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ABSTRACT

RNA RECOMBIANTION AND SELECTION IN BROMOVIRUS NATURAL INFECTIONS

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RNA recombination plays an important role in the evolution of RNA viruses by facilitating modular evolution. Thus, it is not suprising that one of the risks associated with virus resistant transgenic plants (VRTPs) expressing viral sequences is the potential for recombination between the transgene message and a challenging virus. Several independent researchers have already reported instances of such virus/transgene recombination in VRTPs. In particular, Greene and Allison (Science 263: 1423-25, 1994) demonstrated that bromoviruses can recombine with a transgenically expressed message to form viable recombinants with altered phenotypes. However, the evolutionary significance of VRTP recombination is still in question, since viruses have long had the opportunity to interact with and recombine with other viral RNAs in natural situations such as mixed infections. To assess the significance of transgenic recombination, the opportunities for recombination in transgenic plants expressing bromoviral sequences were compared to the opportunities for recombination in different types of bromovirus mixed infections. These experiments have demonstrated that 1) the moat likely scenarion for the formation of bromovirus mixed infections is a delayed inoculation of the two viruses, 2) the level of recombinant recovery in delayed mixed infections is lower than in transgenic plants, 3) the recovery of recombinants is related to the selective fitness of the

parental virus, and 4) the opportunities for recombination in mixed infections do not mirror the opportunities for recombination in VRTPs. In addition, mutational analysis of the bromovirus capsid gene has allowed us to determine the capsid gene requirements for systemic movement, and monitoring reversion mutations in bromovirus recombinants with distinct phenotypes over the course of serial passaging has demonstrated the presence of strong selection pressure for wild type sequence at both the RNA and protein level.

Dedicated to Leonard A. Schneider, for years of devotion to his family, and the field of science education.

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INTRODUCTION

Each year plant viruses cause tremendous amounts of damage to crops in the United States, with estimates of financial loss reaching $60 billion per year (Klausner, 1987). It is not suprising, then, that considerable effort is spent on research and potential control measures for plant virus diseases. Natural resistance to plant viruses has been limited in scope by the genetic resources available to breeders, so considerable amounts of time and money have been spent on the control of insect vectors, usually through expensive and environmentally unfriendly pesticide application. Recent advances in molecular biology techniques have enabled virologists to study the basic biology of plant viruses, which has led to rapid growth in our knowledge of viral biology and the development of new forms of disease control. The work described in this thesis examines some aspects of the basic biology of a model group of plant RNA viruses, the bromoviruses. This in turn allows us to indirectly examine some principles involved in bromovirus evolution, and make suggestions about the impact of RNA recombination in plant virus evolution.

Evolution of RNA Viruses

RNA viruses make up the most abundant group of plant viruses characterized to date (Zaitlin and Hull, 1987). Overall, viruses with RNA genomes or RNA intermediates are the most common group of subcellular parasites (Domingo et al., 1996). A

distinguishing characteristic of RNA viruses is their genetic variability within a given population. RNA viruses increase genetic variability via mutation, just as all prokaryotic organisms do. One mechanism of mutation for RNA viruses is polymerase error during replication. The error rate of RNA-dependent RNA polymerases (RdRP's) which replicate RNA viral genomes is characteristically high. Mutation rates for viral RdRps range from 10^{-4} to 10^{-5} substitutions per nucleotide copied (Holland et al., 1992; Drake, 1993; Mansky and Temin, 1995). This rate is significantly higher than prokaryote and eukaryote DNA polymerase error rates, which range from 10^{-8} to 10^{-11} substitutions per nucleotide copied (Drake, 1991; Beckman and Loeb, 1993). This high error rate is attributed to the lack of 3'to 5' exonuclease activity, which is used for editing by most cellular DNA polymerases. If the error rate for RNA viruses is in fact this high, then RNA viruses, with an average size of 10 kilobases per genome, will on average incur between 0.1 and 1 mistakes per genome replication. Thus, it has been proposed that RNA viruses exist as a spectrum of mutants dominated by a consensus sequence that represents the most competitive form of RNA, known as a quasispecies (Holland et al., 1982).

Quasispecies theory has important implications for the biology of RNA viruses. First, the presence of numerous mutants in a quasispecies represents a reservoir of potentially useful variants which may excel in the face of new selective pressures (Kilbourne, 1991; Holland et al., 1992; Domingo and Holland, 1994). Secondly, the quasispecies becomes the level of population on which selection acts. Thus, individual viral RNAs can sustain mutations without changing the fitness of the viral population. This allows viruses to maintain a consensus sequence over numerous passages despite high mutation rates (Domingo et al., 1978; Steinhauer et al., 1989), a concept referred to as

population equilibrium (Domingo et al., 1996). A third implication of the quasispecies theory is the potential for genetic bottlenecks to affect viral fitness via genetic drift. If an RNA virus with considerable variation in its quasispecies is subject to a tight bottleneck during its life cycle (e.g. transmission from one host plant to the next) there is a greater chance that the RNA sampled at random from the quasispecies will have a deleterious mutation in its genome. As the virus undergoes serial bottlenecks, mutations would accumulate in the viral population. The accumulation of deleterious mutations would result in a loss of fitness in the resulting progeny, a phenomenon that has been observed experimentally in both the tripartite RNA phage $\phi 6$ (Chao, 1990) and the negative strand RNA vesicular stomatitis virus (VSV) (Duarte et al., 1992).

The observed loss in fitness due to polymerase error and serial bottlenecks is known as "Muller's ratchet" (Muller, 1964). Muller's ratchet states that asexual populations (i.e. RNA viruses) of organisms with a small population size and a high mutation rate will tend to incorporate deleterious mutations in an essentially irreversible manner unless compensatory mechanisms can restore the initial mutation free class of genomes. Muller's ratchet has been demonstrated experimentally for RNA viruses (Domingo et al., 1978; Chao, 1990; Duarte et al., 1992; Duarte et al, 1994). The effects of Muller's ratchet on RNA viruses can be countered by reassortment of viral segments, RNA recombination, or selection pressure (Domingo et al., 1996). Subsequently, it has been proposed that multipartite RNA viruses evolved as a means of reversing Muller's ratchet, by undergoing reassortment (Chao, 1988; Chao, 1991; Pressing and Reaney, 1984). Thus, the study of recombination and selection in RNA viruses has immediate relevance to the study of RNA viral evolution.

RNA-RNA recombination, the joining of portions of two genetically distinct RNAs, is widely accepted to have played a major role in the evolution of RNA viruses. In fact, there is considerable evidence for the effects of recombination events in the genomic sequences of both animal and plant RNA viruses (Lai, 1992). RNA recombination is a mechanism by which RNA viruses can incorporate large segments of genetic material into their genomes, facilitating modular evolution (Goldbach, 1987). In addition, as previously mentioned, RNA recombination can counter the effects of Muller's ratchet by joining error free segments of viral RNAs, potentially eliminating deleterious mutations. RNA recombination can either be homologous (recombination between two RNAs with identical sequence) or non-homologous. Homologous RNA recombination in viruses is widely accepted to occur by a template switching or 'copy choice' mechanism (Kirkegaard and Baltimore, 1986), wherein a viral replicase begins replication on one RNA template, dissociates from the original template during replication and switches to a second distinct template (Lai, 1992). However, recent work with phage Qβ indicated that the mechanism of non-homologous recombination is not related to RNA replication and cannot be template switching. (Chetverin et al., 1997).

Virus resistant transgenic plants

Powell-Abel et al. (1986) were the first to demonstrate that the expression of a virus capsid gene by a transgenic plant could lead to resistance to the virus from which the gene was derived. The phenomena of pathogen derived resistance was of immediate importance to industry and agriculture, since numerous cloned and sequenced viral genes were readily available and genetic engineering represented a consistent means of

producing virus resistant plant lines. Since then, genetic engineering has been used to transform many different species of plants with numerous virus derived genes (for review see Grumet, 1995). In some cases, expression of the viral protein is required for resistance, while in other cases expression of the transgenic RNA is sufficient. The exact mechanism of pathogen derived resistance remains unknown, and considerable work continues in this area, even as the first virus resistant transgenic plants (VRTPs) are being approved for commercial release.

Despite obvious advantages, there are specific risks associated with the release of VRTPs, namely transencapsidation and the potential for recombiantion between transgenic RNA and a challenging virus (de Zoeten, 1991). Viral transgenes engineered into plants are usually transcribed in a constitutive manner, which provides a steady supply of potential templates for RNA recombination in every cell. In addition, most viruses are capable of replication in the cells of numerous plant species, including non-hosts, indicating that a wide variety of viruses may replicate in the vicinity of the transgenically expressed viral RNA. As a result, the issue of recombination becomes particularly significant, since RNA recombination can lead to the creation of genetically distinct progeny viruses, and perhaps contribute to the evolution of new viral pathogens (Banner and Lai, 1991; Makino et al., 1986; Nagy and Bujarski, 1992; for review see Simon and Bujarski, 1994). To address this possiblility, a series of experiments were designed to establish whether transgenically expressed viral RNAs were available to the viral replicase of a challenging virus. These experiments were carried out using well characterized members of the bromovirus family.

Bromoviruses

Bromoviruses are small icosahedral single-stranded positive-sense RNA plant viruses belonging to the bromoviridae. Members of the bromovirus group include brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), broad bean mottle virus (BBMV), cassia yellow blotch virus (CYBV), melandrium yellow fleck virus (MYFV) and spring beauty latent virus (SBLV) (Rybicki, 1995). Bromoviruses are among the most thoroughly studied plant viruses, and as such have been model organisms for the study of many aspects of RNA virus biology (for review, see Schneider and Allison, 1994). BMV 1) was the first example of an icosehedral virus (Kaesberg, 1956), 2) was among the first viruses to be sequenced completely (Ahlquist et al., 1981; Ahlquist et al., 1984), 3) was the first example of a subgenomic messenger RNA (Shih and Kaesberg, 1973) and 4) was the first virus for which biologically active transcripts were obtained *in vitro* from cDNA clones (Ahlquist et al., 1984). The sequence of the BMV capsid protein determined the first eukaryotic ribosome binding site (Dasgupta et al., 1975). One of the first examples of RNA/RNA recombination in plant viruses was demonstrated in BMV (Bujarski and Kaesberg, 1986).

Particle characteristics

Bromoviruses have small icosehedral capsids, about 25 nm in diameter (Jarcot et al., 1977; Chauvin et al., 1978). The protein shell has 180 capsid subunits, arranged in T=3 symmetry (Finch and Klug, 1967). Bromovirus virions are most stable between pH 3.0 and 6.0 (Bancroft et al., 1968) and swell to less stable forms above pH 6.5. Although

the encapsidated viral RNA partially penetrates the protein shells, virions appear to have a hollow core approximately 10 nm in diameter. RNA 1 and RNA 2 are encapsidated individually in independent particles, while RNA 3 and subgenomic RNA 4 are encapsidated together (Lane and Kaesberg, 1971; Bancroft and Flack, 1972). Recently the crystal structure of the CCMV virion has been determined (Speir et al., 1995). The N-terminal and C-terminal ends of the capsid protein extend away from a central β-barrel, tying individual subunits together into the capsid structure.

Genomic Strategy

Bromoviruses have a tripartite, single-stranded, positive sense RNA genome consisting of RNA 1 (3.2 kb), RNA 2 (2.9 kb) and RNA 3 (2.1 kb) (Lane and Kaesberg, 1971) (Fig. 1.). A subgenomic messenger RNA, RNA 4, is transcribed from the RNA 3 component during infection, and is not required in the initial inoculum (Lane, 1974). The genomic RNAs from BMV, BBMV, and CCMV have all been cloned and sequenced (Ahlquist et al., 1981; Ahlquist et al., 1984; Allison et al., 1989, Dzianott and Bujarski, 1991; Romero et al., 1992), and *in vitro* transcripts made from full length clones of these three viruses are infectious (Ahlquist et al., 1984; Allison et al., 1989; Romero et al, 1992). All four bromovirus RNAs are capped with 7-methyl guanosine 5'-ppp-5'guanosine. However, in contrast to host encoded mRNAs, the penultimate 5' nucleotide is not methylated (Dasgupta et al., 1976). Bromoviruses RNAs have a characteristic homologous sequence of approximately 200 nucleotides in their 3' untranslated region (Ahlquist et al., 1981). The terminal 140 nucleotides of this region can be folded to resemble a tRNA-like structure (Ahlquist et al., 1981) with a pseudoknot forming the

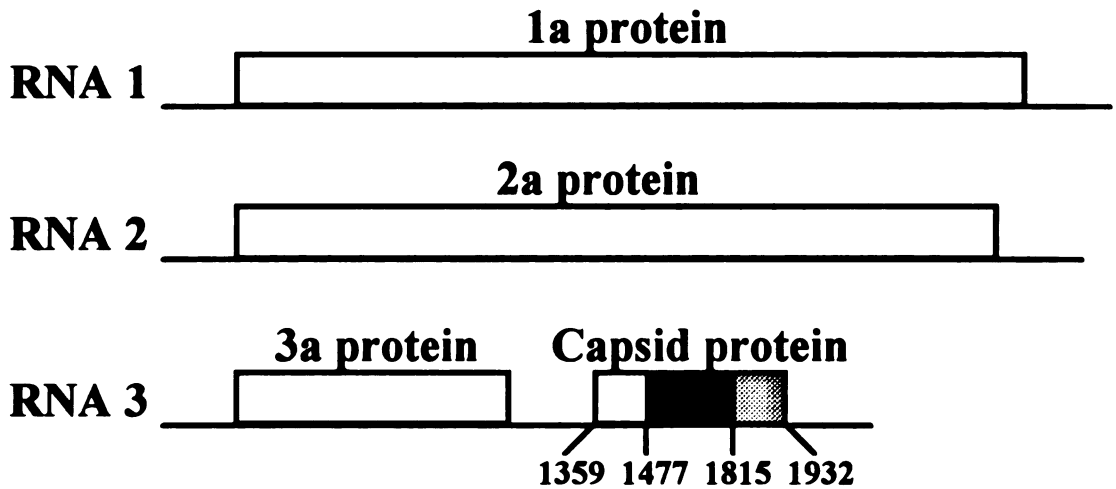

Figure 1. Genomic map of bromvirus RNAs with open reading frames indicated by boxes. Nucleotide positions in the capsid gene indicated by numbers below RNA 3 diagram.

acceptor stem (Reitvald et al., 1983; 1984) The 3' terminus is aminoacylated with tyrosine by a host-encoded aminoacyl-transferase both *in vivo* and *in vitro* (Hall et al., 1972., Kohl and Hall, 1974., Loesch-Fries and Hall, 1982). In addition, the three genomic RNAs of BMV have a homologous region of roughly 40 bases in their 5' untranslated region (Ahlquist et al., 1984; Marsh and Hall, 1987).

Bromovirus genomes encode four proteins. RNAs 1 and 2 are monocistronic, encoding proteins of 109 kD and 94 kD respectively (Ahlquist et al., 1984). These two proteins are required for viral replication (Kisbertis et al., 1981) and are included in the viral replicase complex (Haselhoff et al., 1984; Ahlquist et al., 1985; Kamer et al., 1984; Kao et al., 1992). RNA 3 is dicistronic, with an intercistronic region of approximately 250 nucleotides (Ahlquist et al., 1984), which includes a poly (A) sequence of variable size (Ahlquist et al., 1984). RNA 3 encodes two proteins: the 32 kD cell-to-cell movement protein (Ahlquist et al., 1984; Haselhoff et al., 1984), and the 21 kD capsid protein (Ahlquist et al., 1981; Dasgupta and Kaesberg, 1982). The 3a protein, which shows homology to the 30 kD movement protein of tobacco mosaic virus (Haselhoff et al., 1984), is involved in determining bromovirus host range (Mise et al., 1993; Mise and Ahlquist, 1995; Fujita et al., 1996). Although neither the 3a nor the capsid protein is required for replication in protoplasts (Kisbertis et al., 1981; French et al., 1986), both are required for systemic infection (Allison et al., 1990; Sacher and Ahlquist, 1989). This makes RNA 3 of bromoviruses an ideal target for recombination studies of recombination, since its functions are not required for replication and the inhibition and subsequent recovery of the systemic infection phenotype allows for a very sensitive bioassay.

Life Cycle

As with all plant viruses, bromoviruses require a wounding event to enter the host cell and initiate the viral life cycle. In particular, a membrane lesion is required for entry of CCMV into cowpea protoplasts (Roenhorst et al., 1988), and it is generally accepted that transmission of bromoviruses in nature occurs via wounding by the beetle vector (Lane, 1981). Although little is known about the relationships between viruses and their beetle vectors, beetle transmission is generally considered inefficient, and bromovirus transmission appears to be even less efficient than other beetle transmitted viruses (Gergerich et al., 1983). While beetles acquire bromoviruses rapidly, they transmit them for only a few days (Lane, 1981). CCMV has been detected in the hemolymph of a few individual beetles (Hobbs and Fulton, 1979). Outside of the normal beetle transmission, it has been shown that nematodes can transmit BMV in the laboratory (Schmidt et al., 1963; Fritzche, 1975), and there is one report of BBMV seed transmission (Gibbs, 1977).

Once the virus has entered the cell, the virion RNA must be uncoated and translated. At pHs above 6.0, the CCMV virions swell by 10%, allowing ribosomes access to viral RNA (Spier et al., 1995). Simultaneous release and translation of viral RNA, a process termed co-translational disassembly (Wilson, 1984; Shaw et al., 1986; Gallie et al., 1987), has been observed *in vitro* for CCMV (Roenhorst et al., 1989; Albert et al., 1997). Translation of the 1a and 2a genes enables the virus to begin replication. The capsid protein is translated from the subgenomic message, RNA 4, and newly replicated viral RNA is encapsidated into progeny virions. Cell-to-cell movement requires translation of the 3a protien, and likely occurs through the plasmodesmata.

Bromoviruses have relatively narrow host ranges. With few exceptions, the host range of BMV is limited to grasses, while BBMV and CCMV are pathogens of legumes and several other dicotyledonous plants (Lane, 1981) However, all three viruses infect *Nicotiana benthamiana* (Lane, 1981) and several species in the genus *Chenopodium* (Lane, 1974). Bromoviruses tend to induce mosaic symptoms on their hosts, although there is variation among strains of the same virus depending on the specific host (Lane, 1981). BMV causes mild mosaic symptoms on grasses (Lane, 1977), while CCMV symptoms vary from a systemic mottle to veinal necrosis, depending on the host (Bancroft, 1971). BBMV causes chlorotic mosaic symptoms and vein clearing in legume hosts (Gibbs, 1977). Viral symptoms will appear one to two weeks post inoculation (Lane 1981), as bromoviruses reach and maintain high titers (0.3 to 3 mg per gram of leaf tissue) in their respective hosts (Matthews, 1991). In trace studies, the rate of ^{32}P incorporation into CCMV increases rapidly during the first few days after inoculation, and then drops off rapidly, suggesting that the virus replicates and moves quickly within the host (Dawson and Kuhn, 1974).

Replication

Due to the availability of infectious transcripts made from full length cDNA clones, the replication of bromoviruses has been studied extensively (for review see Ahlquist, 1992). Bromovirus proteins 1a and 2a are responsible for replication of viral RNAs, and as previously mentioned, RNAs 1 and 2 are sufficient for replication in protoplasts (Ahlquist et al., 1984; Kisbertis et al., 1981; French et al., 1986; Loesch-Fries and Hall, 1980). Three functional domains have been identified in bromovirus 1a and 2a proteins; a methyl-

transferase domain and a helicase domain in the 1a protein, and a polymerase motif in the 2a protein. These motifs are also found in replication proteins of other plant viruses, as well as the replication proteins of animal alphaviruses (Haselhoff et al., 1984; Ahlquist et al., 1985; Cornelissen and Bol, 1984; Goldbach, 1987). Three distinct phases of RNA synthesis are necessary for completing a replicative cycle in bromoviruses: (-) strand synthesis using the (+) sense inoculum RNA as a template; full length genomic (+) strand synthesis using the newly formed (-) strands as a template; and synthesis of subgenomic RNA 4 from the (-) strand RNA3 template (Dreher and Hall, 1988, Ahlquist, 1992).

