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RISK ASSESSMENT OF TRANSGENIC PLANTS: RNA RECOMBINATION IN TRANSGENIC PLANTS EXPRESSING VIRAL SEQUENCES

By

Ann Elizabeth Greene

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ABSTRACT

RISK ASSESSMENT OF TRANSGENIC PLANTS:
RNA RECOMBINATION IN PLANTS EXPRESSING VIRAL SEQUENCES

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RNA recombination has played a significant role in the evolution of plant viruses. Therefore, one risk associated with the environmental release of virus resistant transgenic plants is that the transgenic viral mRNA could recombine with challenging viral RNA to produces a chimeric virus with unique properties. To test this hypothesis, a sensitive bioassay was developed to test for recombination in transgenic plants. *Nicotiana benthamiana* was transformed with a portion of the cowpea chlorotic mottle bromovirus (CCMV) capsid gene. Plants were challenged with a movement defective CCMV deletion mutant lacking a segment of the capsid gene present in the transgene. RNA recombination restored the gene in 3% of the transgenic plants. Sequence analysis revealed that the recovered viruses arose by aberrant homologous recombination between the transgenic CCMV message and CCMV challenging RNA, which introduced mutations in the capsid gene. These mutations affected symptom development, but had no effect on host range or movement within non-transformed plants. In a related experiment, RNA recombination within the capsid gene restored function to movement defective brome mosaic bromovirus (BMV) in 2.3% of plants expressing the CCMV transgene. Recombinants were hybrids of the BMV and CCMV genomes. Hybrid 5-61

distinguished itself from either parent by systemically infecting both cowpea and barley. Thus, recombination resulted in a chimeric virus with altered host range. Several hybrid RNAs were isolated that contained non-bromovirus sequence suggesting that host RNAs also participate in RNA recombination with challenging viruses. In an effort to reduce the involvement of the transgene in RNA recombination events removal of the viral replication initiation site from the transgene decreased the recovery of recombinants in transgenic plants. This work indicates that viable recombinant viruses with altered properties can arise by RNA recombination in transgenic plants expressing viral sequences. Experiments excluding viral control sequences from the transgene reduced recombinant recovery and suggest a practical means of reducing transgenic recombination in virus resistant transgenic plants destined for environmental release.

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INTRODUCTION

The modification of plants using genetic engineering, as opposed to traditional breeding methods, has applications in a number of areas. The main applications are to obtain herbicide resistance, resistance to herbivores and pathogens, male sterility and product quality improvement (Dietz, 1993). Examples of product improvement include changes in the constitution of plant storage proteins, modification of plant oils and improvement of transportability and shelf life in fruits (Mitten et al., 1992). Regulatory agencies have approved the release of Calgene's 'Flavr-Savr' tomato with an extended shelf life. Release of other transgenic plants have followed and numerous products are currently under consideration.

Transgenic virus resistance is acquired by introducing a portion of a plant virus genome into the genome of the host plant. Introduction of viral genes can be done using *Agrobacterium tumefaciens* mediated transformation, particle gun transformation or other methods of DNA transfer. Since most plant viruses have RNA genomes, a cDNA is made to the portion of the viral genome to be introduced. The transgenic

construct contains a plant viral gene placed under direction of the 35S transcription promoter of cauliflower mosaic virus, or another constitutive promoter. Most often the DNA insert is arranged as a cassette, which also contains a selectable marker gene to aid in identifying transformed tissue. In many cases the neomycin phosphotransferase (NPTII) gene is used and transformants are selected for kanamycin resistance in tissue culture.

Two viral genes, the capsid protein and replicase genes, have provided resistance for numerous plant viruses. Other viral genes have been used to engineer virus resistance with varying success. Effectiveness of movement protein, antisense RNA and ribozymes for pathogen derived resistance appear to differ among specific viruses and will not be discussed in detail. At the start of this project, the capsid gene was the predominant gene used for pathogen derived virus resistance, and capsid protein mediated resistance has been demonstrated in many plant viruses (Grumet, 1990; Beachy et al., 1990; Fitchen and Beachy, 1993). In general, this resistance is very narrow and only protects the plant against the virus from which the capsid protein was derived and a few closely related viruses. In many cases this type of resistance can be overcome by inoculation with high virus concentrations or by inoculation with naked RNA. Protein expression is required for resistance in some viruses (Cuozzo et al., 1988; Hemenway et al., 1988), while expression of transgenic RNA is sufficient

in others (De Haan et al., 1992; Kawchuk et al., 1991; Lindbo and Dougherty, 1992). The mechanism of resistance remains unknown and may differ among virus groups and constructions. Field tests for release of transgenic plants expressing a capsid protein are on going. Recently, Asgrow Seed, Inc. received approval for release of transgenic squash expressing capsid gene for protection against two potyviruses.

Recently, replicase mediated resistance has become a more attractive method of conveying resistance. Plants expressing a viral polymerase have shown a strong resistance to challenge with high concentrations of that virus or closely related viruses. Early attempts of engineering plants to express replicase did not provide resistance as expression of the entire protein provided a functional replicase complex which complemented replication deficient viral RNAs (Mori et al., 1992). In later attempts, scientists expressed a modified replicase gene or subdomain of the viral replicase to provide resistance (reviewed in Fitchen and Beachy, 1993; Wilson, 1993; Scholthof et al., 1993). This type of resistance was also narrow, however the resistance was not overcome by inoculation with high virus concentrations. Although replicase mediated resistance in TMV requires protein expression (Carr et al., 1992) as does resistance to pea early browning virus (MacFarlane and Davies, 1992), the role of transgenic RNA in protection cannot be ruled out. In general, the mechanism of

replicase mediated resistance is also unknown and may differ among viruses.

With the success of genetically engineered resistance and the imminent commercial release of transgenic plants, several questions with regard to environmental risk have be raised. Many concerns are universal to all transgenic plants and involve potential ecological effects. For instance, the transgene or selectable marker genes may be toxic to humans or other animal species. Calgene was required to demonstrate that the NPTII gene product for kanamycin resistance was non-toxic before the release of its genetically engineered tomato (Calgene, 1990). Further, scientists at Monsanto showed that the NPTII gene is degraded in rodent guts (Fuchs et al., 1992). As for the toxicity of viral proteins, the ubiquitous nature of plant viruses ensures us that humans and animals have digested viral proteins at multiple times in their lifetime without ill effect. Produce exhibiting viral symptoms is frequently observed at grocery stores or produce stands. Therefore, it is unlikely that the ingestion of plant viral proteins expressed in transgenic plants would have ill affect on humans or other organisms. Although the composition of the transgenic inserts are well defined, changes in plant metabolism may occur upon plant transformation. Therefore, toxicity tests should be completed before release of plants (Dietz, 1993).

Two additional concerns raised for all transgenic plants include outcrossing and potential phenotypic changes of the transformant itself. First, outcrossing between the transgenic plant and wild relatives may create wild populations that express the transgenic trait. Through natural selection the introduction of a new gene may influence the evolution of the ecosystem surrounding the transgenic field. The likelihood of outcrossing occurring depends on presence of wild or related species in the area of cultivation. For example, outcrossing of maize is less likely in Europe where there are no native wild relatives than in Mexico or the Southern United States. Ploidy level, flowering time and genetic compatibility influence the risks anticipated in outcrossing. Tests performed in potato (Dale et al., 1992) and rapeseed (Dale et al., 1990; Darmency and Renaud, 1992) to measure pollen spread from transgenic plants help determine recommendations for a minimal distance between transgenic plants and their wild relatives and for border rows surrounding transgenic crops.

The second concern arises due to the process of genetic engineering. Most methods insert the foreign sequence randomly into the host genome. The disruption of a plant gene may affect specific plant characteristics. Potential effects include weediness, genetic instability and expression of undesirable phenotypic traits (Dietz, 1993). Other undesirable phenotypic traits may arise from the expression of the transgene itself. For example, sorghum bred for a higher

tannin content to protect against birds also had a negative effect on the nutritional value of the seed (Hahn et al., 1984). Undesirable traits can often be removed by backcrossing with non-transgenic relatives by traditional breeding methods.

Two potential risks are specifically associated with pathogen derived virus resistance. The first deals with transencapsidation of viral RNA by capsid protein expressed in a transgenic plant. Transencapsidation refers to the encapsidation of viral RNA with capsid protein of a heterologous virus. Transencapsidation has been demonstrated in mixed luteovirus infections (Creamer and Falk, 1990). Capsids of vector transmitted viruses contain determinants of vector specificity on their surface. Therefore transencapsidation can alter vector transmissibility allowing the virus to be introduced to species beyond its normal host range. Encapsidation by a transgenic capsid protein has been demonstrated. Coat protein defective tobacco mosaic virus (TMV) mutants were encapsidated by transgenic TMV capsid protein allowing for recovery of intact virion particles (Osbourn et al., 1990). Transgenic potato leaf roll virus (PLRV) capsid protein transencapsidated potato viruses X, Y, and S (Martin, 1992), however, none of these transencapsidated viruses was aphid transmissible. Farinelli et al. (1992) reported heterologous encapsidation of potato virus Y strain O (PVYO) in transgenic potato virus Y strain N (PVYN) capsid protein. Aphid transmission of transencapsidated zucchini

yellow mosaic potyvirus (ZYMV) has been reported in transgenic plants expressing capsid protein from plum pox potyvirus (Lecoq et al., 1993). Expression of truncated capsid protein from a partially deleted gene, or expression of a non-translatable RNA have been suggested as methods of avoiding transencapsidation. Since some virus resistance requires the intact capsid protein, these strategies may not be appropriate. Although transencapsidation could result in the movement of virus to new hosts, there is no change in the nucleic acid content of the virus. Therefore, further movement of a virus by the alternate vector is unlikely.

The second potential risk unique to virus resistant transgenic plants is RNA recombination. Recombination is important to the evolution of RNA viruses. Similarities among plant and animal RNA viruses suggest modular evolution in which segments of one viral genome are incorporated into another (Goldbach, 1987). RNA recombination may provide the mechanism for modular evolution. Sequence analyses provide evidence that recombination has occurred among numerous plant viruses. These include the *Bromoviruses* (Allison et al., 1989), *Hordeiviruses* (Edwards et al., 1992) and *Tobraviruses* (Angenent et al., 1989). RNA recombination has been demonstrated in the laboratory in many single stranded and double stranded RNA viruses. Genetic exchanges are common between strains of the single stranded minus-sense RNA virus, influenza (Kilbourne, 1983; Orlich et al., 1994) and the

double stranded RNA bacteriophage ϕ6 (Onodera et al., 1993; Mindich, 1995). Recombination in single stranded positive (+) sense RNA viruses has been reviewed by Lai (1992). Recombination has been demonstrated in animal viruses, including poliovirus (Romanova et al., 1980; King et al., 1982), *Coronaviruses* (Lai et al., 1985) and Sindbis virus (Weiss and Schlesinger, 1991; Schlesinger and Weiss, 1994) and in bacteriophage Qß (Biebricher and Luce, 1992; Palasingam and Shaklee, 1992). Among the plant viruses, RNA recombination has been extensively studied in *Bromoviruses* and will be discussed later. Recombination has also been demonstrated in the satellite virus of turnip crinkle virus (Cascone et al., 1990). RNA recombination is responsible for the formation of defective interfering particles in both animal and plant viruses (Holland, 1992; White and Morris, 1994).

Two potential mechanisms for recombination have been proposed. First, recombination could occur by breaking and rejoining of RNA strands by mechanisms similar to eukaryotic mRNA splicing. Although the enzymatic machinery for splicing RNA is present in eukaryotic hosts, there is no evidence to support cleavage and religation in RNA recombination. Rather, recombination appears to occur by a template switch between RNA molecules during viral genome replication (Lai, 1992). This 'copy choice' model was first proposed for poliovirus, where the ability of the replicase to participate in a template switch was demonstrated (Kirkegaard and Baltimore,

1986). Evidence exists that template switching occurs during minus (-) strand replication in poliovirus (Kirkegaard and Baltimore, 1986), brome mosaic bromovirus (BMV) (Nagy and Bujarski, 1993) and *Tombusviruses* (White and Morris, 1994). Recombination occurs during plus- and minus- strand synthesis in turnip crinkle virus (Cascone et al., 1993; Carpenter and Simon, 1994) and mouse hepatitis virus (Lai and Lai, 1992). Mutations in the helicase-like domain of the 1a replication protein of BMV affects RNA recombination (Nagy et al., 1995). This provides evidence for the role of replication in viral RNA recombination.

RNA sequences and structures required for template switching varies among viruses. Recombination in double stranded bacteriophage ϕ6 and in negative, single stranded influenza virus has little dependence on sequence homology at or near the crossover sites. As few as two complementary nucleotides are sufficient for template switching (Mindich, 1995; Orlich et al., 1994). In homologous recombination in picornaviruses, a heteroduplex or secondary structure, may form at regions of self complementation that facilitate recombination (Romanova et al., 1986; Tolskaya et al., 1987). Similar structures have been proposed for parental molecules in coronavirus recombination (Makino et al., 1986). Short sequence homologies, have also been proposed to facilitate non-homologous recombination events in Qβ (Bierbricher and Luce, 1992) and tombusviruses (White and Morris, 1994).

Specific secondary structures are required for recombination in turnip crinkle virus (Cascone et al., 1993). Presence of secondary structure at recombination sites suggests that the replicase may dissociate from one template at these sites and switch to another template. Studies of BMV suggest that heterologous recombination is facilitated by heteroduplex formation between template RNAs (Nagy and Bujarski, 1993). In this case 60 or more nucleotides were required to facilitate heterologous recombination. All recombination events took place at the left side of the heteroduplex region. Homologous recombination in bromoviruses, in contrast to heterologous recombination, requires small regions of homology between templates; secondary structures do not appear to be involved in homologous recombination (Nagy and Bujarski, 1995). Thus recombination can be explained by a model proposed by Jarvis and Kirkegaard (1992) where the replicase can back up during replication of the primary template exposing a short non-base paired region of the nascent strand. The replicase can then switch and begin copying a new template. This ability of polymerase enzymes to back up on a template has been proposed for Klenow DNA-dependent DNA polymerase (Freemont et al., 1988) and human RNA polymerase II (Kassavetis and Geiduschek, 1993; Wang and Hawley, 1993).

