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Consequences of gene transfer between distantly related tombusviruses

(Plant virus; CyRSV-AMCV hybrid virus; long-distance movement of infection; recombinant RNA)

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SUMMARY

Hybrid cDNA clones were constructed by fusing the coat protein-encoding gene and/or the 3′-terminal region (including the 22- and 19-kDa protein-encoding genes) derived from a clone of artichoke mottled crinkle tombusvirus to the 5′-terminal region of a full-length clone of cymbidium ringspot tombusvirus. In vitro transcripts from recombinant clones were infectious when inoculated into *Nicotiana clevelandii* plants. Inoculated plants showed symptoms different from those induced by parent viruses. In particular, systemic invasion depended very much, although not exclusively, on the type of protein that coated progeny viral RNA, suggesting a role of the capsid protein in the long-distance movement of tombusvirus infections.

INTRODUCTION

Cymbidium ringspot virus (CyRSV) and artichoke mottled crinkle virus (AMCV) are members of the tombusvirus group (Martelli et al., 1989). Their genomes, like those of other tombusviruses, contain five ORFs [Grieco et al., 1989; Rochon and Tremaine, 1989; Tavazza et al., 1989 and unpublished results (GenBank accession No. X62493); Grieco and Gallitelli, 1990; Hearne et al., 1990]. The product of ORF 1 is a 33-kDa protein with an unknown function. A 92-kDa protein synthesized due to readthrough of an amber stop codon (ORF 2) is tentatively identified as the viral replicase, and the product of ORF 3 is the capsid protein (Martelli et al., 1989). The functions of the other two proteins encoded by the viral genome (22 kDa and 19 kDa) are not clear. Rochon and Johnston (1991) reported that replication of a cucumber necrosis virus (CNV) mutant containing a mutation in the start codon of the 22-kDa protein was not detectable in inoculated plants, suggesting the role of this protein in virus movement. The 3′-terminal genes are expressed via two subgenomic RNAs, coding for CP and 22-kDa proteins. The 19-kDa product of ORF 5 can occasionally be identified in in vitro translation studies (Hayes et al., 1988; Rochon and Tremaine, 1989).

CyRSV and AMCV are very distantly related serologically (Koenig and Gibbs, 1986), have essentially the same host range, but can be distinguished by the differential ability to invade systemically *Nicotiana clevelandii*, based on the severity of symptoms they induce. A constant proportion of AMCV in inoculated *N. clevelandii* failed to invade the upper, noninoculated leaves. These data suggest that the two genomes can be differentiated. However, their similarity could allow the creation of hybrid molecules through an exchange of genes which could help in understanding the function of tombusvirus genes.

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Abbreviations: aa, amino acid(s); AMCV, artichoke mottled crinkle virus; bp, base pair(s); cDNA, DNA complementary to RNA; CP, coat protein; CyRSV, cymbidium ringspot virus; kb, kilobase(s) or 1000 bp; MOPS, 3-(*N*-morpholino)propanesulfonic acid; *N.*, *Nicotiana*; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; p, plasmid; PCR, polymerase chain reaction.

RESULTS AND DISCUSSION

(a) Sequence relationship between CyRSV and AMCV

Both viruses belong to the tombusvirus group, and the two sequences show a closer relationship than expected on the basis of the serological properties (Koenig and Gibbs, 1986). The overall nt sequence identity of the two viruses is 76%. The highest degree of homology was found between the putative polymerases and gene products of 22- and 19-kDa (91%, 83%, and 75%, respectively). Two CPs show the lowest degree of homology (47%), in particular in the R and P domains (Fig. 1A), which are thought to account for the antigenic properties (Martelli et al., 1989). Moreover, AMCV CP has 8 more aa than CyRSV CP (Fig. 1A). Additional minor differences, shown in Fig. 2, reflect the difference in genome size (4733 nt for CyRSV and 4789 nt for AMCV). These differences consist of: (*i*) a 20 nt shorter 5′ noncoding region of CyRSV, (*ii*) a 6 nt shorter leader of the first subgenomic RNA (sg1) of CyRSV, (*iii*) a 4 nt shorter intergenic region between CP and the 22-kDa protein of CyRSV.