Minus Strand Synthesis:

The 3'-terminal tRNA-like structure common to all bromovirus genomic RNAs acts as a promoter for the initiation of (-) strand synthesis (Ahlquist et al., 1984; Miller and Hall, 1985). Deletion mutants have been used to map (-) strand promoter activity of BMV RNAs both *in vitro* (Miller et al., 1986) and *in vivo* (Bujarski et al., 1986; Dreher and Hall, 1988), and to map the sequence and structural requirements for aminoacylation and 3' adenylation (Bujarski et al., 1985; Bujarski et al., 1986; Dreher and Hall; 1988; Rao et al., 1989). The (-) strand promoter core sequence has been mapped to the 134 3' terminal bases, which are integral in forming the tRNA-like structure. Minus strand synthesis begins with the G complimentary to the last C of the 3' CCA^{OH} (Miller and Hall, 1985), and the terminal adenosine is added to plus strands after replication by an interaction with the host tRNA nucleotidyl transferase (Miller and Hall, 1985; Rao et al. 1989). The RNA-dependent RNA polymerase may initiate after binding of short oligonucleotide primers, since the addition of such primers greatly increases (-) strand

synthesis (Kao and Sun, 1996; Sun et al., 1996). Based on the products recovered from *in vitro* replication experiments (Hardy et al. 1979), it is suspected that double-stranded replicative intermediates are the initial products of (-) strand synthesis (Marsh et al., 1991). These double-stranded products, which consist of the (+) strand template bound to the progeny (-) strands, are resistant to mild ribonuclease treatment (Hardy et al., 1979). Double-stranded BMV RNA complexes have been found both *in planta* (Bastin and Kaesberg, 1976) and in protoplasts (Loesch-Fries and Hall, 1980).

Plus strand synthesis:

Following (-) strand synthesis, (+) strand genomic RNA is synthesized using the (-) strand template. Although less is known about the initiation of (+) strand replication, the promoter for bromoviral (+) strand synthesis has been localized in the sequence corresponding to the 5' untranslated region of the (+) strand. Sequence motifs at the 5' termini of BMV RNAs and within the intercistronic region of RNA 3 resemble the internal control regions (ICRs) 1 and 2 that promote transcription of tRNA genes (Marsh and Hall, 1987). Mutations in the ICR2-like region cause reductions in viral RNA accumulation and preferential decreases in the synthesis of (+) strand RNA Pogue et al., 1990; Pogue et al., 1992). In particular, any mutations that destabilize a predicted stem-loop structure in the 5' terminus of the (+) strand debilitate replication (Pogue and Hall, 1992). This predicted stem loop structure likely binds with a host factor that facilitates initiation of (+) strand synthesis (Marsh et al., 1987; Pogue and Hall, 1992).

RNA 4 replication is initiated internally on (-) sense RNA 3 strands (Miller et al., 1985). The subgenomic promoter is approximately 90-110 nucleotides long with four

functional regions (Marsh et al., 1988; French and Ahlquist, 1988; Marsh et al., 1987). The core sequence lies immediately downstream of the poly (A) tract and upstream of the initial base of RNA 4. Regions 5' of the poly (A) tract and A-U rich regions 3' to the core promoter are also required for promoter activity and accurate initiation. There is significant promoter sequence homology both among the bromoviruses, and among bromovirus subgenomic promoters and other viral subgenomic promoters (Marsh et al., 1988).

Like other positive sense RNA viruses, bromoviruses accumulate more (+) strand RNA than (-) strand RNA. In BMV the ratio of (+):(-) strands has been estimated to be 100:1 (Marsh et al., 1991). The presence of RNA 3 affects the regulation of (+) and (-) sense RNA levels, since the ratio of (+):(-) strands was nearly equal in protoplasts inoculated with RNAs 1 and 2 alone (Pogue et al., 1990). When wild type RNA 3 was included in the inoculum, the (+):(-) strand ratio returned to the expected level of approximately 100:1 (Marsh et al., 1991). Various mutations in RNA 3 which debilitated production of the capsid protein reduced the (+):(-) ratio by approximately 50%, and mutations which deleted the subgenomic promoter reduced the (+):(-) ratio to 1.8:1.0 (Marsh et al., 1991).

Viral Replicase

RNA-dependent RNA polymerases (RdRps) have been isolated from both BMV and CCMV (Horikoshi et al., 1987; Miller and Hall, 1984; Hardy et al., 1979; Bujarski et al., 1982; Miller and Hall, 1983; Maekawa and Furusawa, 1984; White and Dawson, 1978; Quadt et al., 1995). The replication complex of bromoviruses is membrane

associated, and the BMV 1a and 2a proteins have been colocalized to the endoplasmic reticulum (Restrepo-Hartwig and Ahlquist, 1996). Solubilization by detergents generally yields RdRp preparations consisting of the 1a and 2a proteins with 5-6 host factors (Quadt and Jaspers, 1990; Quadt et al., 1993). Preparations of BMV RdRp initiate *de novo* synthesis of (-) strand RNA complementary to BMV RNAs 1, 2, 3 and 4 (Miller et al, 1986), as well as subgenomic RNA 4 *in vitro* (Miller et al., 1985). The initiation of bromovirus (-) strand and (+) strand replication is known to be dependent upon at least one and probably several of the host proteins associated with the replicase complex (Pogue and Hall, 1992; Hall et al., 1987; Quadt and Jaspers, 1990).

Bromovirus 1a and 2a proteins form a complex *in vitro*, and the interaction is also required for replication *in vivo* (Kao et al., 1992, Dinant et al., 1993; O'Reilly et al., 1995; Smirnyagina et al, 1996). Analysis of BMV 1a and 2a proteins expressed in yeast cells indicates that viral RNA is also required for replicase formation (Quadt et al., 1995). The 1a-2a interaction is virus specific (Allison et al., 1988; Dinant et al., 1993), and the domains required for interaction have been mapped to the carboxy-terminal helicase like domain of the 1a protein and a 115 amino acid domain in the N-terminal end of the 2a protein (Kao and Ahlquist, 1992). Mutations in these areas of the BMV 1a and 2a proteins effectively blocked viral replication *in vivo*, further indicating that the 1a-2a interaction is required for BMV replication (Kroner et al., 1990; Traynor et al., 1991, Kao and Ahlquist, 1992). However, recent work has demonstrated that a BMV 2a core polymerase region which lacks the amino-terminal 115 amino acid region associated with 1a-2a binding can function as a fusion with the 1a protein or as a separate protein, indicating that 1a-2a interactions are more complex than originally thought (Smirnyagina et al., 1996).

Bromovirus replicases show a strong preference for bromoviral RNAs as templates (Hardy et al., 1979, Miller and Hall, 1984). However, the BMV replicase can use CCMV RNA as a template at about one-third of BMV RNA efficiency (Miller and Hall, 1983). In protoplasts, replication of BMV RNA 3 is supported by either BMV or CCMV RNAs 1 and 2; similarly, replication of CCMV RNA 3 is supported by either BMV or CCMV RNAs 1 and 2. However, a heterologous combinations of RNAs 1 and 2 (e.g. BMV 1 and CCMV 2) are incapable of supporting any replication (Allison et al., 1988). These heterologous combinations are partially capable of functioning when suitable BMV/CCMV 2a hybrids are involved (Traynor and Ahlquist, 1990).

RNA recombination in bromoviruses

The multipartite nature of the genome and the availability of infectious transcripts have facilitated the study of RNA recombination in bromoviruses (for review see Bujarski et al., 1994). The initial report of plant viral recombination described the repair of a 20 base deletion in the 3' UTR of BMV RNA 3 by recombination with the corresponding regions of either RNA 1 or 2 (Bujarski and Kaesbeg, 1986). Since this pioneering work, several examples of RNA recombination in the 3' UTR of BMV have been reported (Bujarski and Dzianott, 1991; Nagy and Bujarski, 1992; Rao and Hall, 1993). Like recombination in other viruses, RNA recombination in bromoviruses is believed to occur via template switching during replication, since mutations in the 1a protein can affect the location of recombination sites (Nagy et al., 1995). Recombination in bromoviruses is frequently homologous (Nagy and Bujarski, 1992), and even the heterologous recombination events occur at localized heteroduplex regions formed between

recombining RNAs (Nagy and Bujarski, 1993). Homologous recombination in BMV is dependent on both the length and identity of the homologous sequence at the crossover site (Nagy and Bujarski, 1995), and is correlated with the presence of A-U rich regions immediately preceded by upstream G-C rich regions (Nagy and Bujarski, 1997). The current model for recombination involves a replicase switching event during positive strand synthesis (Nagy and Bujarski, 1997).

Recombination within the intercistronic region of CCMV RNA 3 is capable of generating full length RNA 3's from non-infectious deletion mutants. Two RNA 3 mutants were constructed, one with a deletion in the 3a gene and the other with a capsid gene deletion. Both mutants were suitable templates for replication in protoplasts, but neither mutant was capable of supporting a systemic infection of cowpeas when co-inoculated with RNAs 1 and 2. However, when both mutants were included in the inoculum, 50% of the plants became systemically infected. Analysis of the virion RNA 3 component isolated from these co-inoculated plants revealed that recombination between the two deletion mutants had restored a full length RNA 3 (Allison et al., 1990).

Recombination in transgenic plants

The extensive knowledge base for bromoviruses and the previous documentation of bromoviral RNA recombination made bromoviruses a logical subject for studies of recombination in transgenic plants expressing viral sequences. There were several questions to be answered with regards to the transgenically expressed viral mRNA in plants which would be challenged by viruses in the field. First, was the transgene mRNA available to the viral replication complex as a template for recombination? Secondly, could

recombination between viral RNAs and the transgene message restore viable recombinants? Thirdly, would these recombinants differ from the wild type virus in terms of competitve ability and/or host range? Finally, and perhaps most importantly, what steps could be taken to prevent recombination between transgenically expressed viral RNA and a challenging virus?

Greene and Allison (1994) designed a sensitive recombination bioassay to determine the answer to the first and second questions using CCMV. As with all bromoviruses, the 3a and capsid proteins are required for systemic movement of CCMV, but are dispensible for replication, thus the capsid gene is a suitable target for examining recombination in transgenic plants. In the initial study, *Nicotiana benthamiana* plants, a systemic host of CCMV, were transformed with a construct which represented the 3' end of CCMV RNA 3, including the 3' two-thirds of the capsid protein gene and the entire 3'UTR (fig. 2). Expression of the transgene RNA under the control of the constitutive 35S promoter was insufficient to provide virus resistance. The transgene contained three silent mutations, two of which introduced a Not I restriction site at the 3' end of the partial capsid protein gene. These mutations would distinguish eventual recombinants from potential wild type CCMV contaminants. The transgenic *N. benthamiana* were selected for high expression levels of the transgene.

A deletion mutant, pCC3AG3, was constructed which lacked 121 bases from the 3' terminus of the capsid protein gene. The deletion mutant shares 338 nucleotide overlapping region with the transgenically expressed viral construct, as well as maintaining the entire 3' UTR (Fig. 2). Transcripts from AG3 in combination with wild type CCMV RNA 1 (C1) and CCMV RNA 2 (C2) transcripts were unable to infect systemically,

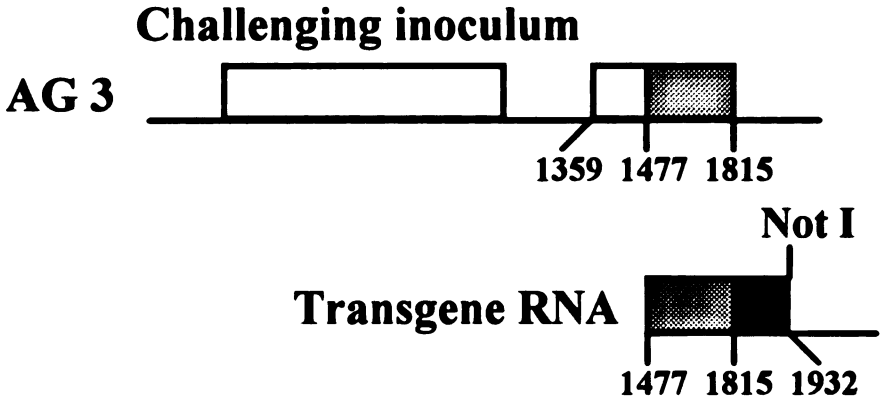

Figure 2. CCMV RNA 3 constructs used in the transgenic recombination study. AG3 is the CCMV RNA 3 deletion mutant used as the inoculum. The CCMV transgene consisted of the 3' two-thirds of the capsid gene and the 3' UTR. Nucleotide positions in the capsid gene indicated by numbers below RNAs. Overlapping region indicated by shading.

despite the fact that the combined transcripts replicated in protoplasts. Transgenic *N. benthamiana* plants were challenged with C1, C2 and AG3. The only way a systemic infection could occur in plants inoculated with this combination was via a recombination event between the challenging deletion inoculum and the transgenically expressed viral RNA that restored a functional capsid protein gene. This bioassay created a high selection pressure for the formation of viable recombinants.

Seven out of 235 (3%) of the transgenic *N. benthamiana* plants inoculated in this manner became systemically infected. Virions were isolated from six of the potential recombinants, viral RNA was extracted, cDNA synthesized and cloned. These clones were sequenced to confirm that the infections were the result of a recombination event. All of the cloned recombinants contained the silent mutations derived from the transgene, including the Not I site, indicating that they had resulted from a recombination event between the trangene RNA and the challenging viral RNA. In addition to these changes, the recombinants also had various other mutations, ranging from large deletions up to 42 nucleotides to point mutations (Fig. 3). These mutations indicated that the recombination events between transgene RNA and viral RNA were occurring in a aberrant homologous manner.

A majority of the mutations occurred within a 225 nucleotide region within the 338 nucleotide overlap. This region could either represent a recombination hot spot, or it may represent a region of the capsid protein gene encoding a portion of the capsid protein amenable to change. Analysis of the crystal structure of the CCMV virion indicates that this portion of the capsid protein forms an alpha helix near the surface of the capsid

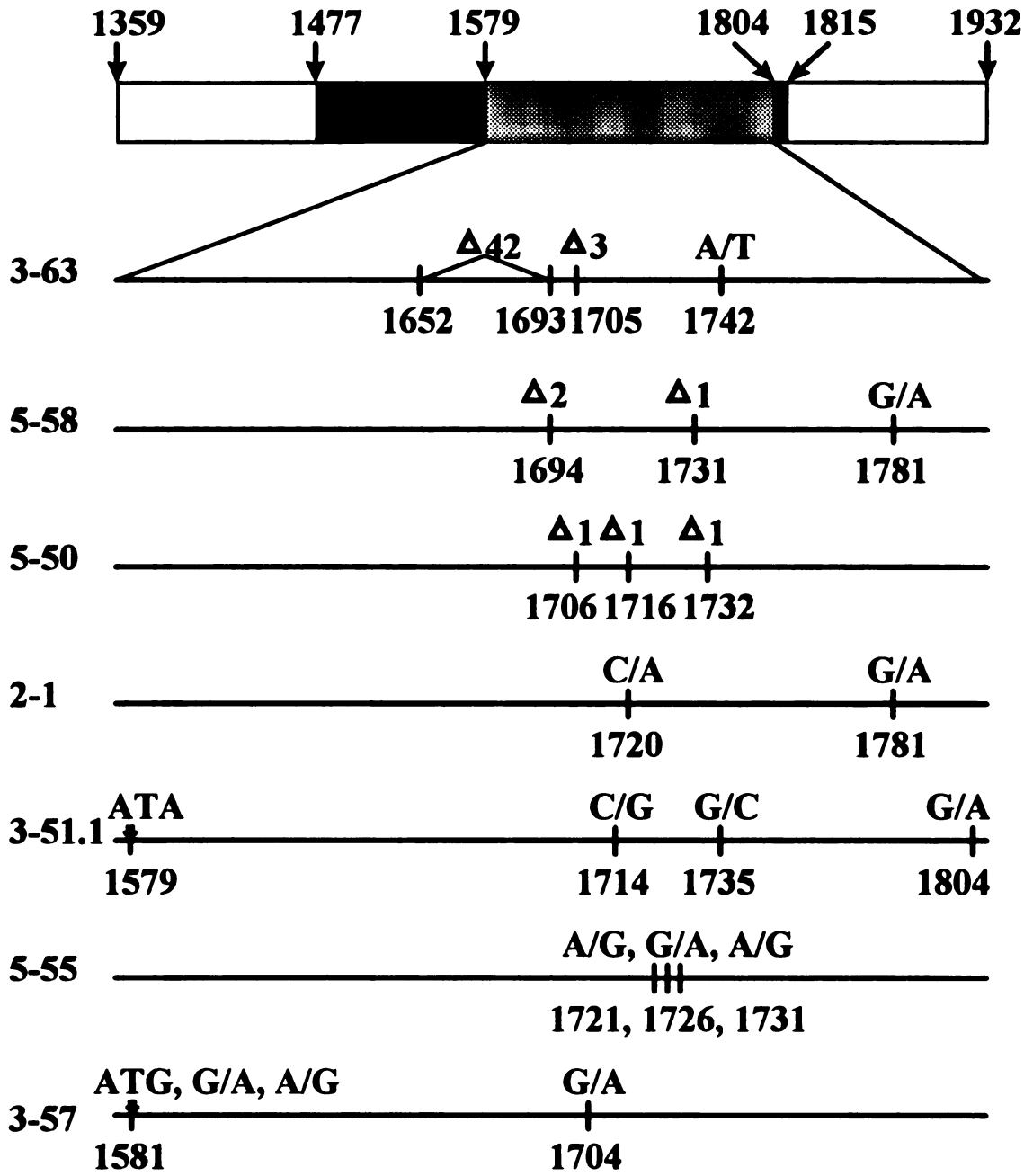

Figure 3. Mutations in the capsid gene region of CCMV recombinants.

subunit (Speir et al., 1995). It is interesting to note that the mutations always occurred in such a manner that the 3' end of the capsid gene remained translatable.

One of the recombinants, 3-57, was of particular interest. Dot blot analysis of plant 3-57 indicated the presence of systemic infection, however, repeated attempts to isolate virions from infected plants were unsuccessful. In addition, virion particles were not detected by electron microscopy. Analysis of total RNA from infected plants revealed the presence of all four viral RNAs. The capsid protein gene was cloned from total RNA of infected plants and sequenced. Sequence analysis revealed that a single nucleotide deletion near the 5' end of the capsid protein gene caused a frameshift which introduced a termination codon at the ninth codon. Additionally, mutations further downstream in the capsid gene had the net effect of adding an in-frame initiation codon. This suggested that a segment of the capsid protein may facilitate systemic movement of CCMV, and that neither the complete capsid protein nor virions were required for systemic movement. Further experiments detailing the capsid protein requirements for systemic infection of CCMV are described in Chapter One.

Characterization of recombinants

Plant virus capsid proteins have roles in several phases of the infection, including replication of the virus, movement of the virus, symptom formation, host specificity, and vector specificity (Ruesken et al., 1995; Flasinski et al., 1995; Dolja et al., 1994; Hacker et al., 1992; Heaton et al, 1991; Rao and Grantham, 1996). Thus, it was of interest to determine if the recombinant viruses recovered from the transgenic plants were phenotypically distinct from the wild type CCMV. From an applied standpoint, it was also of great interest to examine the competitive ability of the recombinants. In theory, most

mutations are deleterious, but the potential for the development of more efficient pathogens via recombination in transgenic plants must be considered.

Passaging the recombinants to legume hosts revealed several differences in symptom formation between the recombinants and wild type CCMV. Recombinants, 5-58, 3-51.1 and 5-50, provided symptoms comparable to wild type CCMV on cowpea, bean and pea, but recombinants 3-57 and 3-63 were symptomless on all legume hosts and recombinants 2-1 and 5-55 caused more severe symptoms on cowpea than wild type CCMV. Inoculating two week old cowpea with recombinant 2-1 resulted in rapid senescence of the whole plant, and inoculation of older plants resulted in severe stunting. In addition, *Phaseolis vulgaris* inoculated with recombinant 2-1 showed more severe symptoms than *P. vulgaris* inoculated with wild type CCMV. Inoculation of cowpeas with recombinant 5-55 resulted in wide spread severe chlorosis, much more severe than the mottling typically associated with CCMV. It is interesting to note that the two recombinants which produced the most severe symptoms are most similar to wild type CCMV at the amino acid level, with only one to two amino acid differences. It is also significant to note that none of the CCMV recombinants had any variation in the typical CCMV host range (Greene, 1995). This is not surprising, since it had been previously demonstrated that the 3a protein is important in determining bromovirus host range (Mise et al., 1993; Mise and Ahlquist, 1995; Fujita et al., 1996).

