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RNA Recombination in the Genome of Barley Stripe Mosaic Virus

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Barley stripe mosaic Hordeivirus (BSMV) is a positive-strand RNA virus requiring three single-stranded RNAs (α, β, and γ) for infectivity. A terminal-sequence-dependent cloning strategy was used to clone the entire genome of the CV17 strain. Full-length γ cDNA clones were obtained when oligonucleotides specific for the 5'-terminal sequence of RNA α were used in the cloning procedure, but not when RNA γ-specific oligonucleotides were used. Sequence analysis of six putative γ cDNA clones revealed that nucleotides 1–70 possess 89% homology with the first 70 nucleotides of RNA α. This leader region is separated from the γ-specific coding region by an eight-base intervening sequence common to both CV17 RNAs α and γ. Northern and Southern hybridization with oligonucleotide probes specific for either α or γ leader sequences indicated that CV17 γ cDNA clones are representative of native CV17 γ RNAs. Furthermore, bioassays indicated that *in vitro* transcripts derived from these γ cDNA clones were infectious when coinoculated with *in vitro* transcripts of full-length α and β cDNA clones. Thus, the evidence suggests that RNA γ of BSMV strain CV17 is a recombinant molecule which may have arisen as a result of natural recombination between RNAs α and γ. © 1992 Academic Press, Inc.

Barley stripe mosaic virus (BSMV), a Hordeivirus, encodes seven major polypeptides in a segmented genome composed of three (+)-sense, single-stranded (ss) RNAs designated α (3.8 kb), β (3.3 kb), and γ (3.2 or 2.8 kb) (*1*). The length of RNA γ varies with the presence or absence of a tandem repeat near its 5' terminus. RNAs α and γ are able to replicate in protoplasts in the absence of RNA β (*2*), although the presence of all three RNAs is essential for infection of intact plants (*3*). Nucleotide sequence analyses confirm that the αa and γa genes encode the essential viral-derived replicase components (*4, 5*). Although the sequence of each RNA is largely unique, the 3'-terminus of each RNA is composed of a highly conserved sequence that can be folded into a tRNA-like structure and aminoacylated with tyrosine *in vivo* (*6*).

A "bandaid" cloning strategy, which exploits the conservation of the 3' terminal sequence, as well as the unique nature of the 5' ends of the BSMV RNAs, has been used previously to obtain full-length cDNA clones from which infectious *in vitro* transcripts can be produced (*3, 7*). We have now used this strategy to clone the entire genome of BSMV strain CV17. Analysis of the resulting cDNA clones suggested that CV17 RNA γ is a naturally occurring chimeric recombinant, composed of a 70-nucleotide (nt) α-specific leader sequence preceding a γ-specific coding region. Furthermore, hybridization and bioassay data indicate that these clones are representative of native CV17 γ RNAs. One of these CV17 γ cDNA clones has been used previously to investigate the effects of short ORFs in the 5' leader of RNA γ on the systemic movement of BSMV in *Nicotiana benthamiana* (*8*). The present paper provides evidence for the recombinant nature of CV17 RNA γ.

BSMV strain CV17 was propagated in barley (*Hordeum vulgare* cv. "Black Hulless," C.I. 666) and purified as previously reported (*9*). Virion RNA was extracted using a phenol–SDS procedure and was subsequently recovered by ethanol precipitation, followed by 3 *M* sodium acetate precipitation, and finally by ethanol precipitation again (*7*). This mixture of RNAs α, β, and γ was then used for cDNA synthesis and cloning as previously described for the ND18 and Type strains of BSMV (*7*). Briefly, linearized single-stranded phagemid DNA with a T7 promoter sequence at its 5'-end and a short 3'-terminal sequence complementary to the 3'-end of BSMV RNA was used as a primer for first-strand cDNA synthesis. Phagemid-linked full-length cDNA was then circularized by annealing to a bandaid oligonucleotide with sequence complementary to both ends of the phagemid:cDNA. The use of three different bandaid oligonucleotides, each with 5'-ends complementary to the T7 promoter sequence and with 3'-ends complementary to the 5'-ends of either full-length α, β, or γ cDNAs, provided a means of isolating specific full-length cDNA clones in a single cloning operation with-