Viral transgenic RNA may participate in RNA recombination. Recombination in transgenic plants has now been shown in cauliflower mosaic virus (Gal et al., 1992). Movement

defective red clover necrotic mosaic virus (Lommel and Xiong, 1991) and alfalfa mosaic virus (van der Kuyl et al., 1991) have recombined with viral transgenes to rescue their respective movement genes. Depending on the templates involved, recombination may result in formation of new viral species with altered properties.

The work in this thesis addresses potential risks of RNA recombination in transgenic plants using a bromovirus system. *Bromoviruses* belong to the family *Bromoviridae* (Rybicki, 1995) and have a tripartite, single stranded, positive (+) sense, RNA genome. The 5' termini of the three genomic RNAs are capped, while the 3' terminal untranslated region folds into a tRNA-like structure that is amino acylated. RNA 1 and RNA 2 each code for single proteins that interact with each other, and host factors, to form the replication complex. Bromovirus replication has been well characterized (Ahlquist, 1992). RNA 3 is dicistronic; the 3a cell to cell movement protein is expressed from the RNA 3 and the capsid protein from a subgenomic message, RNA 4. Studies using deletions of the 3a and capsid genes of cowpea chlorotic mottle virus (CCMV) indicated that both genes are required for systemic movement (Allison et al., 1988). Bromoviruses provided a logical system to study recombination in transgenic plants. Preliminary evidence existed that recombination had occurred in bromovirus evolution (Allison et al., 1989). Additionally, a number of studies demonstrated RNA recombination in the intercistronic

region of CCMV RNA 3 (Allison et al., 1990) and between the 3' untranslated regions (UTRs) of all three genomic BMV RNAs (Bujarski and Kaesburg, 1986; Rao et al., 1990; Nagy and Bujarski, 1992). Also, chimeric virus containing a 3' UTR from TMV recombined with wild type BMV 3' UTRs (Ishikawa et al., 1991). BMV and CCMV are closely related and contain a high degree of homology at both the amino acid and nucleic acid level (Schneider and Allison, 1995). Genetic exchanges between these two viruses indicated that BMV replicase recognizes the replication initiation site within the 3' UTR of CCMV, and supports replication of CCMV RNAs in protoplasts (Allison et al., 1988). Although these viruses have nearly distinct host ranges, CCMV infects legumes and BMV infects grasses, both viruses share a common local lesion host *Chenopodium quinoa* (L.) and a single systemic host *Nicotiana benthamiana* (Domin).

Recombination between viral transgenes and challenging viral RNA had not been addressed at the undertaking of this project. The original goal was to establish if transgenes were available for recombination with challenging virus and determine if viable recombinant virus could be produced. If recombinants were detected experiments would be designed to reduce the involvement of the transgene in recombination events. To this end, different transgenic constructs would be tested to determine if the presence of viral replication signals on the transgenes enhanced the involvement of the transgene in recombination. This work addresses these basic

questions of RNA recombination in transgenic plants expressing CCMV capsid protein gene sequences. The ultimate goal has been to relate the results of those risk analyses to regulatory agencies, and those constructing virus resistant transgenic plants so that the design and release of such plants can take place in a responsible manner.

CHAPTER 1

Recombination between viral RNA and transgenic plant transcripts

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 (Lai, 1992). Recombination has generated mosaic-type defective interfering RNAs in cymbidium ringspot tombusvirus (Burgyan et al., 1989; Hillman et al., 1987) and variants of tobacco rattle tobravirus (Angenent et al., 1989; Robinson et al., 1987). Recombination has been reported in the 3' untranslated and intercistronic sequences of bromoviruses (Bujarski and Kaesberg, 1986; Rao et al., 1990; Rao and Hall, 1993; Allison et al., 1990). The mechanism of plant viral RNA recombination has been addressed experimentally in both brome mosaic virus and turnip crinkle virus subviral RNAs (Nagy and Bujarski, 1992 and 1993; Cascone et al., 1993).

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 (Mayo and Jolly, 1991). Additionally, a deletion mutant of red clover necrotic mosaic virus was restored by recombination with transgenically expressed viral RNA (Lommel and Xiong, 1991). 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 to transgenic plants by expressing segments of viral genome, such as capsid genes (Beachy et al., 1990). Transgenic plants expressing a viral capsid protein exhibit resistance to that virus and closely related strains (Grumet, 1990) but remain susceptible to other viruses.

Plants frequently resist viral attack by restricting virus movement rather than inhibiting replication (Matthews, 1991). Therefore, plants challenged by viruses that are not pathogens 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 (de Zoeten, 1991).

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 cDNA clones of these RNAs

(Allison et al., 1988). 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.

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). To avoid potential spurious mutations, a 359 base SacII-XbaI fragment containing the introduced mutations was substituted for the similar fragment in the original plasmid, pCC3TP4, to form pCC3AG1. The fidelity of all constructs was confirmed by sequence analysis. Effects of mutations on virus infectivity was ascertained by inoculating both cowpea, *Vigna sinensis* (Torner) Savi, and *Nicotiana benthamiana* (Domin) with full length plasmid derived transcripts of wild type CCMV RNAs 1 and 2 and either wild type RNA 3 or pCC3AG1, referred to hereafter as C1, C2, C3 and AG1. Plants became infected within 14 days and no differences were observed in either the quantity or stability of recovered virions or viral RNA. Plasmid pCC3AG1 was used for

construction of both the transgenic *N. benthamiana* and the challenging CCMV inoculum.

Deletion inoculation plasmid pCC3AG3 [transcript AG3 (Fig. 1)] was prepared by deleting 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 co-inoculated 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 (An et al., 1988) to generate pGACCMV (Fig. 1). This construct, which contains the neomycin phosphotransferase (NPT II) marker gene, was used to transform *N. benthamiana*. This placed the CCMV sequence under the control of the constitutive 35S promotor and within the T-DNA region of pGA643. pGACCMV 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.

From 57 kanamycin resistant regenerated plants, 6 were selected based on high levels of NPT II expression as judged by ELISA. A CCMV specific probe (Allison et al., 1990) hybridized to a single band on northern blots of total RNA

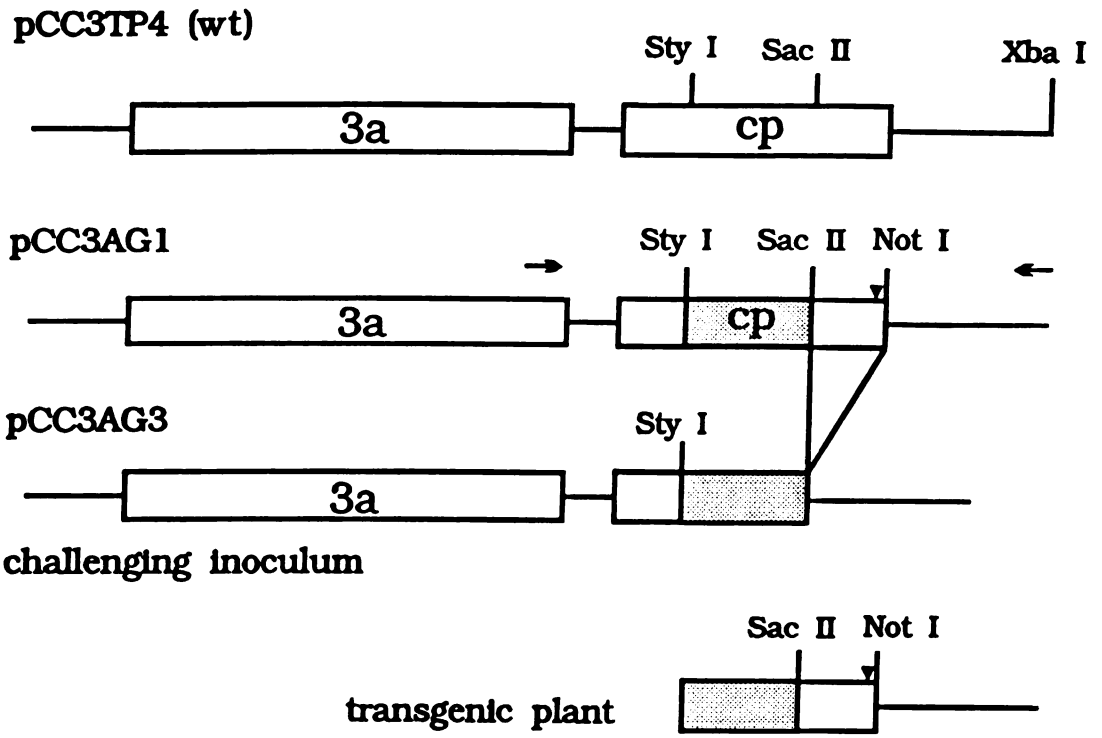

Fig. 1: Plasmid construction. The oligonucleotide TCTTCAGCGGCCGCTAATAGACCGGAGT was used to direct mutagenesis of pCC3TP4. The resulting plasmid, pCC3AG1, contained a silent mutation (G to C) at position 1926 (triangle) and changes at positions 1933 and 1935 (T to C, C to G) which introduced a Not I site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

extracted from the 6 plants which were clonally propagated for recombination experiments. Transgenic plants challenged with either CCMV virions or wild type (WT) RNA transcripts, C1, C2, C3, became systemically infected. Thus the transgenic transcript was insufficient to provide *N. benthamiana* with CCMV resistance.

The 2 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 PCR amplified (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

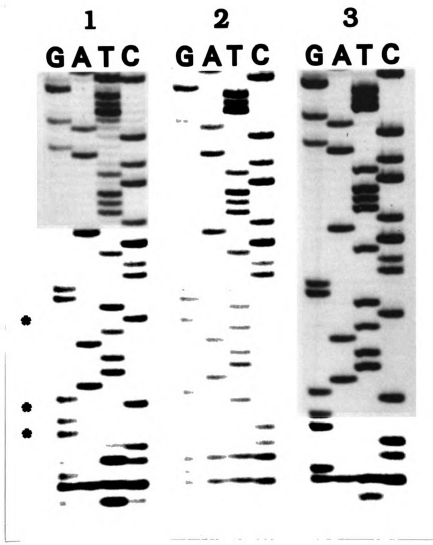

Fig. 2: Sequence analysis of PCR products generated from virion RNA of a transgenic plant infected with WT CCMV and plant 5-58. Full length first strand cDNA was made from virion RNA using oligonucleotide 3'CCMV, CAGTCTAGATGGTCTCCTTAGAGAT. A 1096 base fragment containing the intercistronic region of RNA3 was PCR amplified using oligonucleotides TAAAATCGCCGTAACCGC and 3'CCMV. The amplified product was cloned into the SmaI/XbaI sites of pUC18 and sequenced using USB Sequenase. The sequences of plasmid pCC3AG1, and cloned PCR products from the WT infected plant and plant 5-58 are shown in panels 1-3 respectively. Asterisks (*) denote positions of marker mutations present only in pCC3AG1 and viral RNA recovered from 5-58.

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 regions of the transgenic mRNA and the viral inoculum.

Of 125 transgenic plants tested, four recombinant viruses have been verified from 3 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 non-coding regions (Bujarski and Kaesberg, 1986; Rao et al., 1990; Rao and Hall, 1993; Allison et al., 1990). 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

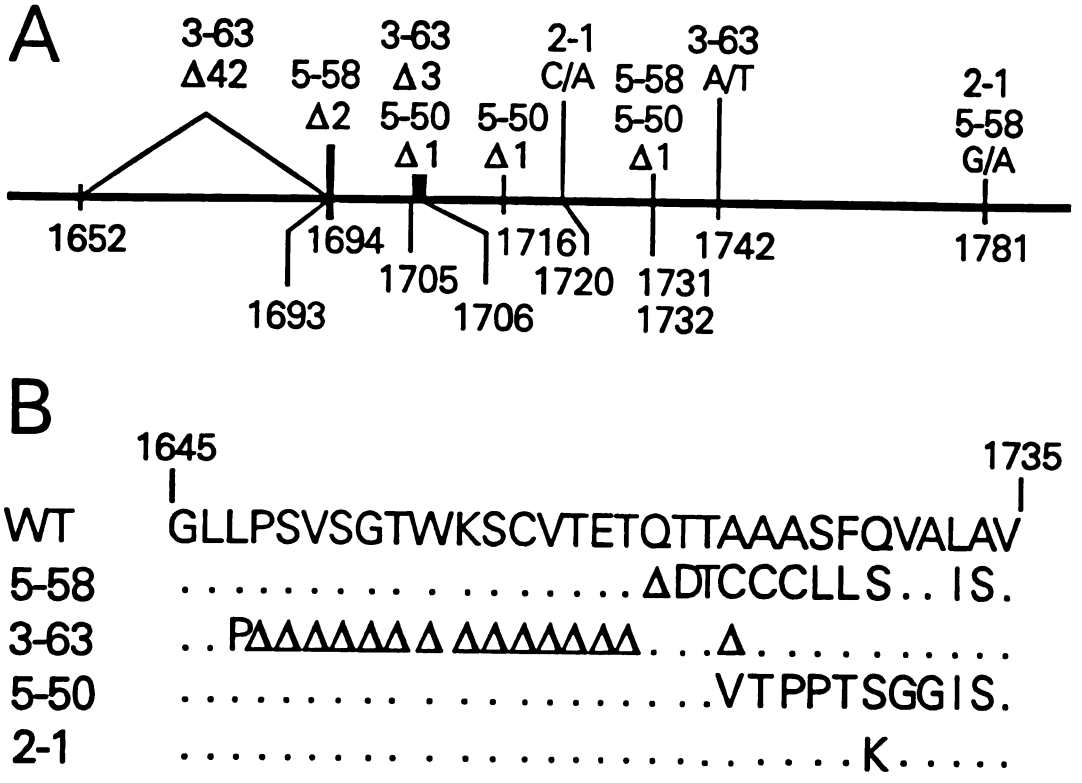

Fig. 3: Mutations in capsid gene of recombinant viruses: 5-58, 3-63, 5-50 and 2-1. **(A)** The nature and extent of each nucleotide mutation is denoted above the horizontal line which represents a segment of the WT capsid gene between nucleotides 1652 and 1781. A delta indicates deleted nucleotides.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

initiates minus strand RNA synthesis on this terminal sequence (Miller et al., 1986), its presence may enable replication to begin on the mRNA transcript and then switch 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 (Kirkegaard and Baltimore, 1986). 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 (Dawson and Hilf, 1992). 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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CHAPTER 2

Analysis of recombinant viruses recovered from transgenic plants

INTRODUCTION

RNA recombination, an important process in the evolution of RNA viruses, has been demonstrated in a wide range of RNA viruses encompassing animal viruses, bacteriophage and plant viruses (Lai, 1992). Recombination is proposed to occur via a template switching mechanism where the viral replicase complex moves between templates during viral replication (Kirkegaard and Baltimore, 1986). The resulting product is the complement of two previously independent RNA templates. Evidence for recombination has been found in sequence analysis of several plant viruses including the *Bromoviruses* (Allison et al, 1989), *Tobraviruses* (Angenent et al., 1989 and *Hordeiviruses* (Edwards et al., 1992).

