was needed for virion assembly rather than the encoded protein, we generated a frameshift mutant, HSP70hFS. This mutant contained a +1 frameshift at nt 12168, which resulted in the expression of a truncated protein of *N*-terminal 40 amino acid residues (Fig. 5A). The failure of HSP70hFS to serially passage in protoplasts suggested that the HSP70h protein, but not the RNA sequence, was required for efficient virion assembly (Fig. 5B).

The requirement of the ATPase activity of HSP70h for virion assembly was examined by introducing two mutations targeting key amino acid residues in "phosphate 1" and "connect 1" motifs in the ATPase domain (Agranovsky *et al.,* 1991), similar to mutations introduced in HSP70h of BYV to examine cell-to-cell movement ac-

FIG. 5. Analysis of HSP70h in virion formation. (A) Schematic diagrams of the HSP70h ORF with *N*-terminal ATPase and *C*-terminal domains, and the mutants with deletions (gaps) in *N*-terminal (ΔATPase), *C*-terminal (Δ*C* domain), and in both domains (Δ*NC* domains), +1 frameshift (HSP70h-FS), and with changes of Asp^7 and Glu^{180} of "phosphate 1" and "connect 1" motifs, respectively, to Lys and Arg (HSP70h-ΔPho1 and HSP70h-ΔCon1). Boxes and solid lines represent translatable and nontranslatable sequences, respectively. (B) Replication of HSP70h mutants from *in vitro* transcripts (TR), 1st (1) and 2nd (2) serial passage of sap from 4-dpi protoplasts. Northern blot hybridizations were probed using a 3′ positive-stranded RNA-specific probe. (C) Electron micrographs of negative-stained virus particles from Δ*NC* domains-transfected protoplasts and (D) decoration with 10-nm gold particles after treating with CP antibodies. Bar, 100 nm.

tivity (Peremyslov *et al.,* 1999). The conserved negatively charged Asp^7 and Glu^{180} residues of phosphate 1 and connect 1 motifs, respectively, which are critical for ATP binding through interaction with Mg^{2+} ions (Bork *et al.,* 1992), were replaced with positively charged lysine and arginine residues in HSP70hΔPho1 and HSP70hΔCon1 mutants, respectively (Fig. 5A). These mutants either failed or were weakly passaged to the second batch of protoplasts and failed to be transferred further, suggesting that the predicted ATPase domain of the HSP70h needs to remain intact for efficient virion formation (Fig. 5B).

Further analysis of p61

The homologs of CTV 61-kDa protein are conserved among all *Closteroviridae* members. To further examine the functional requirement of p61 in virion formation, we introduced deletions, frameshift, and stop codons to cause premature termination of protein translation. Mutant Δp61N had an in-frame deletion of 198 amino acid codons (13843–14438 nts) in the *N*-terminal region (Fig. 6A). A second mutant, Δp61C, contained a deletion (14510–15073 nts) followed by a +1 frameshift with predicted translation of a truncated protein with the *C*-terminal half of the protein deleted (Fig. 6A). These mutants either failed or passaged at low efficiency to the next batch of protoplasts (Fig. 6B) and thus were similar to the HSP70h mutants. SSEM examination of the primary protoplasts extract revealed that Δp61C produced only 0.2 ± 0.4 complete virus particles and 2.2 ± 1.1 small particles per mesh (Fig. 6C; Table 1). We failed to detect any complete virion particles from Δp61N-infected protoplasts and detected only 6.2 ± 2.3 small particles per mesh (Fig. 6C; Table 1). These results confirm that the intact p61 ORF was needed for efficient virion formation.