(b) Construction of recombinant viruses

A full-length clone of CyRSV RNA from which infectious RNA can be obtained (Burgyán et al., 1990) was modified by substituting the last three nt (GGG) with CCC (pCyRG11), which led to a several-fold increase in infectivity of the in vitro prepared transcripts (Dalmay et al., 1993). As for AMCV, a partial clone pAMCV-19 was used representing the 3′ half from nt 1979 in the 92-kDa gene to the 3′ end. It was constructed by priming first-strand synthesis with the oligo 5′-ctcgaGGGCT GCATTGCTGCAATG complementary to 19 nt of AMCV RNA. It contained five additional nt (small letters) forming a restriction site *Xho*I, which does not occur in the AMCV RNA sequence. Second-strand synthesis was done in the presence of RNase H and DNA polymerase I as described by Gubler and Hoffman (1983), ligated with T4 DNA ligase (Boehringer Mannheim, Germany) to *Sma*I-digested pGEM-4Z (Promega, Madison, WI), and cloned in *Escherichia coli* JM101.

(c) Replication and analysis of progeny viruses

All hybrid transcripts were able to replicate in inoculated leaves with similar efficiency. Northern blots probed with nick-translated clone pCyR-7, corresponding approx. to 1000 nt of the 3′ end of CyRSV (Russo et al., 1988), revealed genomic and subgenomic RNAs typical of tombusvirus infections (Fig. 3A; Martelli et al., 1989). The presence of virus-specific sequences in the exchanged genes was shown by hybridization to cloned probes containing 202 nt (pCCP) and 108 nt (pACP) of the CyRSV

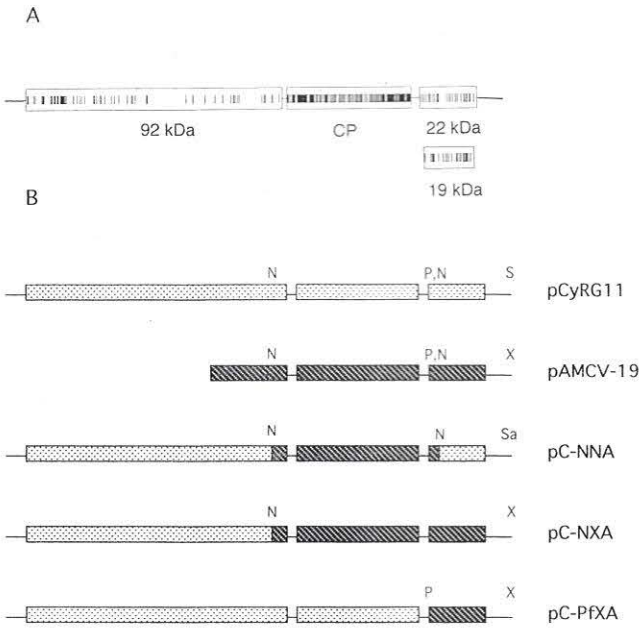

Fig. 1. Comparison of proteins encoded by parental viruses and the construction of hybrid virus genomes. (**A**) Alignments of the aa sequences of each ORF of CyRSV and AMCV. Vertical lines indicate mismatched aa. (**B**) Schematic representation of the hybrid virus constructs aligned with the parental clones (pCyRG11 and pAMCV-19). Light and dark shaded boxes illustrate ORFs of CyRSV and AMCV, respectively. N, *Nco*I; P, *Pfl*MI; S, *Sma*I; Sa, *Sal*I; X, *Xho*I. **Methods:** Three hybrid viral genomes were constructed by substituting fragment of DNA of the full-length CyRSV cDNA clone pCyRG11 with AMCV genes obtained from clone pAMCV-19 (**B**). Plasmid pC-NNA was constructed by substituting the DNA sequence between two *Nco*I sites (nt 2309–3844) of pCyRG11 with the *Nco*I fragment (nt 2327–3901) from the AMCV clone pAMCV-19. This fragment included 286 nt of the C terminus of the 92-kDa gene, the entire CP gene, 31 nt of the 22-kDa gene, and the two intergenic regions. Plasmid pC-NXA was obtained by fusing the entire second half of the AMCV genome at the first *Nco*I site, i.e., the C terminus of polymerase, CP, 22-kDa and 19-kDa genes, and the 3′ noncoding region. Finally, pC-PfXA was prepared by substituting a *Pfl*MI-*Sma*I fragment (nt 3813–4733) from pCyRG11 with the *Pfl*MI-*Xho*I fragment (nt 3870–4789) from pAMCV-19. This sequence consisted of the 22-kDa and nested 19-kDa genes and the 3′ noncoding region. The *Nco*I and *Pfl*MI restriction sites in the two genomes were in the same position with respect to the coding regions; therefore, no alterations were introduced in the sequence of hybrids other than the gene substitution. Hybrid plasmids were used to transform *E. coli* strain JM101. Recombinant DNA was extracted according to Hattori and Sakaki (1986).