Cowpea chlorotic mottle bromovirus (CCMV), a member of the *Bromoviridae,* contains a tripartite, single stranded, positive (+) sense RNA genome. The first two, RNA 1 and RNA 2 are monocistronic and encode replication proteins 1a and 2a respectively. Dicistronic RNA 3 encodes the 3a movement protein and the capsid protein. The 3a and capsid genes of CCMV are required for systemic infection of host plants (Allison et al., 1988). Both homologous and non-homologous recombination have been demonstrated in bromoviruses (Allison

et al., 1990, Nagy and Bujarski, 1993). Studies on the *Bromovirus* type member brome mosaic bromovirus (BMV) suggest that heteroduplex formation may facilitate heterologous recombination (Nagy and Bujarski, 1995).

Lai (1992) defines different types of recombination that occur between RNA molecules. Homologous recombination occurs at precisely the same position in two similar RNA molecules such that the sequence of the recombinant at the recombination site is indistinguishable from the templates. Aberrant homologous recombination events also involve similar RNA molecules, but the sequence of the recombinant molecule is unique to the recombinant due to base changes introduced during the recombination event. Heterologous recombination occurs between unrelated RNA molecules.

The original experiment was designed to determine if transcripts from viral transgenes were available to a replicating virus for RNA recombination. *Nicotiana benthamiana* was transformed with the 3' two thirds of the capsid gene along with the entire 3' untranslated region (UTR) of CCMV RNA 3. Transgenic plants were challenged with transcripts for wild type CCMV RNA 1 and RNA 2 and a systemic movement defective RNA 3 that lacked the 3' 121 nucleotides of the capsid protein gene (AG3). Systemic infection was restored in 3% of the challenged transgenic plants. The viruses recovered from these plants contained markers originally present in only the transgene. Although shared sequence between the transgene and

the inoculating virus provided an opportunity for homologous recombination, analysis revealed that recombinants were derived from aberrant homologous recombination events (Greene and Allison, 1994). Here we report the sequence of three additional recombinants recovered from this system and compare the phenotypes of all seven recombinants to wild type CCMV.

MATERIALS AND METHODS

Recovery of recombinants from transgenic plants.

Construction of transgenic *Nicotiana benthamiana* (Domin), bioassay for RNA recombination, recovery of virion RNA, PCR amplification and sequence analysis of recombinants were performed as previously described (Greene and Allison, 1994). Total RNA was extracted using guanidinium thiocyanate extraction (Puissant and Houdebine, 1990). Leaf dip analysis for the presence of virions in infected tissue was performed by the Plant Diagnostic Clinic, Michigan State University.

Recombinant virus nomenclature

Recombinants are referred to by the tissue culture line and plant from which they were recovered. For example, recombinant 5-58 was recovered from clonally propagated plant 58 from transformed tissue culture line 5. Sequence numbering refers to the corresponding nucleotides and amino acids described for wild type CCMV (Allison et al., 1988).

Transmission and symptomology tests

Mutant viruses recovered from transgenic plants were tested for sap and virion transmission to nontransgenic *N.*

benthamiana and cowpea (*Vigna sinensis* (Torner) Savi) plants. Approximately 0.3 g of leaf tissue from originally infected *N. benthamiana* was ground in 1 ml phosphate buffer, pH 5.5, in a mortar and pestle. Cowpea plants were inoculated at emergence of the first two primary leaves. Two fully expanded leaves of *N. benthamiana* were inoculated at the 5 leaf stage of development. Inoculated leaves were dusted with carborundum and 10 µl of sap was rubbed onto each leaf. Virion isolation was performed as previously described (Allison et al., 1989). Mechanical inoculation with virions was performed as described above using 20 µg virions per leaf. Systemic infections were detected by dot blot analysis of tissue sampled from leaves above the inoculation site. Blots were probed with CCMV specific probe RA518 (Allison et al., 1990). Symptoms arising from systemic infections with recombinant viruses were compared to wild type CCMV controls.

Comparison of mutant to wild type CCMV

Leaf prints were used to determine the efficiency of movement of recombinant viruses within inoculated leaves. Fully expanded primary leaves of cowpea were inoculated as described above. Virions were used as the inoculation source for all recombinants except 3-57. Mutant 3-57 was compared to wild type CCMV using sap from infected *N. benthamiana*. Inoculum was confined to an area of 2 cm^2 per leaf. Leaf prints were made at daily intervals from one to four days post inoculation. Sap from expanded inoculated leaves were pressed

onto nylon membrane (Micron Corp.) under seven thousand pounds per square inch pressure resulting in sap print of the intact leaf. Prints were probed with CCMV specific probe RA518 to look for movement from the inoculated area.

Host Range test

Wild type and recombinant virus were inoculated onto beans (*Phaseolus vulgaris L.*), cucumber (*Cucumis sativis L.*), barley (*Hordeum vulgarae* L. cv. Morex), pea (*Pisum sativum L.*) and *Chenopodia quinoa* (L.) for a limited host range test. The primary leaves of bean and the cotyledons of cucumber were inoculated with sap and virions as described above. All expanded leaves at 3 days post emergence of peas, barley leaves at 7 days post emergence and two leaves of Chenopodia at the 6 leaf stage of development were inoculated as described above. Plants were observed for symptom development and screened by dot blot for virus movement in the inoculated and non-inoculated leaves at two weeks post inoculation.-Non-inoculated leaves were screened again at four weeks post inoculation.

Comparison of recombinant and wild type CCMV in mixed infections

Cowpea plants were inoculated with a single recombinant plus wild type virions for all recovered recombinants. Equal amounts of recombinant and wild type virions (20 μg) were inoculated on opposite primary leaves and, in separate experiments, combined in the inoculation of both primary leaves of cowpea at the two leaf stage. Plants were analyzed

at 14 days post inoculation for systemic infection. Virion or total RNA was isolated and subjected to cDNA synthesis and PCR amplification as previously described (Greene and Allison, 1994). The PCR product was digested with Not I restriction endonuclease and separated by agarose gel electrophoresis on a 0.8% agarose gel.

RESULTS

Analysis of virus recovered from transgenic plants

Systemic movement was detected in 7 of 235 transgenic *N. benthamiana* plants that were inoculated with deletion inoculum AG3 (Greene and Allison, 1994). Sequence analyses of viruses isolated from four plants (5-58, 2-1, 3-63 and 5-50) were reported earlier (Greene and Allison, 1994). Virion RNA was recovered from two of the remaining three plants (3-51.1 and 5-55). Sequence analysis of cDNA copies of the viral RNA corresponding to the 3' terminus of the 3a protein (Fig. 1) gene through the 3' untranslated region (UTR) showed that both viruses contained the three marker mutations that were present originally only in the transgenic RNA indicating that systemic infection resulted from recombination between the challenging deletion inoculum and the transgenic message. Additionally, 3-51.1 and 5-55 contained changes within the capsid gene region that were unique to both the transgenic RNA and inoculum RNA.

Mutant virus recovered from 5-55 contained point mutations resulting in amino acid substitutions at positions 1721 and 1731 and a silent mutation at position 1726 of the

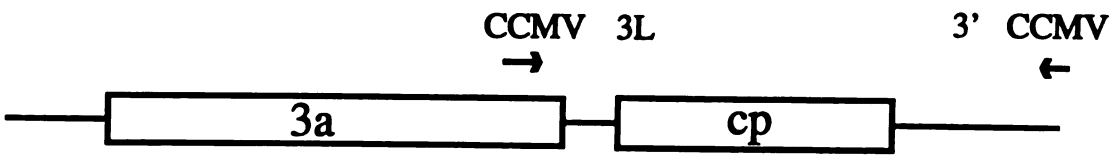

Fig. 1: Schematic diagram of CCMV RNA 3. Boxes represent wild type CCMV movement protein gene (3a) and capsid gene (cp). Single lines represent non-coding sequences. Arrows indicate position of oligonucleotide 3'CCMV used to prime first strand cDNA synthesis and in PCR amplification, and primer CCMV 3L used in PCR amplification to viral or total RNA recovered from transgenic plants.

capsid protein gene (Fig 2). Mutant virus recovered from plant 3-51.1 contained an in frame insertion of 3 nucleotides following position 1579 and point mutations at positions 1714, 1736 and 1801. This resulted in insertion of an isoleucine residue following amino acid 75 and two substitutions at amino acids 109 and 116 respectively (Fig. 3A). The capsid open reading frame (ORF) was not disrupted in either virus.

Systemic infection of plant 3-57 was detected by dot blot, however attempts to isolate virions were unsuccessful. Leaf dip analysis by scanning electron microscopy did not detect any virion particles in this plant (data not shown). Total RNA isolated from 3-57 showed the presence of all four CCMV genomic RNAs when probed with CCMV specific probe RA518. Sequence analysis of cDNA revealed the presence of the marker mutations indicating recombination between the transgene and challenging inoculum. Complete analysis of the capsid gene sequence showed a single nucleotide deletion nine bases from the capsid start codon (n.t. 1369), a three nucleotide insertion followed by two nucleotide substitutions at position 1587 and an additional point mutation at position 1704 in the region shared by the transgene and inoculum RNA (Fig. 2). This sequence was confirmed in independently derived cDNA clones. Translational analysis indicated that the frame shift caused by the deletion mutation introduced a stop codon at codon twelve of the capsid gene thus the ORF is disrupted and translation of a capsid is unlikely (Fig 3B).

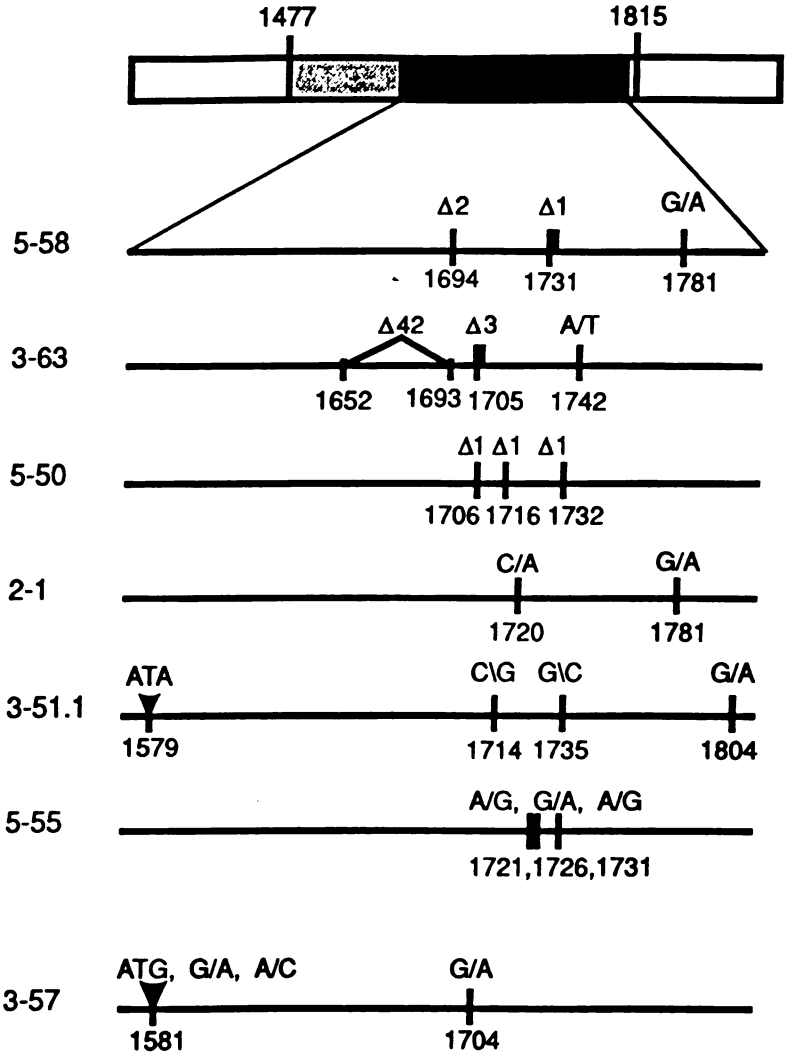

Fig. 2: Representation of mutations recovered in the capsid genes of recombinant viruses. The open box represents the capsid protein open reading frame. Grey shaded box represents the homologous regions between the transgene and inoculum (n.t. 1477-1815). The black box superimposed on the grey box represents region between nucleotides 1579 and 1804 and is blown up to indicate mutations found in recombinant viruses. The nature and extent of each nucleotide mutation is denoted above the horizontal line. A delta indicates deleted nucleotides. Substitution mutations are separated by slanted line with the wild type to the left and substitution on the right. Insertions are indicated by inverted triangles.

Host range, symptomology and movement tests.

All seven recombinant viruses moved systemically in *N. benthamiana*, cowpea, bean and pea when inoculated with sap or purified virions. Recombinant 3-57 moved systemically in cowpea using total purified plant RNA or crude sap from infected transgenic *N. benthamiana* as the inoculum source. Symptom development varied among these recombinants.

As with wild type CCMV, all recombinants were symptomless on *N. benthamiana* and formed local lesions on *Chenopodia quinoa*. Recombinants 5-58, 3-51.1 and 5-50 provided symptoms comparable to wild type CCMV on cowpea, bean and pea. Two recombinants, 3-57 and 3-63 were symptomless on cowpea and bean. Recombinants 2-1 and 5-55 caused more severe symptoms than wild type CCMV on cowpea. Recombinant 2-1 showed severe stunting (Fig. 4) and, inoculation of leaves just after emergence, the typical manner of inoculating cowpeas with wild type CCMV, resulted in rapid senescence of the whole plant. In addition recombinant 2-1 caused more severe mottling on bean when compared to wild type CCMV infection. Recombinant 5-55 generated large patches of severe chlorosis as compared to a much milder mottling of wild type CCMV (Fig. 4). None of the recombinants moved in the inoculated leaves or systemically infected barley or cucumber as consistent with wild type CCMV host range.

