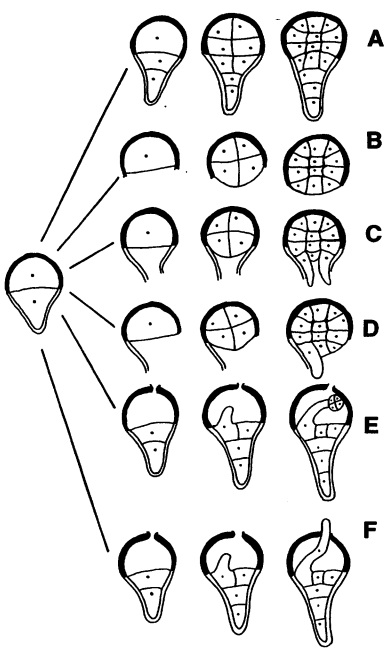


**Fig. 4.** Schematic representation of the development of (**A**) intact embryos and after laser ablation at (**B**) the two-cell stage of the rhizoid cell and wall, (**C**) rhizoid cell contents, (**D**) half of the rhizoid cell wall, and (**E** and **F**) thallus cell contents. (E and F) Also shown is the fate of a rhizoid (E) produced within the thallus cell compartment after prolonged contact with the thallus cell wall or (F) growing through a hole previously cut in the cell wall. Bold lines represent original thallus wall and double lines represent original rhizoid wall.

with the inner thallus cell wall can alter the fate of rhizoid cells.

Experiments have demonstrated the relative importance of position-dependent factors rather than cell lineage in the direction of plant embryogenesis (*8*, *9*), although inductive effects have not been demonstrated directly. Plant embryos are known to dedifferentiate and redifferentiate (*8*–*13*). Somatic embryos of *Daucus* respond to surgical tissue removal by reorganization of the cut region and tissue regeneration (*9*, *12*). In addition to the postulated roles of diffusible morphogens (*14*), indirect evidence has implicated cell surface– and wall-related molecules as fate determinants (*15*). We have demonstrated positionally regulated induction in a plant embryo and shown that fate-determining information is associated with the cell wall, most likely the inner face of the mature wall. If the original rhizoid-thallus dividing wall does not possess fate-determining properties, the mature rhizoid or thallus wall should have an overriding effect on the fate of recently divided cells bounded predominantly by primary, immature wall. The factors involved in these inductive processes must be long-lived and not readily diffusible because isolated cell wall retains its inductive properties for several days. The factors must act through the wall of the induced cell and must therefore be rendered diffusible by cell contact, possibly in response to secretions from the cell itself. The evidence presented here implicates the plant cell wall both in maintaining the differentiated state and in directing the pattern and cell fate in plant development.

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# Recombination Between Viral RNA and Transgenic Plant Transcripts


Ann E. Greene and Richard F. Allison*



**Transformed plants expressing the 3′ two-thirds of the cowpea chlorotic mottle virus (CCMV) capsid gene were inoculated with a CCMV deletion mutant lacking the 3′ one-third of the capsid gene. Although the deletion inoculum replicates in inoculated cells, systemic infections occur only if recombination restores a functional capsid gene. Four of 125 inoculated transgenic plants, representing three different transgenic lines, became systemically infected. Analysis of viral RNA confirmed that RNA recombination had united the transgenic messenger RNA and the challenging virus through aberrant homologous recombination.**


The evolution of plus sense RNA viruses proceeds by natural mechanisms including errors by viral RNA polymerase, which lacks proofreading capabilities, and by homologous and heterologous RNA recombination (*1*). Recombination has generated mosaic-type defective interfering RNAs in cymbidium ringspot tombusvirus (*2*) and variants of tobacco rattle tobravirus (*3*). Recombination has been reported in the 3′ untranslated and intercistronic sequences of bromoviruses (*4*–*6*). The mechanism of plant viral RNA recombination has been addressed experimentally in both brome mosaic virus and turnip crinkle virus subviral RNAs (*7*, *8*).

There are indications that plant RNAs have recombined with replicating viruses. Several potato leafroll virus isolates contain sequences homologous to an exon of tobacco chloroplast RNA (*9*). Additionally, a deletion mutant of red clover necrotic mosaic virus was restored by recombination with transgenically expressed viral RNA (*10*). The rarity of reported recombination events between viral RNA and host mRNA may reflect their infrequency or the failure of products to be viable.

Virus resistance can be conferred on transgenic plants by expression of segments of viral genome, such as capsid genes (*11*). Transgenic plants expressing a viral capsid protein exhibit resistance to that virus and closely related strains (*12*) but remain susceptible to other viruses.