and AMCV genes encoding CP, respectively; these sequences are unique to the two genomes (Grieco et al., 1989; Tavazza et al., 1989; Fig. 3B,C). Stability of the progeny of recombinant viruses was confirmed by RNase protection assays (Sambrook et al., 1989) using as cRNA probe a sequence complementary to nt 2327–4789 of the AMCV sequence, i.e., the AMCV sequence which was substituted in the CyRSV clone. RNA extracted from AMCV or hybrid viruses, but not from pCyRG11 infected plants, was protected. The size of protected RNA corresponded to the substituted sequences (Fig. 4). Cross-

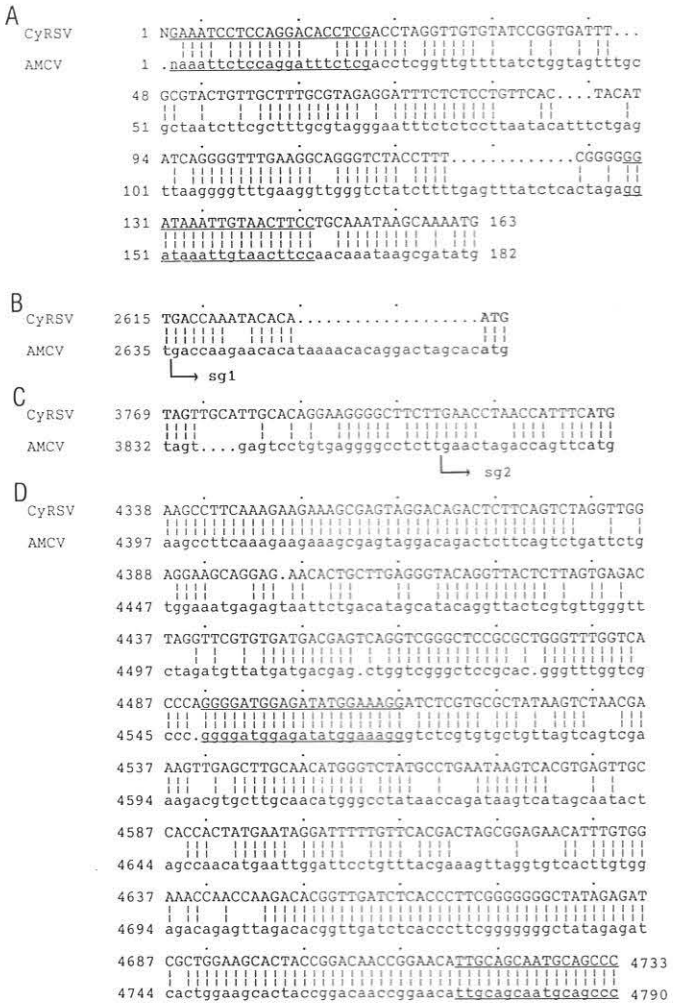

Fig. 2. Alignment of 5′ non-coding sequences (**A**), intergenic regions between genes encoding 92-kDa and CP (**B**), and between CP and 22-kDa (**C**), and 3′ noncoding region (**D**) of CyRSV and AMCV. The underlined sequences indicate oligos used in PCR amplification for sequence analysis. Bold-face letters indicate the stop and start codons at the extremity of the intergenic regions. Arrows show the first nt of subgenomic RNAs. Broken lines indicate matched nt. Dots show the gaps introduced to obtain the maximum sequence homology. N and n are unknown nt.

contamination from the two parent viruses was excluded by sequence analysis of the 5′ end 146 nt and the 3′ end 242 nt, which differ in the two viruses sufficiently for discrimination (Fig. 2). This was done by amplifying the cDNA of viral RNA in total RNA extracts by PCR, cloning, and sequencing as described (Burgyán et al., 1990). Specific 17- or 21-mer oligos at the termini of these regions were used (Fig. 2). Sequencing revealed that progeny had the expected composition corresponding to the inoculum.

