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Replication and Movement of a Coat Protein Mutant of Cymbidium Ringspot Tombusvirus

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The spread of cymbidium ringspot tombusvirus (CyRSV) in host tissue was studied by using a coat protein gene mutant with a six-nucleotide deletion; the deletion removes two amino acids from the shell domain (S) of the capsid protein. Mutated protein subunits were synthesized in infected cells but could not assemble into virus particles. Virions were formed, however, with inoculation of mutated RNA in transgenic plants expressing normal CyRSV coat protein. The mutant is restricted in long-distance movement in *Nicotiana clevelandii*, whereas it spreads systemically in *N. benthamiana*. These results suggest that tombusviruses may spread either as complete virions or in a nonvirion form, depending on the host plant species.

Tombusviruses have a genome composed of one single-stranded positive-sense molecule of about 4,700 nucleotides (nt), which contains five open reading frames that predict the production of at least five proteins of molecular weights of 33, 92, 41, 22, and 19 kDa (Martelli *et al.* 1989). The 33- and 92-kDa proteins are probably involved in the replication of viral RNA, because both the GDD motif of viral replicases described by Kamer and Argos (1984) (Martelli *et al.* 1989) and the NTP-binding consensus sequence of nucleic acid helicases (Habili and Symons 1989) are present in the read-through domain of the 92-kDa protein. The 41-kDa protein is the capsid protein. The 22- and 19-kDa proteins, which are encoded by the same region of genomic RNA, but in a different frame, have not been assigned a precise function, although evidence was provided that both are expressed *in vivo* and are either indispensable (22 kDa) or important (19 kDa) in the virus replication cycle (Rochon and Johnston 1991). Coat, 22-, and 19-kDa proteins are expressed via generation of two subgenomic RNAs (Martelli *et al.* 1989).

The best characterized members of the tombusvirus group are cymbidium ringspot (CyRSV), tomato bushy stunt (TBSV), and cucumber necrosis (CNV) viruses, because their genome organization and expression are known, and infectious *in vitro* RNA transcripts of all were obtained (Rochon and Tremaine 1989; Grieco *et al.* 1989a; Hearne *et al.* 1990; Burgyán *et al.* 1991; Rochon and Johnston 1991). Rochon *et al.* (1991) reported that CNV is able to spread in infected *Nicotiana clevelandii* A. Gray plants in the absence of detectable levels of encapsidated RNA.

A mutation was introduced into a portion of the coat protein of CyRSV that is essential for the maintenance of the virion structure; it was introduced without impairing the synthesis of the almost full-length coat protein. The effect of this mutation on virus assembly, replication, and spread in infected plants is reported in the present paper.

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MATERIALS AND METHODS

***In vitro* mutagenesis and transcription.** The full-length clone (pCyR-G) of CyRSV RNA used in these studies was the same as described by Burgyán *et al.* (1990), except that the last three nucleotides (GGG) were replaced by CCC. This clone, denoted G11, produces *in vitro* RNA transcripts with infectivity that is virtually as high as that of native viral RNA (T. Dalmay *et al.*, unpublished). *In vitro* mutagenesis was done essentially as described by Kunkel *et al.* (1987); a Bio-Rad Mutagenesis Kit (Bio-Rad, Richmond, CA) was used. A deletion of six nucleotides was introduced into the coat protein gene from nt 3,352 to 3,357. Mutants were identified by sequencing DNA in the region of interest; a 21-mer oligonucleotide starting from nt 3,176 (i.e., 176 nt upstream from the modified region) was used as primer. Transcription was carried out as previously described (Burgyán *et al.* 1990). Two micrograms of DNA template was used, which yielded approximately 8–10 μg of RNA.

Growth and inoculation of plants. Seedlings of *Nicotiana clevelandii* and *N. benthamiana* Domin. were grown in a growth chamber (14-hr/21° C light and 10-hr/16° C dark periods). Plants with three to four (*N. benthamiana*) or six to seven (*N. clevelandii*) expanded leaves were inoculated with *in vitro* transcribed RNA (estimated concentration 100 ng/μl); each leaf was rubbed with a sterile glass spatula with 10 μl of a mixture (1:1) of RNA and inoculation buffer containing Celite and bentonite (Heaton *et al.* 1989). All expanded leaves of each plant were inoculated. Subinoculations were done by using infected tissue ground in a small volume of the appropriate buffer.