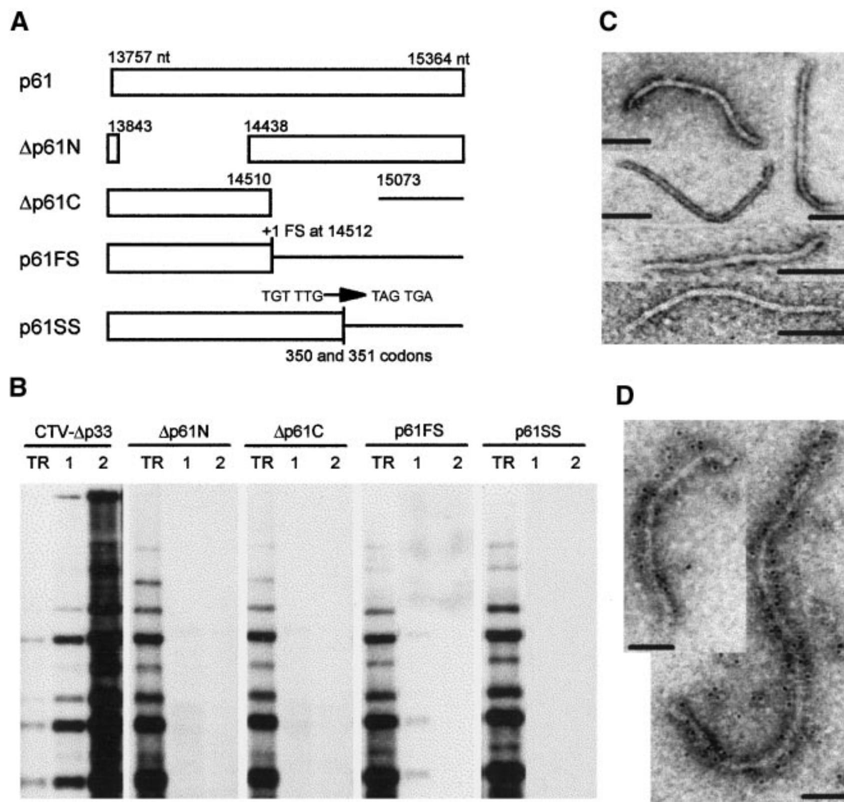

FIG. 6. Analysis of the p61 requirement in virion formation. (A) Schematic diagrams of mutants with deletions (gaps) (Δp61N, Δp61C), +1 frameshift (p61FS), and two stop codons (p61SS). Boxes and solid lines represent translatable and nontranslatable sequences, respectively. (B) Replication of p61 mutants from *in vitro* transcripts (TR), 1st (1) and 2nd (2) serial passage from sap of 4-dpi protoplasts. Northern blot hybridizations were carried out using a 3′ positive-stranded RNA-specific probe. (C) Negative stained and (D) 10-nm gold-labeled virus particles from p61 mutants (Δp61N and Δp61C) after treating with CP antibodies. Bar, 100 nm.

It is possible that the deletions removed *cis*-acting elements in the RNA sequence that affected assembly rather than the mutation of the encoded protein. We generated two mutants, p61FS and p61SS, to determine whether virion assembly would occur in the absence of the functional protein but with retention of the RNA sequence (Fig. 6A). Mutant p61FS contained a +1 frameshift at nt 14512 and could express a truncated protein containing the *N*-terminal 252 amino acids of p61. Mutant p61SS contained two stop codons at the position of amino acid codons 350 and 351. This mutant should express the *N*-terminal 349 amino acids of p61 (Fig. 6A). Neither p61FS nor p61SS was efficiently transferred via sap beyond the first passage (Fig. 6B), suggesting that the p61 protein rather than a *cis*-acting sequence was required for efficient virion assembly.

DISCUSSION

The objective of this work was to examine CTV genes involved in virion formation, because production of progeny virions by encapsidation of the viral genome in a proteinaceous capsid/nucleocapsid is one of the most important steps in the virus life cycle. However, large RNA viruses like CTV present special problems in the examination of the genetics of virion formation. Full-length RNAs of CTV are extraordinarily inefficient as inoculum for protoplasts. In our hands, the inoculation of protoplasts with CTV deletion mutants that reduce the size of the RNA from 19.3 to 12–17 kb, or the use of full-length BYV transcripts (Peremyslov *et al.,* 1998) which are 15.5 kb, results in substantially greater infection. This appears not to be due to the inability of full-length CTV to replicate in protoplasts, because inoculation of sap-containing virions from infected cells which can infect up to 80% of protoplasts results in maximal levels of progeny RNA accumulation. “Good” virions in sap were very infectious and could be passaged at low dilutions, but the RNA of incomplete virus particles apparently was degraded in the crude sap. We used two approaches to examine virions in the few primary infected protoplasts. The first was to examine infectivity of virions incubated in crude sap by serially passaging in *N. benthamiana* protoplasts. The second approach was to examine virus particles using SSEM to concentrate the particles from the few infected protoplasts.

CTV contains ORFs 1a and 1b of the “replication gene

block" in the 5′ half, and a "signature gene module" of five genes plus other five genes in the 3′ half of the genome. Of the latter five genes, the p33, p18, and p13 have unknown functions, while p20 and p23 were reported as an inclusion body and an RNA binding protein, respectively (Gowda *et al.,* 2000; López *et al.,* 2000). We could not examine the effect of the replication-associated proteins on assembly, because mutations within these sequences were lethal or greatly decreased replication. We examined the involvement of the 3′ genes in CTV virion formation by a series of mutations. Deletion mutagenesis revealed that the ORFs outside of the signature gene module (p33, p18, p13, p20, and p23) were not required for virion formation. The reduced efficiency of passage of the mutants with deletions in p18 and p13 ORFs was probably due to involvement of these sequences in amplification of free positive-stranded genomic and sgRNAs (unpublished data). However, excluding p6 gene, the other genes of the "signature gene module" (HSP70h, p61, CPm, and CP) appear to be involved in virion assembly. If this "signature gene module" was primarily involved in assembly, it would be expected that all the genes in this module would be required for virion formation, but the p6 gene appeared to have no significant effect on virion formation.