(d) Encapsidation assay

All three recombinant RNAs were encapsidated as shown by RNase resistance assays of virus-specific RNA

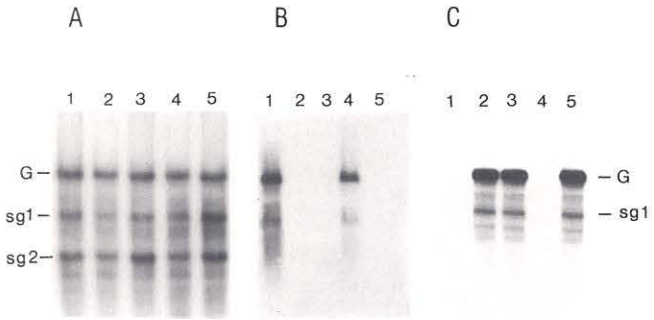

Fig. 3. Analysis of total nucleic acids extracted from plants inoculated with 1.5 μg RNA from cloned viruses. Total RNA from infected leaves was isolated essentially according to White and Kaper (1989) with some modifications, i.e., leaf tissue (100–200 mg) was rapidly homogenized with ice-cold mortar and pestle, resuspended in 600 μl buffer (100 mM glycine/100 mM NaCl/10 mM EDTA/2% sodium dodecyl sulfate), and the RNA extracted with phenol and chloroform, precipitated with ethanol, and resuspended in sterile water. Lanes: 1, pCyRG11; 2, pC-NXA; 3, pC-NNA; 4, pC-PfXA; 5, 1.5 μg purified genomic RNA of AMCV. The presence of virus-related RNA was assessed by Northern blot analysis. Samples were denatured with formamide and formaldehyde, electrophoresed in 1.5% agarose gel in MOPS buffer containing 2.2 M formaldehyde (Sambrook et al., 1989), and blotted onto nylon membranes (Amersham). Blots were hybridized with probes pCyR-7 (**A**) (Russo et al., 1988), pCCP (**B**), and pACP (**C**), respectively. G, sg1, and sg2 indicate the positions of genomic or subgenomic RNAs.

carried out according to Jupin et al. (1990). However, pC-NXA showed a lower proportion of encapsidated RNA (Fig. 5). The latter observation was in line with the finding that fewer virus particles could be extracted from pC-NXA-infected tissue (50 μg/g tissue) than from pC-NNA- and pC-PfXA-infected material (500 μg/g tissue). We do

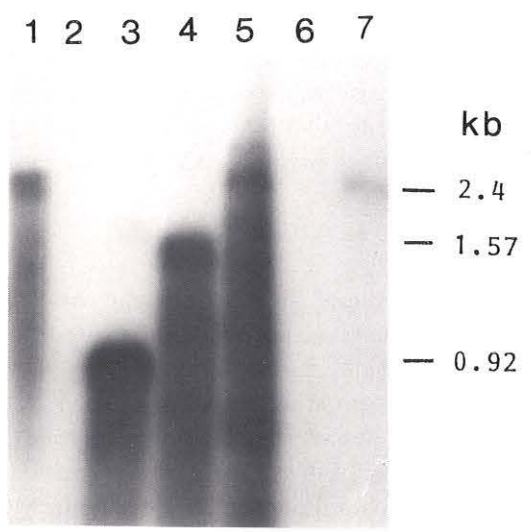

Fig. 4. RNase protection assay of viral-specific RNAs extracted from healthy plants (lane 2) and from plants infected with AMCV (lane 1), pC-PfXA (lane 3), pC-NNA (lane 4), pC-NXA (lane 5), and pCyRG11 (lane 6), respectively. Total nucleic acid extracts were hybridized to ^{32}P-labelled RNA probe complementary to nt 2327–4789 of AMCV (lane 7), digested with RNase, denatured with formamide and formaldehyde, and analysed on 1% denaturing agarose gel (2.2 M formaldehyde). The sizes of protected RNAs are indicated.

