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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.
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 M13K07, 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 γ 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 γ RNA (3.8 kb). Fourteen additional clones with inserts of this size were identified in subsequent cloning experiments utilizing the γ bandaid oligonucleotide, whereas only one full-length α clone was isolated.

Analysis of Rsal 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'-CGTGCGAGCA-CAGTA). 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'-TTTACCGTTTTGGCAAGC) 32P-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
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
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 tobaviruses 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.

ACKNOWLEDGMENTS

This work was supported in part by USDA Competitive Grant 9037282-5533, awarded to A.O. Jackson.