Analysis of progeny viral RNA. RNA was extracted from 200 mg of inoculated or uninoculated leaves by grinding tissues in liquid nitrogen and resuspending the powder in glycine buffer containing 2% sodium dodecyl sulfate (SDS) and 1% Sarkosyl (White and Kaper 1989). RNA was resuspended in 100 μl of water after ethanol precipitation. Northern blotting of formaldehyde-denatured 5-μl samples was done by standard techniques (Sambrook *et al.* 1989). Probing was done with the ^{32}P-labeled nick-translated clone

pCyR-7, which represents the last 1-kb region of CyRSV RNA (Russo *et al.* 1988). Sequence analysis of progeny RNA in the mutated region was done by making a DNA copy with reverse transcriptase with a 21-mer oligonucleotide complementary to nt 3,560–3,580 in genomic RNA as primer, and amplifying it by polymerase chain reaction (PCR) with a 21-mer oligonucleotide homologous to nt 3,176–3,186 in genomic RNA as second primer. PCR was carried out in a Perkin Elmer Cetus DNA Thermal Cycler with the GeneAmp kit (Perkin Elmer, Norwalk, CT). Melting, annealing, and polymerizing steps were at 94° C (1 min), 45° C (1 min), and 72° C (2 min), respectively. dsDNA was made blunt-ended with Klenow enzyme, electrophoresed in agarose gel, electroeluted, ligated into the *Sma*I site of plasmid pUC18, and cloned in *Escherichia coli* strain DH5α. Other details of cDNA synthesis, PCR amplification, and cloning are in Burgyán *et al.* (1991). Recombinant DNA was prepared with the alkaline lysis method (Hattori and Sakaki 1986) and sequenced with T7 DNA polymerase (Sequenase, US Biochemicals, Cleveland, OH).

Protein extraction and Western blots. About 50 mg of leaf tissue was rapidly ground in 2 vol of sample buffer (Laemmli 1970), incubated at 100° C for 3 min, and fractionated by SDS-PAGE in 12.5% polyacrylamide gels without prior quantitation of protein content. Proteins were transferred to nitrocellulose, and virus coat protein was detected by antibodies raised in rabbit against purified virus particles and alkaline-phosphatase-conjugated anti-rabbit antibodies raised in goat (Sigma, St. Louis, MO). Blocking of membrane, incubation with antibodies, washings, and reaction with BCIP-NBT (5-bromo-4-chloro-3-indoyl phosphate para-toluidine salt [BCIP] and para-nitro blue tetrazolium chloride [NBT]) were essentially as described by Hibi and Saito (1985), except that skimmed milk was used as blocking agent.

Electron microscopy. Leaf tissue was fixed in glutaraldehyde, postfixed in osmium tetroxide, embedded, sectioned, and viewed as in Martelli and Russo (1977). Immune electron microscopy was as described by Milne and Luisoni (1977). Negative staining was with uranyl acetate.

RESULTS

Construction of a coat protein mutant. CyRSV RNA clone G11 was constructed by site-directed mutagenesis of the full-length clone pCyR-G (Burgyán *et al.* 1990) with substitution of CCC for the 3′ terminal GGG (T. Dalmay *et al.*, unpublished). Clone G11 was mutated with the oligo 5′-CTGCGTTTGTCCCTCCATACGTC-3′, which, by introducing a deletion of six nucleotides (lowercase letters) in the sequence 5′-GACGTATGGAGGGcccggaACAAACGCAG-3′, results in the deletion of two amino acids (proline 243 and glycine 244). This mutant was designated CP1 (Fig. 1). There was no change in the reading frame, and the expected product had virtually the same size as the native product. This was shown by SDS-PAGE of *in vitro* translation products of full-length G11 and CP1 RNA transcripts in rabbit reticulocyte lysates, in which a polypeptide corresponding in size to capsid protein was formed as well as the expected 33-kDa protein (not shown).