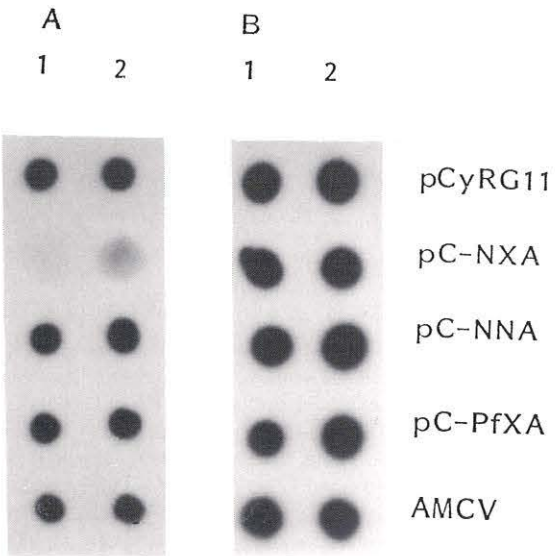

Fig. 5. Dot blot analysis of encapsidated viral RNA of parental and hybrid viruses. *N. clevelandii* plants were inoculated as described in Table I, and nucleic acid was extracted from the same amount of infected plant material. Plants samples were homogenized in 100 mM Tris·HCl/10 mM $MgCl_2$ pH 7.5 incubated at 37°C for 30 min to digest all nonencapsidated viral RNAs by plant RNases (Jupin et al., 1990), and then protected RNA was phenol-chloroform extracted (**A**), or total RNA was extracted as described in Fig. 3 (**B**). Vertical rows 1 and 2 contain RNA samples from two different leaves on the same plant. The blotted RNAs were hybridized to ^{32}P-labelled pCyRG-7 (Russo et al., 1988).

not have a clear explanation for this observation, but the less efficient encapsidation of pC-NXA than pC-NNA could be a consequence of structural differences between the two genomic RNAs. Purified virus particles from pCyRG11-and pC-PfXA-infected plants reacted with CyRSV antiserum, but not with AMCV antiserum in agar double diffusion tests. On the contrary, purified virus particles extracted from pC-NNA-, pC-NXA-, and AMCV-infected plants reacted only with AMCV antiserum (data not shown).

(e) Symptoms

Symptoms produced by recombinant viruses were compared with those caused by parent viruses (Table I). Constructs pCyRG11, pC-NNA, pC-PfXA, and AMCV caused similar chlorotic lesions in inoculated *N. clevelandii* leaves 4–5 days after inoculation. Chlorotic lesions turned necrotic in 3–4 days. Leaves inoculated with pC-NXA developed chlorotic-necrotic lesions much faster, i.e., 2–3 days after inoculation. The amount of viral RNA in inoculated leaves was comparable in all combinations as shown by Northern blot (Fig. 3A) and dot blot assay of total RNA extracts (Fig. 5 B).

Systemic symptoms varied according to the inoculum composition. Plants inoculated with pCyRG11 transcripts obtained in vitro showed distortion of the inoculated leaves 5–7 days after inoculation. This was followed by apical necrosis and death of the plants within 2–3 weeks. The same effect was obtained after inoculation of the plants with virus purified after the first passage. This is in line with the known effect of CyRSV infections in the absence of defective interfering RNA (Burgyán et al., 1990). Similar systemic symptoms were induced by AMCV infections, but they appeared 2–3 days later. Contrary to CyRSV infections, a low, but constant proportion (approx. 20%) of inoculated plants failed to show systemic symptoms. This characteristic of AMCV infection is not an effect of natural defective interfering RNA accumulation as shown by Northern blot analysis (Fig. 3A, lane 2). Plants infected with in vitro transcripts of the hybrid clone pC-NNA showed systemic symptoms similar to those produced by pCyRG11, but not the apical necrosis. Plasmid pC-NXA did not cause any symptom on upper uninoculated leaves for at least six weeks. No virus-specific RNA was detected in total RNA extracts from such leaves (data not shown). Occasionally, only leaves just above the inoculated ones developed chlorotic/necrotic patches. Virus could be extracted from this tissue and, when inoculated in other plants, reproduced the pattern of infection described above. Finally, plants infected with in vitro transcripts of clone pC-PfXA developed symptoms similar to those produced by CyRSV, but less severe. These plants did not show apical necrosis up to six weeks after inoculation.

(f) Conclusions

(*1*) Putative RNA polymerase (92 kDa) derived completely (pC-PfXA) or mostly (pC-NNA, pC-NXA) from CyRSV can recognize the 3′ end of AMCV RNA, replicate it, and form subgenomic RNAs, notwithstanding the different length of the leader sequences of the subgenomic RNAs of the two viruses.