Experiments conducted to study movement of the recombinants revealed no apparent differences between the recombinants and wild type CCMV in the rate of spread in either the initially inoculated leaf or systemic movement. In an effort to determine how the recombinants competed with wild type CCMV, plants were inoculated simultaneously

with both the wild type CCMV and one of the recombinants. cDNA synthesized from viral RNA isolated from infected cowpeas was PCR amplified and screened for the Not I marker characteristic of the recombinant. Of the doubly inoculated plants, only those inoculated with either recombinant 2-1 or recombinant 5-55 and wild type CCMV, showed any trace of PCR product with the Not I site, indicating that wild type CCMV dominated in the double infections. Upon passaging, recombinants 2-1 and 5-55 were lost and only wild type viral RNA was amplified. Control RT-PCR reactions using approximately equal levels of wild type and recombinant RNA resulted in approximately equal levels of Not I digested and undigested PCR product, indicating that the PCR product from whole plant competitions represented a quasispecies where the wild type CCMV was dominant. This suggests that within the tested conditions, wild type CCMV was a more efficient virus than any of the recombinants (Greene, 1995).

Effect of deletions in the 3' untranslated region on recombination

The 3' untranslated region plays a critical role in bromovirus replication as the binding site for the viral replicase during minus strand synthesis (French and Ahlquist, 1987; Dreher and Hall, 1988). Since recombination is thought to be a result of template switching during viral replication it is logical to predict that deletions in the 3' untranslated region of a potential recombination template would affect the level of recombination. To address this hypothesis, three additional CCMV constructs were designed for transformation of *N. benthemiana* (Greene and Allison, 1996). A series of deletions of 69, 83 and 214 bases were made to the 3'UTR of the original transgenic construct. *N. benthemiana* transformed with these constructs were challenged in the same manner as the

original transgenic plants, with transcripts of wild type CCMV RNAs 1 and 2, and the deletion mutant AG3. Once again, a recombination event between the transgene RNA and the challenging RNA 3 inoculum was necessary to restore the capsid gene open reading frame and allow systemic infection. Despite the fact that similar numbers of each transgenic line expressing the truncated viral RNA were inoculated as in the initial study using the full length 3' UTR construct, no systemic infections were detected in any of the 3' UTR deletion transgenic lines. All three plant lines were susceptible to infection by wild type CCMV, indicating that the lack of recovery of recombinants was not due to resistance.

These results indicated that the 3' UTR plays an important role in the frequency of recombination in transgenic plants. Recent evidence has indicated that most RNA/RNA recombination in bromoviruses occurs during plus strand synthesis (Nagy and Bujarski, 1997). If this is the case, the recombination seen in transgenic plants may not be due to a template switch by the replicase from the plus strand inoculum of the virus to the transgene RNA during minus strand synthesis. Rather, the virus may synthesize a minus strand copy of the transgene RNA, which would then be available as template during plus strand synthesis.

Recombination between BMV and a CCMV transgene

All previous reports of recombination between viruses and transgenic messages involved recombination within a single virus species (for review, see Allison et al., 1996). To determine if a transgenically expressed CCMV message could recombine with a different challenging virus, the transgenic plants described above were inoculated with

BMV RNA1, BMV RNA2, and BMV RNA3 deletion mutant AG5. AG5 was similar to the CCMV deletion mutant used in the original study, since it lacked a portion of the 3' end of the capsid gene required for systemic movement. Again, the only way systemic infection could occur in the inoculated transgenic plants was via a recombination event between AG5 and the CCMV transgene that restored a viable capsid gene to the BMV RNA3. Four of the 170 (2.3%) transgenic plants inoculated with the BMV deletion mutant became systemically infected. Infectious clones of a viable recombinant RNA 3 were recovered from two of the systemic infections, indicating that a heterologous challenging virus (BMV) could recombine with a CCMV transgenic message to form a viable recombinant. In addition, one of the BMV/CCMV recombinants had an expanded host range, systemically infecting cowpea in addition to the normal BMV host range. However, it is not clear whether the expanded host range is due to the recombination event or to other mutations elsewhere in the genome (Greene, 1995).

The research on bromovirus recombination in transgenic plants clearly indicated that the transgenically expressed viral message was available to challenging viruses as a template for recombination, and that this recombination could produce viable recombinants with distinctive phenotypes. However, these observations alone do not provide us with a clear picture of the risks associated with VRTP's. It is important to remember that the bioassays used to measure recombination in VRTP's created extremely high selection pressure for viable recombinants. In addition, it has been proposed that the opportunites for recombination in transgenic plants essentially mimicked the opportunities for recombination in natural mixed infections, where more than one virus infects the same

plant at the same time (Falk and Bruening, 1994). The experiments described in this thesis attempt to analyze the impact of potential recombination in natural infections of bromoviruses by investigating 1) the capsid protein requirements for CCMV movement, 2) the levels of recombination in more natural viral settings of mixed infections and 3) the strength of selection pressure for wild type capsid gene sequence.

The experiments in Chapter One describe the capsid protein requirements for systemic movement of CCMV. The capsid protein requirements were determined by mutational analysis of the CCMV capsid gene.

The experiments described in Chapter Two establish a viable recombinant recovery rate for a pseudo-mixed infection of deletion mutant transcripts. Recombination was measured between deletion mutants that mimicked the constructs used for the transgenic recombination study, thus maintaining the high selection pressure for recombination. By using the same bioassay system and looking at recombination in exactly the same segment of the same gene, the viable recombinant recovery rate from these pseudo-mixed infections can be compared directly to the viable recombinant recovery rate from the transgenic plants.

Chapter Three broadens the scope of the mixed infection study by measuring recombination in true mixed infections of bromoviruses, where both of the parental viruses were infectious by themselves. Theoretically, this would be more represenitive of the recombination opportunites available in the field. In addition, the experiments were expanded to determine three things: 1) the basal level of recombination between viruses in true mixed infections, 2) the viable recombinant recovery rate for true mixed infections of

bromoviruses, and 3) how the selective fitness of the parental viruses affect the level of recombinant recovery from mixed infections of bromoviruses.

Chapter Four describes experiments designed to look at the selection pressure for wild type sequence in the capsid gene region of CCMV. Recombinants derived from the original transgenic recombination study were serially passaged and analyzed for reversions to the wild type CCMV sequence. The rate and precision of reversion mutations allows us to assess the strength of selection pressure for the wild type CCMV capsid gene sequence.

The role of RNA recombination in natural infection situations and the effects of selection on recombinants have not been investigated at this time. The experiments described in this thesis are designed to address these questions about recombination and selection in bromoviruses. The ultimate goal is to apply the data gathered to risk analysis for VRTP's.

CHAPTER 1

Carboxy-terminal Two-thirds of the Cowpea Chlorotic Mottle Bromovirus Capsid Protein is Incapable of Virion Formation yet Supports Systemic Movement

INTRODUCTION

Plant viruses typically have genomes of limited sizes which produce as few as four proteins, a surprisingly small number in comparison to the numerous functions required for infection. Multifunctional proteins may provide a mechanism to maximize the use of virally encoded proteins. Previous studies of plant viruses have uncovered several multifunctional viral proteins. The tobacco etch virus helper component (HC-Pro), for example, is involved in movement and aphid transmission (Cronin et al, 1995). The alfalfa mosaic virus capsid protein functions both in genome activation and virion formation (Ruesken et al., 1995), and the presence of both a capping motif and a helicase motif suggest a multifunctional role for the bromovirus 1a protein (Ahlquist et al., 1990).

Plant virus capsid proteins, in particular, often have secondary roles in addition to virion formation. Capsid proteins of several plant viruses are involved in movement, including potyviruses (Dolja et al., 1994), cucumoviruses (Suzuki et al., 1991), comoviruses (Wellink and van Kammen, 1989), carmoviruses (Hacker et al., 1992; Heaton et al., 1991), luteoviruses (Ziegler-Graff et al., 1996), and bromoviruses (Allison et al., 1990; Sacher and Ahlquist, 1989). Within bromoviruses, the 3a gene and the capsid

protein gene are required for systemic movement of cowpea chlorotic mottle virus (CCMV) and brome mosaic virus (BMV) (bromoviruses (Allison et al., 1990; Sacher and Ahlquist, 1989). Mutational analysis of the closely related BMV capsid protein demonstrated that various regions of the capsid protein are important in virus movement in different hosts (Flazinski et al., 1995). In previous reports, however, no attempt was made to distinguish between the structural role of the protein and its role in systemic movement.

While using an *in vivo* assay to measure recombination in transgenic plants, Greene and Allison (1994) recovered a number of genetically distinct CCMV recombinants with mutations in the capsid protein. One recombinant, 3-57, systemically infected plants without formation of detectable levels of virions. The 3-57 infection was symptomless on cowpeas, and virions were undetectable in infected plants using either standard virion preparations or electron microscopy. Northern blots of total RNA from infected plants provided the normal banding pattern of three genomic RNAs plus the subgenomic RNA 4. Sequence analysis of the capsid gene in 3-57 cDNA clones revealed a single nucleotide deletion nine nucleotides from the capsid protein initiation codon. This deletion caused a frameshift which introduced a termination codon at the ninth codon. In addition, a three nucleotide insertion, approximately 1/3 of the way into the capsid protein gene, created a translation initiation codon within the original capsid protein reading frame (Greene, 1995).

The 3-57 infection suggested that virion formation and perhaps the capsid protein itself is not required for systemic movement of CCMV in cowpea and non-transgenic *Nicotinana benthamiana*. However, it was possible that the sequenced clones did not

represent the functional infectious RNA 3 from the 3-57 infection even though these mutations were confirmed in several cDNA clones derived from independent RNA extractions. The 3-57 infection may have been due to a structurally unstable virion that did not survive isolation techniques, or the capsid protein may have been expressed at such low levels that virions were undetectable. Based on sequences revealed in the 3-57 recombinant, a series of CCMV capsid protein mutants was designed to test the capsid protein requirements for systemic movement. Analysis of these mutants indicates that the CCMV capsid protein is multifunctional, with a systemic movement function independent of its structural role in virion formation.

MATERIALS AND METHODS

Clone Construction and *in vitro* transcription

Three CCMV RNA 3 mutant clones were constructed for use in this study (Figure 1). pCC3AW1 was constructed by making a single nucleotide deletion of the 12th nucleotide of the capsid protein gene in the infectious CCMV RNA 3 clone pCC3TP10 using a PCR mutagenesis primer (TTATCATGTCGACAGTGGAACAGGG). pCC3AW2 was constructed by the addition of an in frame initiation codon at the same location described for the recombinant 3-57. The original initiation codon of pCC3AW2 (nucleotides 1359-1362) was mutated from ATG to TTG using a PCR mutagenesis primer (CCACTGTCGACAAGATAAATTAC) to create pCC3AW3. All mutations were verified by sequencing before being used as templates for *in vitro* transcription. *In vitro* transcripts of clones pCC1TP1, pCC2TP2, pCC3TP4, pCC3AW1, pCC3AW2, and pCC3AW3 were

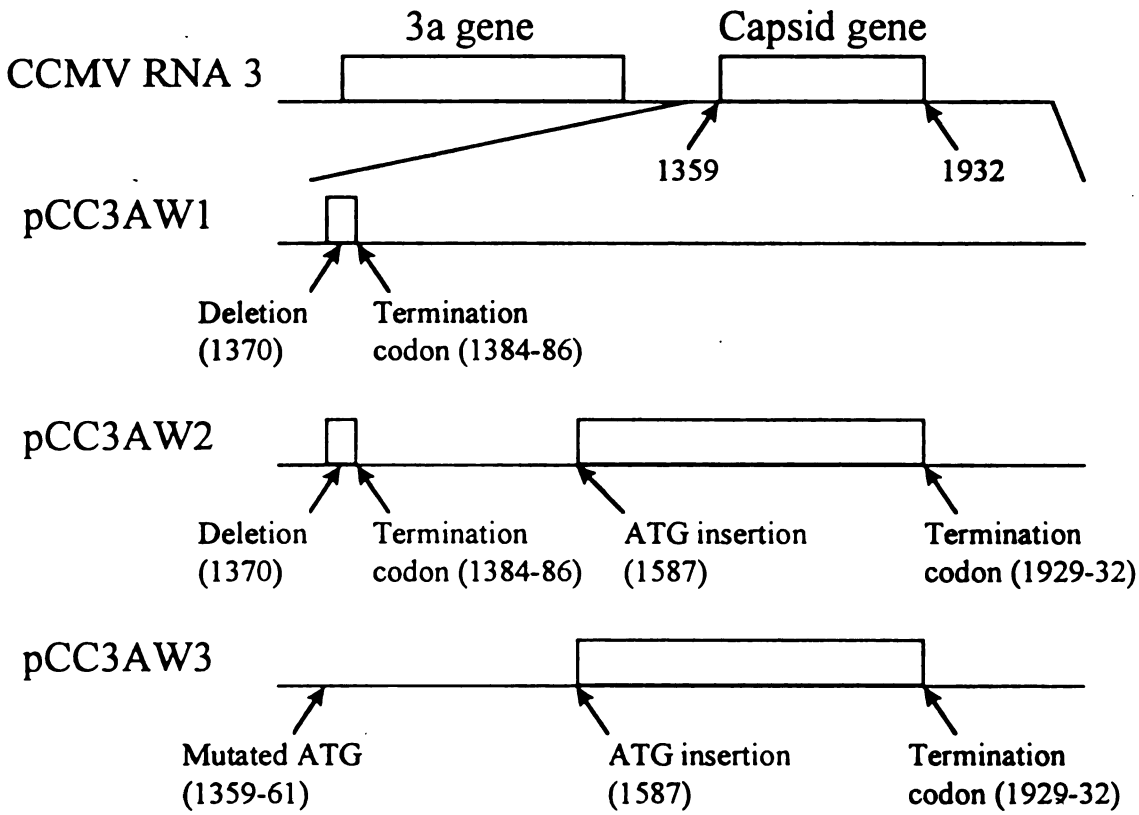

Figure 1. CCMV RNA 3 mutant construction. In pCC3AW1 the ninth nucleotide of the capsid protein gene has been deleted, which results in a translational frameshift and early translational termination. pCC3AW2 is similar to pCC3AW1, but has an added internal initiation codon (ATG insertion). pCC3AW3 lacks the normal capsid gene initiation codon, but has the internal initiation codon of pCC3AW2. Functional open reading frames are indicated by boxes and nucleotide positions are shown in parenthesis.

made as described by Allison et al. (1988). Transcripts derived from these clones are here after referred to as C1, C2, C3, AW1, AW2 and AW3 respectively.

Inoculation of plants/Assay for systemic infection

Leaves of two week old cowpea (*Vigna sinensis* Torner) or *Nicotiana benthamiana* (Domin) were dusted with carborundum. One µg of both CCMV RNA 1 and 2 transcripts (C1, C2) were mixed with one µg of CCMV RNA 3 (C3), AW1, AW2 or AW3 transcripts. Combined transcripts were mixed with 2.5 µl of 50 mg/ml bentonite, final volume raised to 25 µl, and the transcript mix was rubbed onto primary leaves of cowpea or two fully expanded leaves of *N. benthamiana*. Uppermost leaves of inoculated plants were collected two to four weeks post inoculation and used for a crude total RNA dot blot as described by Greene and Allison (1994). Dot blots were probed with dig-labeled RA 518 (Allison et al., 1990), which is specific for the 3' untranslated region of CCMV. Probed dot blots were washed, blocked, incubated with anti-dig fab fragments and lumiphos 530 was applied following manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). Blots were exposed to film for 1-2 hr. Virion RNA was isolated from infected plants as described by Greene and Allison (1994) and analyzed on 0.8% agarose gels. Leaf dip preparations were examined for presence of virions using an electron microscope.

Local lesion assay

Full sized leaves of one month old *Chenopodium hybridum* (L.) were dusted with carborundum. One µg of CCMV RNA 1 and 2 transcripts (C1, C2) were mixed with 1-2 µg of C3, AW1, AW2 or AW3 transcripts. Combined transcripts were mixed with 10 µl

of 50 mg/ml bentonite, final volume raised to 100 µl, and 50 µl of transcript mix was rubbed onto one half of each leaf.

Total RNA isolation and northern blotting

Total RNA was isolated from infected plants (Puissant and Houdebine, 1990) 28 days post inoculation and separated on a 1.0% denaturing agarose gel. Equal amounts of total RNA from each plant (25µg) were loaded in each lane. The RNA was transferred to a nylon membrane (Leharch et al., 1977) and probed with dig-labeled RA 518. Blots were treated as described above and exposed to film for 22 min.

Polyclonal CCMV Antibody

Polyclonal antibodies, raised against CCMV virions in rabbit, were conjugated directly to alkaline phosphatase (Avraneas, 1969). Conjugated antibodies were cross-reacted against healthy plant tissue to remove non-pathogen-specific antibodies (Ball et al., 1990), and used for enzyme-linked immunosorbant assays (ELISA) and western blotting.

ELISA

Polyclonal CCMV antibodies were used to detect CCMV capsid protein using double antibody sandwich ELISA (Converse and Martin, 1990). Wells of a microtiter plate were coated with unconjugated polyclonal CCMV antibody and washed three times with phosphate buffered saline (PBS). Protein extracts from infected and non-infected plants were added to wells, incubated 30 min. and removed. Wells were rinsed three times with PBS. Alkaline phosphatase conjugated CCMV polyclonal antibody was added to wells, incubated for 30 min. and removed. Wells were rinsed five times with PBS. Substrate solution was added to the wells, incubated for 45 min. and removed.

Absorbance at 405 nm was read using an ELISA reader (Minireader II, Dynatech, Alexandria, VA).

Western blot

Total protein was extracted from *N. benthamiana* plants that were either mock inoculated, infected with wild type CCMV, infected with AW2, or infected with AW3. Proteins were separated on a 18% SDS/polyacrylymide gel for 24 hrs. Equal amounts of total protein from mock inoculated, wild type CCMV infected, AW2 infected, or AW3 infected plants (from 10 to 100μg, depending on the gel) were loaded in each lane. Proteins were electro-transferred to a nylon membrane (Hammond, 1990). Blots were washed twice with PBS, incubated with 10% dried milk for 1.5 hr, incubated in 10% dried milk with the CCMV polyclonal antibody for 1.5 hr, washed twice for 15 min. in PBS to remove excess antibody, and equilibrated for 5 min. in 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ pH 9.5. Lumiphos substrate was added, the blot covered by plastic and exposed to film for 1.5 hr.

RESULTS

Characterization of the internal initiation codon in the 3-57 mutant

The mutations in recombinant 3-57 introduced a termination codon at codon nine of the capsid protein reading frame, and an in frame initiation codon approximately one-third of the way into the capsid protein gene (Figure 2). These mutations would explain the apparent lack of virions in a 3-57 infected plant. However, it was still necessary to determine if a truncated capsid protein was being translated from the internal initiation site, and if this truncated capsid protein was sufficient for systemic movement of CCMV.

	1359											1385
w.t. CCMV	**ATG**	**TCT**	**ACA**	**GTC**		**GGA**	**ACA**	**GGG**	**AAG**	**TTA**		
Rec. 3-57	**ATG**	**TCT**	**ACA**	**GT . G**		**GAA**	**CAG**	**GGA**	**AGT**	**TAA**		**stop**

	1582											1593
w.t. CCMV	**CCU**	**AAU**	.	.	.		**GAG**	**CUA**				
Rec. 3-57	**CCU**	**AAU**	**AUG**		**ACG**		**CUA**					

Figure 2. Comparison of mutated regions in recombinant 3-57 (Rec. 3-57) to homologous regions in wild type CCMV (w.t. CCMV). Deletion of nucleotide 1370 in the 3-57 recombinant introduces an early termination signal. Mutations in the region between nucleotides 1582 and 1593 introduce and in frame initiation codon (underlined). The A at the -3 position (nucleotide 1585) indicates that the internal initiation codon in recombinant 3-57 is a likely site for the initiation of translation.

Comparison of the sequence surrounding the inserted initiation codon with functional translation initiation sites (Cavener and Ray, 1991) suggested that this newly introduced initiation codon may be a likely place for the initiation of translation (Figure 2). There were no other in frame initiation codons upstream of the inserted start codon. To demonstrate that a truncated protein expressed from this initiation codon would support systemic movement, three CCMV capsid protein gene mutants were made (Figure 1). pCC3AW1 has a single nucleotide deletion of the twelfth nucleotide of the capsid protein gene, causing a frameshift and early termination at codon nine, and subsequently should produce no capsid protein. pCC3AW2 and pCC3AW3 both have the interior initiation codon and should produce a truncated 15 kD capsid protein. In addition, the original capsid protein initiation codon in pCC3AW3 (nucleotides 1359-1361) was changed to prevent translation of a full length capsid protein via a frameshift in the first nine codons.