Ability of CP1 mutant to replicate and spread in infected plants. Plants of *N. clevelandii* and *N. benthamiana* were inoculated with uncapped RNA from *in vitro* transcriptions of clones G11 and CP1. Leaves of *N. clevelandii* reacted to inoculation with CP1 RNA within 4–5 days; numerous rounded necrotic lesions that enlarged into irregular patches of necrotic tissue were observed. Leaves inoculated with G11 RNA showed diffuse chlorotic lesions and fewer necrotic lesions (Fig. 2A,B), followed by systemic leaf distortion and apical necrosis. Upper uninoculated leaves of CP1-infected plants remained either symptomless or occasionally showed necrotic patches and distortion very similar to those of inoculated leaves (Fig. 2C). Systemic symptoms, when present, were limited to only one lateral shoot above the inoculated leaves. All plants that did not show systemic symptoms 7–10 days after inoculation remained symptomless.

Plants of *N. benthamiana* reacted similarly to both G11 and CP1; local lesions on inoculated leaves followed by crinkling of younger leaves and top necrosis were observed.

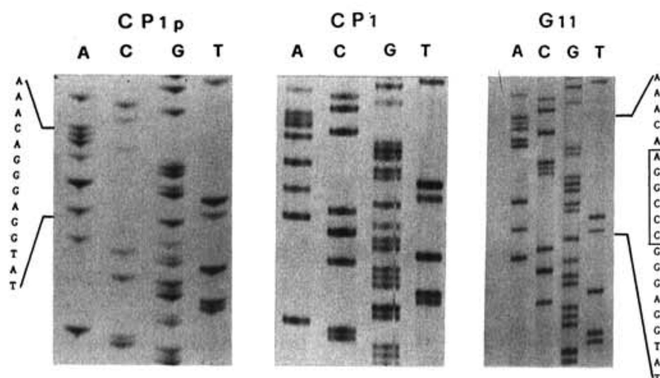

Fig. 1. Sequence of the progeny of cymbidium ringspot tombusvirus (CyRSV) coat protein mutant (CP1p) compared with the mutated (CP1) and original (G11) clones. Part of the deduced sequence is shown. Boxed nucleotides in the G11 sequence are deleted in CP1.

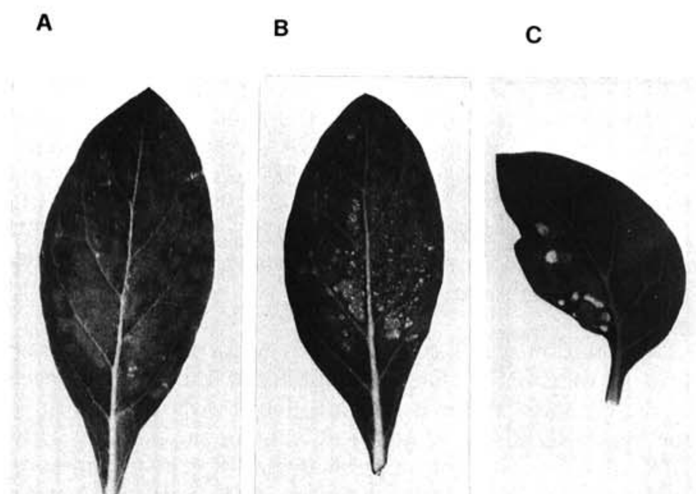

Fig. 2. A, Chlorotic mottle and **B,** necrotic lesions in inoculated leaves of *Nicotiana clevelandii* infected with G11 and CP1, respectively. **C,** Patches of necrotic tissue surrounding lesions in a leaf systemically infected with CP1.

Plants infected with CP1 developed symptoms 1–2 days after plants infected with G11 (not shown).

Total RNA was extracted from inoculated and upper leaves, and equal quantities were electrophoresed in agarose gels, blotted, and analyzed for the presence of CyRSV genomic, subgenomic, and defective interfering (DI) RNAs. Genomic and subgenomic RNAs were present in inoculated and upper leaves of *N. benthamiana* infected with either G11- or CP1-derived RNA transcripts. As judged from the intensity of bands in Northern blots and gels stained with ethidium bromide (not shown), the amount of genomic and subgenomic RNAs in leaves inoculated with either of the two transcripts was comparable. In contrast, the amount of viral RNA in leaves systemically infected with CP1 was consistently less than in the corresponding leaves infected with G11 (Fig. 3A). No RNA species migrating ahead of the smaller subgenomic RNA (approximately 0.9 kb) was detected, indicating that no *de novo* generation of DI RNA had occurred. As for CP1-inoculated *N. clevelandii*, CyRSV CP1 RNAs were found in extracts from necrotic tissue of inoculated leaves but not in preparations from green tissue of the same leaves or from upper, symptomless, uninoculated leaves. In the few cases in which systemic symptoms had developed, viral RNA was detected in extracts from necrotic tissue, but not from healthy looking surrounding tissue (not shown).