Experiments to study movement of mutants in the initially inoculated leaves of cowpea indicated that the recombinants

A

WT	N_ELSSERNKQLKVGRVLLWLGLLPSVSGTVKSCVTETQTTAAASFQVALAVADN
5-58	._.....ΔDTCCCLLS.....
3-63	._.....PΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔ...Δ.....
5-50	._.....VTPPTSGG.....
2-1	._.....K.....
3-51.1	.I.....E.....H.
5-55	._.....R.A.....

B

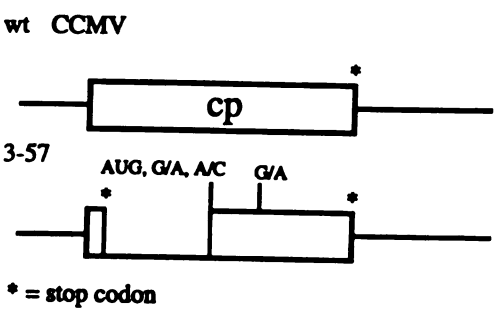

Fig 3: Capsid protein amino acid sequence of recovered CCMV recombinants. **(A)** Representative amino acid deletions, insertions and substitutions in recombinants 5-58, 3-63, 5-50, 2-1, 3-51.1 and 5-55. Deletions are represented by deltas. Changes are compared to wild type capsid amino acid sequence for amino acids. Single letter amino acid codes are used to denote substitutions. Dots represent unchanged amino acids. Insertion of isoleucine residue in recombinant 3-51.1 is denoted by I in the 3-51.1 and underline in wild type and all other recombinants. **(B)** Representation of wild type and recombinant 3-57 capsid open reading frames. Open reading frames are denoted by an open box. Single lines denote noncoding sequence. Stop codons are denoted by asterisks (*). Additional start codon and point mutations in 3-57 are denoted above the line. Substitutions are separated by slanted lines with the wild type nucleotide on the left and substituted nucleotide on the right.

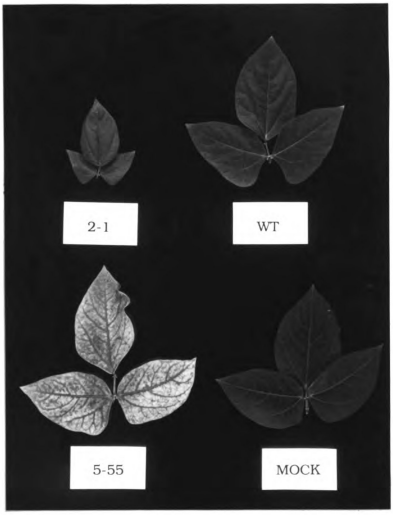

Fig. 4: Tri-foliate leaves from systemically infected cowpeas inoculated with wild type and recombinant CCMV. Leaves are from comparable positions on concurrently inoculated plants. Recombinant 2-1 (top left) produces stunted leaves compared to leaves of the same age from plants inoculated with wild type CCMV (top right) or mock inoculated with buffer (bottom right). Recombinant 5-55 (bottom left) produced severe chlorosis versus the mottling symptom of wild type CCMV.

did not differ from wild type CCMV. Systemic infection of cowpea occurred at the same rate as wild type CCMV with infection in upper, non-inoculated leaves detectible at approximately 14 days post inoculation.

Comparison of mutant and wild type CCMV

To determine how the recombinants competed with wild type CCMV in a mixed infection, plants were inoculated simultaneously with both the wild type and one of the recombinants. A cDNA copy was synthesized to virus isolated from infected cowpeas, PCR amplified and digested with Not I. All recombinants were distinguished from wild type CCMV by a Not I restriction site in the capsid gene. PCR product was made to virion RNA for all recombinants except 3-57 which was made from total RNA from infected cowpea. In both same leaf and alternate leaf inoculations, PCR products for all recombinant plus wild type inoculated plants gave wild type pattern when digested with Not I (Fig. 5). This indicated selection for the wild type infection in these plants. PCR product for virus isolated from 2-1 and 5-55 inoculated plants showed faint bands at the size expected for the mutant virus containing the Not I site (data not shown). Subsequent analysis of virus that was passaged to cowpeas showed only the wild type PCR product. Control PCR products of individual recombinants or wild type CCMV gave expected Not I restriction patterns. PCR control reactions using both wild type CCMV and recombinant RNA showed the Not I restriction patterns for both

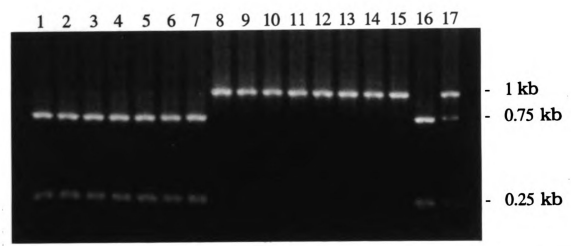

Fig. 5: NotI digestion of PCR product from cowpeas inoculated with recombinant and wild type CCMV. First strand cDNA synthesis and PCR amplification was performed against virion or total RNA isolated from plants inoculated with wild type and recombinant CCMV. NotI restriction site was present in products derived from cowpea inoculated with recombinants alone (Lanes 1-7: 3-63, 3-51.1, 3-57, 5-58, 5-50, 5-55 and 2-1), but not in product derived from cowpea inoculated with wild type CCMV alone (Lane 8). PCR product derived from cowpeas inoculated with wild type CCMV and recombinant viruses (Lanes 9-15, corresponding recombinant same as lanes 1-7) showed wild type restriction pattern for NotI digestion. Control derived from equal amounts of virion RNA from recombinant 5-58 and wild type CCMV (Lane 17) showed NotI restriction pattern of both wild type and recombinant PCR product. Lane 16 contained NotI digested PCR product derived from cDNA clone pCC3AG1 which was used in the original bioassay constructions.

products in approximately equal amounts indicating that the PCR product recovered from the test plants was due to predominant wild type CCMV infection in the test plants rather than preferential PCR amplification of wild type CCMV.

DISCUSSION

In our previous study we recovered recombinant virus in 4 of 125 transgenic plants that were challenged with the deletion inoculum. Since then, we have recovered 3 additional recombinants from an additional 115 challenged transgenic plants. The bioassay used in this study was developed to screen for only those recombination events that restored systemic movement. Thus we maintained a recombinant recovery frequency of 3% in these transgenic *N. benthamiana*.

In all plants, aberrant homologous recombination occurred within the overlapping regions of the transgenic RNA and inoculum RNA. In 6 of 7 recovered viruses recombination restored a translatable capsid ORF. Stable virions were recovered from all 6 of these recombinants. Theoretically, homologous recombination events were free to occur within the 338 nucleotides shared between the transgene and inoculum. However, analysis of recombinants indicated that recombination had occurred within a more limited 225 nucleotide region (n.t. 1579-1804) corresponding to amino acids 75 through 129 of the capsid protein. No obvious secondary structure is predicted in this region to facilitate recombination. The majority of the mutations leading to amino acid changes occurred in a smaller

region corresponding to amino acids 97 to 129. This region forms an alpha helix near the surface of the capsid subunit (Speir et al., 1995). These results suggest that this particular region of the capsid protein is not essential to the integrity of the virion. Disruption of this helix did not affect systemic movement. It is not known if this region of the capsid gene represents a hot spot for recombination or merely represents a region in which genetic changes are tolerated without effect on virus function. Nagy and Bujarski (1995) reported aberrant homologous recombination in BMV when the parental RNAs had little sequence homology at the crossover points. These results are consistent as no significant secondary structure or complementary regions were noted in the region of recombination.

Virus recovered from plant 3-57 contained a single nucleotide deletion nine nucleotides from the beginning of the capsid ORF (n.t. 1369). This shifted the reading frame and resulted in the introduction of a stop codon 12 amino acids into the capsid gene. It is unlikely that this mutation occurred during recombination since the region was not included in the transgene, but it probably occurred by polymerase error during replication. Other mutations were found in the same 92 nucleotide region as noted in all other recombinants. The introduction of an in frame AUG at position 1587 provides a second site for translation initiation. If initiation were to occur from this additional start codon, a

truncated carboxyl terminal protein may provide the movement function to the virus. These results suggest that the full length capsid protein is not required for systemic CCMV movement since plants inoculated with 3-57 became systemically infected. This virus was efficiently sap passaged to *N. benthamiana* or cowpea, however virion particles were never recovered in any plant inoculated with 3-57. This is consistent with the lack of the full length capsid ORF. Wild type virus formed the predominant infection in plants co-inoculated with 3-57 as the diagnostic NotI site of the recombinant was not detected in virion or total RNA isolated from these plants. Further characterization of this mutant is underway to examine the role of the capsid protein gene in systemic movement of CCMV.

Host range of recombinant CCMV viruses did not differ from wild type. Previous studies indicated that the 3a movement protein determines bromovirus host range (Mise et al., 1993; Mise and Ahlquist, 1995). Exchanges of the capsid genes between BMV and CCMV have not altered host range (Allison, unpublished results). These results agree that CCMV capsid protein plays little role in establishment of host specificity. Mutations present in recombinant viruses also had little effect on the rate of movement or time required for establishment of systemic infection suggesting that the 225 nucleotide region in which mutations accumulated is dispensable for establishment of infection.

Several recombinants showed changes in symptomology on cowpea and bean hosts. Virus recovered from plants 3-63 and 3-57 were symptomless on these hosts. Recombinant 3-63 contained a large deletion in the capsid protein while 3-57 did not make virions. Two of the viruses, 2-1 and 5-55, gave more severe symptoms compared to wild type and recombinant 2-1 was lethal if plants were inoculated at a very young stage. Recombinants 2-1 and 5-55 contained few changes in their capsid genes. However, both contained substitutions at amino acid 111 indicating that this position is important to symptom development in cowpea. These results are consistent with reports in other viral systems where small changes in the capsid protein produce severe symptoms. Single amino acid change in tobacco mosaic tobamovirus (Banerjee et al., 1995) was sufficient to cause severe symptoms. These results together demonstrate that the capsid protein plays a role in the physical manifestation of CCMV infection in host plants.

All cowpea plants inoculated with both recombinant and wild type CCMV showed predominant CCMV infection. While recombinant viruses performed comparable to wild type when inoculated independently they were suppressed when wild type virus was present. It is possible that the recombinant virus may not interact as well with host or viral determinants during the infection cycle. Results with 2-1 and 5-55 indicate that the more subtle changes enabled the recombinant to

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survive the initial infection, but even those were eliminated by wild type upon subsequent passage.

This work further demonstrates that viral transgenic RNA is available for recombination with challenging viral RNA. Our results suggest that mutations introduced into the CCMV capsid gene through aberrant homologous recombination did not significantly alter the characteristics of the virion, although changes in symptomology were noted. In this study, changes in phenotype do not predict a selective advantage of recombinant over wild type virus. These recombinants were at a disadvantage in plants where wild type CCMV was also present, suggesting that changes in the capsid gene may affect some interaction between the virus and host or within the virion itself. Further study may lend insight to the role of capsid protein in CCMV infection.

CHAPTER 3

RNA recombination in transgenic plants produces hybrid viruses.

INTRODUCTION

RNA recombination, an important evolutionary tool of RNA viruses, has been demonstrated in a number of animal viruses, bacteriophage and plant viruses (reviewed in Lai, 1992). Homologous and non-homologous recombination have been demonstrated in brome mosaic bromovirus (BMV) (Rao and Hall, 1993; Nagy and Bujarski, 1995) and cowpea chlorotic mottle bromovirus (CCMV) (Allison et al., 1990).

Recombination in the bromoviruses appears to occur via the copy choice mechanism that was first proposed for poliovirus (Kirkegaard and Baltimore, 1986). This model proposes that a template switch occurs during minus (-) strand replication resulting in the union of two previously separate RNA molecules. Nagy and Bujarski (1993) have shown that heterologous recombination in BMV is facilitated by heteroduplex formation near the site of crossover. Additionally, it was demonstrated that CCMV sequences may also interact to facilitate recombination (Dzianott et al., 1995).

The *Bromoviruses* belong to the *Bromoviridae* and are single stranded, positive (+) sense RNA viruses. The genome consists of three RNAs designated RNA 1-3. Monocistronic RNAs 1 and 2 encode replication proteins (Kiberstis et al., 1981;

Kroner et al., 1989, 1990; Traynor et al., 1991) and RNA 3 encodes the putative movement protein and the capsid protein (French and Ahlquist, 1987). The 3a and capsid protein are dispensable for RNA replication, but are required for systemic infection of host plants (Allison et al., 1990). CCMV has a host range restricted mainly to legumes, while BMV infects grasses. Both viruses infect *Nicotiana benthamiana* (Domin) and form local lesions on *Chenopodia quinoa* (L.).

We have established that aberrant homologous recombination between viral CCMV RNA expressed in a transgenic plant and mechanically inoculated CCMV generates virus variants (Greene and Allison, 1994). This original study demonstrated that transgenic RNA was able to recombine with viral RNA. Transgenic plants expressing viral sequences will be challenged by numerous plant viruses in the field. Many of these viruses can replicate in the plant as evidenced by the ability of viruses to replicate in non-host protoplasts. Therefore, recombination between the CCMV transgene and a different virus may also generate hybrid virus. CCMV and BMV genomes have sufficient homology at the nucleic acid level to promote interactions to facilitate a template switch between the CCMV transgene and BMV inoculum. Additionally, BMV replicase can recognize the replication initiation site in the 3' untranslated region (UTR) of CCMV RNAs and amplify CCMV RNAs in protoplasts (Allison et al., 1988). Recombination between the CCMV transgene and the mutant BMV could result in

a hybrid BMV/CCMV molecule with restored systemic movement. To test this hypothesis, *N. benthamiana* was transformed with the 3' two thirds of the CCMV capsid gene and challenged with a movement deficient BMV mutant lacking a 3' segment of its capsid gene. We describe recovery and analysis of virion RNA resulting from recombination between BMV inoculum and both CCMV transgene and host RNA.