As expected, both coat proteins were needed for efficient virion formation. Mutations in the CP gene resulted in the loss of ability to be passaged in sap and no full-length virus particles were detected by SSEM (Figs. 2B and 2C). However, the detection of some smaller virus-like particles suggests that the CPm might have attempted assembly in the absence of the CP. Although we did not detect any full-length particles with CPm deletion, there was a low level of passage in one experiment suggesting some protection of genomic RNA. Results obtained from deletion of CPm suggest that the CP possibly was more efficient in initiating assembly than CPm in the absence of the other coat protein, but by itself was not capable of efficient assembly. This is similar to a previous report that the BYV CP can encapsidate the genomic RNA in the absence of CPm (Alzhanova *et al.,* 2000). One possibility is that CP normally assembles first, producing a "fire cracker-like" structure with a small portion of unassembled RNA at one end. This could be followed by completion of assembly by CPm. However, in absence of CPm we did not find particles of the expected length of "fire cracker" structures. It appears that some assembly could occur in the absence of CPm, but most particles were short, and the lengths apparently were variable. However, some particles must have had the entire genome protected since there was some infectivity in crude sap from these protoplasts.

The HSP70h protein appears to be required for efficient virion formation. Mutations in the HSP70h gene resulted in large decreases in the ability of the virus to be passaged in crude sap and in substantial reductions in the proportion of full-length particles (Figs. 5B and 5C; Table 1). The other gene that was required for efficient virion formation was p61, which possesses limited homology with the cellular HSP90 (Pappu *et al.,* 1994). The HSP70h and p61 genes appeared to be equally required for the efficient formation of virus particles, suggesting that they might work together. Cellular proteins function jointly as molecular chaperones, either by forming complex macromolecular machines or by providing separate but tandem functions (Kimura *et al.,* 1995; Hutchison *et al.,* 1994; Buchner, 1999). The cellular HSP90 acts together with HSP70 for reverse transcription and assembly of hepadnavirus (Hu *et al.,* 1997).

If the HSP70h and p61 function as chaperones, it is puzzling that closteroviruses would encode their own chaperones instead of using host chaperones. There are numerous examples of viruses utilizing host chaperones in function and/or assembly (Macejak and Sarnow, 1992; Jindal and Young, 1992; Cripe *et al.,* 1995; Hu *et al.,* 1997). It is possible that the formation of some full-length virus particles with mutations in HSP70h and/or p61 might be due to the involvement of cellular chaperones in CTV assembly in the absence of virus-encoded chaperones. It is equally puzzling why the coat proteins of these rod-shaped viruses would need molecular chaperones for assembly. The other rod-shaped viruses do not encode chaperone-like proteins, and there is little evidence that they are needed for assembly. Yet, correct assembly of *Tobacco mosaic virus* (TMV) coat protein into virions with heterologous RNAs in bacteria has been associated with host chaperones (Hwang *et al.,* 1998). In addition, the formation of several cellular macromolecules such as actin, tubulin, and bacterial pili have been shown to be dependent on molecular chaperones (Sakellaris and Scott, 1998; Purich and Southwick, 1999). Even though the wild-type CP of TMV assembles into virions *in vitro* and presumably in the plant without the help of molecular chaperones, numerous single amino acid substitutions in the TMV coat protein causes some of it to misfold. This results in the coat protein being proportioned between correct folding into virions and incorrect folding into amorphous inclusion bodies, which have been correlated with leaf yellowing due to chloroplast disorganization (Culver *et al.,* 1991; Lindbeck *et al.,* 1992). Although it would appear that normal TMV assembly, and presumably that of other rod-shaped plant viruses, might occur without the aid of molecular chaperones, there might be conditions that need them.