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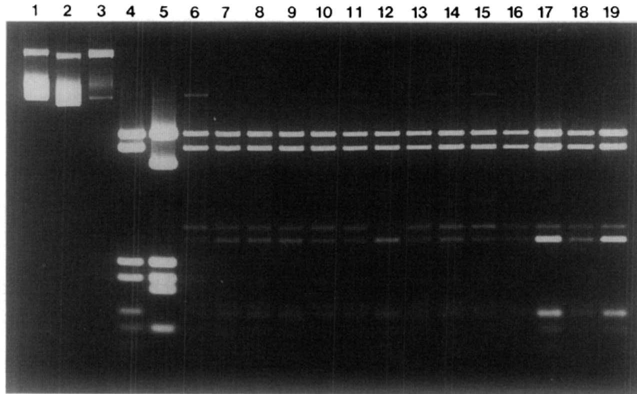

FIG. 1. Comparison of putative barley stripe mosaic virus strain CV17 γ cDNA clones (lanes 6–19) with γ cDNA clones of the Type (lane 4) and ND18 strains (lane 5) after digestion with *Rsa*I. Lanes 1–3: uncut Type, ND18, and CV17 γ cDNA clones, respectively.

out the need for prior separation of the α, β, and γ RNAs. Full-length clones were initially identified by colony color and gel electrophoresis of ssDNA obtained after superinfection of transformed *Escherichia coli* strain DH5αF′ with bacteriophage M13KO7, as described previously (*7*). The full-length nature of individual clones and the integrity of the T7 promoter/BSMV 5′-end junction were verified by dideoxynucleotide sequencing of ssDNA templates (*10*).

Full-length CV17 α and β cDNA clones were successfully isolated using the α and β bandaid oligonucleotides, respectively. However, all attempts to isolate full-length γ clones using the γ bandaid oligonucleotide were unsuccessful. Instead, the initial size screening of cDNA clones obtained with the α bandaid oligonucleotide resulted in the identification of one with an insert equivalent in size to a 3.2-kb γ RNA rather than a full-length α RNA (3.8 kb). Fourteen additional clones with inserts of this size were identified in subsequent cloning experiments utilizing the α bandaid oligonucleotide, whereas only one full-length α clone was isolated.

Analysis of *Rsa*I RFLPs in these fifteen putative γ-specific CV17 cDNA clones indicated that all were similar to each other, but that they differed from the BSMV Type or ND18 strain γ cDNA clones previously isolated by Petty *et al.* (*3*) (Fig. 1). Using an M13 universal primer, six of these putative CV17 γ clones were then sequenced in the region of the cDNA insert corresponding to the 5′-end of CV17 RNA γ. A CV17 α clone was sequenced in a similar manner and also by direct RNA sequencing with reverse transcriptase (*4*) and a primer which bound to nt 195–209 (5′-CGTGCGAGCACAGTA). The sequences of all six γ clones were identical for at least the first 100 nt and the first 70 nt of these cDNAs matched the 5′-terminal 70 nt of CV17 RNA α in 62 positions (Fig. 2). However, no significant sequence alignment could be made between this region of the putative CV17 γ cDNA clones and the first 70 nt of the Type strain RNA γ. The 5′ terminal 70 nt of the α-specific sequence in the CV17 γ cDNAs were followed by an eight-base segment common to both CV17 α and γ RNAs. This sequence was, in turn, followed by a region that is identical to the sequence reported (*4*) for the Type strain RNA γ for at least an additional 100 nt (Fig. 2).

Since the CV17 RNA γ contains a ca. 370-nt tandem repeat near its 5′-terminus similar to that described for the Type strain (*4*) and a variant of the ND18 strain (*3*), the authenticity of the CV17 γ cDNA clones was not readily verifiable by direct RNA sequencing. Instead, the authenticity of these clones was assessed by hybridization with oligonucleotide probes specific for CV17 α and Type γ leader sequences. Northern blots of native RNAs of BSMV strains CV17, CV42, and ND18 and Southern blots of the fifteen CV17 γ cDNA clones were prepared according to standard protocols (*11*). Blots were probed with either an α leader-specific (5′-AAGAATCGATTACGATTATG) or a γ leader-specific (5′-TTTAGCGTTATTTGGCAAGC) ^{32}P-end-labeled oligonucleotide probe complementary to nt 41–60 of the CV17 α and Type γ RNAs, respectively. Results evident in Fig. 3 demonstrate that native CV17 γ RNAs typically possess an α leader sequence, as do all fifteen of the CV17 γ cDNA clones.