MATERIALS AND METHODS

Construction of transgenic *Nicotiana benthamiana* expressing the 3' two thirds of the capsid gene and the complete CCMV 3' UTR was previously described (Greene and Allison, 1994). A BMV RNA 3 deletion mutant was constructed by removing a 26 nucleotide XbaI-StuI fragment, nucleotides 1754 through 1779, from the capsid gene of wild type BMV RNA 3 plasmid pB3TP8 (Janda et al., 1987) (Fig. 1). Sticky ends were filled in with Klenow DNA polymerase and ligated using T4 DNA polymerase to form plasmid pB3AG5, referred to here after as AG5. All modifying enzymes were purchased from Boehringer Mannheim Biochemical and used according to manufacturer's instructions.

Transcripts from wild type (wt) CDNA clone of BMV RNA 1 and RNA 2 (Ahlquist et al., 1984; Janda et al., 1987) and modified RNA 3 (AG5) were inoculated onto two leaves of transgenic *N. benthamiana* at the 5 leaf stage of development. Inoculation constituents were as previously described for CCMV (Greene and Allison, 1994). Controls were inoculated with all

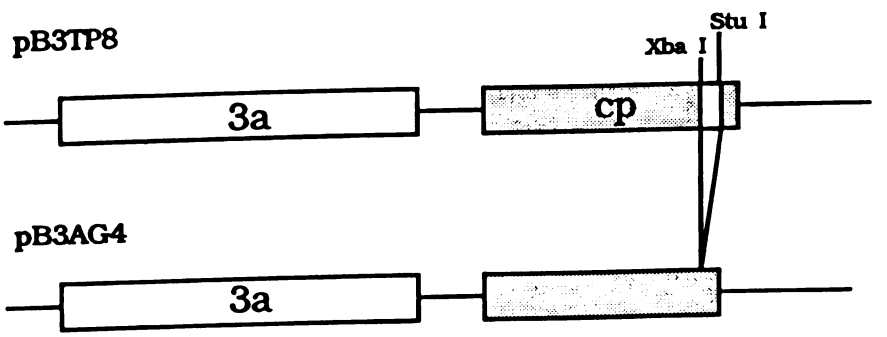

Fig. 1: BMV RNA3 deletion inoculum. Deletion mutant of BMV RNA 3 was constructed by removing a 26 nucleotide region between the StuI and XbaI restriction sites in the capsid gene of BMV RNA 3 clone pB3TP8. Sticky ends were filled in with Klenow DNA Polymerase and blunt end ligated with T4 DNA ligase to form plasmid pB3AG5. The first open box represents the movement protein gene (3a). Grey shaded box represents capsid protein gene (cp), deleted area is represented by the open box superimposed on the grey box. Single lines represent non-coding sequence.

three wild type BMV RNAs or mock inoculated with buffer. BMV systemic movement was determined by dot blot analysis to crude extracts using BMV specific probe HE1 (Pacha and Ahlquist, 1991) which was DIG labeled using Genius RNA labeling system (BMB). HE1 is complementary to the 200 3' terminal nucleotides conserved in all three genomic BMV RNAs.

Virion RNA was isolated from plants demonstrating systemic movement of BMV. First strand CDNA was primed with oligonucleotide ΩE (De Jong and Ahlquist, 1995) or 3'CCMV-(5'-CAGTCTAGATGGTCTCCTTAGAGAT-3'). ΩE anneals to the 3' terminal nucleotides of all three BMV genomic RNAs. 3'CCMV anneals to the 3' terminal nucleotides of CCMV genomic RNAs. Full length RNA 3 was PCR amplified using the 5' BMV primer oligonucleotide 3.11 (De Jong and Ahlquist, 1995) which anneals to the 5' terminal nucleotides of BMV RNA3, and the appropriate 3' oligonucleotide from first strand CDNA synthesis. The resulting cDNA was cut with BglII (n.t. 1218) producing two fragments, one approximately 1.3 kb, the second approximately 0.9 kb which corresponded to the capsid protein and the 3' untranslated region of RNA 3. Sticky ends of the smaller fragment were filled in with Klenow and fragments were blunt end cloned into the EcoRV site of Bluescript SK+ vector (Stratagene). The capsid protein gene and 3' untranslated region (UTR) was sequenced using Sequenase version 2.0 (USB). Sequences were compared with GenBank sequences using GCG analysis programs.

A limited host range test was performed to establish host range of the recovered virus. The two primary leaves of Cowpea (*Vigna sinensis*), both cotyledons of cucumber (*Cucumis sativis*) and two leaves of untransformed *N. benthamiana* were inoculated with sap from infected transgenic plants. Barley (*Hordeum vulgarae* cv. Morex) was inoculated with sap at 7 days post emergence. Plants were screened by dot blot for movement of virus in the inoculated and non-inoculated tissue of the plant using BMV probe HE1.

RESULTS

BMV deletion inoculum, AG5, was not infectious on non-transgenic *N. benthamiana* or barley when inoculated with wt BMV RNA 1 and RNA 2. This confirmed that the 26 nucleotide deletion in the capsid gene inhibited systemic movement. Transgenic *N. benthamiana* was not virus resistant and became systemically infected when inoculated with wild type BMV. Two of 76 transgenic plants inoculated with BMV RNA 1 and RNA 2 and AG5 became systemically infected within two months of inoculation. Sequence analysis of virus isolated from these plants (3-22.10, 6-25.1) revealed RNA recombination had occurred between the CCMV transgene and BMV challenging inoculum.

The sequence of cDNA clone 6-25.1 revealed the 5' terminal 42 nucleotides of BMV capsid protein gene followed by 177 nucleotides that were homologous to the 3' UTR of CCMV RNA3 (n.t. 1986-2163; Fig. 2). The next 201 nucleotides shared

no sequence homology to either BMV or CCMV genome sequences. The remainder of this sequence contained 202 nucleotides homologous to the BMV capsid gene (n.t. 1545-1747) followed by the 49 terminal nucleotides of CCMV capsid gene. This region included marker mutations present on the CCMV transgene (Greene and Allison, 1994). The region corresponding to the 3' UTR of this cDNA consisted of 97 nucleotides from 3' UTR of CCMV RNA 3 (n.t. 1993-2030), 75 nucleotides homologous to the 3' UTR of BMV RNA 3 (n.t. 1999-2074) and 74 terminal nucleotides of the 3' UTR of CCMV RNA 3 (n.t. 2099-2173). Similarly, the 3-22.10 cDNA clone contained the 5' 350 terminal nucleotides of BMV capsid gene (Fig. 2). This was followed by 329 nucleotides of sequence that were not derived from either bromovirus. The region corresponding to the 3' untranslated region (UTR) contained 52 nucleotides of CCMV sequence (n.t. 1986-2038) followed by the 3' terminal 136 nucleotides (1977-2113) of BMV RNA 3 (Fig 2). Further analysis of these sequences revealed that neither contained a functional capsid ORF. Search of GenBank did not reveal the origin of non-viral RNA included in these recombinants although greater than 90% homology was noted to several sequences (Table 1). Attempts to passage these hybrid viruses derived from plants 6-25.1 and 3-22.10 to nontransgenic *N. benthamiana*, barley and cowpeas were unsuccessful.

A second set of inoculated plants revealed 2 systemically infected plants out of 94 that were inoculated. Virus from

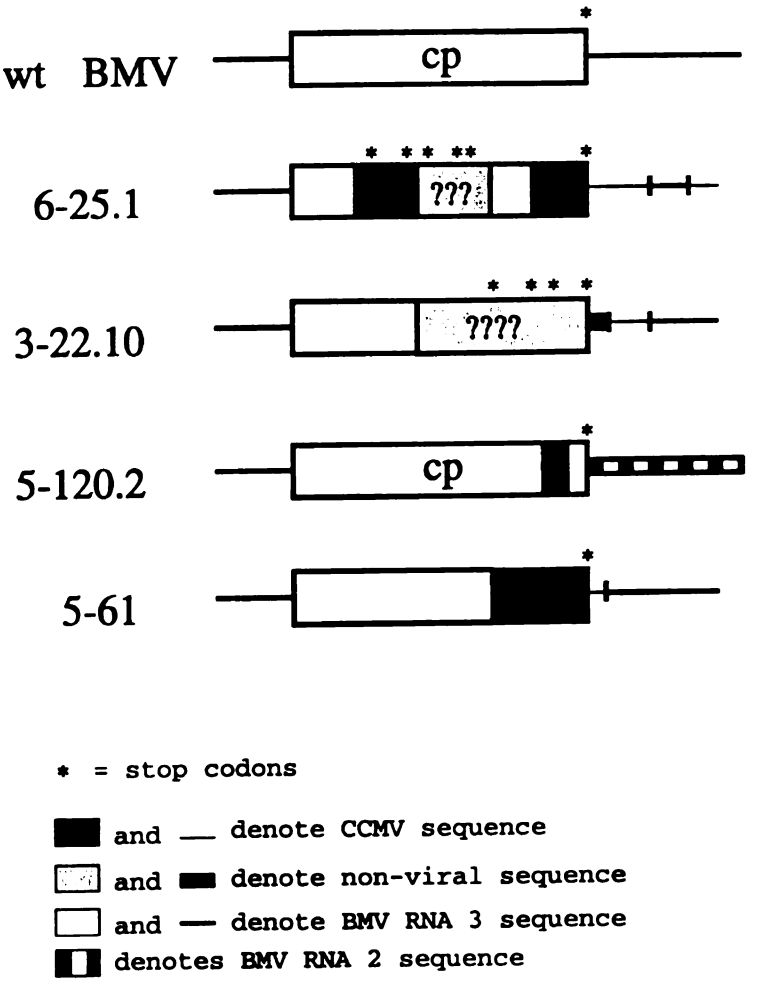

Fig. 2: Schematic representation of chimeric viruses recovered from transgenic plants. Wild type BMV capsid gene (open box) and non-coding sequences (single lines) are shown at the top. Corresponding sequence derived from hybrid viruses are shown below. Asterisks (*) denote location of stop codons. Source of recovered sequence is denoted by line thickness or shaded boxes (see key above). Length of sequence is explained in the text. Full sequence of these clones can be found in Appendix A.

Table 1: Results of GenBank search of non-bromovirus derived sequences recovered in hybrids 3-22.10 and 6-25.1. The top four sequences showing greater than 90% identity by fasta analysis are presented with the number of nucleotides in the identical region.

Genbank sequence	% identity	nucleotides in overlap
3-22.10		
1.) *Chlamydomonas gelatinosa* chloroplast P700 chlorophyll-a A1 gene exon	94.4	71
2.) *C. longicaudatus* mRNA for cyclin B	95.6	68
3.) *Homo sapiens* cDNA clone hbc002C 5' end	92.8	69
4.) *Arabidopsis thaliana* transcribed sequence FAFH57 3' end	90.1	71
6-25.1		
1.) *Streptococcus pneumoniae* ciaR/ciaH genes (histidine kinase; response regulator	92.3	52
2.) *Homo sapiens* Alu repeat region, chromosome 19	94.2	52
3.) *Homo sapiens* glucokinase gene associated satellite repeat	95.0	40
4.) Plasmid cloning vector pFL260 bla gene (beta-lactamase)	100.0	18

these plants was passaged to non-transgenic *N. benthamiana* before virus isolation, cloning and sequencing. Sequence analysis of virus from these plants, 5-120.2 and 5-61, revealed hybrid virus that arose by recombination between the transgenic RNA and challenging inoculum (Fig.2). Only bromovirus sequence was recovered in these viruses. The capsid gene of 5-120.2 was predominantly of BMV origin, however the deletion had been repaired with a 28 nucleotide fragment from the CCMV transgene (n.t. 1769-1797). The 3' terminus of 5-120.2 was derived from BMV RNA 2. Transgenic marker mutations were not incorporated into the hybrid as in 5-120.2. Hybrid 5-5-61 contained the 3' terminal two thirds of the BMV capsid gene (n.t. 1247-1600) followed by a 6 nucleotide insertion and the 3' terminal 222 nucleotides of the CCMV capsid gene (n.t. 1710-1932). This region contained the marker mutations present on the transgene. The 3' UTR of this hybrid contained 30 nucleotides of the 3' UTR of CCMV RNA 3 (n.t. 1933-1963) followed by 3' UTR terminus of BMV RNA 3 (n.t.1850-2113).

Virus recovered from plants 5-120.2 and 5-61 was sap inoculated to the limited host range plants. Both viruses infected barley, however infection was low compared to wild type BMV. Wild type BMV infected 70% of the inoculated plants, while 5-61 and 5-120.2 infected 30% and 40% of inoculated plants respectively. Neither hybrid virus produced symptoms on barley. Five of six cowpea plants inoculated with hybrid virus 5-61 produced symptoms of systemic virus infection. Dot blot

analysis using BMV specific probe HE1 indicated that the 5-61 hybrid had moved systemically in these plants. Symptoms resembled the chlorotic mottling symptoms acquired by cowpeas inoculated with CCMV but were more severe (Fig.3). Further hybridization analysis provided no evidence of CCMV contamination. We concluded that hybrid 5-61 has an expanded host range and now infects cowpeas and barley.

DISCUSSION

These experiments demonstrate that RNA recombination between a CCMV transgene and challenging BMV inoculum can generate hybrid viruses. Sequences of the initial recombinants, 3-22.10 and 6-25.1, provided evidence that host RNAs participate in viral RNA recombination. This supports previous findings from Mayo and Jolly (1991) where potato leaf roll luteovirus isolates contained sequences homologous to an exon of tobacco chloroplast RNA. Nagy and Bujarski (1995) also reported insertion of a non-homologous sequence during recombination that had homology to rice chloroplast sequences. Satellite Y of cucumber mosaic virus contains a complementary sequence to chloroplast tRNA (Masutsa et al., 1992). In animal viruses, insertion of a portion of the 28S ribosomal RNA into influenza virus resulted in a change in virus host range (Katchikian et al., 1989). Lethal biotypes of bovine viral diarrhea pestivirus contained mRNA sequences derived from the ubiquitin gene (Meyers et al., 1989). These reports together with the sequences found in recombinants 3-22.10 and 6-25.1

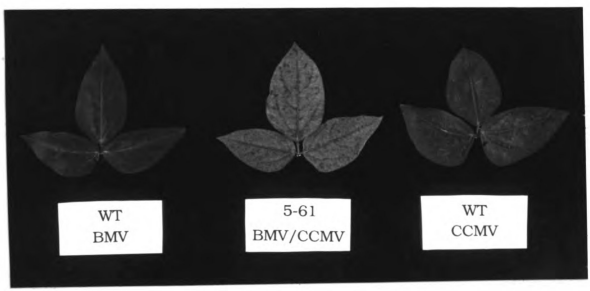

Fig. 3: Infection of cowpea with BMV/CCMV hybrid 5-61. Trifoliate leaves from cowpea inoculated with wild type BMV (left) are not systemically infected and do not exhibit symptoms. Leaf from cowpea inoculated with wild type CCMV show typical chlorotic mottling symptom (right). Leaf from cowpea inoculated with hybrid 5-61 produce severe chlorotic symptoms compared to wild type CCMV (center).

indicate that RNA recombination between viruses and host RNA are not uncommon, but may not result in viable viral RNAs.