(*2*) The observation that the two recombinant viruses expressing CP of AMCV are defective (pC-NXA) or delayed (pC-NNA) in long distance movement can be taken as an indication of the involvement of CP in this function. However, the difference between pC-NNA, pC-NXA, and pC-PfXA in the ability to invade upper leaves may also indicate that this function is not determined by a single gene (encoding CP) and may rather depend on the integrated action of other viral genes, e.g., that encoding 22-kDa protein which is suggested to be involved in the virus movement (Rochon and Johnston, 1991). The suggestion that the CP plays an important role in tombusvirus spreading long distances is further supported from experiments with a mutant of pCyRG11 in which 2 aa in the shell domain of the CP were deleted. This CP mutant exhibits very similar characteristics to pC-NXA, namely, does not produce virus particles at a detectable

TABLE I

Symptom development on *Nicotiana clevelandii* plants after infection with pCyRG, AMCV, and recombinant viruses

Construct or virus[a]	Symptoms after inoculation[b]			
	After 10 days		After 20 days	
	Inoculated leaves	Upper leaves	Inoculated leaves	Upper leaves
pCyRG11	dchL	D,NLL,M+++	LD	LD[c]
pC-NNA	chLL	D±	LD	D,NLL,M+++
pC-NXA	NLL	(chs)[d]	LD	–
pC-PfXA	dchL	D,M+++	LD	D,M+++
AMCV	chLL	D±	LD	D,NLL,M+++

[a]Groups of ten plants were inoculated with pCyRG11, AMCV, and recombinant viruses.

[b]dchL, diffuse chlorotic lesions; chLL, chlorotic local lesions; chs, chlorotic spot; D, leaf distortion; NLL, necrotic local lesions; M, mosaic symptoms; LD, leaf death; number of (+) indicate the severity of systemic symptoms.

[c]First appeared as systemic necrosis on apical leaves; it culminated in death of the plants (6 out of 10).

[d]On upper leaves recombinant pC-NXA occasionally caused a few chlorotic spots which became necrotic but were not found on leaves formed later.

Methods: Plants were inoculated with in vitro synthesized genomic RNA except for AMCV. In this case RNA was extracted from virion, fractionated by electrophoresis in 1% low-melting-point agarose gel and visualized by staining with ethidium bromide. The band corresponding to the genome-length RNA of AMCV was excised, and the RNA was extracted (Sambrook et al., 1989). In the case of pCyRG11 and hybrid constructs, DNA template (2 μg) was transcribed using a T7 transcription system (Amersham) after linearization in the appropriate restriction site: pCyRG11 was digested with *Sma*I, pC-NNA with *Sal*I, pC-NXA and pC-PfXA with *Xho*I. The run-off transcripts of *Sma*I-digested pCyRG11 had no extra nt, whereas transcripts from *Sal*I-digested pC-NNA and *Xho*I-digested pC-NXA and pC-PfXA had 9 and 4 additional, nonviral nt, respectively, at the 3′ end. In vitro synthesized RNA was inoculated to *N. clevelandii* plants (transcripts from 2 μg DNA/plant) as described (Burgyán et al., 1990). Further inoculations were done by grinding infected leaf tissue in an equal volume of inoculation buffer (50 mM glycine/30 mM K_2HPO_4 pH 9.2). Plants were kept in a growth chamber set for 14 h/21°C light (intensity 200 μE) and 10 h/18°C dark period, observed for symptoms, and analysed for RNA content starting one week after inoculation.

level and is not able to invade the upper uninoculated leaves of *N. clevelandii* (Dalmay et al., 1992). Similar observations were recently published by Heaton et al. (1991), who showed that a CP mutant of turnip crinkle carmovirus, which is related to tombusviruses (Martelli et al., 1989), was unable to form virus particles and move systemically in host plants. A CP mutant of CNV failed to form virus particles but was able to spread systemically in *N. clevelandii* (Rochon et al., 1991). These results suggest that tombusviruses most likely move systemically in the form of virus particles, but encapsidation does not appear to be required for systemic movement by all tombusviruses in all hosts. Based on our observations, CyRSV probably belongs to those viruses which required encapsidation-competent CPs for spreading systemically (Lazarowitz et al., 1989; Quillet et al., 1989; Sacher and Ahlquist, 1989).

(*3*) Finally, the possibility that parts of the genome can be exchanged between tombusviruses to produce viable recombinants gives experimental support to the suggestion of Hearne et al. (1990) that most tombusviruses should be regarded as derivatives of the same virus.

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