Further verification of the authenticity of the CV17 γ cDNA clones was obtained by infectivity assay. Capped RNAs were produced by *in vitro* transcription of four of the CV17 γ cDNA clones exactly as previously described (*3*). Each was combined with *in vitro* transcripts of either CV17 or ND18 α and CV17 β cDNA clones and inoculated onto 5- to 6-day-old barley seedlings. Inoculated seedlings developed symptoms typical of BSMV infection within 4 to 7 days, thus verifying that these CV17 γ cDNA clones represent full-length and biologically active copies of CV17 RNA γ. (Table 1)

Our results strongly suggest that RNA γ of BSMV strain CV17 is a naturally occurring product of recombination between RNAs α and γ. Although the mechanism of recombination in BSMV is not known, two possible models for RNA recombination have been commonly proposed: (I) template switching (copy choice) and (II) enzymatic cutting/splicing. Kirkegaard and Baltimore (*12*) provided convincing evidence for a copy choice mechanism of poliovirus recombination; it appears likely that the recombination reported here proceeded in a similar manner. Although template switching may occur in the absence of sequence homology, it should be noted that a short homologous sequence at

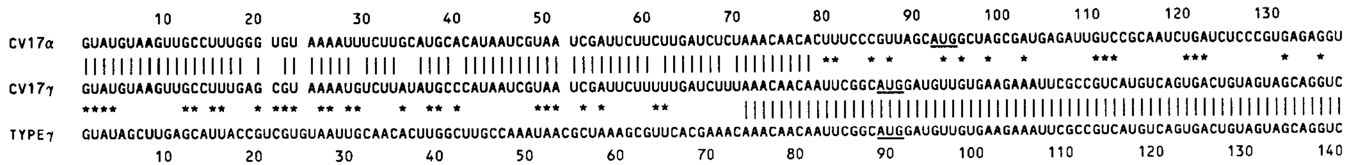

FIG. 2. Comparison of the 5′ terminal sequence of barley stripe mosaic virus strain CV17 RNA γ with that of CV17 RNA α and Type strain RNA γ. Initiation codons for the αa and γa coding regions are underlined. Vertical bars denote homology between sequences believed to be evolutionarily related. Asterisks denote nucleotide matches that may have arisen by chance between molecules believed to be distinct.

the putative crossover point is present in CV17 RNAs α and γ, but not in RNA β. The presence of a tandem repeat in CV17 RNA γ which is similar to the 372-nt tandem repeat present in the Type strain γ is also suggestive of a copy choice mechanism.

We have no conclusive explanation for the minor differences observed between the CV17 α and γ leader sequences. Considering the high degree of sequence conservation among BSMV strains (e.g., the α leader sequences of the Type and CV17 strains differ by only 1 nt), the sequence divergence between the CV17 α and γ leaders may be due to selection for ability to replicate rather than random drift. The normal accumulation of RNA γ to much higher levels than RNA α in all strains studied thus far, including CV17, is an indication that differences in replication efficiency do exist among these RNAs (Fig. 3). Alternatively, natural selection pressures on RNA α may not apply to RNA γ, hence allowing genetic drift of the two leader sequences. As a result of this divergence, only one of the two small overlapping ORFs present in the CV17 γ leader is present in the CV17 α leader. It has been shown that the small γ leader ORFs play a significant role in the efficiency of *in vitro* translation of the CV17 γa gene and can affect systemic movement and pathogenicity of ND18/CV17 pseudorecombinants in *N. benthamiana* (*8*). However, the α origin of these small leader ORFs is not significant, since the Type strain γ leader also contains a small ORF with unrelated sequence that has a similar effect on translation and systemic movement (*8*). Thus, phenotypic effects, if any, resulting from recombination of the CV17 RNA α and γ leader regions have not yet been observed.

Substantial evidence for the occurrence of RNA recombination now exists. Recombination has been well documented in RNA viruses, especially the picornaviruses, coronaviruses, and alphaviruses (*12–15*). Altered pathogenicity as a result of incorporation of host