Inoculation of cowpea and *Nicotiana benthamiana* with AW mutant transcripts

Wild type CCMV RNA 1 and 2 (C1,C2) transcripts were used in combination with either AW1, AW2, AW3 or C3 transcripts to inoculate cowpea, *N. benthamiana* and *Chenopodium hybridum* plants. Inoculated plants were tested for the presence of virus by probing dot blots of total RNA (Table 1). All transcript combinations caused local lesions on *C. hybridum*. In numerous attempts, transcripts from AW1, the construct designed to express no capsid protein, infected neither cowpea nor *N. benthamiana*, despite the fact that it caused local lesions on *C. hybridum*. AW2 and AW3 transcripts in combination with C1 and C2 infected cowpeas at equal percentages to wild type transcripts, while the percentage of *N. benthamiana* infected by AW2 and AW3 was slightly reduced. In both cases the development of systemic infection by AW2 and AW3 progressed at a slower rate

Table 1. Results of inoculation tests using C1, C2 and either AW1, AW2, AW3 or C3 transcripts. The number of plants systemically infected is compared to the total number of plants inoculated. Systemic infections were determined by blotting total RNA of inoculated plants at either 14 or 28 days post inoculation (d.p.i.). All transcript combinations induced local lesions on *Chenopodium hybridum*, a CCMV local lesion host.

		Inoculum			
Host	d.p.i.	AW1	AW2	AW3	wt CCMV
Cowpea	14	0/25	3/25	0/22	10/10
Cowpea.	28	0/12	12/12	16/16	10/10
N. benthamiana	14	0/22	2/20	0/15	10/10
N. benthamiana	28	0/14	13/16	10/15	10/10
C. hybridum	14	local lesions	local lesions	local lesions	local lesions

than a wild type infection, taking 7-14 days longer (Table 1). In addition, the cowpea infections caused by AW2 and AW3 were symptomless, as was the infection caused by the 3-57 recombinant.

Attempts to recover virions from AW2 and AW3 infected plants were unsuccessful, despite the recovery of virions from wild type CCMV infected plants using the same techniques (data not shown). In addition, no virions were observed using electron microscope analysis of infected leaf sap (data not shown). The sap from the AW2 and AW3 infected plants that was used for electron microscope analysis was infectious on both cowpea and *N. benthamiana*. Repeated northern analyses of total RNA extracted from AW2 and AW3 infected plants consistently confirmed the presence of all four viral RNAs. The northern blots also indicated that AW2 and AW3 viral RNA accumulated to levels roughly equal to wild type CCMV infections (Figure 3).

Expression of the Truncated Capsid Protein

ELISA tests, using a polyclonal CCMV antibody made to intact virions, were performed on total protein extracts from CCMV and AW2 infected cowpeas. ELISA absorbance values from AW2 infected plants averaged 0.63 at 450 nm, lower than that of wild type CCMV infected plants (1.05), but significantly higher than the values obtained for the mock inoculated control plants (0.00). The ELISA values suggested that the AW2 infection was indeed producing a protein that maintained antigenic epitopes common to CCMV virions.

To determine if the AW2 and AW3 infections produced the expected truncated capsid protein, total protein extracts from wild type CCMV, AW2 and AW3 infected plants were separated on a SDS/polyacrylimide gel and analyzed by western blotting using

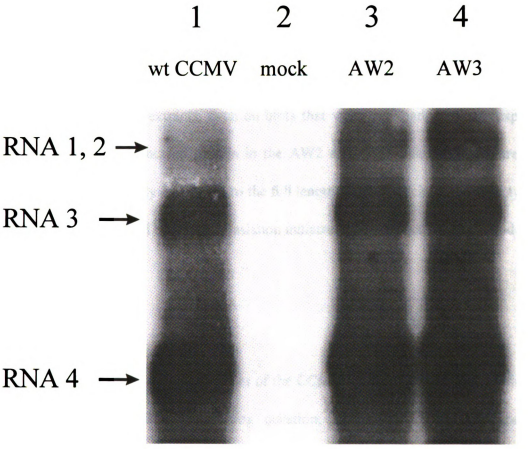

Figure 3. Northern blot of total RNA from wild type CCMV infected *Nicotiana benthamiana* (lane 1), mock inoculated *N. benthamiana* (lane 2) AW2 infected *N. benthamiana* (lane 3) and AW3 infected *N. benthamiana* (lane 4). The blot was probed with RA 518, a CCMV 3' untranslated region specific probe.

the polyclonal CCMV antibody (Figure 4). Repeated blots revealed that the band corresponding to the 21 kD full length capsid protein was present in the lane corresponding to the wild type infection, but absent in both AW2 and AW3 protein extractions. A 15 kD band, corresponding to the predicted size of the truncated capsid protein, was present in both the AW2 and AW3 protein extracts, but absent in the wild type CCMV protein extracts, even on blots that were overloaded or overexposed (data not shown). The truncated protein in the AW2 and AW3 infections appeared to be at concentrations roughly equivalent to the full length capsid protein in the wild type CCMV infection. The method by which translation initiates at the internal initiation codon of AW2 remains unknown.

DISCUSSION

In light of the previous studies of the CCMV capsid gene, the lack of virions in the 3-57 infection posed an interesting question; exactly what are the capsid protein requirements for systemic movement of CCMV? The sequence data from 3-57 cDNA clones suggested that no full length capsid protein was being produced, hence virion formation might not occur in the 3-57 infection. This would correlate with recent work with the ultrastructure of the CCMV virion, which indicated that the amino terminal end of the CCMV capsid protein is necessary for virion formation (Speir et al., 1995). The apparent lack of virions and the 3-57 sequence data predicted that virion formation was not required for systemic spread of CCMV. There are a number of plant viruses which do not require virion formation for systemic movement, including members of the tobraviruses (Hamilton and Baulcombe, 1989), tobamoviruses (Dawson et al. 1988),

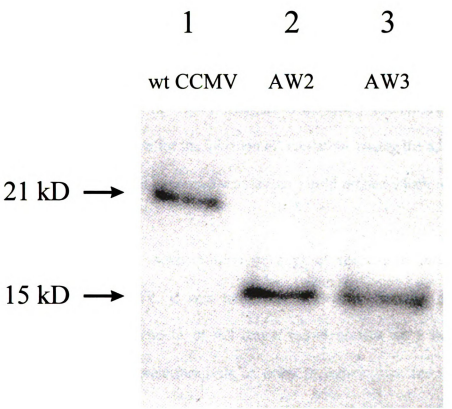

Figure 4. Western blot of total protein extracts from wild type CCMV infected cowpeas (lane 1), AW2 infected cowpeas (lane 2) and AW3 infected cowpeas (lane 3). 21 kD band in lane 1 is the full length capsid protein, 15 kD band in lanes 2 and 3 are the carboxy-terminal portion of the capsid protein translated from the internal initiation codons of AW2 and AW3 respectively. The blot was probed with polyclonal antibody made to purified CCMV virions.

dianthoviruses (Xiong et al., 1993) and tombusviruses (Dalmay et al., 1992; Rochon et al., 1991; Scholthof et al., 1993), although in a number of these cases the coat protein deficient viruses are less efficient than the wild type. However, the ability of 3-57 to move systemically without virion formation did not exclude the possibility that the CCMV capsid protein gene was required in some form for systemic movement as previously described (Allison et al., 1990). Analysis of the newly inserted initiation codon revealed that it was a likely site for the initiation of translation, raising the additional possibility that the 3-57 recombinant expressed a truncated capsid protein which was sufficient to enable systemic movement.

To conclude what portion (if any) of the capsid protein enables systemic movement of CCMV, it was necessary to establish whether the 3-57 mutant was producing a trace amount of full length capsid protein via a frameshift, producing a truncated capsid protein incapable of virion formation from the newly inserted interior initiation codon, or producing no capsid protein at all. If no capsid protein was produced in the 3-57 infection, the capsid protein of CCMV would be dispensable for systemic movement. If the 3-57 mutant was relying on a truncated, virion deficient capsid protein for systemic movement, the capsid protein of CCMV must be multifunctional. Finally, if a low level of full length capsid protein was being expressed, the 3-57 virus could move in a manner functionally identical to the wild type virus. These possibilities were tested using the AW mutant series.

The AW1 construct was designed to test the possibility that no CCMV capsid protein was required for systemic movement. AW1 transcripts in combination with C1 and C2 formed local lesions on *C. hybridum* indicating that it was replication competent, but it

failed to move systemically, indicating that at least some portion of the CCMV capsid protein is required. The capsid protein requirements for systemic movement were tested using the AW2 and AW3 constructs, which were designed to express the carboxy terminal two-thirds of the capsid protein. The only difference between these two constructs was the deletion of the original initiation codon in AW3. If 3-57 moved systemically using a small amount of full length capsid protein made by frameshift during translation, AW2 would be infectious and AW3 would not. Both AW2 and AW3 were infectious, indicating that the full length capsid protein was not required for systemic movement. Like 3-57, AW2 and AW3 infections were symptomless on cowpea, which suggests that either the complete capsid protein or the virion plays some role in symptom development.

Both the ELISA and western blot data indicated the presence of a truncated capsid protein in AW2 and AW3 infections. There was no evidence of truncated capsid protein expression in wild type CCMV infections, even when western blots were overloaded or overexposed (data not shown). The movement function in wild type infections is likely fulfilled by full length capsid protein. The fact that AW2 and AW3 infections proceed at a slower rate than wild type CCMV may indicate that either the full length capsid protein or the virion itself has some effect on the systemic movement of the virus *in planta*, perhaps in stabilizing viral RNAs.

In conclusion, mutational analysis of the CCMV capsid protein indicates that it plays a multifunctional role in the virus life cycle *in planta*. The carboxy terminal two-thirds of the capsid protein is sufficient for systemic movement of CCMV, although the infection proceeds at a slower rate than the wild type virus and is symptomless. This indicates that at least some portion of the carboxy-terminal 2/3 of the capsid protein has a

role in systemic movement, while the full length capsid protein is responsible for virion formation. Additionally, this demonstrates that virion formation is not required for the systemic movement of CCMV. It remains to be seen exactly what role the complete CCMV capsid protein plays in systemic movement, and whether the capsid protein interacts with other host or viral proteins to accomplish this role.

This chapter was published in modified form as Schneider et al., 1997.

CHAPTER 2

Recombination between inoculation transcripts of CCMV RNA 3 deletion mutants

INTRODUCTION

Since the advent of virus resistant transgenic plants (Powell-Abel, 1986), the capsid proteins of many viruses have been expressed in numerous plant species. (for review see Grumet, 1990; 1995). Along with the advent of capsid protein mediated protection comes the possibility for recombination between a transgenically expressed viral RNA and a challenging virus (de Zoeten, 1991). This possibility was tested experimentally, leading to several reports in the literature of recombination between transgenically expressed viral RNA and a challenging virus (Schoeltz and Wintermantel, 1993, Gal et al., 1992; Greene and Allison, 1994; for review see Allison et al., 1996).

RNA-RNA recombination is widely accepted to have played a role in the evolution of RNA viruses (Lai et al., 1992), and there is considerable evidence of RNA recombination in the genomic sequences of several plant RNA viruses (Angenant et al., 1989; Gibbs et al., 1991; Mayo and Jolly, 1991; Edwards et al., 1992). Recombination enables RNA viruses to incorporate large sections of RNA into their genomes, allowing for modular evolution (Domingo et al., 1996). The similarities among functionally homologous genes of different plant virus groups indicates that modular evolution has occurred in the evolutionary history of many plant RNA viruses (Goldbach, 1987). In

theory, recombination between a challenging virus and transgenically expressed viral RNA could support or enhance the evolution of new viruses (de Zoeten, 1991).

Greene and Allison (1994) successfully demonstrated that a cowpea chlorotic mottle bromovirus (CCMV) RNA 3 deletion mutant can recombine with a transgenically expressed CCMV message intragenically to restore a functional capsid protein gene. Further, Greene and Allison (Greene, 1995) demonstrated that in these recombinants genetic alterations that occur during recombination can lead to distinct phenotypic variations. Recombinants were recovered from three percent of the transgenic plants inoculated, a significant amount considering the numbers of transgenic plants that would be released in an agricultural setting. Additionally, when the study was repeated using a BMV deletion mutant as the inoculum, viable recombinant hybrid viruses were recovered at approximately the same frequency (2.3%) (Greene, 1995).

These results have immediate relevance to the risk assessment of virus resistant transgenic plants. However, there are two important points to consider when assessing the impact of Greene and Allison's study on recombination in transgenic plants. First, the very nature of the bioassay used to measure recombination resulted in extremely high selection pressure for functional recombinants. Secondly, the opportunities for recombination in transgenic plants could theoretically be the same as the opportunities for recombination that already exist in natural mixed infections (Falk and Bruening, 1994), where two or more viruses infect the same host at the same time.

Recombination has been widely studied in bromoviruses, where recombination between transcripts has been repeatedly demonstrated in the 3' untranslated region (3' UTR) (Bujarski and Kaesberg, 1986; Rao et al., 1990; Ishikawa et al., 1991). In addition,

recombination in the intercistronic region of CCMV RNA 3 restored a functional RNA 3 component in 50% of plants inoculated with two movement defective CCMV RNA 3 deletion mutants lacking either the 3a or the capsid gene (Allison et al., 1990). To estimate the level of recombination in bromovirus mixed infections, experiments were designed to measure the recovery of viable recombinants from plants inoculated with two CCMV RNA 3 deletion mutant transcripts which mimicked the constructs used in the original transgenic recombination study (Greene and Allison, 1994). This allows for a direct comparison between the viable recombinant recovery rates in transgenic plants and in more natural pseudo-mixed infection systems.

MATERIALS AND METHODS

Clone construction

Two CCMV RNA 3 deletion mutants were constructed for use in this study (Fig. 1). pCC3SR1 was constructed by digesting pCC3TP10 with Sal I and Sty I and religating the plasmid. This had the net effect of deleting the first 118 bases of the capsid protein gene. pCC3SR2 was created by digesting pCC3AG1 with Not I and Sac II, blunting the plasmid fragment with Klenow fragment (Ausebel et al., 1994), and religating the plasmid. This had the net effect of deleting the 3' 119 nucleotides from the capsid protein gene. pCC3SR2 also maintained the characteristic Not I site, which would distinguish recombinants from wild type virus. The integrity of the plasmids was confirmed by sequencing before they were used as templates for *in vitro* transcription reactions (Allison et al., 1988). The replication ability of both SR1 and SR2 was tested in barley protoplasts.

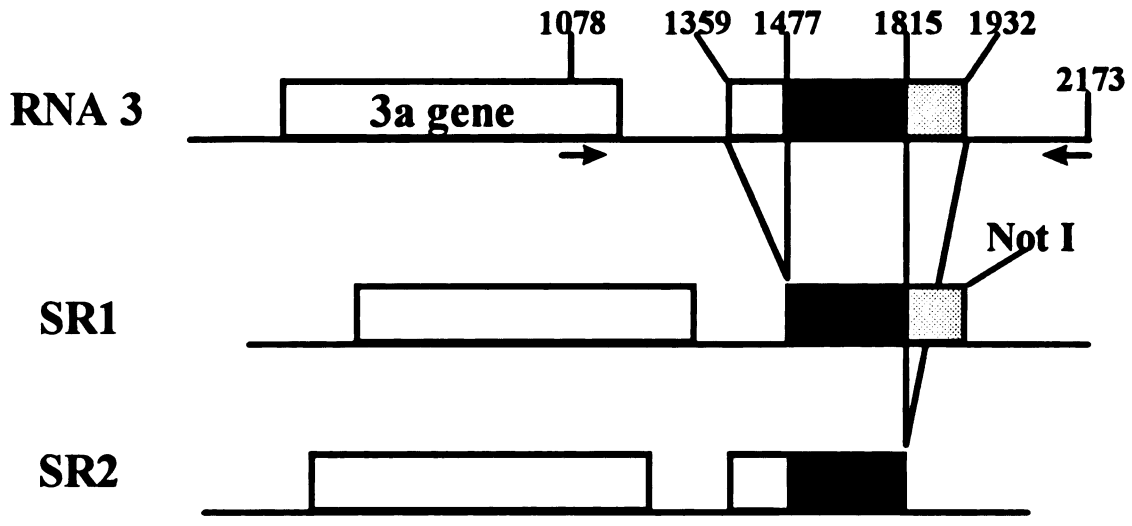

Figure 1. CCMV RNA 3 deletion mutants SR1 and SR2. SR1 has a deletion of the 5' end of the capsid protein gene. SR2 has a deletion of the 3' end of the capsid protein gene. The two deletion mutants share an overlapping region between bases 1475 and 1813 where recombination can occur (crosshatched region). Potential recombinants are screened by Not I restriction site unique to SR1. Nucleotide positions indicated by numbers above RNA 3. PCR primer location indicated by arrows below RNA 3.

Inoculation of cowpea

Five microliters (approximately 2 μg) of transcripts made from the plasmids pCC1TP1, pCC2TP2, and either pCC3TP4 or pCC3SR1 and/or pCC3SR2 (referred to as C1, C2, C3, SR1 and SR2 respectively hereafter) were diluted in 50 μl of distilled water with 0.5 mg/ml bentonite. These transcript combinations were then used to inoculate the primary leaves of seven day old cowpea (*Vigna sinensis* (Torner) Savi) plants. Two weeks post infection the cowpea plants were tested for systemic infection by dot blotting total RNA from non-inoculated leaves and probing with the CCMV 3'UTR specific probe RA518 (Allison et al., 1990).

Analysis of potential recombinants

Virion RNA was extracted from cowpea plants that provided a positive signal when total RNA was probed with RA518 (Allison et al., 1989), and analyzed on a 0.8% agarose gel. Viral RNA was then used as a template for reverse transcription reactions (Ausebel et al., 1994) primed with CCMV 3' primer (CAGTCTAGATGGTCTCC-TTAGAGAT). First strand cDNA from these reactions was used as a template for PCR, using CCMV specific 5' and 3' primers. PCR products were digested with Not I and analyzed on agarose gel as previously described (Greene and Allison, 1994).

Host range test

Wild type and recombinant viruses were sap inoculated onto cowpea, bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), cucumber (*Cucumis sativus* L.), barley (*Hordeum vulgarae* L. cv. Morex) and tobacco (*Nicotiana tabaccum* L. and *Nicotiana benthamiana* Domin). In addition, a local lesion host, *Chenopodium hybridum* (L.), was inoculated. In all cases approximately 0.5 grams of infected cowpea tissue was ground in

600 µl of 0.01M sodium phosphate buffer (pH 7.0) with a mortar and pestle. Either primary leaves (for legumes) or mature leaves of 2-3 week old plants (for barley, tobacco species and *C. hybridum*) were dusted with carborundum (600 mesh) and rub inoculated with 10-50 µl of sap. Plants were observed for symptom development and screened for systemic infection by dot blot as described above. Crude total RNA isolations from inoculated plants were tested by dot blotting at both three and six weeks post inoculation.

RESULTS

Clone construction and inoculation experiments

To compare the recovery of recombinants in transgenic plants with the recombinant recovery between inoculated viral RNAs, two RNA 3 deletion mutants were constructed which mimicked the constructs used in the previous transgenic recombination studies (Greene and Allison, 1994). pCC3SR1 lacked the 5' one-third of the capsid gene, and pCC3SR2 lacked the 3' one-third of the capsid protein gene (Fig. 1). As in the previous transgenic recombination experiment, the two RNA 3 deletion mutants shared the same 336 nucleotide overlapping region where recombination would be required if a functional capsid gene were to be restored. Additionally, pCC3SR1 contained the same silent marker mutations as the original transgene. When inoculated separately, transcripts from either mutant, designated SR1 and SR2, were not infectious in combination with C1 and C2, despite the fact that both mutants replicated in protoplasts (data not shown).

The primary leaves of fifty-six two week old cowpea plants were inoculated with a combination of C1, C2, SR1 and SR2 transcripts. In addition, fifteen plants were inoculated with a combination of C1, C2 and wild type C3 transcripts as a positive

control. After two weeks the plants were tested for the presence of systemic CCMV infections by probing dot blots of crude total RNA isolations using the CCMV specific probe RA518. Twelve of the fifty-six plants inoculated with the transcript combination of C1, C2, SR1 and SR2 were systemically infected. All of the plants inoculated with wild type CCMV became systemically infected.