Stability of the CP1 mutant. CP1 could be propagated serially from plant to plant by sap inoculation. Infected *N. benthamiana* tissue was ground in a small volume of high pH (glycine) inoculation buffer, used for inoculation of RNA transcripts, and inoculated immediately to fresh *N. benthamiana* plants. RNA was extracted from infected plants at the third passage, and cDNA of the region of interest was prepared. This was amplified by PCR, cloned, and sequenced. As shown in Figure 1, the mutation was maintained with no evidence of revertants.

CP1 coat protein synthesis. Total proteins from leaves infected with CP1 or G11 were analyzed by SDS-PAGE and Western blotting. CyRSV coat protein was detected in CP1- and G11-infected samples from inoculated and systemically infected leaves of *N. benthamiana* (Fig. 4A,B) and from inoculated leaves of *N. clevelandii* (Fig. 4C), and in upper leaves of G11- but not CP1-infected *N. clevelandii* (not shown).

Because the CP1 mutant synthesizes a protein only two amino acids shorter than authentic coat protein, its ability to form virions was investigated. The results provided evidence that both CP1 moieties (RNA and coat protein) were synthesized in host tissues, but they were apparently unable to assemble into virions. This conclusion was based on the following: highly infectious sap from CP1-infected tissues extracted in 0.02 M acetate buffer, pH 5.5, lost infectivity within 1 hr, whereas sap from G11-infected controls retained a high level of infectivity, thus supporting the view that CP1 infectivity was due to unencapsidated RNA (Fig. 3B); no virus particles could be identified in thin sections of CP1-infected leaf tissue, although many multivesicular bodies (i.e., the possible site of viral RNA synthesis) (Russo *et al.* 1987) were present with patches of electron-dense material reminiscent of the intracellular accumulations of TBSV coat protein (Fig. 5D) (Martelli *et al.* 1989); and many virus particles were detected by immune electron microscopy in sap of G11-infected leaves, but none in CP1-infected tissues (Fig. 5A,B).

To determine whether the obstacle to virion assembly was due to mutated RNA or coat protein, or both, we inoculated *in vitro* transcripts of CP1 RNA into two transgenic *N. benthamiana* plants expressing CyRSV coat protein (L. Rubino and M. Russo, unpublished). Two weeks after inoculation, virus particles were detected in sap from both inoculated plants (Fig. 5C), but not in extracts from uninoculated transgenic plants. Virus was extracted from one of these plants in 0.1 M acetate buffer, concentrated with polyethylene glycol, and inoculated to *N. clevelandii*, which reacted with the local lesions typical of CP1. Analysis of viral RNA extracted from these plants

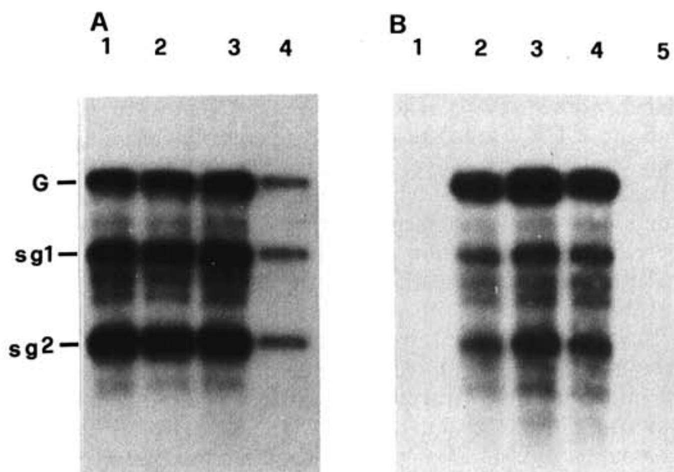

Fig. 3. Northern blots of RNA extracts from *Nicotiana benthamiana* plants infected with G11 or CP1. **A,** Inoculation with *in vitro* G11 (lanes 1 and 2) and CP1 (lanes 3 and 4) transcripts. RNA preparations from inoculated (lanes 1 and 3) and systemic (lanes 2 and 4) leaves. **B,** Inoculation with sap from plants infected with CP1 (lanes 1 and 2) and G11 (lanes 3 and 4). Lanes 1 and 3, sap extracted in acetate buffer and incubated 1 hr before inoculation; lanes 2 and 4, sap extracted in glycine buffer and inoculated immediately. Lane 5, extract from uninoculated control plant. G, sg1, and sg2 indicate the positions of genomic and subgenomic RNAs, respectively.