Analysis of 3-22.10 and 6-25.1 also indicated that a single RNA may be the product of multiple recombination events. Numerous replication cycles may be required to produce the assortment of sequences observed in these cDNA clones. The cDNA sequence derived in this experiment was unlikely to be responsible for systemic infection since a functional capsid gene had not been restored. Our inability to either passage the virus or isolate cDNA with a functional capsid gene suggested the RNA with a gene responsible for encoding a functional capsid protein was a small portion of the viral RNA in the initially infected plant. Subsequent loss of these plants prevented further analysis of these mutants.

To select for viable recombinants responsible for systemic infection, viruses from plants 5-61 and 5-120.2 were analyzed only after passage through non-transgenic *N. benthamiana*. Sequence from these viruses revealed functional open reading frames (ORFs). The 3' UTR of hybrid 5-120.2 had been provided by BMV RNA 2. RNA recombination had been demonstrated previously between the 3' UTRs of the three BMV RNAs (Bujarski and Kaesberg, 1986; Rao and Hall, 1993). BMV RNA 3 containing a 3' terminus of tobacco mosaic tombusvirus was replaced by recombination with BMV RNA 1 or RNA 2 (Ishikawa et al., 1991). Similar selection may have been at work here.

Infection of cowpea by hybrid 5-61 was unexpected. Previous studies of BMV and CCMV suggested that the movement protein 3a plays the major role in host range determination (Mise et al., 1993; Mise and Ahlquist, 1995). While other genes are believed to complement the role of the movement protein in host range, the capsid protein has never been implicated. Valverde (1987) reported isolation of a BMV isolate that infects cowpea. Pseudorecombinants between wild type BMV and this variant indicated that several regions of the genome were involved in this host range modification. RNA 3 appeared to play a major role in host specificity (De Jong and Ahlquist, 1995). Our results suggest that the capsid protein may play a role in host specificity, although we cannot directly attribute the host range change of 5-61 to the recovered hybrid capsid gene. Determination of host range may involve a complex interaction among viral and host proteins. Further study of the complete 5-61 genome may reveal other modifications that specify the unique host range.

This work demonstrates that viable hybrid viruses may result from recombination between transgenic RNA and challenging viral RNA. As members of the *Bromoviridae*, BMV and CCMV contain sufficient homology to form secondary structures, or local regions of hybridization that facilitate RNA recombination in the transformed plant cells. Additionally, BMV replicase can recognize CCMV RNAs which may aid in providing an opportunity for interaction of the transgene and

challenging BMV RNAs in the cell. Recombination produced hybrid BMV/CCMV virus with a unique host range indicating that viable hybrid viruses with altered host range can be recovered from transgenic plants expressing viral sequences.

CHAPTER 4

Deletions in the 3' Untranslated Region of Cowpea Chlorotic Mottle Virus Transgene Reduce the RNA Recombination Frequency in Transgenic Plants

INTRODUCTION

Virus resistant transgenic plants (VRTPs) acquire pathogen derived resistance through the constitutive expression of a segment of a plant virus genome including the capsid (Beachy *et al.*, 1990), polymerase (Carr and Zaitlin, 1993) and modified movement proteins (Cooper *et al.*, 1995). One concern about the release of VRTPs to the environment is that recombination between the viral transgene and a challenging virus could produce chimeric viruses with distinct properties.

RNA recombination appears to be a fundamental evolutionary mechanism of RNA viruses. It occurs when the viral replicase switches RNA templates during synthesis of the complementary RNA strand and effectually, unites two previously distinct RNAs (Lai, 1992). Sequence analysis provides evidence of recombination in several animal RNA viruses, phage (Lai, 1992) and plant RNA viruses (Edwards *et al.*, 1992,; van der Kuyl *et al.*, 1991; Gal *et al.*, 1991).

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, both of which are required for systemic movement (Allison et al., 1990). Infectious transcripts produced from complementary DNA (cDNA) clones of these RNAs (Allison et al., 1988) infect legumes systemically and produce a symptomless systemic infection in *Nicotiana benthamiana* (Domin).

Using CCMV as a model system, we established that viral RNA transcribed in a transgenic plant is available for recombination with challenging viruses (Greene and Allison, 1994). When transgenic *N. benthamiana* expressing the 3' two thirds of the capsid gene and the complete 3' untranslated region (UTR) of cowpea chlorotic mottle virus (CCMV) was inoculated with a CCMV systemic movement defective mutant lacking that RNA segment, 3% of the transformants became systemically infected. Viable recombinant virus with restored capsid genes was recovered in each case.

Given the critical role of the 3' UTR as the viral replicase binding site in bromoviruses (French and Ahlquist, 1987, Dreher and Hall, 1988) and other RNA viruses (Barrera *et al.*, 1993, Zhang *et al.*, 1994, Hagen *et al.*, 1994), this study was undertaken to determine the influence of elimination of the 3' UTR of CCMV transgene on recombination frequency.

MATERIALS AND METHODS

Cowpea chlorotic mottle virus

The 3 CCMV full-length cDNA clones and their corresponding infectious transcripts, designated C1, C2 and C3, used in this study were described previously, (Allison et al., 1988). C1 and C2 were used without modification. Infectious clone pCC3AG1 (Fig. 1a) contained a complete cDNA copy of CCMV RNA3 adjacent to a T7 promoter and differed from the wild type sequence of C3 by two marker mutations: a *NotI* restriction site at the 3' terminus of the capsid gene and a silent mutation within the capsid gene at nucleotide 1926 (Greene and Allison, 1994).

Construction of plasmid deletions

Enzymatic reactions followed published protocols (Henikoff, 1984) and were conducted under conditions specified by the supplier (Boehringer-Mannheim, Indianapolis, IN). One μg of pCC3AG1 was cut within the polylinker with *PstI* and *XbaI* and digested at 30° C with 3 units exonuclease III. Ten equal aliquots were taken at 5 second intervals and frozen in a dry ice/ethanol bath. Enzyme was inactivated by incubating at 70°C for 10 min. To remove 3' single stranded DNA, plasmids were digested with mung bean nuclease at 1 unit per μg of DNA for 20 min. at room temperature. (Henikoff, 1984) Plasmid termini were filled with Klenow and plasmids were religated with T4 DNA ligase at 16°C overnight.

Following transformation and propagation in *E.coli* strain JM101, plasmids were isolated by alkaline lysis (Lee and Rasheed, 1990) and the extent of the 3' deletions was determined by sequence analysis (Sequenase 2.0, USB, Cleveland Ohio). Three plasmids containing deletions of 69 (pCC3AG2▲69), 83 (pCC3AG2▲83) and 214 (pCC3AG2▲214) nucleotides from the 3' terminus of the cDNA insert were selected for plant transformation (Fig. 1b).

Transformation of *Nicotiana benthamiana*

Restriction fragments produced by a *StyI-HindIII* digest, cDNA nucleotide 1476-1477 and polylinker cleavage sites respectively, of the 3' deletion plasmids included the 3' two thirds of the capsid gene and the remaining 3' UTR. Binary transformation vector pGA643 (An et al., 1988) was digested with *HindIII* and treated with alkaline phosphatase. In a two step ligation process, the *HindIII* end of the restriction fragment was ligated to the plasmid. Incompatible sticky ends of the plasmid and insert were filled with Klenow and blunt end ligated. This placed the CCMV sequence between the 35S CaMV promoter and 7' termination sequence of the octopine-type Ti plasmid pTi63 (An *et al.*, 1988). The resulting plasmids, pGACCMV▲69, pGACCMV▲83 and pGACCMV▲214, were mobilized into *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) by tri parental mating using helper plasmid pRK2013 (Ditta *et al.*, 1980). *N. benthamiana* leaf explants were transformed as described (Greene and Allison, 1994).

Transformed tissue was selected on Murashige and Skoog (MS) callus inducing medium containing 150 mg/l kanamycin and 300 mg/l cefotaxime. Shoots were regenerated on medium containing 150 mg/l kanamycin and rooted on basal MS medium. Plants were maintained in clay pots at room temperature under a 16 hr. photoperiod. Transformation was confirmed by enzyme linked immunosorbant assay (ELISA) for NPTII (5 Prime → 3 Prime, Inc. Boulder, CO) and by northern blot hybridization. Total RNA isolated from transformed *N. benthamiana* was electrophoretically separated in a 1% agarose formaldehyde denaturing gel and capillary blotted onto nylon membranes (Micron Separations, Westborough, MA). Blots were probed with a nick translated (Boehringer Mannheim) 456 base pair *StyI-NotI* fragment from pCC3AG1 (Fig. 1a). Hybridization and autoradiography protocols were as described by Ausubel et al. (1994). Transformants with NPTII ELISA absorbance values and transcript hybridization signals comparable to the transformants used in previous recombination experiments (Greene and Allison, 1994) were selected for further study. Antigenic tests for capsid protein were not appropriate since the entire capsid gene was not included in the transgene and the remaining sequence was unlikely to encode a polypeptide recognized by CCMV antisera. Plants were clonally propagated by tissue culture or cuttings for RNA recombination bioassays.

Bioassay for RNA recombination in transgenic plants

Transgenic plants were inoculated at the 5 leaf stage of development with 1 μg each of infectious transcripts C1 and C2 and AG3, a movement defective CCMV RNA3 lacking 119 nucleotides from the 3' terminus of the capsid gene (Fig. 1a) (Greene and Allison, 1994). Plants transformed with the same portion of the capsid gene and a full length 3' UTR (Fig. 1a) were inoculated similarly. Nontransformed control plants were inoculated with wild type CCMV infectious transcripts C1, C2, and C3 or mock inoculated with buffer only. Plants were grown in the laboratory at room temperature under a 16 hr. photoperiod. Uninoculated leaves were tested for systemic infection by dot blot assay with probe RA518 which hybridizes to the 3' UTR of all three CCMV RNAs (Allison *et al.*, 1990). Tests were initiated 14 days post inoculation (dpi) and repeated at two week intervals for 4 months.

RESULTS

Plasmid deletions and transformation

An ordered series of deletions from the terminus of the 3' UTR of the full length cDNA clone of CCMV RNA3, pCC3AG1, was generated by periodically terminating small aliquots of an exonuclease III digestion. Sequence analysis of the resulting plasmids revealed nested deletions which removed from 20 to 250 nucleotides of the 3' UTR. Sequence analysis of the remainder of the 3' UTR and capsid gene indicated that no spurious mutations had been introduced and that the marker

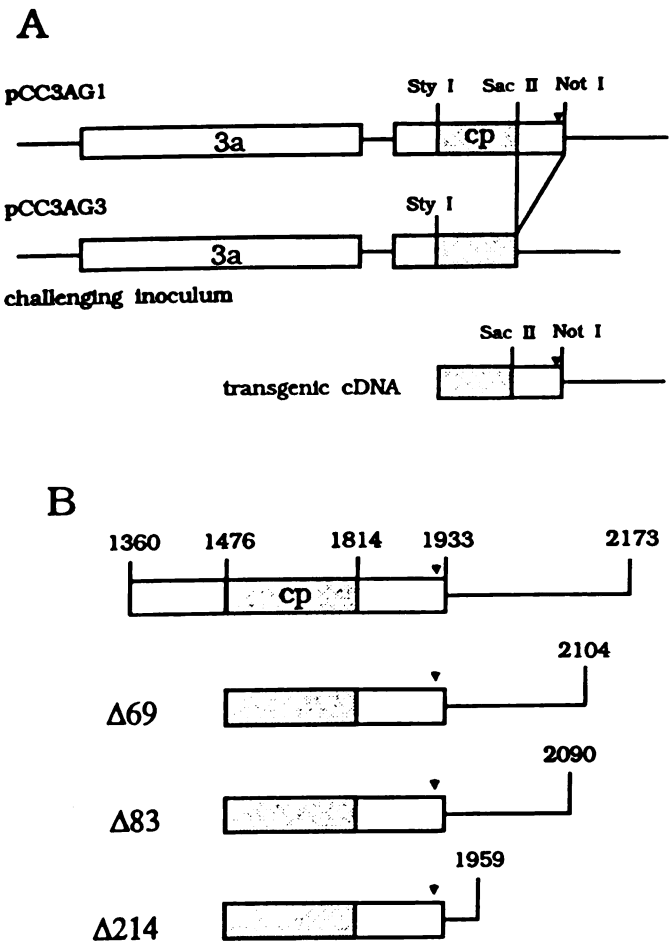

Fig. 1: Inoculum and transgene constructions for bioassay of recombination in transgenic plants. (A) Full length CCMV RNA 3 cDNA clone pCC3AG1 is the parent clone for the inoculum and transformation constructions. The NotI site and silent mutation denoted by the arrowhead, ▼, distinguish pCC3AG1 from wild type CCMV RNA 3. Appropriate restriction sites within the capsid gene, cp, are indicated. RNA 3 inoculum transcribed from pCC3AG3 contains a deletion within the cp gene between SacII (1814-1815) and NotI (1933-1934). Plants expressing transgenic cDNA with a complete 3'UTR served as a control. (B) The complete CCMV cp gene and 3' UTR are represented on the first line, numerals indicate several nucleotide positions. The deletion series with the abbreviated 3'UTRs are shown below. Numbers prefixed by a delta (▲) indicate the extent of the 3' deletion. Gray areas indicate the sequence shared by the transgenic insert and challenging inoculum.

mutations that distinguish pCC3AG1 from wild type CCMV RNA 3 remained intact. Three plasmids with deletions of 69, 83 and 214 nucleotides of the 3' UTR were transformed into *N. benthamiana*. From thirty transformed plants, three independent transformants for each deletion were selected for recombination studies. Since a full length capsid protein was not expressed, all selected transformants were susceptible to CCMV infection.