Analysis of potential recombinants

Virion RNA was extracted from all twelve plants which had become systemically infected following inoculation with C1, C2, SR1 and SR2 transcripts as well as from two of the wild type CCMV infected plants. Virions were recovered from all of the SR1/SR2 plants, and electrophoretic analysis of viral RNA indicated all four CCMV RNAs appeared to accumulate to roughly wild type levels for all of the potential recombinants (data not shown). The virion RNA extracted from the SR1/SR2 plants was used as a template for reverse transcription PCR. Analysis of the PCR product revealed the presence of the predicted 1100 base pair band for all twelve reactions. The PCR product was then digested with restriction endonuclease Not I to confirm that the systemic infections were the result of recombination events involving SR1 and SR2. Despite repeated digestions, only four of the twelve potential recombinants contained a Not I restriction site, indicating that the other eight systemic infections were likely caused by a wild type contaminant (Fig. 2). Thus, the viable recombinant recovery rate in this pseudo-mixed infection study of CCMV capsid protein deletion mutant transcripts was roughly eight percent.

Host range test

The four systemic infections that were caused by confirmed recombinants were passaged to a number of different plant species in a partial host range test (Table 1). All

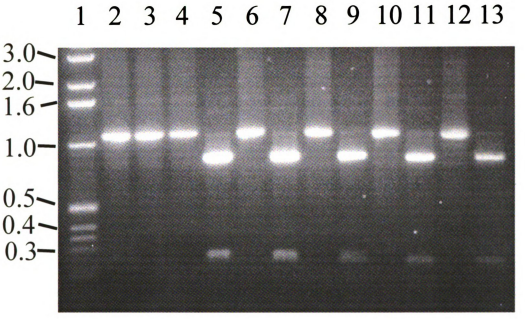

Figure 2. Not I restriction digest of RT-PCR products derived from SR1/SR2 recombinants. Lane 1 = 1 kb ladder. Lanes 2 through 13 represent PCR products that are undigested and Not I digested respectively. Lanes 2 and 3, wild type CCMV RNA 3; lanes 4 and 5, pCC3AG1; lanes 6 and 7, recombinant 3-3; lanes 8 and 9, recombinant 5-2, lanes 9 and 10, recombinant 8-1; lanes 12 and 13, recombinant 12-2. All four recombinants show the presence of two bands in the digested samples, indicating the presence of the Not I marker

Table 1. Results of the limited host range test of SR1/SR2 recombinants. Plus sign (+) represents systemic infection. Minus sign (-) represents non-infection. Multiple plus signs indicate level of symptom development.

	W. T. CCMV	REC. 3-3	REC. 5-2	REC. 8-1	REC. 12-2
Bean *Phaseolis vulgaris*	+++	+++	+++	++	+++
Cowpea *Vigna ungulata*	+++	+++	+++	+++	+++
Pea *Pisum sativum*	+++	+++	+++	+++	+++
Cucumber *Cucumis sativis*	-	-	-	-	-
Barley *Hordeum vulgare*	-	-	-	-	-
Tobacco *Nicotiana tabaccum*	-	-	-	-	-
Nicotiana benthemiana	+	+	+	+	+
Chenopodium hybridum	local lesions	local lesions	local lesions	local lesions	local lesions

four recombinants were able to infect the legumes used for the test (pea, bean, and cowpea) as well as *Nicotiana benthamiana*. None of the four recombinants were able to infect the non-host species, tobacco, cucumber, and barley. This correlated perfectly with the host range observed for the wild type CCMV control. In addition, no distinct differences in symptom development or rate of systemic spread were noted between any of the recombinants and wild type CCMV on legume hosts (data not shown). All four recombinants caused local lesions on the CCMV local lesion host *Chenopodium hybridum* at roughly equal levels to wild type CCMV.

DISCUSSION

These experiments were conducted to compare the viable recombinant recovery rate in transgenic plants with viable recombinant recovery rate between transcripts of viral deletion mutants. The two deletion mutants constructed for this study contained the same deletions and marker mutations as the constructs used in the transgenic recombination studies (Greene and Allison, 1994). pCC3SR1 lacks the 5' one-third of the capsid protein gene and is functionally equivalent to the deletion inoculum AG3; pCC3SR2 lacks the 3' one-third of the capsid protein gene and serves as the functional equivalent of the transgene RNA. As with the transgenic recombination constructs, the two deletions shared the identical overlapping region in the capsid protein gene and had the entire 3' UTR intact. In addition, SR1 has the same silent mutations introduced to the 3' end of the capsid gene, which were used for distinguishing recombination products. This resulted in the two studies being directly comparable, since the RNA 3 constructs have the same replication signals, overlapping regions and additional mutations in both studies.

Previous work with CCMV has indicated that the carboxy-terminal two-thirds of the capsid protein is sufficient for systemic infection of cowpea and *Nicotiana benthamiana* (Schneider et al., 1997). The SR2 construct maintained the original initiation codon, so the possibility existed that systemic infection in SR1/SR2 inoculated plants could be supported by a truncated capsid protein translated from the SR2 transcript. Although this appeared unlikely, since the deletion of the 5' end of the capsid gene had changed the capsid reading frame, the experiments were conducted in such a way as to distinguish SR2 mutants from true recombinants. When co-inoculated with C1 and C2 transcripts, neither SR1 nor SR2 was able to support systemic movement. Thus the remaining segment of the capsid gene was inadequate to support systemic movement. Additionally, all of the SR1/SR2 recombinants were recovered as virions. Previous studies have demonstrated that the portion of the capsid protein encoded by SR2 is incapable of virion formation (Spier et al., 1995; Schneider et al., 1997).

Systemic infections occurred in twelve of the fifty-six cowpeas inoculated with a combination of SR1 and SR2. Of these twelve systemic infections, only four were the result of infection with an RNA 3 containing the Not I restriction site marker, indicating that these four were the result of a recombination event that restored a viable CCMV capsid gene reading frame. The remaining eight infections were probably the result of wild type CCMV contamination. This sets the viable recombinant recovery rate for pseudo-mixed infections of SR1 and SR2 at eight percent, slightly higher than the viable recombinant recovery rate of three percent seen in the CCMV transgenic recombination study (Greene and Allison, 1994) or the BMV/CCMV transgenic recombination study (Greene and Allison, 1997). Further, this study demonstrates that homologous

recombination in the 336 base pair overlapping region can occur in natural infections, and that these recombination events are capable of restoring a viable capsid reading frame. None of the recombinants were moved systemically by using a truncated capsid protein translated from SR2, since virions were present for all four recombinants, and PCR analysis demonstrated the presence of both the full length capsid gene and the Not I site for all recombinants (Fig. 2).

Although a limited number of plants were inoculated in the transcript recombination study, the eight percent viable recombinant recovery rate would most likely remain steady or possibly increase no matter how many plants were inoculated. In all previous recombination studies the viable recombinant recovery rate has been consistent throughout the course of the experiment. The SR1/SR2 recovery rate could possibly be higher than the observed eight percent, since the wild type CCMV contamination may have masked potential recombinants by out competing them in those plants.

The difference in viable recombinant recovery rates between plants inoculated with these SR1 and SR22 deletions mutants and transgenic plants inoculated with the CCMV RNA 3 deletion mutant may reflect the stability of these larger transcripts, and/or the quantity of transcript available to the replication complex. There have been no attempts to quantify the level of transgenic viral message in the cells of the transgenic *N. benthamiana*, but based on northern analysis we conclude that the quantity of the transgenic transcript per unit leaf area is significantly less in the transgenic leaves than the quantity of viral RNA used in these inoculations (data not shown). Logic would suggest that recombinant recovery is directly related to the quantity of transcript available as a template for recombination.

Recombination in virus resistant transgenic plants will have an environmental effect only if the recombination generates selectively advantaged recombinants at a rate higher than what occurs in natural situations. In theory, virus resistant transgenic plants may pose no greater risk than recombination in natural mixed infections (Falk and Bruening, 1994). This study confirms that CCMV is capable of recombination between two deletion mutant transcripts, and that this recombination can restore a viable capsid gene. However, these experiments do not reflect what goes on in a true mixed infection. In a true mixed infection, both viruses are self sufficient in terms of replication, movement and encapsidation. There is no guarantee that the two viruses involved in a mixed infection will be present and replicating in the same cells at the same time, while a transgenic plant expressing a viral RNA provides a steady state level of potential templates for recombination. Although these experiments are helpful in showing that viral recombination is not a phenomena limited to transgenic plants, more studies are necessary to provide a true picture of recombination in mixed infections.

CHAPTER 3

Recombination Opportunities in Mixed Infections of Bromoviruses Differ from the Recombination Opportunities in Transgenic Plants

INTRODUCTION

Mixed infections occur when two or more viruses infect the same host simultaneously. There are numerous examples of mixed infections of plant viruses in the field (Duffus, 1962; Davis, 1987; Zink, 1972; Colinet et al., 1994; Koening, 1995) with as many as five viruses found in the same host (Falk and Bruening, 1994). The presence of two or more viruses in the same host presents opportunities for unique interviral interactions, including transencapsidation (Rochow, 1972; Bourdin and Lecoq, 1991; Maiss et al., 1994), synergy (Vance et al., 1995; Anderson et al., 1996), cross protection (Krstic, 1995; Shukla and Ward, 1989) and recombination (Fraile, 1997). In certain cases, one virus can act as a helper virus to a second virus by facilitating systemic movement in a non-systemic host (Fuentes and Hamilton, 1991; Taliansky, 1995).

Recombination between viruses in a mixed infection presents a unique opportunity for RNA virus evolution to occur. It is widely accepted that recombination has played a role in the evolution of both plant and animal RNA viruses (Lai, 1992; Simon and Bujarski, 1994) and there is considerable circumstantial evidence for past recombination events in the genomic sequences of several plant RNA viruses (Angenent et al., 1989;

Allison et al., 1990, Koonin et al., 1993; Sano et al., 1992; Edwards et al., 1992; LeGall et al., 1995; Gibbs and Cooper, 1995; Mayo and Jolly, 1991; Pappu et al., 1994). Fraile et al. (1997) studied the occurrence of recombinants and reassortants in natural populations of cucumber mosaic virus (CMV) where both subgroup I and subgroup II strains were present in the same field. It was estimated that seven percent of natural infections were the result of CMV isolates with a recombinant RNA 3, presumably arising from a recombination event during mixed infections of CMV strains. However, to date there have been no attempts to measure in mixed infections under controlled conditions.

In previous experiments, Greene and Allison (1994, 1996) challenged transgenic *Nicotiana benthamiana* plants transcribing portions of the 3' end of cowpea chlorotic mottle bromovirus (CCMV) RNA 3 with a CCMV RNA 3 deletion mutant lacking the 3' one-third of the capsid gene. Since the carboxy-terminal portion of the CCMV capsid protein is required for systemic movement (Schneider et al., 1997; Allison et al., 1990), a recombination event between the transgene RNA and the challenging deletion mutant was required for systemic infection. Three percent (7 out of 235) of the transgenic plants transcribing the CCMV RNA with the full length 3' untranslated region (3'UTR) became systemically infected as a result of recombination events. Surprisingly, cDNA clones derived from viral RNA sampled directly from the initially infected transgenic plant represented a variety of nonfunctional RNA 3's, with numerous recombination sites in each clone. However, once the virus was passaged to a non-transgenic host, the derived cDNA clones were genetically identical, and all clones represented the infectious RNA 3. This suggests that within the initially infected transgenic plant, the functional RNA 3 was

not the dominant RNA 3 component in the viral population, and that the clones from the initially infected plant represented the variability within the viral quasispecies. In addition, it appears as though the functional recombinant RNA 3 becomes the dominant RNA in the quasispecies upon passaging to a non-transgenic plant (Greene, 1995).

With the impending release of virus resistant transgenic plants and the corroborating evidence for recombination between transgenic RNA and challenging viruses (Lommell and Xiong, 1991; Gal et al., 1992; Schoelz and Wintermantel, 1993; for review see Allison et al., 1996), it becomes important to be able to compare recombination in natural situations, such as mixed infections, to recombination in transgenic plants. To compare recombination in transgenic plants with recombination between inoculating viral RNAs, CCMV RNA 3 deletion mutants were constructed which mimicked the constructs used in the transgenic recombination study (see Chapter Two). The deletion mutant transcripts, SR1 and SR2, lacked the identical portions of the 5' and 3' ends of the capsid gene as the transgene and deletion inoculum of the transgenic study, and shared the same overlapping region (Greene and Allison, 1994). Once again, the only way for systemic infection to occur was via a recombination event in the overlapping region that restored a viable capsid gene. Eight percent of the plants inoculated with the CCMV RNAs 1, 2, SR1 and SR2 became systemically infected as a result of a recombination event (Allison et al., 1996), compared to the three percent viable recombinant recovery rate seen in transgenic plants. However, the SR1/SR2 experiment may not reflect recombination rates in mixed infections, since in true mixed infections both viruses are capable of replication, movement and encapsidation.

To compare recombination levels in transgenic plants with recombination in bromovirus mixed infections, experiments were designed to measure viable recombination in mixed infections of wild type CCMV and brome mosaic bromovirus (BMV) as well as in mixed infections of BMV and CCMV capsid gene exchange mutants. In addition, we used these mixed infections to estimate a basal level of recombination by sampling the unselected pool of viral RNAs in a mixed infection. Finally, the effects of selection on the recovery of recombinants was assessed by using bromovirus parental virus sets with differing adaptive abilities.

MATERIALS AND METHODS

Construction of capsid gene exchange mutants

The capsid genes of BMV and CCMV were exchanged to form the mutants AJ8 and AJ11 (Figure 1) in the following manner. A Sal I site was added to the 5' end of the CCMV capsid gene in the CCMV RNA3 infectious clone pCC3TP4 (Allison et al., 1989) to make pCC3TP10. The capsid gene and 3'UTR from pCC3TP10 was then replaced with the capsid gene and 3'UTR from the BMV RNA 3 infectious clone pB3TP8 (Ahlquist et al., 1984). The majority of the BMV 3'UTR of the resulting clone was removed with restriction enzymes Nsp I and Sph I, and replaced with the Sac II/Sph I fragment from CCMV clone pCC3TP12, which includes a portion of the CCMV capsid gene and the entire CCMV 3'UTR. This clone was mutagenized to remove the extra BMV 3'UTR bases and the extra CCMV capsid gene bases to form pBC3AJ11. pBC3AJ8 was constructed by replacing the capsid gene of BMV RNA 3 clone pB3TP8 with the Sal I /Bsp1286 fragment (capsid protein gene) from the CCMV RNA 3 clone pCC3TP9

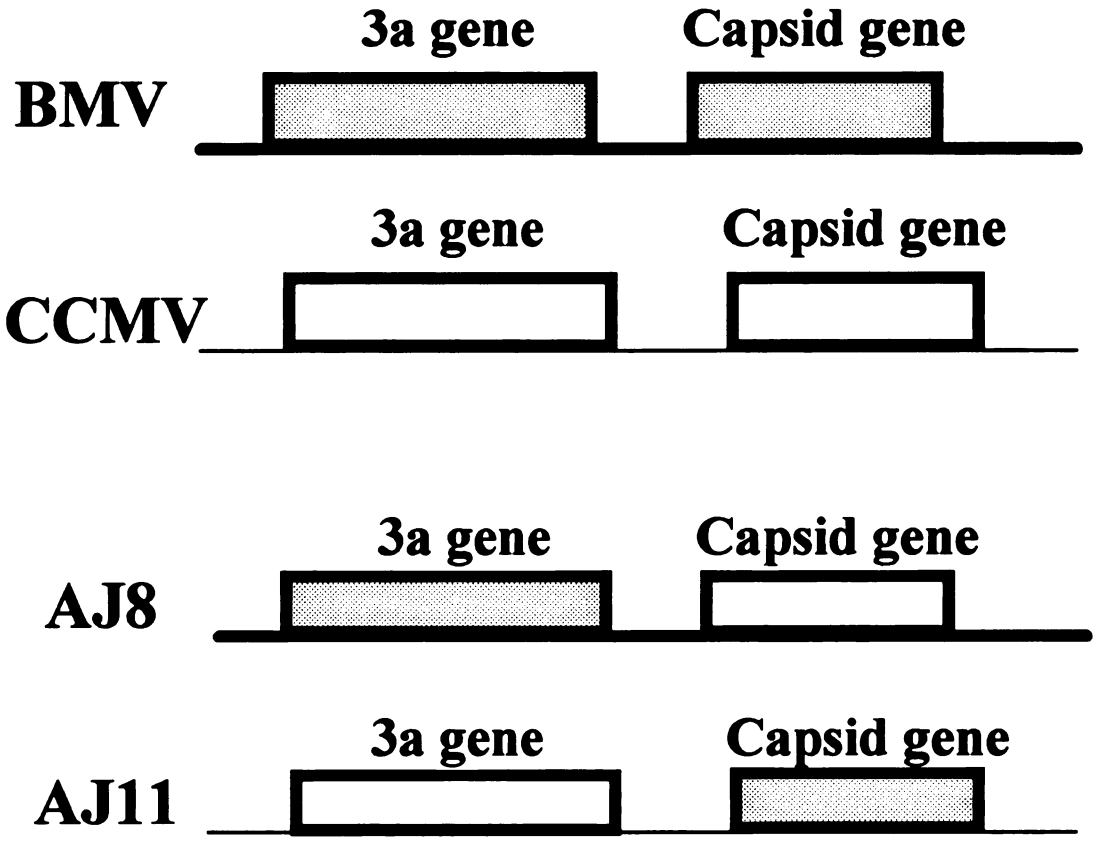

Figure 1. RNA 3 components of the parental bromoviruses used to establish mixed infections. Wild type BMV and CCMV represent one set of parental viruses, and the capsid gene exchange mutants AJ8 and AJ11 represent the other set of parental viruses.

(Allison, personal communication). Transcripts were made from BMV and CCMV clones as described by Ahlquist et al. (1984) and Allison et al. (1989).

Parental Viruses

Nicotiana benthamiana plants were inoculated with wild type BMV RNAs 1,2 and 3; wild type CCMV RNAs 1,2 and 3; BMV RNAs 1,2 and BMV RNAs mutant AJ8; or CCMV RNAs 1,2 and CCMV RNA 4 mutant AJ11 (referred to as wt BMV, wt CCMV, AJ8 and AJ11 infections hereafter). Both AJ8 and AJ11 are infectious clones, causing systemic infections in combination with the appropriate RNAs 1 and 2 (Allison, personal communication). Single infections were confirmed by dot blotting total RNA and probing with BMV and CCMV 3'UTR specific probes HE1 and RA518 (Pacha and Ahlquist, 1991; Allison et al., 1990).

Comparison of AJ8 and AJ11 to wild type infections

After single infections of wt BMV, wt CCMV, AJ8 and AJ11 were established, the viruses were tested for rate of systemic movement using dot blotting and probing with HE1 and RA518 as described above. In addition, symptom development in infected plants was monitored. Four component inoculations were used to directly compare the RNA 3 components AJ8 to BMV RNA 3 and AJ11 to CCMV RNA 3. In these tests, *N. benthamiana* plants were inoculated with either BMV RNAs 1,2,3, and AJ8, or CCMV RNAs 1,2,3, and AJ11. Total RNA of systemically infected plants was dot blotted and probed with BMV and CCMV capsid gene specific probes WS50 and RA517 (Allison et al., 1990) (Figure 2) to assay for the presence of the specific RNA 3 components. WS50 was constructed by subcloning the SacI/XbaI fragment of BMV RNA 3 (bases 1478-1755) into bluescript vector (Pharmacia, Piscataway, NJ).