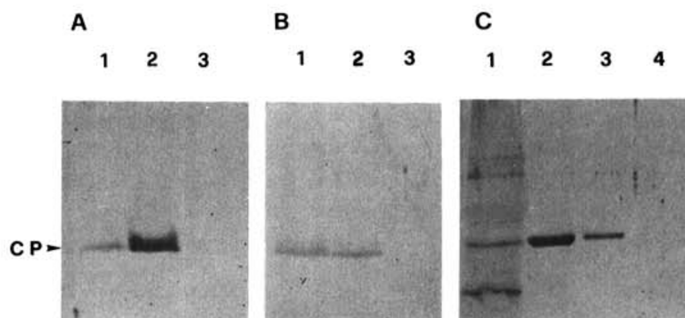

Fig. 4. Western blots of protein extracts from plants infected with G11 or CP1. **A,** Inoculated leaves and **B,** upper leaves of *Nicotiana benthamiana* infected with CP1 (lane 1) and G11 (lane 2) RNA. Lane 3, uninoculated control. **C,** Inoculated leaves of *N. clevelandii* infected with CP1 (lane 1) or G11 (lane 2) RNA. Lane 3, coat protein of purified cymbidium ringspot tombusvirus (CyRSV). Lane 4, uninoculated control. CP, coat protein.

showed that the sequence of the coat protein gene was the same as that of CP1 (not shown).

DISCUSSION

The architecture of tombusvirus particles was deduced from high resolution studies of TBSV (Harrison *et al.* 1978; Harrison 1984). Each of the 180 structural subunits of the viral capsid is folded in three distinct domains: the N-terminal disordered internal domain (R), the shell domain (S), and the protruding C-terminal domain (P), which forms the projections on the virus particles. On the basis of amino acid sequence similarity between the coat protein of TBSV and CyRSV, the same structure was suggested for CyRSV protein subunits (Grieco *et al.* 1989b). By site-directed mutagenesis, six nucleotides were deleted from the coat protein gene of CyRSV, which produced a deletion of two amino acids from the highly ordered S domain. The result was a coat protein unable to encapsidate RNA. Failure to support assembly did not depend on mutated RNA as shown by an experiment in which transgenic plants expressing wild-type CyRSV coat protein formed complete virions with CP1 RNA.