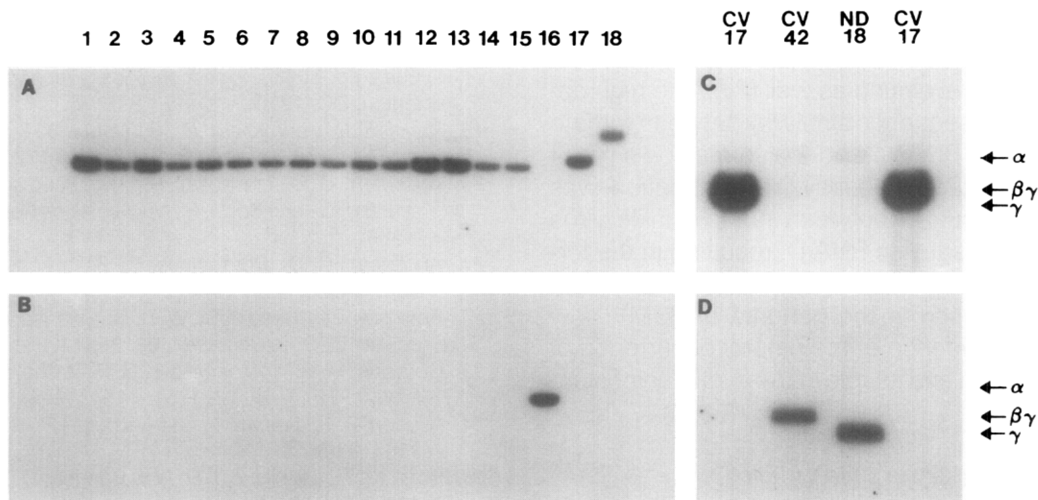

FIG. 3. Identification of barley stripe mosaic virus strain CV17 α and Type strain γ-specific leader sequences in 15 putative CV17 γ cDNA clones and native RNAs of strains CV17, CV42, and ND18. (A) Southern blot of 15 putative BSMV strain CV17 γ cDNA clones (lanes 1–15), a Type strain γ cDNA clone (lane 16), and a CV17 α cDNA clone (lanes 17–18) hybridized to an α leader-specific oligonucleotide probe. Plasmid DNAs in lanes 1–17 were linearized with *Mlu*I. (B) Southern blot identical to A, except that the oligonucleotide probe used was γ leader-specific. (C) Northern blot of native RNAs from BSMV strains CV17, CV42, and ND18 hybridized to the same α leader-specific probe as in A. The γ RNAs of CV17 and CV42 are each 3.2 kb in size and comigrate with RNA β (arrow labeled $\beta\gamma$); ND18 RNA γ is 2.8 kb in size (arrow labeled γ). (D) Northern blot identical to C, except that the γ leader-specific oligonucleotide probe was used. Hybridizations were performed in 6× SSPE + 1% SDS at 54° for the α leader-specific probe and at 58° for the γ leader-specific probe ($T_h = T_m - 9°$).

TABLE 1

INFECTIVITY OF BSMV TRANSCRIPTS

Inoculum[a]	No. Infected/No. Inoculated[b]
ND18α + CV17β + CV17γ2	13/25
ND18α + CV17β + CV17γ12	10/14
ND18α + CV17β + CV17γ58	5/14
ND18α + CV17β + CV17γ60	9/14
CV17α + CV17β + CV17γ2	8/10
CV17α + CV17β + CV17γ12	4/7

[a] Black Hulless barley plants were inoculated with a mixture of *in vitro* transcripts from cDNA clones of both the ND18 and CV17 strains suspended in GKP buffer (50 m*M* glycine, 30 m*M* K_2HPO_4, pH 9.2, 1% bentonite, 1% celite). The number of infected plants was determined by visual inspection. No symptoms were observed on control plants mock inoculated with GKP buffer alone.

[b] Combined data from 3, 2, 2, 2, 1, and 1 experiments, respectively.

sequences has been reported for an influenza virus and a togavirus (*16, 17*). Among plant viruses, recombination was first demonstrated by rescue of deletion mutants of brome mosaic virus (*18*). Other examples, such as the deletion of repeated sequences in tobacco mosaic virus mutants, have been reported more recently (*19*). Although the latter examples demonstrate the potential for recombination in plant RNA virus genomes, they provide no direct evidence for naturally occurring RNA recombination.

Evidence for such natural recombination now exists for several plant virus groups. Recombination among tobraviruses has been reported (*20–23*), and sequence analysis of cowpea chlorotic mottle and brome mosaic bromoviruses suggests that recombination has occurred during their evolution as well (*24*). The high degree of nucleotide sequence similarity between tomato ringspot nepovirus RNAs 1 and 2 also may be a result of recombination (*25*). Recently, recombination was found to have occurred between turnip crinkle virus genomic RNA and satellite RNA D, resulting in the formation of the new satellite RNA CX (*26*). The discovery of RNA recombination in the genome of BSMV now provides further evidence of the natural occurrence of RNA recombination and its role in the evolution of plant viruses.

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