Bioassay for recombination in transgenic plants

Uninoculated leaves from the inoculate transgenic plants were screened for systemic CCMV infection beginning 14 dpi. Dot blot hybridization with a probe specific for the CCMV 3' UTR indicated that of 156 (▲69), 172 (▲83) and 151 (▲214) transgenic plants inoculated with C1, C2 and movement defective AG3 none became systemically infected over the 4 month screening period. In contrast, three percent of transformants expressing the 3' two thirds of the capsid gene plus the full length 3' UTR became systemically infected by viable recombinants (data not shown). All nontransgenic plants inoculated with wild type transcripts became systemically infected while plants inoculated with buffer only remained uninfected. Collectively, these data suggested that RNA recombination had either not occurred in the transformants with 3' UTR deletions or recombination did not result in the restoration of viable virus.

DISCUSSION

In this study, exclusion of the terminal portion of the 3' UTR from the transgenic transcript of VRTPs eliminated recombination events that regenerated viable virus. These transformants expressed 456 nucleotides of the CCMV capsid gene and limited segments of the associated 3' UTR. In our previous study (Greene and Allison 1994) and in controls of this study, similar inoculum yielded recombinant virus in 3% of the transgenic plants expressing the same segment of the capsid gene but the complete 3' UTR.

Many reported virus resistant transgenic plant include the 3' UTR; however, the UTR may not be required for resistance since it is not a characteristic of all successful resistance constructs (Cuozzo *et al.*, 1988; Stark and Beachy, 1989). While it may be included to add stability to the transcript, we identified numerous 3' deletion transformants with transcript quantities similar to those found in plants expressing the full length 3' UTR. Therefore, a complete UTR is likely unnecessary for either transcript stabilization or resistance in the transgenic plants.

The lack of recombination in these experiments suggests that the 3' UTR plays a significant role in the frequency of RNA recombination in transgenic plants. The presence of the complete 3' UTR on transgenic transcripts may enable a viral replicase complex to initiate replication on the transcript

itself, in addition to the viral RNA. This may increase the incidence of RNA recombination in two ways:

First, initiation of replication on the transgenic transcript could result in the synthesis of a complete minus strand RNA complementary to the transgenic viral transcript. In this case, both plus and the minus strand copies of the transgenic insert would be available for RNA recombination.

Second, if replication is, indeed, initiated on the 3' UTR of a transgenic transcript, only one template switch is required to regenerate a complete viral RNA (Fig. 2a). If replication cannot be initiated on the transgenic insert, a minimum of two template switches is required to restore a complete viral RNA. If the viral RNA terminus is absent from the transgenic transcript, recombination events generating full length viral RNA must initiate synthesis on a challenging virus RNA, switch to the viral transcript and return to a viral RNA to complete synthesis of a full length viral RNA with complete 3' and 5' termini.

Thus the elimination of the initiation site from the transgenic transcript appears to significantly complicate the formation of viable recombinants. In closely related brome mosaic bromovirus, the 3' UTR of RNA 3 interacts with tRNA specific host enzymes (Haenni *et al.*, 1982) and may be involved with the recruitment of host factors required for active replication. If the 3' UTR of CCMV plays a similar role, this region may target the transcript to the replication

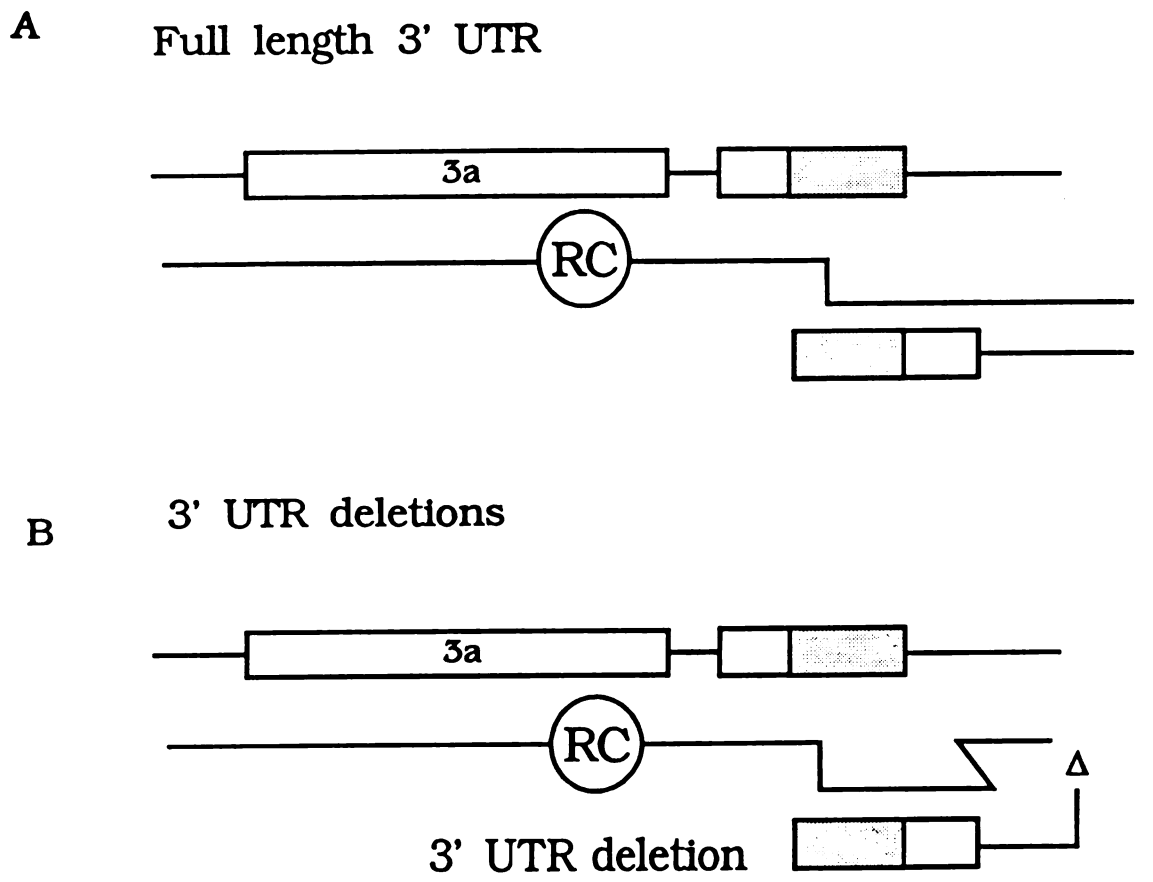

Fig. 2: Models for RNA recombination. CCMV RNA 3 deletion mutant is depicted undergoing recombination with the transgene. In A, the replication complex, RC, has initiated minus strand synthesis on the transgene with the full length 3'UTR. One strand switch within the shaded area to the inoculum permits synthesis of a full length RNA 3 to be completed on the deletion mutant. In B, the deletion, ▲, in the transgene disrupts the replication initiation site. Synthesis of a full length RNA requires initiation on the deletion inoculum, a switch to the transgene and a second switch to the inoculum for completion of minus strand.

complex, thus increasing the possibility of interactions between the challenging virus and the transgene.

Other recombinants recently recovered in our laboratory using a similar bioassay suggest that a 3' UTR is not mandatory for recombination events (manuscript in preparation). Therefore, exclusion or disruption of the 3' UTR does not guarantee that the transgenic transcript will not be involved in recombination, however, inclusion of this region appears to facilitate generation of viable viral RNA products.

SUMMARY AND CONCLUSIONS

The experiments described in this thesis provide direct evidence for RNA recombination in transgenic plants. Analysis of recombinant viruses recovered from transgenic plants revealed that recombination had occurred between challenging viral RNA and transgenic RNA expressed by the plant. Characterization of these recombinants has been described in the preceding chapters. Recombination between CCMV inoculum and transgenic CCMV RNA resulted in several recombinant viruses with symptoms that were distinct from the parental virus. Additionally, recombination between the CCMV transgene and BMV resulted in recovery of two hybrid viruses, one of which had a unique host range. In further study, removal of viral replication recognition signals from the transgene reduced the recovery of recombinants. These results have direct applications in the design and construction of virus resistant transgenic plants.

The initial experiment proved that viral transgenic mRNA is available within the cell in adequate concentrations for recombination with challenging RNA viruses. Recombination between a movement defective CCMV RNA 3 and a segment of CCMV capsid gene expressed in transgenic *Nicotiana benthamiana* (Domin) restored movement in recombinant viruses. All of the recombinants contained unique sequence within a 225 nucleotide region of the capsid gene that distinguished them from wild

type. Thus, aberrant homologous recombination had restored the gene. Many of the mutations were confined to a 92 nucleotide region of the capsid gene. It cannot be distinguished whether this region represents a hot spot for recombination or that it is an area which is more tolerant of mutation. No obvious RNA secondary structure suggested a recombination hot spot. Thus, selection for a functional capsid gene may have driven recovery of these recombinant viruses. Numerous mutations resulting in amino acid substitutions within the capsid protein suggested, that this region of the capsid protein was tolerant of change with no effect on movement within the host plant.

Analysis of the recovered virus also lent insight into the role of the capsid protein gene in systemic movement. While six of the seven recombinants contained mutations that maintained a functional capsid open reading frame (ORF), a seventh virus (3-57) moved systemically within the host without forming capsids. Sequence of this recombinant revealed a deletion that disrupted the capsid ORF. Additional mutations, in the region common to the transgene and inoculum, introduced an in frame start codon 3' to the deletion. Further study is underway to determine if protein expression initiates from this second start codon to produce a functional, but truncated, protein that is involved in systemic movement. This would suggest that the capsid protein is a multifunctional protein and that the carboxyl-terminal portion of the protein

functions independently of the complete protein and is sufficient for virus systemic movement.

CCMV recombinants were similar to wild type CCMV in host range and movement within hosts. No noticeable time lag for establishment of infection in cowpea or *N. benthamiana* was noted. Virus concentrations were similar to wild type when virus was isolated from the same amount of tissue. Interestingly, when the recombinants were competed against wild type CCMV in the same plant, wild type formed the predominant infection. Only recombinants containing minor amino acid changes were partially maintained during co-infection with wild type, and they were overcome upon passage of the infections to new plants. Thus, although CCMV recombinants were similar to wild type in single infections, wild type virus had a distinct selective advantage when both were present simultaneously. The difference may lie in the interaction of the capsid protein with host factors for movement and/or replication, or mutations in the capsid protein may affect protein/protein or protein/nucleic acid interactions in the cell or virion.

Symptom development was altered in several recombinants. Two recombinants, 2-1 and 5-55, formed more severe symptoms on both cowpea and bean when compared to wild type infection. Both recombinants contained a substitution at amino acid 111, suggesting that this position is important for symptom development. Similar results have been observed in tobacco

mosaic virus (TMV) where a single amino acid change in the capsid protein provided more severe symptoms (Banerjee et al., 1995). Mutants with more significant changes, including 3-57 which makes no capsid, were symptomless on the normal CCMV host range.

This work indicated that a transgene is available for recombination with challenging virus. Similar results had been observed in alfalfa mosaic virus (van der Kuyl et al., 1991), cauliflower mosaic virus (Gal et al., 1992) and red clover necrotic mosaic virus (Lommel and Xiong, 1991). In all cases recombination occurred between a transgene and inoculum derived from the same virus. Recombination between a transgene and a heterologous virus had not been reported to date. We postulated that a CCMV transgene could recombine with brome mosaic virus (BMV) since BMV and CCMV are closely related members of the *Bromoviridae*. For the most part these viruses have distinct host ranges, but they both systemically infect *N. benthamiana*. This species provided a convenient host for the recombination experiments. The same type of transgenic plants that were challenged with CCMV in earlier experiments were inoculated with movement defective, truncated BMV. Systemic movement was restored in 2.3% of transgenic plants. Sequence analysis revealed recombination between the CCMV transgene and BMV challenging RNA.

Virions were recovered from 4 infected transformants. Virus concentration was lower than wild type BMV for the same

amount of infected tissue. Attempts were made to analyze two of the viruses prior to passage to non-transgenic plants. Although the sequences derived from the cDNA clones were unlikely to be responsible for virion formation they revealed multiple recombination events in single RNAs. Both viruses contained sequence segments that were unlike either CCMV or BMV. Comparisons with known sequences did not determine the origin of these nucleotides although they most likely were derived by recombination with host RNA. Evidence of recombination with host RNA exists in several RNA viruses. Among the plant viruses host sequences have been detected in potato leafroll virus (Mayo and Jolly, 1991), the satellite Y of cucumber mosaic virus (Masuta et al., 1992) and BMV (Nagy and Bujarski, 1995). Host sequences were also detected in the animal viruses influenza (Katchikian et al., 1989) and bovine diarrhea pestivirus (Meyers et al., 1991). Taken together these results suggest that RNA recombination between viruses and host RNA is not uncommon. Based on numerous viral sequences, these recombination events apparently rarely result in viable virus. The recovery of these non viable recombinants provides a glimpse of the mutational activity of plant RNA viruses and permits speculation of the variety of material available for natural selection.

Two additional transgenic plants also exhibited systemic virus movement. To ensure selection of viable recombinants responsible for systemic infection, virus from these plants

was first passaged to non-transgenic *N. benthamiana* before cloning and sequence analysis. Clones of virus isolated from plants 5-61 and 5-120.2 revealed viable hybrid BMV/CCMV capsid genes and the absence of unidentifiable sequences. Hybrid 5-120.2 contained 27 nucleotides that were homologous to CCMV. The host range of 5-120.2 was similar to wild type BMV. In contrast, hybrid 5-61 contained a much larger insert of CCMV sequence, which corresponded to approximately one third of the capsid protein. This hybrid infected both barley and cowpea which are the hosts of BMV and CCMV respectively. Although the ability of the BMV/CCMV hybrid to infect cowpea may be attributed to the hybrid capsid protein. Other bromovirus experiments suggest that additional changes in this recombinant may have occurred elsewhere in the 5-61 genome. Further study of this hybrid may provide clues to host range determination.