Plants frequently resist viral attack by restricting virus movement rather than inhibiting replication (*13*). Therefore, plants challenged by viruses that are not patho-


Department of Botany and Plant Pathology, Plant Biology Building, Michigan State University, East Lansing, MI 48824–1312, USA.

*To whom correspondence should be addressed.

gens of that particular species may support viral replication. Thus, in virus-resistant transgenic plants, replicating pathogenic and nonpathogenic viruses may come in contact with a pool of viral RNA transcribed by the plant that is available for RNA recombination. Such events could generate a virus with properties that differ from either progenitor virus (*14*).

The following experiments sought to determine if mRNA expressed in a transgenic host is available for recombination with a replicating virus. Cowpea chlorotic mottle bromovirus (CCMV) consists of two monocistronic RNAs 1 and 2 that encode replication proteins and a dicistronic RNA 3 that encodes the putative movement protein, 3a, and capsid protein. Infectious transcripts are produced from complementary DNA (cDNA) clones of these RNAs (*15*). Transformed plants expressing the 3′ two-thirds of the CCMV capsid gene were inoculated with a CCMV deletion mutant lacking the 3′ one-third of the capsid gene. This deletion prohibits systemic infections. If recombination occurs within the central third of the capsid gene, a segment shared by both the transgenic and inoculation RNAs, a functional capsid gene could be restored that supports systemic infection. With this system, we demonstrate RNA recombination between mRNA derived from the host chromosome and replicating viral RNA.

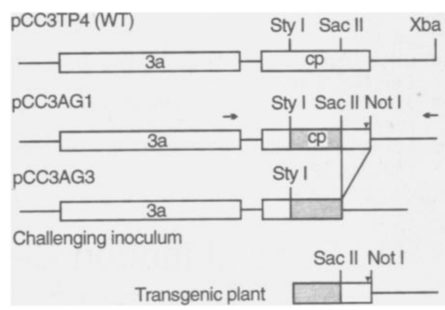


**Fig. 1.** Plasmid construction. The oligonucleotide TCTTCAGCGGCCGCTAATAGACCGGAGT was used to direct mutagenesis of pCC3TP4. The resulting plasmid, pCC3AG1, contained a silent mutation (G to C) at position 1926 (triangle) and changes at positions 1933 and 1935 (T to C, C to G), which introduced a Not I site at the 3′ end of the capsid gene. The 119-nucleotide Sac II–Not I fragment was removed from pCC3AG1, the sticky ends were filled with Klenow, and the plasmid was ligated to form pCC3AG3. Plasmid pCC3AG1 was digested with Sty I and Xba I, and the 697-nucleotide fragment was ligated into the Xba I site of transformation vector pGA643. This ligation was facilitated by first treating the vector ends with alkaline phosphatase, completing the Xba I ligation, then blunting the incompatible ends with Klenow and completing the ligation to form pGACCMV. Nucleotide numbering is given in (*23*). Arrows indicate positions of oligonucleotides used during PCR amplification of virion RNA.

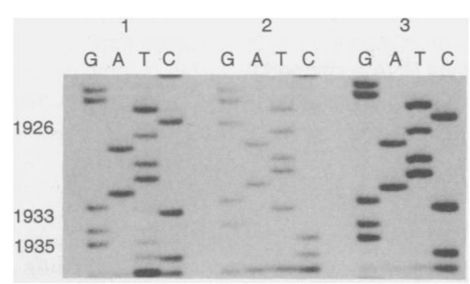


**Fig. 2.** Sequence analysis of PCR products generated from virion RNA of a transgenic plant infected with WT CCMV and plant 5-58. Full-length first-strand cDNA was made from virion RNA with oligonucleotide 3′-CCMV, CAGTCTAGATGGTCTCCTTAGAGAT. A 1096-bp fragment containing the intercistronic region, capsid gene, and 3′ untranslated region of RNA3 was amplified by PCR with oligonucleotides TAAAATCGCCGTAACCGC and 3′-CCMV. The amplified product was cloned into the Sma I–Xba I sites of pUC18 and sequenced with USB Sequenase. The sequences of plasmid pCC3AG1, and cloned PCR products from the WT infected plant and 5-58 are shown in panels 1 to 3, respectively. Nucleotide numbers denote positions of marker mutations present only in pCC3AG1 and viral RNA recovered from 5-58.