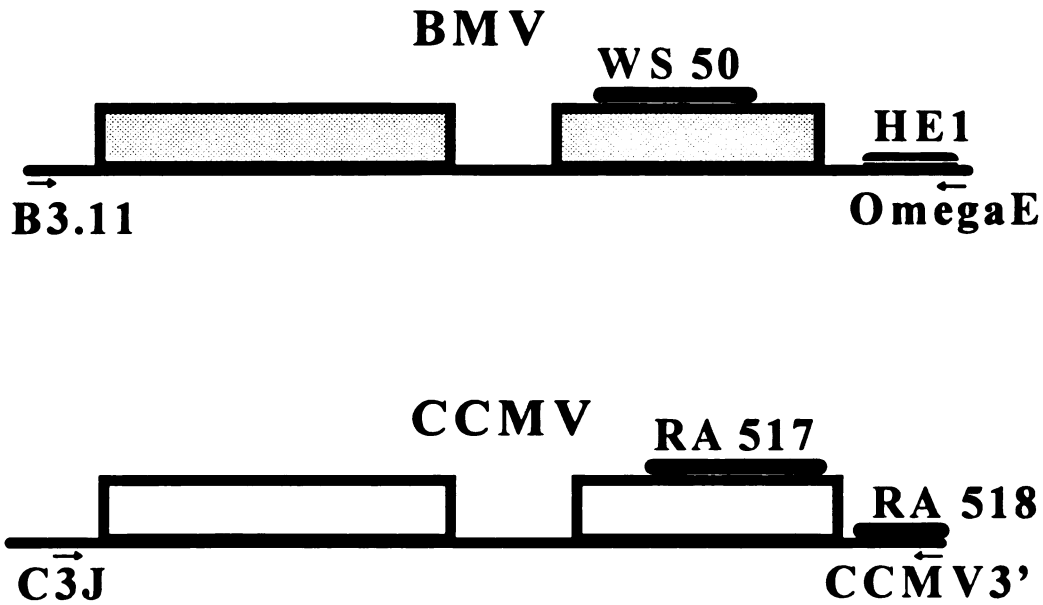

Figure 2. Probes and PCR primers used for recombination analyses. RNA 3s of BMV and CCMV are shown with locations of probes indicated above the RNA 3 and PCR primer locations indicated below RNA 3 with arrows. WS50 and RA 517 hybridize to the capsid gene of BMV and CCMV respectively. HE1 and RA518 hybridize to the 3' untranslated region of BMV and CCMV respectively. B3.11 and OmegaE are the primers for BMV amplification, C3J and CCMV 3' are the primers for CCMV amplification.

Establishing Mixed Infections

Mixed infections were established by inoculating leaves of a two week old *N. benthamiana* either with sap from wt BMV infected *N. benthamiana* and wt CCMV infected *N. benthamiana*, or sap from AJ8 infected *N. benthamiana* and AJ11 infected *N. benthamiana*. Roughly the same amount of infected inoculum source tissue was ground in equal amounts of buffer for each of the parental viruses. *N. benthamiana* plants were inoculated in three ways: 1) both parental viruses were mixed and inoculated on the same leaf at the same time, 2) each parental virus was inoculated onto separate leaves at the same time, or 3) one virus was established in the plant and the second virus was introduced 2-4 weeks later. The presence of mixed infections was established using probes RA518 and HE1 (Allison et al., 1990; Pacha and Ahlquist, 1991).

Measuring basal recombination

Once a mixed infection was established and confirmed by probing, virion RNA was extracted (Greene and Allison, 1994) and used as a template for reverse transcription. During reverse transcription two primers were used to prime cDNA synthesis, CCMV 3' (CAGTCTAGATGGTCTCCTAGAGAT), which anneals to the 3' terminus of all CCMV RNAs and ΩE (Dejong and Ahlquist, 1995), which anneals to the 3' terminus of all BMV RNAs. The cDNA was then used as a template for PCR reactions in which both 3' primers were included (ΩE and CCMV3') and a CCMV specific primer, CCMV 3J (GACTCGAACTCAGGCGG) (Figure 2). PCR product was cloned into bluescript vector (Pharmacia), and clones were denatured and dot blotted onto duplicate nylon membranes. One membrane was probed with 3'UTR probes (either RA518 or HE1), the other was probed with capsid gene specific probes (either RA517 or WS50). Clones that hybridized

to a different combination of clones than the parental virus were selected for di-deoxy sequencing analysis (Sanger et al., 1977). Sequence of potential recombinants was analyzed by GCG sequence analysis program (Devereux et al., 1984).

Selection of viable recombinants

Once mixed infections were established in *N. benthamiana*, the infection was sap passaged to the CCMV systemic host cowpea to separate the CCMV virus in the mixed infection from the BMV virus. To determine if each individual cowpea was infected by a recombinant virus or a parental type virus, total RNA of infected cowpeas was dot blotted onto duplicate membranes and probed with RA517, RA518, HE1 and WS50 as described above. Potential recombinants were cloned and sequenced, and sequences were analyzed by GCG.

RESULTS

Comparison of AJ mutants to wild type RNA 3s

BMV and CCMV are closely related viruses, with considerable sequence homology (Allison et al., 1989). In fact, BMV and CCMV are capable of recognizing the 3' untranslated region (UTR) of the heterologous bromovirus and replicating any such template (Allison et al., 1988). Despite widely differing host ranges, BMV and CCMV share a common host, *Nicotiana benthamiana*, which permitted this study of mixed infections. Two sets of parental viruses were used in this study, wild type BMV and CCMV, and two capsid gene exchange mutants, AJ8 and AJ11. AJ8 is essentially a BMV RNA 3 with a CCMV capsid gene, and AJ11 is a CCMV RNA 3 with a BMV capsid gene (Allison et al., personal communication) (Figure 1). AJ8 and AJ11 are functional RNA 3

components, and the heterologous capsid proteins are capable of transencapsidating and supporting the systemic movement of the coinoculating BMV and CCMV RNAs respectively. AJ8 and AJ11 also replicate to wild type levels in barley protoplasts (Allison et al., personal communication).

To test relative fitness of the wild type bromoviruses and the capsid gene mutant viruses, the systemic movement of wild type BMV and CCMV were compared to AJ8 and AJ11. In plants infected with wild type bromoviruses, systemic movement was detected in 100% of the plants two weeks post inoculation. However, in plants inoculated with the capsid gene mutant viruses the earliest detection of systemic movement ranged from two to four weeks post inoculation (data not shown). In addition, the capsid protein mutants were mechanically transmitted 25-50% less efficiently than wild type bromoviruses (data not shown). Finally, *N. benthamiana* plants were inoculated with a transcript combination of either BMV 1, 2, 3, and AJ8 or CCMV 1, 2, 3, and AJ11. In both cases, the wild type RNA 3 was the only RNA 3 detected in the systemic infections (data not shown).

Establishing mixed infections

Mixed infections were established in three different ways. Both parental viruses (wt BMV and CCMV or AJ8 and AJ11) were either mixed together and inoculated onto the same leaf of young *N. benthamiana* simultaneously, inoculated independently on different leaves of the same plant simultaneously, or one virus infection was established first, followed by a delayed inoculation of the second virus in two to four weeks. In an attempt to make the experimental conditions as true to field conditions as possible, *N. benthamiana* plants were mechanically inoculated with sap from singly infected *N.*

benthamiana, since this would be the most likely means of transmission via beetles in natural situations. In all cases, roughly equal amounts of both parental viruses were used to inoculate uninfected plants.

Interestingly, mixed infections of bromoviruses were relatively rare in cases where both viruses were inoculated simultaneously. Regardless of whether the viruses were inoculated on the same leaf at the same time or on different leaves at the same time the percentage of resulting mixed infections was between 33 and 38 percent (Table 1). This was true for both of the parental virus sets used. However, in situations where one virus was inoculated first, allowed to move systemically, and then the infected plant was challenged with the second virus, mixed infections were detected in 80% of the inoculated plants (Table 1). In these delayed inoculations, the wild type bromoviruses were slightly more likely to form mixed infections than the capsid gene mutants.

Basal recombination rates

Greene (1995) noted that sampling viral RNA directly from the transgenic plant where recombination had occurred led to the recovery of a diverse range of clones, none of which actually represented the infectious RNA 3 component. This suggests that sampling the viral population directly from the originally infected plant provides a snapshot of the quasispecies where recombination is occurring, including both viable and non-vaible recombinants. By sampling viral RNA directly from the plants infected with both parental viruses, the basal level of recombination can be roughly estimated in mixed infections of bromoviruses.

Once mixed infections were established and confirmed viral RNA was extracted directly from the dually infected plant. The viral RNA was used as a template for reverse

Table 1. Percentages of plants with mixed infections resulting from the inoculation methods used in recombination study. The percentages were similar for both mixed infections of wild type bromoviruses and the capsid gene mutants AJ8 and AJ11.

Inoculation method	Mi/total	Mixed infections
Same leaf, same time	23/72	32%
Separate leaves, same time	29/76	38%
One virus established, second virus delayed	32/40	80%

transcription and PCR (RT-PCR). Both BMV and CCMV 3' primers (ΩE and CCMV3') were used during reverse transcription, since potential recombinants could have the 3' untranslated region (UTR) of either virus. The cDNA was PCR amplified using both 3' primers and the CCMV specific primer C3J (Figure 2). This allows separation of CCMV cDNAs from the mixed infection cDNA pool. The PCR product was blunt end cloned and analyzed for evidence of recombination by probing with probes specific for capsid protein genes and the 3'UTRs. Recombination was measured only in the CCMV RNA3 components of the mixed infections.

In control infections where only the CCMV parental virus was inoculated, no recombinants were observed. In addition, no recombinants were recovered from mixed infections of simultaneously inoculated wild type BMV and CCMV, regardless of whether the viruses were inoculated on the same leaf at the same time or on different leaves at the same time. However, when the capsid protein exchange mutants AJ8 and AJ11 were inoculated simultaneously, approximately two percent of the clones derived from the mixed infection viral RNAs represented the products of recombination (Table 2). The percentage of recombinants was roughly equal for both same leaf and different leaf inoculations. The difference between the recombination levels in wild type mixed infections and mixed infections of AJ8 and AJ11 is significant at the P= 0.05 level using a χ^2 test.

All of the recombinants were confirmed by sequencing (Figure 3), and only two of the fourteen recombinants shared the same sequence. This further suggested that the clones represented the viral quasispecies in the original mixed infections. Sequence analysis also showed that none of the clones recovered from the initial mixed infection

Table 2. Recombination recovery rates from unselected pools of viral RNA taken directly from mixed infections of bromoviruses. The infecting viruses are listed in the inoculum column, followed by the method used to establish the mixed infection. In separate inoculations, the viruses were inoculated onto separate leaves of the same plant. In together inoculations, the viruses were pooled and inoculated onto the same leaf. In delayed mixed infections the second virus was inoculated 2-4 weeks after the first. The number of recombinants (Rec.) recovered is shown as the numerator over the total number of viral clones assayed (denominator). The recombinant recovery rate is listed as a percentage in the fourth column. In cases where one virus was established first (delayed), the first virus is noted.

Inoculum	Method	Rec./total	Rate
AJ11	alone	0/323	0.0%
CCMV	alone	0/362	0.0%
AJ8/AJ11	separate	9/396	2.3%
AJ8/AJ11	together	5/243	2.1%
BMV/CCMV	separate	0/315	0.0%
BMV/CCMV	together	0/262	0.0%
AJ8/AJ11	delayed, AJ8 1st	0/153	0.0%
AJ8/AJ11	delayed, AJ11 1st	0/296	0.0%
BMV/CCMV	delayed, BMV 1st	0/192	0.0%
BMV/CCMV	delayed, CCMV 1st	0/283	0.0%

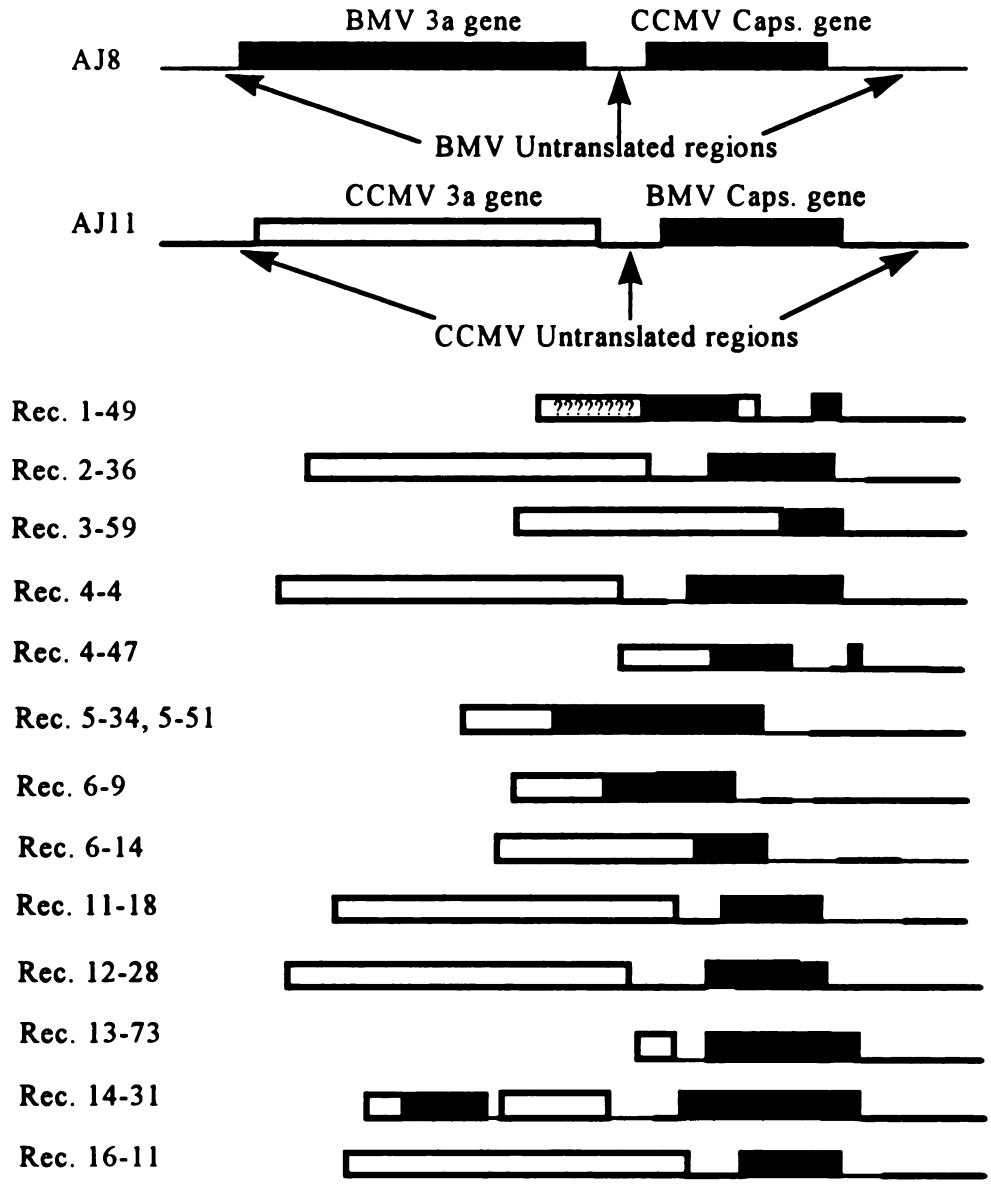

Figure 3. Non-viable recombinants derived from AJ8/AJ11 mixed infecitons. Recombinants are illustrated below AJ8 and AJ11 maps. BMV 3a sequence represented by shaded region. CCMV 3a sequence represented by open boxes. BMV capsid gene represented by horizontal crosshatching. CCMV capsid gene represented by diagonal crosshatching. BMV UTR represented by thin lines, CCMV UTR represented by thick lines. Unknown sequence represented by open box with question marks.

plants were from viable recombinants. None of the clones had a functional capsid gene open reading frame, and many had several recombination sites. Recombinant 1-49 had incorporated a stretch of non-viral sequence, presumably of host origin. Both the recovery of non-viable recombinants and the incorporation of non-viral sequences during viral recombination were noted previously in the transgenic recombination study (Greene, 1995), as well as in other systems (Mayo and Jolly, 1991; Nagy and Bujarski, 1995).

The basal recombination rates were also established for delayed mixed infections, where one virus was inoculated and moved systemically followed by the inoculation of the second virus. Delayed mixed infections were established using either the BMV parental virus (w.t. BMV or AJ8) as the initial inoculum followed by challenge with the CCMV parental virus (w.t. CCMV or AJ11), or in the opposite order. As with the other assays, recombination was measured in the CCMV RNA 3 component. No recombinants were recovered from the delayed mixed infections where the CCMV parental virus was inoculated first, regardless of whether the wild type parents or the capsid exchange mutants were used as the inoculum (Table 2). The same is true when the BMV parental virus was inoculated first.

Measuring viable recombination

In previous recombination studies (Greene and Allison, 1994; Greene, 1995), it was noted that passaging the recombinant from the initially infected plant where recombination occurred selects for the viable recombinants in the next host. This phenomena was used to separate the viable recombinants from non-viable recombinants found in mixed infections. Once mixed infections were established in *N. benthamiana* as described above, the mixed infection was sap transmitted to cowpeas, a CCMV specific

host. This separated the CCMV component of the mixed infection from the BMV component, and also selected for viable viruses.

All three types of mixed infections were passed to numerous cowpeas. Probing total RNA of infected cowpeas with the capsid protein gene and 3'UTR probes revealed that none of the cowpeas inoculated with sap from a plant infected with wild type BMV and CCMV became infected with a recombinant, regardless of how the mixed infection was established. Passaging the sap from AJ8/AJ11 mixed infections to cowpeas resulted in the recovery of one viable recombinant, recombinant 7-2, which was derived from a simultaneous, same leaf inoculated mixed infection. In addition, no viable recombinants have been recovered from delayed mixed infections of any kind, using either wild type or capsid gene mutant parental viruses (Table 3).

The viral RNA from cowpea 7-2 was cloned and sequenced (Figure 4). Sequence analysis determined that recombinant 7-2 resulted from two recombination events, one in the 3' untranslated region and the second presumably in the intercistronic poly A region. Collectively, these recombination events replaced the BMV capsid gene of AJ11 with the CCMV capsid gene from AJ8. The resulting RNA 3 is essentially a wild type CCMV RNA 3 with a BMV subgenomic promoter and a short region of BMV 3'UTR immediately adjacent to the capsid gene. This sequence was confirmed using multiple clones from separate cloning experiments.

DISCUSSION

It is widely accepted that recombination during mixed infections has contributed to the modular evolution of plant RNA viruses (Simon and Bujarski, 1994; Lai, 1992), and

Table 3. Viable recombinant recovery rates in mixed infections of bromoviruses. The CCMV component of bromovirus mixed infections was passaged to cowpeas, and total RNA was assayed for the presence of a viable recombinant.

Mixed infection	Viable Rec./total	Viable Rec. Rate
BMV/CCMV, simultaneous	0/117	0.0%
BMV/CCMV, delayed	0/125	0.0%
AJ8/AJ11, simultaneous	1/56	1.8%
AJ8/AJ11, delayed	0/74	0.0%

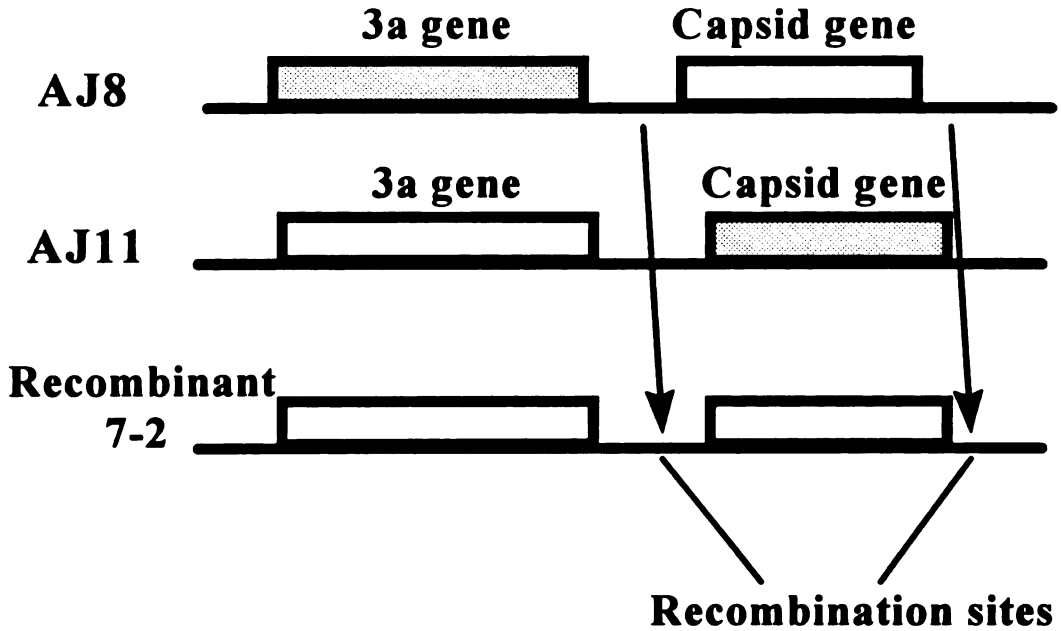

Figure 4. Recombinant 7-2. Recombinant 7-2 is a viable recombinant derived from a mixed infection of AJ8 and AJ11. Two recombination events occurred, one in the poly A region, the other in the 3' untranslated region which replaced the BMV capsid gene with the CCMV capsid gene.

there are numerous reports which describe the recovery of two or more viruses from the same host plant. However, to date there are no reports investigating the establishment of mixed infections, or the rates of interviral recombination during mixed infections. These experiments were designed to look at the recombination that occurs in mixed infections, as well as some of the factors which may influence recombination rates. For convenience, recombination was only measured in the capsid gene and 3'UTR of the CCMV half of the mixed infection. This, along with the combined facts that only interviral recombination was screened for, and only encapsidated viral RNAs were sampled, suggests that the recombination rates detected were probably low estimates of the actual basal recombination rates in mixed infections.