The final study examined the effect of the 3' UTR in the transgene. Many viral capsid protein transgenes contain some viral regulatory sequences (see review in Beachy et al., 1990). These sequences are often included in the transgenes because they were part of the original cDNA clone and they were thought to add stability to the transgene. The 3' UTR of the CCMV transgene contains the replication initiation site for minus-strand synthesis. This site may enable the transgene to participate in recombination in two ways. First, by targeting the transgene to the replication complex, it is more

likely that the transgene and viral RNA would be in close proximity within the cell. Second, a complementary copy of the transgene may be generated if the viral replicase recognizes the transgene as a suitable template. This may provide unexpected recombination because recombination may occur during either plus- or minus-strand synthesis.

The 3' UTR of bromoviruses folds into a t-RNA like structure (Ahlquist et al., 1981) that is amino acylated with a tyrosine residue (Hall et al., 1972; Loesch-Fries and Hall, 1982). This 3' t-RNA like structure is important for the initiation of minus-strand synthesis (Ahlquist et al., 1984; Dreher and Hall; 1988). The core promoter has been mapped to the 3' terminal 134 nucleotides of the 3' UTR (Miller et al., 1986). None of the plants expressing CCMV transgenes lacking either 69, 83 or 214 3' terminal nucleotides provided any recombinant virus. In comparison, 3% of plants expressing the full length 3' UTR provided recombinants in the same number of inoculated plants. Thus it appears that elimination of the replication initiation site from the transgene reduces its involvement in recombination. However, the appearance of host RNAs, which do not have replication initiation sites, in recombinants 3-22.10 and 6-25.1 suggest that the transgene may remain capable of recombination, but the event may be more random.

Taken together the results of this thesis indicate that RNA recombination in transgenic plants is likely to generate

recombinant virus. In this study, there is strong selection for recombination. It is possible that expression of the viral protein, or other conditions that provide resistance, the transgenic RNA may be associated with ribosomes, plant proteins or expressed viral proteins and will be less available for the recombination events described here. Selection pressure appears to play a role in the frequency of recombination. The frequency of recombination in phage ϕ6 increased significantly under conditions where viral replication was reduced (Mindich, 1995). Similarly, recombination occurs frequently between the untranslated regions in the bromovirus under conditions that inhibit replication or movement of the viral RNAs (Allison et al., 1988; Bujarski and Kaesberg, 1986).

Selection pressure in the field for viruses that overcome transgenic resistance will be high. Resistance breaking by pathogens is a natural occurrence for virus resistant plants. MacFarlane and Davies (1992) detected strains of pea early browning virus that overcame transgenic resistance in inoculated plants that were maintained for a prolonged period of time. From this perspective, selection pressure for resistance breaking viral strains is similar for both transgenic and non-transgenic resistant varieties. However, the evolutionary potential is increased in resistant transgenic plants with respect to viruses that can replicate in the plant.

Viral replication is often supported in cells of non-host plants as evidenced by the ability of viruses to replicate in non-host protoplasts. This study demonstrated that movement defective virus can recombine with a transgene derived from a different virus. Recombination was greatly reduced when the replicase binding site was removed from the transgene. Thus the ability of the challenging virus to recognize the transgene may affect recombination in transgenic plants. In the bromovirus system, RNAs containing a BMV 3' UTR can also be replicated by tobacco mosaic tobamovirus (Ishikawa et al., 1991) and BMV replicase can replicate RNAs containing cucumber mosaic virus 3' UTR (Rao and Grantham, 1993). This indicates that viral transgenes may be recognized and replicated by non-related viruses in the field. Since recombination occurs by a template switch during viral replication, replication of the transgene may increase the frequency of recombination events.

This study provides evidence that multiple recombination events, including recombination between host and viral RNAs, can occur within virus infected cells. It is possible then, that presence of a transcribed viral sequence may increase the likelihood of producing viable recombinant molecules. The extent of homology between the transgene and challenging virus may also affect recombination by affecting the interaction between RNA molecules. In bromoviruses, heterologous recombination was facilitated by heteroduplex formation between the recombining RNA components (Nagy and Bujarski,

1993). Heterologous recombination has also been found in other single stranded RNA viruses (Lai, 1992), a negative-strand virus (Bergman et al., 1992) and in double strand RNA phage ϕ6 (Mindich et al., 1992). The amount of sequence homology required to facilitate recombination varies between viruses. Homologous recombination seems to require only small regions of homology at the point of recombination (Nagy and Bujarski, 1995; Bierbecher and Luce, 1992; Wang and Hawley, 1993). In the case of turnip crinkle virus a stem loop structure, rather than homology between templates was required for recombination (Cascone et al., 1993).

The mechanism of pathogen derived virus resistance varies among viruses and differs depending on the gene used to induce resistance (Fitchen and Beachy, 1993; Wilson, 1993; Scholthof et al., 1993). Therefore, it is difficult to make a blanket statement with regard to risk of transgenic plants expressing viral sequences. It seems necessary to study different viral transgenes independently to assess the potential for recombination with challenging viral RNA. RNA replication plays a very important role in viral RNA recombination. The results presented in this thesis indicate that the frequency of recombination decreased when the transgene is unable to interact with viral replicase. Thus, viral transgenes that decrease viral replication can affect the potential for recombination. Viral replicase-mediated protection appears to inhibit replication of the viruses to

which the plant is resistant. It would be interesting to compare levels of viral replication for virus that can still infect the transgenic plant to see if replication levels are decreased for other viruses in these plants.

One recurring argument against the risk posed by RNA recombination in transgenic plants is that mixed infections exist in the field and pose the same potential for recombination. Although mixed infections have been identified, little is known about the behavior of the individual viruses in the host plant. First, it has not been shown if the RNAs in a mixed infection have the opportunity to interact within individual cells of the plant. Both viruses may not be present in the same cell or may not be uncoated and replicating at the same time within an individual cell. Transgenic plants express viral sequences in every cell of the plant which may increase the potential of interaction with RNA of a challenging virus. Additionally, selection pressure may differ for mixed infections, where both viruses have the ability to infect the same plant. In some mixed infections one virus may provide a helper function to allow infection/movement of another virus. In this case it is again, unknown if the RNAs have the potential to interact within the plant cells.

This study addressed basic questions of RNA recombination in transgenic plants using a bromovirus system. Recovery of a hybrid virus with altered host range from this system suggests caution. To date viral transgenes containing capsid gene and

movement gene sequences have been shown to recombine. Viral replicase has yet to be studied. Study of the mechanisms of pathogen-derived resistance and the plant genes involved may provide alternate transgenes that can circumvent the risk posed by virus-derived transgenes.

APPENDIX

APPENDIX

Nucleic Acid Sequence of BMV/CCMV Recombinants

Recombinant 3-22.10 (capsid protein start codon at postion 4)

```
  1  tctatgtcga cttcaggaac tggtaagatg actcgcgcgc agcgtcgtgc
 51  tgccgctcgc agaaatcgtt ggaccgctag ggtccaccaa ccagtaattg
101  tcgaaccact cgctgctggc caaggcaagg ccattaaagc gattgcagga
151  tacagcatat caaagtggga ggcgtcttcg gacgcgatta cagcgaaagc
201  caccaatgct atgagtatca ctctgcccca tgagctctct tctgaaaaga
251  ataaggagct taaggtcggc agggtgctgc tttggttggg acttcttcct
301  agcgttgctg ggaggattaa ggcttgtgtt gctgagaaac aggcacaggc
351  ctcgaaatta accctcacta aagggaacaa aagctggtac ccccccccc
401  ctgaggacga cggtatcgat aagcttgata tccctttcga cagcttcttc
451  gcgctctcaa ccaccatttt cttcgcagct ttaatggtgg tagcaagacc
501  tcgcagttcc agtcttcgcg tagagtgtgg aaagggtgac tttcgcccgt
551  cactcccttg ccgttaatta cactcaatga gtgagtaatc catggggtcc
601  gaaatgtgaa atacgaaggc cgagcataca cacttaacac tcgcctattg
651  ttaaagtgtg tcctttgtcg atactgtgtt tctttaaacg gtaatcgttg
701  ttgaaacgtc ttccttttac aagaggattt ctaggtgcct ttgagagtta
751  ctctttgctc tcttcggaag aacccttagg ggttcgtgca tgggcttgca
801  tagcaagtct tagaatgcgg gtaccgtaca gtgttgaaaa acactgtaaa
851  tctctaaaag agacca
```

Recombinant 6-25.1 (capsid protein start codon at position 4)

```
   1  tctatgtcct aattcagcgt attaataatg tcgacttcag gaactgcgtt
  51  tctttaaacg gtaatcgttg ttgaaacgtc ttccttttac aagaggattg
 101  agctgccctt gggttttact ccttgaaccc ttcggaagaa ctctttggag
 151  ttcgtaccag tacctcacat agtgaggtaa taagactggt gggcagcgcc
 201  tagtcgaaag actaggtgat ctctgtgtgg aaagggtgac ctttcgcccg
 251  tcactccctt gccgttaatt acactcaatc gagtgagtaa tccatggggt
 301  ccgaaatgtg aaatacgaag gccgagcata caacacacct taacactcgc
 351  ctattgttaa agtgtgtcct ttgtcgatac tgtactaatg cttaaattat
 401  gctgagtgat atcccttaag ctcgaggttg ctgggaggat taaggcttgt
 451  gttgctgaga aacaggcaca ggccgaggct gcttttcaag tagccttggc
 501  ggttgcagac tcctcgaaag aggtggtcgc ggccatgtat acggacgcct
 551  ttcgaggggc gactctgggg gatttgctta atctccagat ttatctgtat
 601  gcatctgaag cagtgcctgc taaggcggtt gagcatgtca gacctacgtt
 651  tgacgactct ttcactccgg tctattagcg gccgctgaag agcgttacac
 701  tagtgtggcc tacttgaagg ctagttataa ccgttcttta aacggtaatc
 751  tttgctctct tcggaagaac ccttaggggt tcgtgcatgg gcttgcatag
 801  caagtcttag aatgcgggta ccctagtacc tcacatagtg aggtaataag
 851  actggtgggc agcgcctagt cgaaagacta ggtgatctct aaggagacca
```

Recombinant 5-61 (capsid protein start codon at position 1)

```
   1  ATGTCGACTT CAGGAACTGG TAAGATGACT CGCGCGCAGC GTCGTGCTGC
  51  CGCTCGCAGA AATCGTTGGA CCGCTAGGGT CCAACCAGTA ATTGTCGAAC
 101  CACTCGCTGC TGGCCAAGGC AAGGCCATTA AAGCGATTGC AGGATACAGC
 151  ATATCAAAGT GGGAGGCGTC TTCGGACGCG ATTACAGCGA AAGCCACCAA
 201  TGCCATGAGT ATCACTCTGC CCCATGAGCT CTCTTCTGAA AGAATAAGGA
 251  GCTTAAGGTC GGCAGGGTGC TGCTTTGGTT GGGACTTCTT GCTGGGAGGA
 301  TTAAGGCTTG TGTTGCTGAG AAACAGGCAC AGGCCGAGGC TGCTTCCCGA
 351  TTTCAAGTAT GCCTCCTTTC AGGTGGCATT AGCTGTGGCC CGACAACTCG
 401  AAAGATGTTG TCGCTGCTAT GTACCCCGAG GCGTTTCGGG TATAACCCTT
 451  GAACAACTCG CCGCGGATTT AACGATCTAC TTGTACAGCA GTGCGGCTCT
 501  CACTGAGGGC GACGTCATCG TGCATTTGGA GGTTGAGCAT GTCAGACCTA
 551  CGTTTGACGA CTCTTTCACT CCGGTCTATT AGCGGCCGCT GAAGAGCGTT
 601  ACACTAGTGT GGGTCACAGG CCCCTTGTCT CAGGTAGAGA CCCTGTCCAG
 651  GTAGGACACT TTGGCTAAGG TTAAAAGCTT GTTGAATCAG TACAATAACT
 701  GATAGTCGTG GTTGACACGC AGACCTCTTA CAAGAGTGTC TAGGTGCCTT
 751  TGAGAGTTAC TCTTTGCTCT CTTCGGAAGA ACCCTTAGGG GTTCGTGCAT
 801  GGGCTTGCAT AGCAAGTCTT AGAATGCGGG TACCGTACAG TGTTGAAAAA
 851  CACTGTAAAT CTCTAAAAGA GACCA
```

Recombinant 5-120.2 (capsid protein start codon at position 1)

```
   1  ATGTCGACTT CAGGAACTGG TAAGATGACT CGCGCGCAGC GTCGTGCTGC
  51  CGCTGGCAGA AATCGTTGGA CCGCTAGGGA CCAACCAGTA ATTGTCGAAC
 101  CACTCGCTGC TGGCCAAGGC AAGGCCATTA AAGCGATTGC AGGATACAGC
 151  ATATCAAAGT GGGAGGCGTC TTCGGACGCG ATTACAGCGA AAGCCACCAA
 201  TGCCATGAGT ATCACTCTGC CCCATGAGCT CTCTTCTGAA AAGAATAAGG
 251  AGCTTAAGGT CGGCAGGGTG CTGCTTTGGT TGGGACTTCT TCCTAGCGTT
 301  GCTGGGAGGA TTAAGGCTTG TGTTGCTGAG AAACAGGCAC AGGCCGAGGC
 351  TGCTTTTCAA GTAGCCTTGG CGGATGCAGA CTCCTCGAAA GAGGTGGTCG
 401  CGGCCATGTA TACGGACGCC TTTCGAGGGG CGACTCTGGG GGATTTGCTT
 451  AATCTCCAGA TTTATCTGTA TGCATCTGAA GCAGTGCCTG CTAAGGCGGT
 501  CGTTGTACCC CGAGGCGTTA AAGGGTATAA CACGTTCGAT GACTTCTTCA
 551  CCCCGGTTTA TAGGTAGCGG TTCTATGATA TATGAACCTA AGCTGTGAAC
 601  AGCCCTTTGG TTAAGGTTAA AAACTCCTGG TCAGGCAGAC CACTTTGGCT
 651  AAGTTTAAAA GCTTGTTGAA TCAGTACAAT AACTGATAGT CGTGGTTGAC
 701  ACGCAGACCT CTTACAAGAG TGTCTAGGTG CCTTTGAGAG TTACTCTTTG
 751  CTCTCTTCGG AAGAACCCTT AGGGGTTCGT GCATGGGCTT GCATAGCAAG
 801  TCTTAGAATG CGGGTGCCGT ACAGTGTTGA AAAACACTGT AAATCTCTAA
 851  AAGAGACCA
```

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