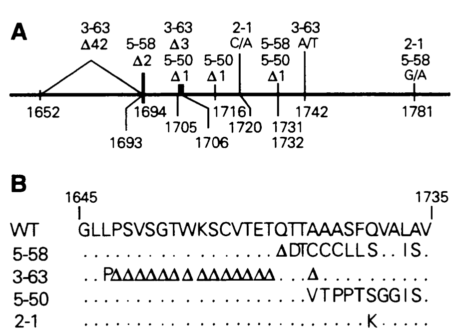


**Fig. 3.** Mutations in capsid genes of recombinant viruses 5-58, 3-63, 5-50, and 2-1. (**A**) The nature and extent of each nucleotide mutation is denoted above the horizontal line, which represents a segment of the WT capsid gene between nucleotides 1652 and 1781. Delta (Δ) indicates deleted nucleotides. Wild-type nucleotides and substitution mutations are separated by slanted lines. Exact positions of mutations are noted below the horizontal lines. Where adjacent or overlapping changes occur, the top recombinant corresponds to the top nucleotide number. (**B**) Amino acid deletions (Δ) and changes in the recombinants are compared with the WT capsid amino acid sequence between the Gly and Val codons beginning and ending at the indicated nucleotides. Dots indicate identity. See (*24*) for amino acid abbreviations.

Three marker mutations were introduced near the junction of the capsid gene and the 3′ untranslated region of the full-length cDNA clone of CCMV RNA3, pCC3TP4, to form pCC3AG1 [transcript AG1 (Fig. 1) (*16*)]. These mutations did not affect the viability of CCMV (*17*), and pCC3AG1 was used for construction of both the transgenic *Nicotiana benthamiana* and the challenging CCMV inoculum.

Deletion inoculation plasmid pCC3AG3 [transcript AG3 (Fig. 1)] was prepared by deletion of 119 nucleotides from the 3′ terminus of the capsid gene of pCC3AG1. When full-length transcripts of CCMV RNAs 1 and 2, C1 and C2, were coinoculated with AG3, neither cowpea nor *N. benthamiana* became systemically infected, but AG3 replication was observed in protoplasts.

A truncated capsid protein gene lacking 118 nucleotides from the 5′ end of the coding region, but containing the full-length 3′ untranslated region of RNA3 was cloned into transformation vector pGA643 (*18*) to generate pGACCMV (Fig. 1). This construct, which contains the neomycin phosphotransferase (NPT) II marker gene, was used to transform *N. benthamiana* (*19*).

From 57 kanamycin-resistant regenerated plants, 6 were selected on the basis of high levels of NPT II expression as judged by enzyme-linked immunosorbent assay. A CCMV-specific probe (6) hybridized to a single band on Northern blots of total RNA extracted from the six plants that were clonally propagated for recombination experiments. Transgenic plants challenged with either CCMV virions or wild-type (WT) RNA transcripts, C1, C2, and C3, became systemically infected. Thus, the transgenic transcript was insufficient to provide *N. benthamiana* with CCMV resistance.

The two youngest expanded leaves of 60 transformants at the six-leaf stage of development were inoculated with C1, C2, and AG3. Fourteen days after inoculation, plants were screened for systemic infections. Total RNA extracted from the fifth leaf above the inoculated leaf was probed for CCMV RNA. A positive hybridization to extracts from plant 5-58 suggested recombination.

Virion RNA was extracted from plant 5-58, and cDNA extending from the 3′ end of the 3a gene to the 3′ terminus of putative recombinant RNA 3 was synthesized and amplified by polymerase chain reaction (PCR) (Fig. 1). The recovered fragment was cloned and sequenced. All three marker mutations that were originally present only in the transgenic RNA were identified in the nucleotide sequence of the capsid gene (Fig. 2). Only wild-type sequence was recovered from control transgenic plants inoculated with WT CCMV. These results indicate that the systemic infection of 5-58 resulted from recombination between mRNA expressed by the plant and the challenging deletion inoculum.

Several deletions within 5-58 shifted the capsid open reading frame (ORF) 13 codons (Fig. 3). Despite these amino acid substitutions, sap extracts from 5-58 initiated typical CCMV systemic infections in both cowpeas and *N. benthamiana*, and normal yields of virion RNA were recovered from both species. Therefore, RNA recombination in 5-58 produced a mutant form of CCMV by aberrant homologous recombination within the overlapping region of the transgenic mRNA and the viral inoculum.

Of 125 transgenic plants tested, four recombinant viruses have been verified from three different transgenic plant lines. Despite attempts to favor homologous recombination by providing 338 overlapping nucleotides between the transgenic viral mRNA and genomic RNA of the challenging virus, sequences derived from recombinants revealed that each resulted from a distinctly different aberrant homologous recombination event (Fig. 3). Therefore, precise recombination was not required to restore virus viability.