The HSP70h of LIYV and BYV, and the p61 analog of LIYV (p59) have been found to be associated with virion preparations (Tian *et al.,* 1999; Napuli *et al.,* 2000). The high stability of the HSP70h-virion complexes of ~10 molecules of HSP70h per virus particle (Napuli *et al.,* 2000) and the association of both proteins with LIYV virions (Tian *et al.,* 1999) suggests that they might be structural components of virions. It also is possible that

the association of these proteins with virion preparations is a result of their previous function during assembly. Their similarity to cellular chaperones supports their possible role as molecular chaperones to prevent aggregation or to assist in proper folding and assembly of coat protein aggregates in virion formation. However, we cannot eliminate the possibility that HSP70h and p61 are needed for virion stability. HSP70h and p61 analogs as components of the "signature gene module" of BYV have been reported to be essential for cell-to-cell movement (Peremyslov *et al.,* 1999; Alzhanova *et al.,* 2000). It is possible that these two gene products function both in virion formation and cell-to-cell movement. However, formation of virions is a prerequisite for cell-to-cell movement of many plant viruses (Vaewhongs and Lommel, 1995; Cruz *et al.,* 1998). With our present system, we cannot test whether HSP70h and/or p61 genes of CTV are also involved in cell-to-cell movement, because we can infect citrus plants only with mutants that are efficiently assembled and amplified by serial passages in protoplasts (unpublished data).

MATERIALS AND METHODS

Generation of CTV mutants in 3′ genes

The full-length cDNA clone pCTV9 and a self-replicating deletion mutant, pCTV-ΔCla (Fig. 1A), were described previously (Satyanarayana *et al.,* 1999). pCTV-Δp33 was obtained by fusing the ORF 1b stop codon to the 3′ of the p33 ORF (Fig. 3A), thus deleting most of p33 sequence (nts 10858–11660), but retaining the 3′ 154 nts of the ORF which were sufficient for the promotion of the p6 and HSP70h sgRNA(s). Nucleotide deletions, frameshifts, and amino acid codon changes were introduced in 3′ genes of pCTV9 or pCTV-Δp33 by polymerase chain reaction using primers with mutagenized sequences, and their corresponding nucleotide numbers are described in the text and appropriate figures. The nucleotide sequence and numbering are according to Karasev *et al.* (1995). When deletions were introduced by restriction enzyme digestion that created noncompatible ends, the single-stranded ends were blunt-ended with T4 DNA polymerase (New England Biolabs, Beverly, MA) prior to ligation. Deletions, frameshifts, and codon changes were confirmed by nucleotide sequencing.

Virion assembly assay in *N. benthamiana* protoplasts

The procedures for the isolation of mesophyll protoplasts from *N. benthamiana* and polyethylene glycol mediated transfection have been described (Navas-Castillo *et al.,* 1997; Satyanarayana *et al.,* 1999). The capped *in vitro* transcripts were generated from *Not*I-restriction enzyme linearized pCTV9 and its derivative mutants using SP6 RNA polymerase (Epicentre Technologies, WI) as described in Satyanarayana *et al.* (1999). Freshly prepared *in vitro* transcripts in 30 μl volume were used directly for inoculation of protoplasts (~1×10^6). Protoplasts were harvested at 4 dpi and divided into two halves; one half was stored at −70°C for subsequent transfer to the next batch of protoplasts and another half was analyzed by Northern blot hybridization as described in Satyanarayana *et al.* (1999). The 3′-terminal 900 nucleotides of CTV T36 in pGEM-7Zf (Promega) were used to make positive-stranded RNA-specific riboprobe with digoxigenin-labeled UTP.

The frozen (−70°C) protoplasts were resuspended in 100 μl of 40 mM sodium phosphate buffer, pH 8.2, and kept on ice for 20–30 min with intermittent gentle mixing. The sap was clarified at 3000 rpm for 3 min and 60 μl of supernatant at room temperature was used to inoculate the next batch of protoplasts. The crude sap containing virions was passaged 2–4 times through protoplasts. Each serial passage experiment with mutants included the wild-type CTV9 and/or CTV-Δp33 as a positive control and was repeated at least 3–5 times using two to three independent constructs of each mutant.

SSEM analysis and immunogold labeling

Crude extracts from protoplasts inoculated with CTV9 and its derivative mutants, at 4 dpi, were prepared by suspending the frozen protoplasts pellet in 40 mM sodium phosphate buffer, pH 8.2. Virus particles were adsorbed to carbon-coated copper grids using Protein A (0.1 mg/ml, Sigma) followed by treatment with CP- (709–11 IgG, at 1:1000 dilution) or CPm-specific (at 1:250 dilution) antiserum and stained with 2% aqueous uranyl acetate as described by Derrick and Brlansky (1976). In addition, virus particles were examined by coating with CTV CP-specific antibodies (3DFI monoclonal antibodies, 1:1000 dilution) and by decorating with 10-nm gold particles conjugated to goat anti-mouse IgG (Sigma) followed by staining with 2% aqueous uranyl acetate. The number of small (~100–1200 nm) and large (~1600–2000 nm) virus particles in proportion to the length of the corresponding RNA mutant per mesh was counted from at least 10 to 15 meshes. All the mutants assayed for virus particle formation were tested by SSEM and immunogold labeling from at least two independent transfections.

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