In an attempt to keep the experiments as true to field conditions as possible, and to thoroughly investigate the initiation of mixed infections, the parental viruses were established in multiple ways. Interestingly, mixed infections of BMV and CCMV were not as easily established when both viruses were inoculated simultaneously as when the times of inoculation were staggered (Table 1). This may indicate that the most common means of establishing mixed infections in the field is by delayed inoculation, where one virus is established prior to inoculation with the second virus. Despite their similarities, BMV and CCMV have widely different host ranges, with BMV infecting grasses and CCMV infecting mostly legumes (Schneider and Allison, 1994). The beetles that vector bromoviruses, which belong to the chrysomelid family of leaf eating beetles (Hobbs and Fulton, 1979), are specialist feeders, which means they prefer to feed on one or two specific plant species, usually within the same taxanomic family (Jolivet, 1988). BMV and CCMV have very distinct plant host ranges, therefore, it is unlikely that a single beetle

could acquire and vector both BMV and CCMV to the same plant simultaneously. Thus, the formation of simultaneous mixed infections of BMV and CCMV in the field may be quite rare.

In addition, it appears that recombination recovery rates are related to selection pressure. Previous recombination studies (Greene and Allison, 1994; 1996; Greene, 1995; Allison et al., 1996) have maintained high selection pressure for recombinants that could move systemically. In contrast, these experiments reduced selection pressure for recombinants by using parental viruses already capable of systemic infection. Both capsid gene mutants, AJ8 and AJ11, were infectious viruses (Allison, personal communication). However, when either AJ8 or AJ11 was compared to the wild type RNA 3s in terms of movement, mechanical transmissibility, and direct *in planta* competition, the wild type RNA 3 always dominated the final systemic infection, replacing the chimeric RNA 3 of the mutant AJ viruses. This suggests that both BMV and CCMV are best suited to interact with their own capsid protein. Thus, in mixed infections of AJ8 and AJ11 there should be selection pressure for a recombinant that replaces the heterologous capsid gene and restores the wild type virus. In contrast, there should be no selective advantage for a recombinant in mixed infections of wt BMV and CCMV, since each virus is best suited to interact with its own capsid protein.

Recombination could not be detected for mixed infections of wild type BMV and CCMV, either in the unselected viral RNA pool from the initial mixed infection, or in the mechanically passaged new infections. In contrast, in mixed infections of the capsid gene mutants AJ8 and AJ11, non-viable recombinants were recovered from the initial mixed infection, and a viable recombinant was recovered from passaged virus populations. This

difference between the recombination levels of wild type and mutant viruses is statistically significant. The difference in recovery rates can be explained in one of two ways. First, the wild type RNA 3 components may be so highly adapted in the systemic host that the selection pressure against recombinants (i.e. the selection pressure for the wild type RNA 3) is high enough to keep the number of recombinants in a wild type viral RNA population below the detection levels of this experiment. AJ8 and AJ11 are less efficient forms of BMV and CCMV RNA 3, so in these cases the selection pressure against recombinants may be reduced to the point where recombinants can reach detectable levels. A second possibility involves recombination by breaking and ligating (Chetverin et al., 1997). If the RNA secondary structures of the AJ mutants are less stable due to the presence of a foreign capsid gene, the AJ RNAs may be more prone to cleavage by RNA endonucleases, leading to increased opportunities for ligation to other RNAs. A final possibility which cannot be discounted is that recombination rates in mixed infections of wild type bromoviruses are actually lower than in mixed infections of the capsid gene mutants.

The difference in recombination recovery levels between wild type bromovirus mixed infections and capsid gene mutant mixed infections also extends to the recovery of viable recombinants. No viable recombinants were recovered by passing the CCMV portion of wild type mixed infections to cowpeas, but a viable recombinant was recovered from a mixed infection of AJ8 and AJ11 by passaging to cowpea. Although the difference in recovery levels was not quite significant at the P=0.05 level (P value is less than 0.1), the trend toward higher recovery in the mutant mixed infections is still evident.

Perhaps the most important recombination measurements are the data recorded for the delayed mixed infections, because this seems to be the most likely scenario for the

formation of mixed infections of bromoviruses (Table 1). This is significant from a recombination standpoint, because evidence from other virus systems indicates that viral replication is a temporal process, occurring at the front of infection (Wang and Maule, 1995). If this is in fact the pattern of replication, it reduces the chances of recombination in delayed mixed infections, since the two parental viruses are less likely to be replicating in the same cells at the same time.

In delayed bromovirus mixed infections there was no recombination between wild type BMV and CCMV, either in the unselected viral RNA pools or the passaged viable virus populations. Similarly, in delayed mixed infections of AJ8 and AJ11 no recombinants were recovered in mixed infections when AJ11 was inoculated first or when AJ8 was inoculated first. If there is a temporal replication pattern for bromoviruses, where the virus replicates as it moves through the plant, and after systemic infection is complete replication is markedly reduced, we would expect to see a higher AJ11 recombinant recovery rate in mixed infections where AJ8 was inoculated first. The delayed virus should be more likely to undergo interviral recombination, since it is actively replicating as it moves systemically through the plant cells already containing the viral RNA from the first virus. The lack of recombination in delayed mixed infections could be due to a combination of encapsidation sequestering the viral RNA of the first virus and host degradation of the remaining cellular viral RNA.

The data collected here can be used to make a comparison between the opportunites for viral recombination in mixed infections and the opportunities for recombination in transgenic plants expressing viral transgenes. Greene and Allison (1994) observed viable recombinants in three percent of the transgenic plants inoculated with a

CCMV deletion mutant. The highest viable recombinant recovery rate observed for simultaneous mixed infections of AJ8 and AJ11 was 1.8%, slightly lower than the transgenic recombinant recovery rate. For all other mixed infections, including delayed mixed infections which appear to be the most likely scenario for the formation of mixed infections, the viable recombinant recovery rate was zero percent. Thus, the opportunities for recombination in mixed infections do not appear to mimic the opportunities for recombination in transgenic plants, since in transgenic plants a steady state level of transgenically expressed, unencapsidated viral RNA is available in every cell for recombination with a challenging virus.

CHAPTER 4

Mutations in CCMV RNA 3 Recombinants Rapidly Revert to Wild Type Sequence During Serial Passaging

INTRODUCTION

RNA viruses are known for having error prone RNA-dependent RNA polymerases (RdRp's), with error rates estimated as high as 10^{-4} per nucleotide copied (Holland et al., 1992; Drake, 1993; Mansky and Temin, 1995). The RdRp's high error rate can be attributed to the lack of a 3' to 5' exonuclease proof-reading activity. This high error rate has led to the theory that virus populations exist within the host as a heterogeneous mixture of related sequences known as a quasispecies (Holland et al., 1982). Not suprisingly, high error rates have also been observed for the RdRp's of plant RNA viruses (Drake, 1993), leading to the conclusion that plant RNA viruses exist in a quasispecies form as well.

Plant viruses undergo thousands of rounds of replication within a single host, and are subject to selection for the ability to use host products for replication, translation, cell-to-cell movement and long distance movement. Despite the potential evolutionary ramifications, there is little research investigating the role of selection on the evolution of plant RNA viruses. Bacher et al. (1994) examined the mechanisms that maintain the sequence identity between the nearly identical 1390 base 3' terminal untranslated region

(3'UTR) of RNAs 1 and 2 of blueberry leaf mottle nepovirus (BBLMV). It had been proposed that replication of nepovirus RNAs began on the 3' UTR of only one of the RNAs, and that replication of the other RNA occurred via template switching (Rott et al., 1991). While this would account for the near identity of the 3' UTR's of RNAs 1 and 2, Bacher et al. (1994) were unable to detect recombination using marker mutations in multiple 3'UTR clones of RNA 1 and 2. In the absence of detectable recombination, it is likely that selection pressure is playing a strong role in maintaining the sequence identity of the BBLMV 3' UTR. This indicated that selection may be playing a more significant role in the evolution of RNA viruses than previously thought, even in non-coding regions.

In addition, there is evidence for strong selection pressure for bromoviruses to maintain wild type sequences. For example, bromovirus capsid gene exchange mutants do not compete well against wild type bromoviruses *in planta* (see Chapter Three), and numerous unrelated experiments have demonstrated mutations are rarely recovered from cDNA clones derived from wild type bromoviral infections. Greene and Allison (1994) recovered several genetically distinct cowpea chlorotic mottle bromovirus recombinants from an *in vivo* assay designed to measure recombination in transgenic plants. All of the recombinants contained point mutations in the capsid gene, presumably resulting from the recombination event, and some of the recombinants had distinctive phenotypes as a result of mutations. In theory, if there is strong selection pressure for the wild type bromovirus sequence, and if the RdRp of CCMV is as prone to error as other viral RdRP's, then we should be able to observe reversions to wild type CCMV sequence during passage of the recombinant virus. Using recombinants with unique symptom phenotypes enabled us to utilize symptom formation as an indicator of possible reversions.

MATERIALS AND METHODS

Recombinant viruses

Two recombinants, 5-55 and 3-57 derived from the initial recombination experiments (Greene and Allison, 1994; Greene, 1995) were used in this study. Recombinant 5-55 has three substitution mutations from wild type CCMV, resulting in two amino acid changes (Fig. 1). In addition, 5-55 has three silent mutations at the 3' end of the capsid gene derived from the CCMV transgene which were used to distinguish recombinant 5-55 from wild type contamination (Greene and Allison, 1994). Recombinant 5-55 causes severe chlorosis on infected cowpea leaves (Greene, 1995), which is quite distinct from the mild mottling associated with wild type CCMV. Recombinant 3-57 systemically infects cowpea and *Nicotiana benthamiana* without the formation of virions, and does not cause detectable symptoms on cowpeas. A single base deletion in the 5' end of the 3-57 capsid gene resulted in a frameshift which introduced a termination codon after twelve codons (Fig. 2). A newly inserted initiation codon allowed translation of the carboxy-terminal two-thirds of the capsid gene (Fig. 2), and this portion of the capsid protein is sufficient for systemic movement of CCMV (Schneider et al., 1997). Recombinants 5-55 and 3-57 accumulate viral RNA to wild type levels (Greene, 1995), but recombinant 3-57 shows slower systemic movement than wild type CCMV (Schneider et al., 1997).

Passaging of recombinants

The recombinant virus from transgenic plant 5-55 was originally mechanically passaged to non-transgenic cowpeas, and virions were purified from the first passage

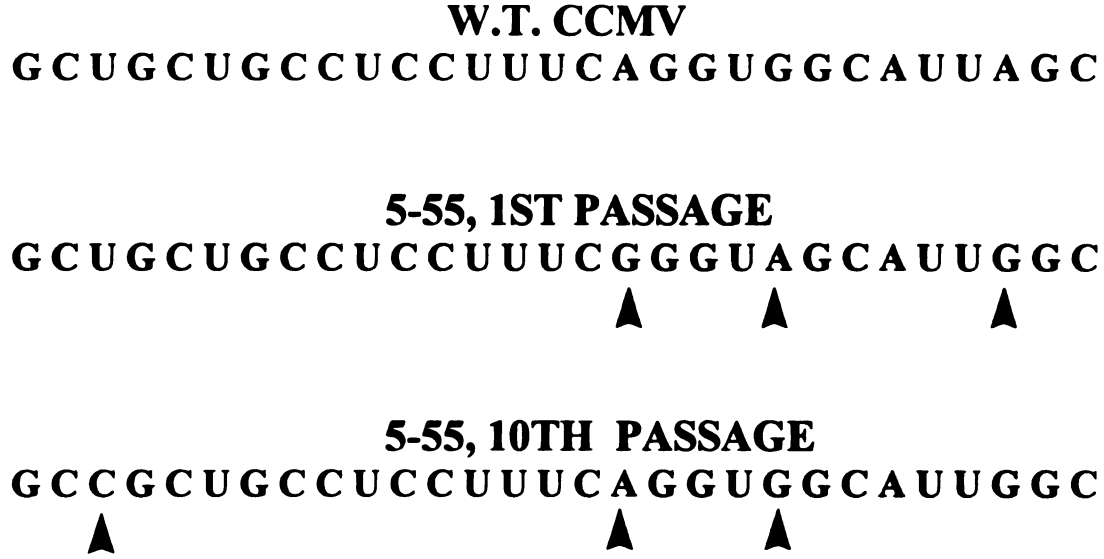

Figure 1. Sequence comparison of the mutated region in wild type CCMV RNA 3 to the same region in recombinant 5-55 first and tenth passage clones. Mutations marked by arrows underneath sequence. The mutations in the tenth passage clones revert two substitutions to wild type sequence, restoring wild type amino acid sequence. The third mutated base aligns with the remaining unreverted substitution in a potential stem loop.

A

Wild type CCMV

AAUUUGAUAGUAAUUUAUC AUG UCU ACA GUC GGA ACA GGG AAG UUA

M S T V G T G K L

3-57, passage 1

AAUUUGAUAGUAAUUUAUC AUG UCU ACA GU.G GAA CAG GGA AGU UAA

M S T V E Q G S Stop

3-57, passage 4

AAUUUG AUG AUA AUU UAU CAU GUC UAC AGU. GGA ACA GGG AAG UUA

M I I Y L V Y S G T G K L

B

Wild type CCMV CCU AAU . . . GAG CUA

P N E L

3-57, passage 1 CCU AAU AUG ACG CUA

M T L

3-57, passage 4 CCU AAU . . . GAG CUA

P N E L

C

Wild type CCMV ACU GCU GCU GCC

T A A A

3-57, passage 1 ACU ACU GCU GCC

T T A A

3-57, passage 4 ACU GCU GCU GCC

T A A A

Figure 2. Sequence comparison of wild type CCMV RNA 3, recombinant 3-57 first passage and recombinant 3-57 fourth passage. The regions with mutations intorduced by recombiantion are shown. Panel A is the sequence between bases 1340-1390, panel B is the region between bases 1582-1593, panel C the region between bases 1700-1710. Amino acid sequence is listed under the RNA sequence in bold.

plants. These purified virions were used to inoculate cowpeas to begin the passaging experiments. The second recombinant, 3-57, was established using *in vitro* transcripts derived from an infectious CCMV clone (pCC3AW2) containing mutations specific to the recombination event (Schneider et al., 1997). Recombinant viruses were mechanically passaged from cowpea to cowpea, with infection confirmed by dot blotting total RNA from inoculated plants followed by probing with CCMV specific probe RA518 (Allison et al., 1990) as described by Schneider et al.(1997). In addition, wild type CCMV infections were established on cowpeas using *in vitro* transcripts (Allison et al., 1988), and mechanically passaged as described for the recombinants. At each passage symptoms caused by the recombinants were observed and compared to the wild type infections.

Viral isolation, cloning and sequencing

Plants with passaged recombinant virus were observed for the development of wild type symptoms. If wild type symptoms formed, the recombinant virus was passaged one additional time, and virion RNA was isolated from this plant as described by Allison et al. (1989). If wild type symptoms failed to form during the course of passaging, the viral RNA was isolated from the tenth passage plant. cDNA copies were made from viral RNA using reverse transcriptase as described by Greene and Allison (1994), and then amplified using PCR. PCR products were cloned into Bluescript plasmid (Phamacia, Piscataway, NJ) and the entire capsid gene and 3' untranslated region were sequenced using dideoxy-termination sequencing (Sanger et al., 1977) The potential RNA secondary structure of the capsid genes of brome mosaic bromovirus (BMV) and CCMV were analyzed using GCG program RNAFold (Zuker, 1989).

RESULTS

Recombinant 5-55

Recombinant 5-55 (Fig 1) was established on cowpeas and passaged mechanically from cowpea to cowpea. At each subsequent passage, the symptoms on cowpea were observed, looking for a reversion to the mild mottling typically caused by wild type CCMV. After ten passages on cowpea, the severe chlorosis phenotype was still evident, so viral RNA was extracted from infected plants and cloned using reverse transcription/PCR. The entire capsid gene and the 3' untranslated region (UTR) of both passaged 5-55 clones and passaged wild type CCMV RNA 3 clones were sequenced.

Analysis of the clones derived from serially passaged wild type CCMV clones revealed no mutations in six clones derived from different cloning events. The clones of serially passaged recombinant 5-55, however, had three substitution mutations in the capsid gene region originally affected by the recombination event (Fig. 1). Two of the substitutions, bases 1721 and 1726, were reversions of original mutations back to the wild type sequence, which also restored the amino acid sequence to wild type. The third mutation, which changed base 1707 from an T to a C, was silent. This change becomes more significant when it is aligned with the one remaining silent mutation that was not reverted during the course of serial passage. The cytosine residue is capable of binding the guanosine residue introduced at position 1731 during recombination, lowering the free energy of a potential stem loop structure (Fig. 3). There were no other mutations in the

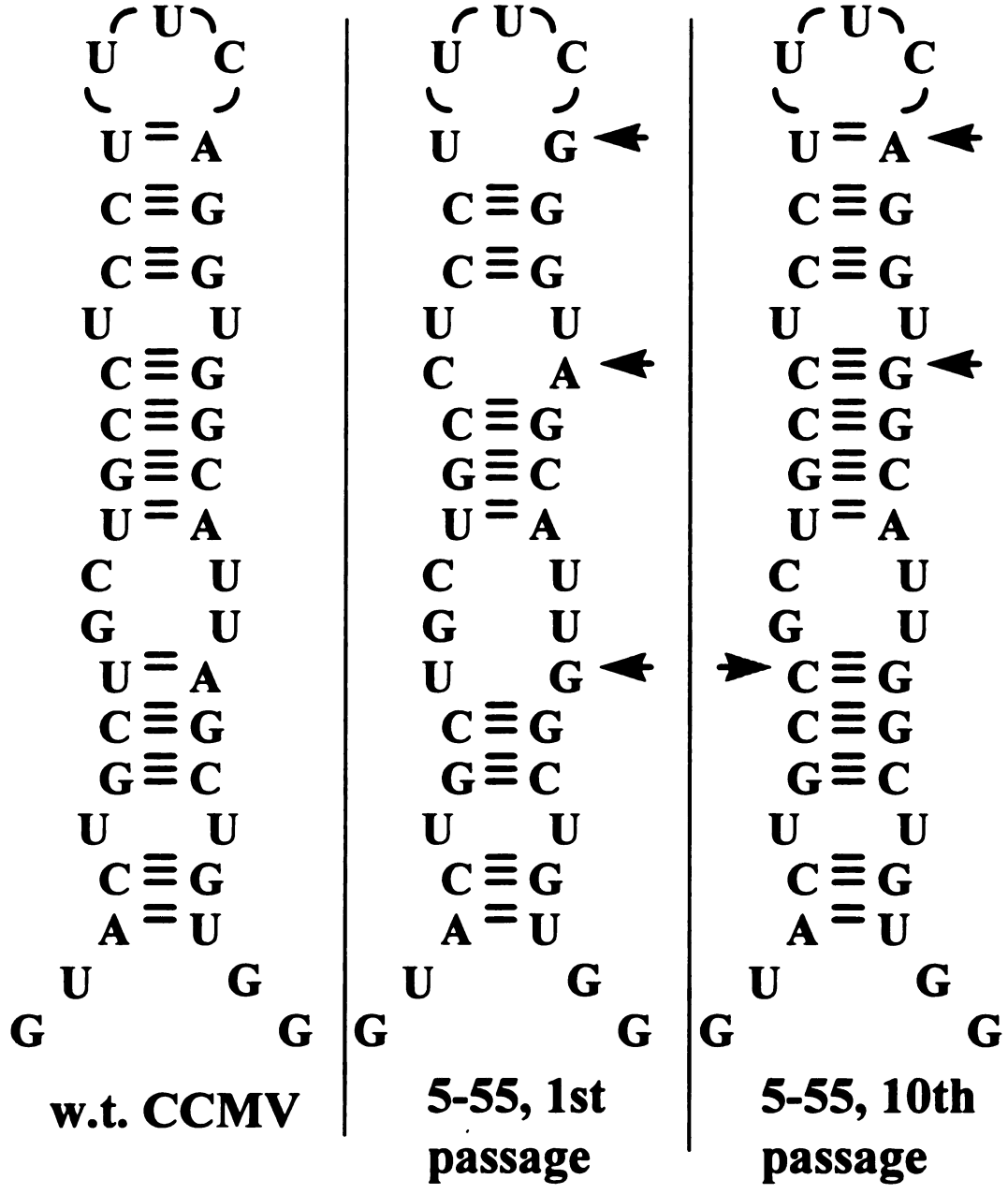

Figure 3. Proposed stem loop structure in capsid gene of wild type CCMV, 5-55 1st passage and 5-55 10th passage. All three mutations in 5-55 after ten passages restore bonds to the potential stem loop.

remainder of the cloned sequence, and the mutations described were present in multiple clones derived from separate cloning events. In addition, the silent mutations engineered at the 3' end of the capsid gene remained in the sequence of the passaged 5-55.