Previous bromovirus studies have demonstrated RNA recombination only within noncoding regions (*4–6*). This report demonstrates intragenic recombination in 3% of the transgenic plants inoculated. Regeneration of a functional ORF must provide stringent selection pressure on recombination products.

One factor that may contribute to recombination is the presence of the complete 3′ untranslated sequence from CCMV RNA3 in the mRNA transcript. Since the viral replicase complex initiates minus strand RNA synthesis on this terminal sequence (*20*), its presence may enable replication to begin on the mRNA transcript and then switch to the RNA inoculum to complete synthesis. Thus, the presence of 3′ untranslated sequence may target the transcript to the replication complex and enhance the possibility of recombination. This would be consistent with the template-switching model for RNA replication (*21*). Because the 3′ untranslated region of the virus may lend stability to the viral RNA, it is frequently included in transgenic constructions.

Recombination during RNA virus replication contributes to the rapid evolution of RNA viruses and could affect host range or vector specificity, traits that have been attributed to capsid proteins of several plant viruses (*22*). As transgenically expressed viral mRNA is available to recombine with replicating RNA viruses, RNA recombination should be considered when analyzing the risks posed by virus-resistant transgenic plants.

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16. To avoid potential spurious mutations, we substituted a 359–base pair (bp) Sac II–Xba I fragment containing the introduced mutations for the similar fragment in the original plasmid, pCC3TP4, to form pCC3AG1. The fidelity of all constructs was confirmed by sequence analysis.
17. Effects of mutations on virus infectivity were ascertained by inoculation of both cowpea, *Vigna sinensis* (Torner) Savi, and *Nicotiana benthamiana* (Domin) with full-length plasmid-derived transcripts of WT CCMV RNAs 1 and 2 and either WT RNA 3 or pCC3AG1, referred to hereafter as C1, C2, C3, and AG1. Plants became systemically infected within 14 days, and no differences were observed in either the quantity or stability of recovered virions or viral RNA.
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19. This placed the CCMV sequence under the control of the constitutive 35*S* promoter and within the T-DNA region of pGA643. The pGACCMV plasmid was introduced by tri-parental mating into *Agrobacterium tumefaciens* strain LBA4404 for use in leaf disk transformation of *N. benthamiana*. Transformed explants were selected for kanamycin resistance in tissue culture.
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24. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


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# High-Resolution Molecular Discrimination by RNA


Robert D. Jenison, Stanley C. Gill, Arthur Pardi, Barry Polisky*



**Species of RNA that bind with high affinity and specificity to the bronchodilator theophylline were identified by selection from an oligonucleotide library. One RNA molecule binds to theophylline with a dissociation constant $K_d$ of 0.1 μM. This binding affinity is 10,000-fold greater than the RNA molecule's affinity for caffeine, which differs from theophylline only by a methyl group at nitrogen atom N-7. Analysis by nuclear magnetic resonance indicates that this RNA molecule undergoes a significant change in its conformation or dynamics upon theophylline binding. Binding studies of compounds chemically related to theophylline have revealed structural features required for the observed binding specificity. These results demonstrate the ability of RNA molecules to exhibit an extremely high degree of ligand recognition and discrimination.**


The conformational complexity of libraries of random-sequence single-stranded oligonucleotides offers the opportunity to search for molecules that show high-affinity binding to biomedically important targets (*1*). A procedure called SELEX (systematic evolution of ligands by exponential enrichment) permits the iterative isolation and amplification of RNA or DNA oligonucleotides with selective affinity for defined targets, which represents a route to drug discovery (*1*). With this technique, RNA oligomers have been isolated that have high affinity and specificity for a variety of both protein and small molecule targets, including bacteriophage T4 DNA polymerase (*1*), R17 coat protein (*2*), human immunodeficiency virus (HIV) reverse transcriptase (*3*), HIV Rev protein (*4*), basic fibroblast growth factor (*5*), adenosine triphosphate (*6*), and several amino acids (*7*). Oligomers of DNA that recognize thrombin (*8*) and oligomers of RNA and DNA that bind to organic dyes (*9*) have also been identified.

Many of these SELEX-generated oligo-


R. D. Jenison, S. C. Gill, B. Polisky, NeXagen, Inc., 2860 Wilderness Place, Boulder, CO 80301, USA.
A. Pardi, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA.

*To whom correspondence should be addressed.