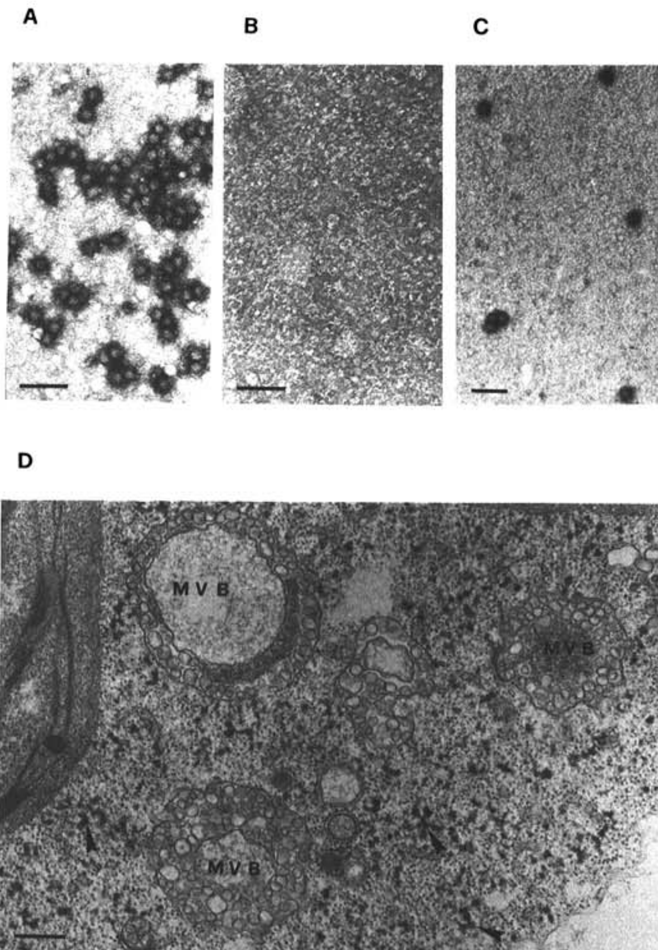

Fig. 5. A–C. Immune electron microscopy of extracts from nontransgenic *Nicotiana benthamiana* inoculated with *in vitro* RNA transcripts of clones **A,** G11; **B,** CP1; or **C,** cymbidium ringspot tombusvirus wild-type coat protein transgenic *N. benthamiana* inoculated with CP1 *in vitro* RNA transcript. **D,** Thin section of a CP1-infected *N. benthamiana* cell showing multivesicular bodies (MVB) and clumps of electron-dense amorphous material (arrowheads). Scale bars, 100 nm (A–C); 400 nm (D).

Availability of this CyRSV mutant allowed a study of virus replication and spread in the presence of coat protein, which was synthesized normally but in the absence of complete virions. Absence of virions did not prevent cell-to-cell movement in *N. clevelandii* and *N. benthamiana* or long-distance movement in *N. benthamiana*. However, long-distance spread of the CP1 mutant in infected *N. clevelandii* plants was affected. CP1 RNA produced a hypersensitive response in inoculated leaves, which may be responsible for the lack or limitation of long-distance movement. In plants in which a limited spread had occurred, viral RNA was localized in necrotic patches, which may have inhibited its movement to the vascular system for long-distance spread.

A viral product directly involved in short-distance movement (movement protein) has not been identified with certainty in tombusviruses, although for CNV it was suggested that the 21-kDa protein may be involved in cell-to-cell spread (Rochon and Johnston 1991). The present results show that a coat protein mutation impairing production of complete particles does not affect cell-to-cell movement of CyRSV, but, depending on the host, it may affect long-distance movement. Long-distance spread of CyRSV, therefore, seems possible in a nonvirion form, although transport involving complete particles is more dependable and has less stringent host requirements. *N. benthamiana*, which has a remarkable susceptibility to systemic infection by most plant viruses (see discussion in Dawson 1990), seems more permissive than *N. clevelandii.*

There is no consensus whether plant viruses spread in host tissues as virions or in a nonvirion form (Hull 1989). With turnip crinkle virus, a member of the closely related carmovirus group, encapsidated RNA is required for long-distance movement in one plant species (*Brassica campestris* L.), whereas in *N. benthamiana* there is not even an accumulation of unencapsidated viral RNA in inoculated leaves (Heaton *et al.* 1991; Hacker *et al.* 1992). A coat protein mutant of CNV failed to produce detectable virus particles but was able to move long distance in *N. clevelandii* (Rochon *et al.* 1991), which contrasts with the results of our study. However, because details of the experiments were not given (Rochon *et al.* 1991), a comparison is not possible to confirm a difference in the systemic movement of CNV and CyRSV in *N. clevelandii.*

Several coat protein mutants of tobacco mosaic tobamovirus (TMV) have been examined and, in all cases, the involvement of viral coat protein in long-distance spread was suggested (Dawson *et al.* 1988; Saito *et al.* 1990). In particular, a TMV coat protein mutant, modified in such a way that assembly into virus particles was impaired, exhibited a greatly reduced ability for long-distance movement. Its residual ability to spread was attributed to those few particles that did assemble. RNA and capsid protein did not act efficiently *in trans* to promote spreading (Saito *et al.* 1990). The same situation may exist with CyRSV, in which both viral components are synthesized in infected cells, but they do not assemble; thus, spreading in *N. clevelandii*, a less permissive host than *N. benthami-*

ana, is affected.

Transgenic *N. benthamiana* plants expressing CyRSV coat protein were useful in establishing that formation of virus capsids depends on the capsid protein rather than the viral RNA. On the other hand, besides genomic RNAs, tombusvirus coat protein can encapsidate a variety of virus-related RNA species, such as subgenomic, satellite, and, to a lesser extent, DI RNAs. It ensures that, regardless of RNA type, encapsidation occurs, as long as the coat protein is functional.

Finally, no recombination was detected in transgenic plants between infecting CP1 RNA and RNA transcripts of the wild-type coat protein gene inserted into the plant genome under the control of the CaMV 35S promoter; all progeny sequenced retained the CP1-RNA mutation.

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