The RNA sequence of the 3' end of the capsid gene was analyzed using the GCG folding program FoldRNA (Zuker, 1989). Analysis of the region indicated that a stem loop was optimal for the region affected by the recombination mutations (Fig. 3). All three mutations in the passaged 5-55 clones, including the silent mutation in the wild type sequence, restored bonds in this potential stem loop, thus minimizing the free energy. Analysis of the same region in the BMV capsid protein gene predicted that a similar stem loop with the identical sequence at the top of the loop is the optimal secondary structure for the region (Fig. 4). RNAs 1 and 2 of both CCMV and BMV were analyzed looking for a similar stem loop in the 3' end of the coding region, but none were found that were similar to the stem loops described for RNA 3 (data not shown).

Recombinant 3-57

Recombinant 3-57 was passaged in an identical manner as recombinant 5-55 and the wild type controls. After three passages on cowpea, the infection caused by recombinant 3-57 began to show mild mottling symptoms characteristic of wild type CCMV. The recombinant 3-57 was passaged one additional time, and then viral RNA was extracted. At this point, virions could be isolated from the 3-57 fourth passage plants, suggesting that the full length capsid protein was now being produced and virions were being formed (data not shown).

Viral RNA from fourth passage 3-57 plants was isolated and cloned. Sequence analysis of the 3-57 clones indicated that the single base substitution at base 1704 had

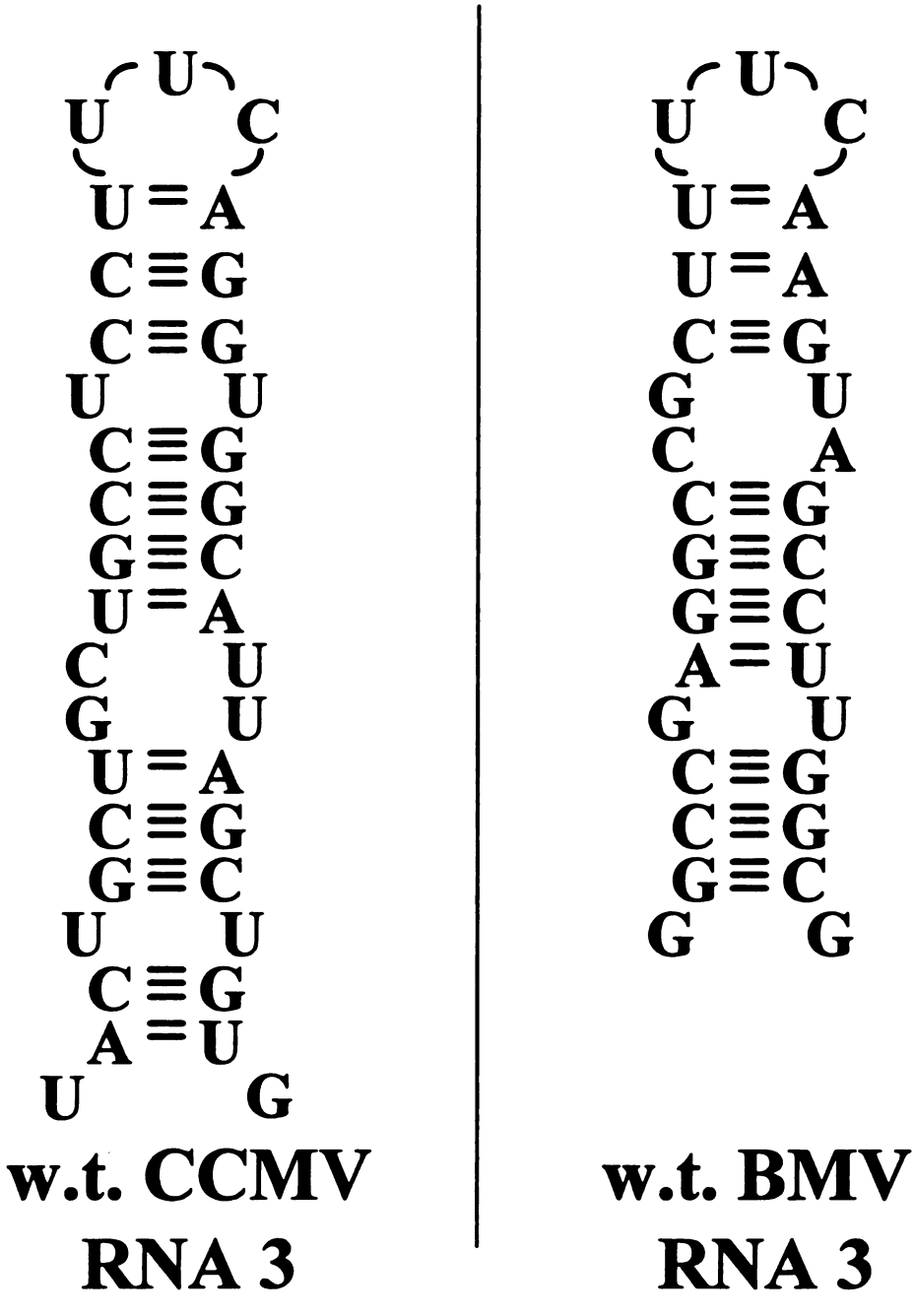

Figure 4. Comparison of the regions in CCMV and BMV capsid protein genes containing proposed stem loop structures.

reverted back to wild type sequence. In addition, the three base insertion at bases 1581-1583, which had introduced an in frame start codon, had been removed precisely, resulting in reversion to wild type sequence. The single base deletion in the 5' end of the capsid gene, however, had not been corrected (Fig. 2). Two additional changes were made in the sequence upstream of the capsid gene start codon, which resulted in the creation of a new initiation codon (Fig. 2). The new initiation codon was out of frame with the original capsid gene reading frame, but was brought into frame by the single base deletion that occurred in 3-57 (Fig. 5). This allowed for translation of a full length capsid protein, which fulfilled the movement and encapsidation functions.

DISCUSSION

All of the recombinants generated in the transgenic recombination study were genetically diverse isolates arising from aberrant homologous recombination (Greene and Allison, 1994; Greene, 1995). Some of the recombinants also caused unique symptom development on cowpeas, presumably as a result of mutations incurred during recombination. The two recombinants used for these experiments were chosen based on the selectable phenotypes that distinguished 5-55 and 3-57 from wild type CCMV. Recombinant 5-55 caused unique chlorosis symptoms on cowpeas, indicating that it was likely interacting with the host in a manner different than wild type CCMV. Previous studies have linked the BMV and CCMV capsid gene to symptom development (Greene, 1995; Rao and Grantham, 1996), so it appeared as though the mutations in the capsid gene of recombinant 5-55 were creating a selectable phenotype. Recombinant 3-57 was incapable of producing full length capsid protein, and subsequently was unable to form

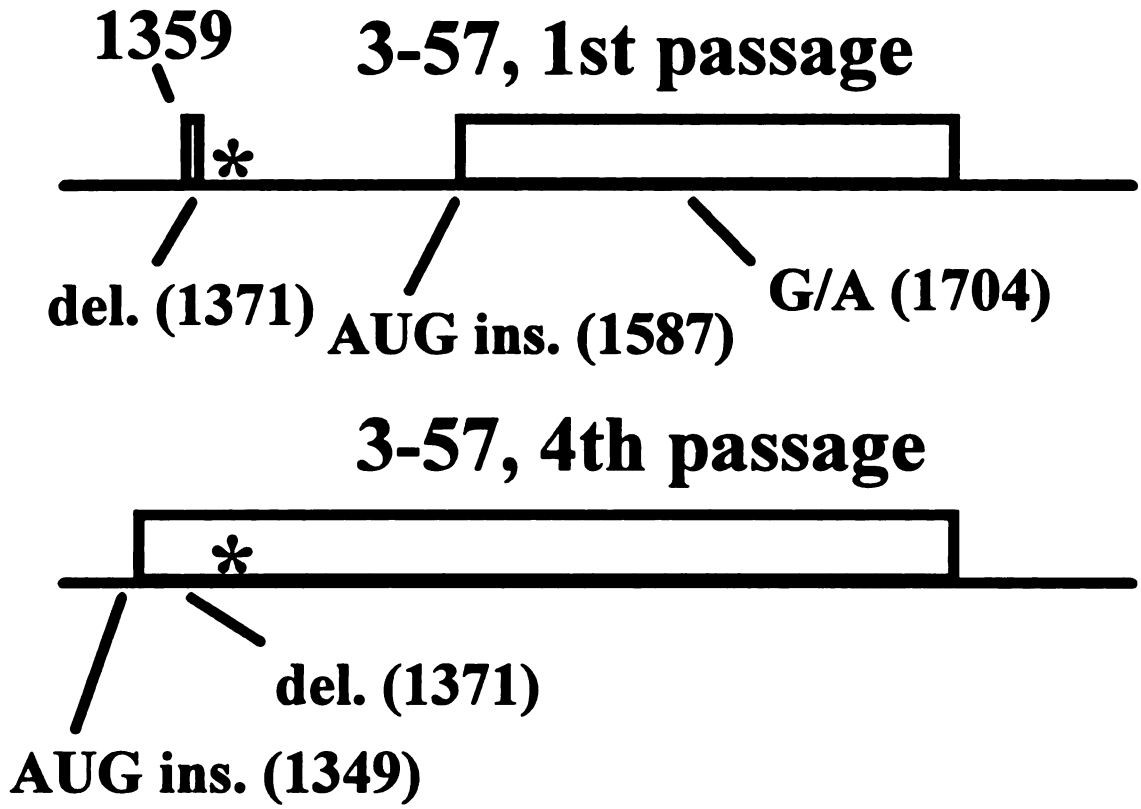

Figure 5. Translational strategies of the capsid gene of recombinant 3-57 after the first and fourth passages. After the first passage the truncated capsid protein is translated from the internal initiation codon (1587). After four passages, the extended full length capsid protein is translated from an introduced intiation codon ten bases upstream (1349-1351) of the original initiation codon (1359-1361). All other mutations have reverted to wild type sequence except the single base deletion of base 1371. Base positions indicated by numbers.

virions. As such, recombinant 3-57 caused no symptoms on cowpeas, and moved systemically at a slower rate in cowpeas and *N. benthamiana* than the wild type virus. Therefore, we predicted that there should be selection pressure for mutations in 3-57 which restore the full length capsid gene.

Both recombinants were mechanically transmitted from the initially infected transgenic plant, where recombination had occurred, to cowpeas immediately after identification. Sequencing multiple clones of either recombinant from this initial passage resulted in identical sequences, indicating that the identified mutations were fixed in the viral population. If the mutations arising from recombination were advantageous or selectively neutral, the mutations should remain in the population over the course of serial passages. However, if the mutations were deleterious, any revertants to the wild type sequence should be selectively advantaged, and replace the original recombinants over the course of time and serial passaging.

Recombinant 5-55 retained its severe chlorosis phenotype over the course of ten passages, so the RNA 3 component of the passaged 5-55 infection was cloned to see if the mutations introduced by recombination were still present. Subsequent sequence analysis indicated that two of the mutations in recombinant 5-55 had reverted to wild type. Both of these mutations also restored changes in the wild type amino acid sequence, indicating possible selection pressure for the wild type capsid protein (Fig. 1). There were no other mutations in the capsid gene reading frame, possibly indicating that the severe chlorosis phenotype associated with the 5-55 recombinant is not associated with the capsid protein, and may be the result of a mutation elsewhere in the CCMV genome that was carried along during passaging. Although bromovirus symptom formation is usually associated

with the capsid protein (Greene, 1995; Rao and Grantham, 1996), there are instances where mutations in other bromovirus proteins have resulted in altered symptom development (Fujita et al., 1996; Traynor et al., 1991).

The third silent substitution mutation in recombinant 5-55 remained unchanged after ten passages, but there was an additional silent substitution mutation in all clones which lay in a position that could align with the remaining unchanged mutation from the 5-55 recombination event in a potential stem loop structure (Fig 3). Analysis of potential RNA secondary structure for the region indicated that such a stem loop was likely to form, and analysis of the BMV capsid gene indicated a similar structure was likely to form at the same relative position in the BMV capsid gene (Fig 3). The fact that all three changes in the passaged 5-55 would restore bonds in the potential stem loop indicates that there is selection pressure to maintain the structure. The stem loop can sustain the loss of bonds without affecting the ability of the virus to infect, but there must be selection pressure for the chemical bonds in the stem loop to remain intact.

The apparent selection pressure to maintain the stem loop in the capsid gene of CCMV indicates that it probably has a function in the life cycle of the virus. Stem loops have previously been identified as signals for encapsidation (Zimmern, 1977, Qu and Morris, 1997) and replication (Pogue and Hall, 1992) in plant RNA viruses, as well as contributing to RNA stability. A similar stem loop structure could not be found in RNAs 1 or 2 of BMV or CCMV, possibly indicating that its function is specific to RNAs 3 and 4. It has been shown that RNAs 3 and 4 are encapsidated together at equimolar concentrations significantly higher than the levels of encapsidated RNAs 1 and 2 (Loesch-Fries and Hall, 1980), and RNAs 3 and 4 are translated more efficiently than either RNA 1

or 2 (Brisco et al., 1986), and that BMV and CCMV can transencapsidate the RNA of the other bromovirus (Allison et al., 1988) In addition, the potential BMV and CCMV stem loops are in the same region of the same gene as the origin of assembly for turnip crinkle virus (Qu and Morris, 1997). The stem loop could represent the CCMV encapsidation signal or a translational signal, but we have no evidence to assign function to the CCMV stem loop at this time.

In contrast to recombinant 5-55, recombinant 3-57 began to display wild type symptoms on cowpeas after only three passages, possibly indicating changes in its genetic makeup. At this point the 3-57 virus moved systemically at wild type rates, and virions could be isolated after four passages, indicating that full length capsid protein was being produced. Sequence analysis of fourth passage 3-57 clones determined that all of the mutations in the capsid gene reading frame had been reverted to wild type, with the exception of the single base deletion responsible for early termination (Fig. 2). Analysis of the region upstream of the capsid gene indicated two mutations had resulted in the insertion of an initiation codon, which is in frame when the single base deletion is present (Fig 5). These mutations compensated for the single base deletion and resulting frameshift, allowing for the translation of full length capsid protien and the formation of virions. Although the 3' two-thirds of the CCMV capsid gene can support systemic movement, the specific changes and the number of passages required to achieve them indicate strong selection pressure for mutations that restore a full length capsid gene to the 3-57 recombinant.

Both recombinants incurred specific mutations over the course of serial passaging that brought the sequence closer to wild type CCMV, indicating strong selection pressure

for the wild type capsid gene at both the RNA and protein level. In addition, no mutations were ever recovered from multiple clones of passaged wild type CCMV RNA 3, further confirming selection pressure for wild type sequence. The reversion mutations seen in 5-55 and 3-57 were consistent in numerous clones from independent cloning experiments, indicating that the changes observed in the passaged recombinants were selectively advantageous in comparison to the sequence of the original recombinant. Interestingly, 5-55 maintained the silent mutations at the 3' end of the capsid gene, suggesting that this region of CCMV RNA 3 may not be under as strong of selection pressure as the remainder of the capsid gene.

Perhaps most interesting is the speed at which selection affected recombinants 5-55 and 3-57. Both recombinants were dramatically changed in ways that brought them more in line with wild type CCMV in less than ten passages. The reversions were always extremely precise, and no additional mutations were found that did not seem to have a significant biological function. The recombinants were passaged alone, and no transgenic source of wild type CCMV capsid gene was available, so error repair could not have occurred via recombination. There is no evidence to suggest that the CCMV RdRp is less error prone than the RdRp's of other RNA viruses, especially considering the substitution mutations that occurred in a small number of passages. If the RdRp of CCMV is no more accurate than other RdRp's, the only remaining possible explanation for the reversion to wild type sequence is high selection pressure to restore or maintain the wild type CCMV capsid gene and protein in the regions where 5-55 and 3-57 had mutations due to recombination.

In conclusion, there appears to be strong *in vivo* selection for the wild type CCMV capsid gene sequence. The selection pressure for wild type sequence is so strong, the mutations generated by recombination or other mechanisms will quickly revert to near wild type sequence. Theoretically, strong selection pressure such as this would reduce the quasispecies cloud size. If selection pressure for wild type bromovirus sequence is as strong as our experiments indicate, the CCMV quasispecies that theoretically exists for all RNA viruses may have significantly less sequence variation than what has been reported for other RNA viruses.

SUMMARY AND CONCLUSIONS

The goal of this research was to examine recombination and selection in natural infections of bromoviruses and apply what was learned to risk assessment of virus resistant transgenic plants (VRTPs). Transgenic viral resistance is economically attractive, but has raised concerns regarding the possibilities of transgenically expressed viral RNA may participate in the evolution of new pathogens via recombination. This thesis builds on the work of Greene and Allison (1994; 1996), which demonstrated the potential for recombination between transgenically expressed viral RNA and challenging viruses.

Greene and Allison (1994) took advantage of the CCMV requirement for the capsid gene to set up a sensitive bioassay for recombination in transgenic plants. The requirement for the capsid gene had been established by testing the biological activity of several RNA 3 deletion mutants (Allison et al., 1990). Although these experiments demonstrated the need for the capsid gene for systemic movement, they did not distinguish between the role of the capsid protein and the virion itself in systemic movement, since the deletions always prohibited translation of the remaining capsid gene. The recovery of a recombinant that moved systemically but did not form virions led to questions about the capsid gene requirements for CCMV systemic movement.

Chapter One experimentally determined that the carboxy-terminal two-thirds of the CCMV capsid protein is sufficient to allow systemic movement. Thus, the CCMV capsid protein is multifunctional, with a distinct long distance movement function in addition to

role in virion formation. The CCMV capsid protein is one of many multifunctional plant viral proteins, a fact which is not suprising, considering the limited genome capacities of plant viruses and the numerous functions required for infection. This also emphasizes a potential problem with capsid gene mediated resistance. Many plant virus capsid proteins have roles in the systemic movement of the virus. It is also known that plant viruses that are systemic pathogens of a given host are capable of acting as a helper virus by facilitating the systemic movement of otherwise non-pathogenic viruses. The expression of capsid gene RNA in transgenic plants may create recombination opportunities that convert non-systemic viruses to systemic pathogens. Thus, it would be prudent to thoroughly examine the biological roles of viral genes selected for transformation purposes.

Publication of the original CCMV transgenic recombination research raised concerns about the possibilities for the evolution of new pathogens in virus resistant transgenic plants. Other researchers, however, proposed that such opportunities were no different than the opportunities for recombination that had already existed naturally in mixed infections (Falk and Bruening, 1994). After all, viruses constantly come into contact with the RNA of other viruses all the time in mixed infections, providing recombination opportunities. Certainly there is ample evidence for past recombination events in the genomes of many RNA viruses (for review, see Simon and Bujarski, 1994). This led us to examine the opportunities for recombination in bromovirus mixed infections.

The experiments in Chapters Two and Three were designed to examine bromovirus mixed infections, with the goal of comparing the recombination opportunities in mixed infections to those in transgenic plants. As predicted, we found that recombination did occur in mixed infections. However, recombination was only observed

in situations where there was obvious selection for recombinants, never in mixed infections of wild type bromoviruses. Perhaps more importantly, recombination was observed in mixed infections only when both viruses were inoculated at the same time. Our data indicates that mixed infections of bromoviruses were most likely to happen when the viruses were inoculated in a staggered fashion, which quite likely may be the case for viruses in nature as well. In delayed mixed infections, no recombination was detected even in the mixed infections of capsid protein gene mutants. