

Recombination in plants expressing viral transgenes

Richard F. Allison, William L. Schneider* and Ann E. Greene†



When a segment of a specific viral genome is expressed in a transgenic plant, the plant is often resistant to that virus. This promising method of deriving virus resistance appears also to provide an opportunity for RNA recombination. Several reports confirm that the viral transgenic transcript is available to a challenging virus for recombination. Using cowpea chlorotic mottle virus as a model, modifications of the transgene significantly reduced the recovery of viable recombinants from transgenic plants. Several recommendations are made for reducing the involvement of the transgene in RNA recombination events.

Key words: bromoviruses / plant viruses / risk assessment / RNA recombination

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TRANSGENIC PLANTS that express a segment of a plant virus genome are often resistant to that specific virus.¹ Such resistance has been demonstrated with several different viral genes but the replicase and capsid genes appear to be most useful in delaying or preventing the onset of virus infection. Presently, virus resistant transgenic plants (VRTP) provide a most promising means of combating plant viral disease, particularly for plant species where natural virus resistant genes are unavailable. During consideration of VRTPs, it was suggested that these plants may provide a unique opportunity for virus recombination.²

As addressed in other articles of this issue, RNA recombination involves the exchange of RNA templates during virus replication and results in a replication product that represents the union of two previously distinct RNA templates. Such recombination events have undoubtedly contributed to the evolution of numerous plant and animal viruses.³ If transgenic transcripts were available to a replicating virus, then all or portions of the transcript could be

incorporated into the genome of that replicating virus through RNA recombination. Current transgenic constructions assure the constitutive expression of the viral transgene in virtually every plant cell and predictions suggested that these transcripts would be available for RNA recombination with a challenging virus. Survival of recombinants would depend on selection for any useful characteristics that a recombinant virus may have acquired, its stability and its competitiveness. Several independent investigations have demonstrated that transgenic transcripts are available to replicating viruses.

Recovery of complete viral genes by recombination with a transgene

The initial report of incorporation of a viral transgene into a challenging virus involved red clover necrotic mosaic dianthovirus (RCNMV)⁴ which has a bipartite RNA genome. *Nicotiana benthamiana* was transformed with the movement protein gene of the monocistronic RNA2 and challenged with RNA1 which encodes the replication and capsid proteins. Several transgenic plants became systemically infected. Although the transgene lacked the 5' untranslated region (UTR) of RNA2, virus recovered from systemically infected plants contained a complete RNA2 which had recovered a functional 5' UTR from RNA1 of the inoculum through RNA recombination.

The second demonstration of transgene recombination was in the DNA plant virus cauliflower mosaic caulimovirus (CaMV).⁵ In these experiments *Brassica napus* was transformed with gene VI derived from the S strain of CaMV. The CaMV inoculum, derived from strains 4184 and D/H, contained a deletion in gene VI and was consequently incapable of systemically infecting *B. napus*. The termini of the transgene shared 818 and 1033 nucleotides of nearly homologous overlapping sequence with the inoculum and these overlapping regions were available for homologous recombination. Four of the 12 transgenic plants inoculated became systemically infected with recombinant virus derived from the inoculum and the transgene. As a pararetrovirus, CaMV uses an RNA

From the Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824, *Genetics Program, Michigan State University, East Lansing, MI 48824, and †USDA/ARS, Agronomy Department, 1150 Lilly Hall of Life Sciences, West Lafayette, IN 47907-1150, USA

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intermediate and reverse transcriptase during viral replication. Nucleotide sequences of three recombinants suggested that the circular genome had been restored by two RNA recombination events. Sequence of the fourth recombinant provided evidence of both RNA and DNA recombination events.

Another incidence of CaMV transgene's involvement in recombination was reported by Schoelz and Wintermantel.⁶ In their strains the ability to systemically infect *Nicotiana bigelovii* is encoded by gene VI. When transgenic *N. bigelovii* plants expressing gene VI from the strain which infects that species were challenged with the noninfecting CaMV strain, systemic infections developed in all 10 plants. Theoretically, complementation could have facilitated the systemic infection, but analysis revealed that the challenging virus had acquired the gene VI transgene through recombination, presumably during the reverse transcriptase phase of replication. Passaging revealed that the recombinant virus had acquired the capacity to infect nontransgenic *N. bigelovii*.

Repair of deletions in viral genes by recombination with a transgene

The experiments outlined above involved recovery of a complete viral gene by a challenging deletion mutant. The following experiments examined intergenic repair of small deletions by RNA recombination. In a study of tombusviruses (Jackson A.O., Rubio T., Borja M., Scholthof H.B., unpublished results), *N. benthamiana* was transformed with the capsid gene of tomato bushy stunt virus (TBSV). Although transgene mRNA was detected, transgenic plants were not resistant to TBSV, which causes plant death. These transgenic plants were inoculated with a TBSV mutant lacking 50 nucleotides of the capsid gene. This mutant systemically infects *N. benthamiana* but causes only mild symptoms. Initially all plants displayed these mild symptoms, however, within four weeks several plants died. The presence of virions in the necrotic plants suggested that recombination may have repaired the capsid deletion. To ensure that recombination could re-establish the capsid gene, transformants were inoculated with a chimeric virus consisting of the related cucumber necrosis tombusvirus (CNV) that contained a defective TBSV capsid gene. Again, plant death signaled the presence of a restored virus and the recovery of virion RNA which contained a complete TBSV capsid gene within the CNV RNA confirmed recombination. In contrast to

most other plant RNA viruses, the capsid genes of tombusviruses are not 3' terminal but are located internally. The tombusvirus transgenes described above consisted of the capsid gene; no additional viral untranslated sequences were included in the transgenic construct. Consequently, a minimum of two recombination events was required to repair the TBSV capsid gene. This is in contrast to many transgenic constructs derived from viruses in which the capsid gene is the 3' terminal ORF. In these cases the transgenic construct frequently includes the 3' UTR of the viral genome which may simplify the restoration of these deletion genomes (see below).

Intergenic repair of a small capsid gene deletion was also studied in cowpea chlorotic mottle bromovirus (CCMV).⁷ Like all bromoviruses, CCMV is a tripartite single-stranded positive sense RNA virus (Figure 1A). Transcripts from the three full-length cDNA clones, designated C1, C2 and C3, systemically infect the natural CCMV host range which includes many legumes and *N. benthamiana*.⁸ RNAs 1 and 2 encode single proteins which are responsible for viral replication. Dicistronic RNA3 encodes the movement

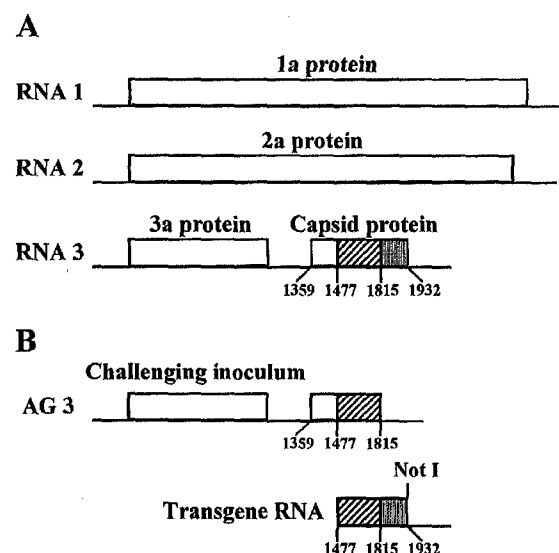


Figure 1. Genomic map of CCMV RNAs used in this recombination study. Panel A is a map of the three genomic CCMV RNAs with their ORFs shown as open boxes. Numbers indicate nucleotide positions within the capsid gene. Panel B depicts the CCMV RNA 3 deletion mutant AG3 used in the inoculation of plants expressing the transgene RNA that is indicated at the bottom. The Not I restriction site that is present only in the transgene is indicated.

and the capsid proteins which are both required for systemic movement but are not involved in replication.⁹ The requirement for a functional capsid gene became the basis for detection of recombination in the following studies.

Nicotiana benthamiana was transformed with the 3' 2/3 of the capsid protein gene and its natural 3' UTR (Figure 1B). The transgene could be distinguished from the wild type CCMV capsid gene by three silent mutations which introduced a Not I restriction site. The 35S promoter provided constitutive detectable transcription of the CCMV transgene, but since no initiation codon was added to the truncated gene, translation was unlikely and undetectable. Plants with high levels of transgenic RNA were selected for recombination experiments; these transgenic plants were not resistant to CCMV.

An RNA3 deletion mutant, pCC3AG3, was constructed which lacked the 3' terminal 121 nucleotides of the capsid gene (Figure 1B). This deletion mutant shared 336 nucleotides of the capsid gene and the entire 3' UTR with the transgene. Transcripts from pCC3AG3 (AG3) plus C1 and C2 were unable to systemically infect nontransgenic plants despite their replication in protoplasts. If recombination between the transgene of the transgenic plant and the inoculum restored a functional capsid gene, then the virus would systemically infect the plant.

When transgenic *N. benthamiana* plants were challenged with C1, C2 and AG3, seven of the 235 transgenic *N. benthamiana* plants inoculated (3%) became systemically infected. The infection was passed to nontransgenic plants from which viral RNA was recovered, converted to cDNA, cloned and sequenced to confirm recombination events. All of the cloned recombinants contained the Not I restriction site, indicating that segments of the transgene had been incorporated into the virus. The sequence of each recombinant capsid gene was uniquely different from wild type (Figure 2); differences ranged from single point mutations to a 42-base deletion. In some cases scattered point mutations briefly changed the reading frame but in all recombinants the reading frame of the 3' terminal portion of the capsid gene was preserved. The numerous mutations that were introduced during recombination indicated that recombination was occurring in an aberrant homologous manner, as defined by Lai.³ This is in contrast to the previous reports outlined above where mutations at or near the recombination sites were not reported.

A majority of the mutations occurred within a span

of 225 nucleotides within the 336 nucleotide overlapping region shared by both the inoculum and the transgene. While this span may include sequences which facilitate recombination, no distinct secondary RNA structures that may cause pausing of the replicase, or repeated nucleotide sequences that may encourage recombination, were predicted for that area. Alternatively, the region could encode a segment of the capsid protein that is amenable to change. Analysis of the crystal structure of the CCMV virion indicates that this segment of the capsid protein forms an alpha helix near the surface of the capsid

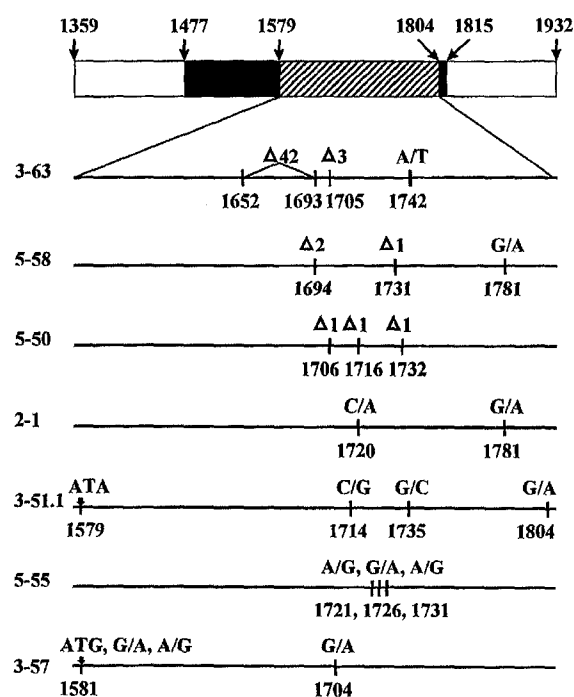


Figure 2. Variability in recombinant capsid genes recovered from transgenic plants. The box at the top represents the capsid open reading frame. The blackened portion, nucleotides 1477 to 1815, represents the homologous sequences shared by both the inoculum and the transgene. The hashed line box superimposed on the black box represents the region where variation in the recombinant sequences was observed, nucleotides 1579 to 1804. Each line below represents the sequence of a cDNA clone of a specific recombinant virus, identified by the number at the left. The nature and extent of each nucleotide mutation is denoted above the vertical lines. A delta (Δ) indicates deleted nucleotides; substitutions are indicated by slanted lines with the wild type nucleotide to the left and the substitution on the right. Insertions are indicated by arrows. Mutation positions are denoted below each line.

subunit.¹⁰ This region may contribute little to the physical structure of the virion and thereby may be more tolerant of modification than regions that are responsible for the virion's structural integrity. Symptom variations observed among these recombinants suggest that this region may interact with the host.

Effect of deletions in the 3' UTR on recombination

In several of the experiments described above the transgenes that were involved in RNA recombination contained a replicase binding site. In RCNMV for instance,⁴ the transgene consisted of RNA2 lacking only the 5' UTR and likely contains a replicase binding site for minus strand RNA synthesis. In Gal's CaMV experiments the gene VI transgene contained the site where reverse transcriptase normally switches templates during CaMV replication.⁵

The CCMV transgene described above includes the complete 3' UTR which is characteristic of many of the capsid protein transgenic constructs reported to date. In CCMV the 3' UTR contains the replicase binding site which is required for the initiation of minus strand synthesis.^{11,12} Since the transgene includes the replicase binding site, it is conceivable that the viral replicase of a challenging virus could initiate replication on the transgene itself.² In the case of the capsid deletion repair,⁷ only a single recombination event would have been required to restore the capsid gene. Furthermore, the presence of the replicase binding site in the transgene may facilitate the synthesis of a complete minus strand copy of the transgene by the challenging virus replicase which could also be involved in recombination events during plus strand RNA synthesis. Our preliminary data indicate that the CCMV transgene is recognized as a viral template by a challenging bromovirus and a complete minus strand copy of the transgene is produced during infections.

The significance of the 3' UTR in replication led to our examination of CCMV transgenes which contained deletions in this region.¹³ Three new transgenes were constructed which contained the same segment of the capsid gene as used in the original experiments but differed from the original transgene by 3' terminal deletions of 69, 83 and 214 nucleotides. These deletions were intended to disrupt or delete the replication binding site. *Nicotiana benthamiana* plants were transformed with these constructs and transformants were selected which revealed transcript

quantities similar to those observed in the original transformation experiment.⁷ These plants were inoculated with the same transcripts, C1, C2 and AG3, as in the previous experiment and tested for systemic infections. In the 479 transgenic plants evaluated, none became systemically infected. In our original experiments, recombinants were recovered from 3% of the inoculated transgenic plants. So by varying only the 3' UTR, the detection of recombinants fell significantly. Reduction may have occurred by limiting the size of the recombination target or by disrupting the replicase binding site and thereby inhibiting the initiation of RNA synthesis on the transgene.

Recombination between CCMV inoculation transcripts

To compare recombination in transgenic plants with recombination among inoculating viral RNAs, two RNA3 deletion mutants were constructed which mimicked the constructs used in the transgenic recombination studies. pCC3SR1 lacked the 5' 1/3 of the capsid gene, and pCC3SR2 lacked the 3' 1/3 of the capsid protein gene (Figure 3). As in the previous transgenic recombination experiment,⁷ the two RNA3 deletion mutants shared the same 336 nucleotide overlapping region where recombination would be required if a functional capsid gene were to be restored. Additionally, pCC3SR1 contained the same silent marker mutations as the original transgene. Transcripts from either mutant, designated SR1 and SR2, were not infectious in combination with C1 and

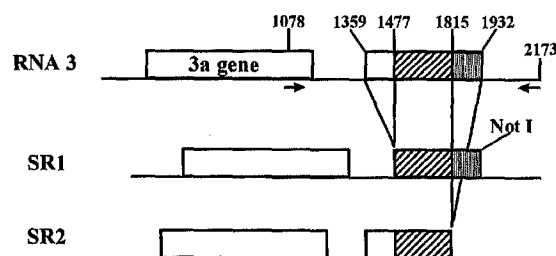


Figure 3. CCMV RNA3 deletion mutants SR1 and SR2. Genomic RNA3 is shown at the top, various nucleotide positions used in the construction of the deletion mutants are indicated. SR1 lacks the 5' 118 nucleotides of the capsid gene and contains marker mutations which provide a Not I restriction site. SR2 lacks 117 nucleotides from the 3' terminus of the capsid gene. Shading is similar to that of Figure 1A. Arrows denote the location of oligonucleotide primers used for RT/PCR.

C2, despite the fact that both mutants replicated in protoplasts.

Leaves of non transgenic cowpeas were inoculated with a combination of C1, C2, SR1 and SR2 at a concentration of 0.5 µg of each transcript per plant leaf. Systemic infection occurred in 7% (four of 56) of the plants inoculated. Virion RNA extracted from these plants was amplified by reverse transcription and PCR (RT-PCR), and digested with Not I (Figure 4). Presence of the Not I restriction site distinguished the infection from WT while the size of the restriction fragments confirmed that all four infections were derived from recombinant virus. The greater percentage of viable recombinants recovered from plants inoculated with these SR1 and 2 deletions mutants, as compared to the transgenic experiment, may reflect the stability of these larger transcripts, and/or the quantity of transcript available to the replication complex. Based on northern analysis, the quantity of the transgenic transcript per unit leaf area is significantly less in the transgenic leaves than the quantity of viral RNA used in these inoculations. These experiments demonstrate that recombination in the 336 nucleotide overlapping sequence is not unique to the transgenic plants, and they suggest that recombinant recovery is proportional to the quantity of transcript available.

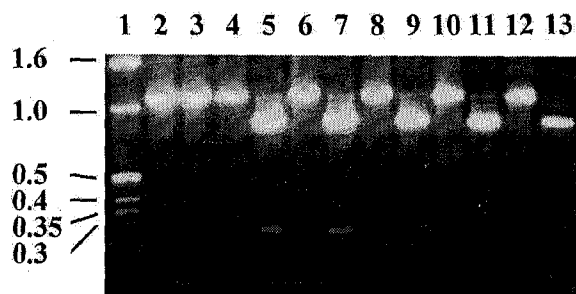


Figure 4. Not I restriction digest pattern of RT-PCR products derived from plants that became systemically infected following inoculation with the SR1/SR2 deletion mutants. Lane 1 of this ethidium bromide stained 1.0% agarose gel contains the 1 kb ladder, fragment sizes are indicated at the left in kilobases. The following pairs of lanes represent undigested and digested cDNA respectively; lanes 2 and 3, WT CCMV; lanes 4 and 5, pCC3AG1. The remaining four pairs of lanes represent cDNA recovered from systemically infected, SR1/SR2 inoculated cowpeas.

Transgene recombination in the field

Collectively, the experiments above demonstrate that the viral transgene is available within transgenic plants for recombination with a challenging virus and that RNA recombination can restore a virus deletion. All of the experiments have required a virus to recombine with either itself or a different strain of the same virus and many of the recovered recombinants have been genetically distinct from the wild type virus.

How do these experiments differ from what may happen in a field of virus-resistant transgenic plants? Since recombination is known to be involved in virus evolution,¹⁴ it is conceivable that all or a portion of the transgene could be incorporated into a challenging virus. Viruses challenging VRTPs are either known pathogens or non pathogens. Known pathogens are already adapted to that host and may have had previous recombination opportunities during mixed infections. So the concern most unique to VRTPs is that recombination could occur between the transgene and a virus that is capable of replicating in the transgenic plant but is not capable of systemic movement. Many viruses that are considered non pathogens of a plant species replicate in the initially inoculated cells but their spread is limited. Consequently, if the non pathogenic virus is to be converted to a systemically invading pathogen, recombination must result in the non pathogen acquiring movement functions specific for that host. Selection pressure for such a viable recombinant appears to be quite high and similar to that seen in the above experiments. While specific movement proteins have been identified or implicated in many plant viruses, other viral proteins, including the capsid protein of some viruses, contribute to systemic movement.¹⁵⁻¹⁹ Determinants of host specificity, which may be directly linked to systemic movement, have not been established. Consequently, it is difficult to predict if recombination involving a transgene could convert a non pathogenic virus to a pathogen. However, functional chimeric viruses have been assembled in the laboratory^{20,21} and we have preliminary evidence that they may also form by recombination in transgenic plants.

At this point in time, early in the development and release of virus-resistant transgenic plants, it seems prudent to discourage recombination events involving the viral transgene by designing transgenes that are less likely to recombine than the ones studied above. Accumulated results may suggest several ways of accomplishing this.

1. Exclude sequences known or predicted to be replication complex initiation sites. Based on our data, exclusion of this region reduced recombination significantly. Although such sites have been identified in only a few plant viruses, they are likely near the termini of linear viral RNA's. If such regions are not required for resistance, they can be excluded or disrupted.
2. The transgene should be the smallest viral fragment that provides useful resistance. Larger fragments provide not only larger recombination targets but they are more likely to encode functional modules that could be incorporated into another virus as a single unit.
3. Select transgenic plants that provide the maximum resistance with the minimal amount of transgene expression. The fewer the transcripts, the less likely they are to be involved in recombination.

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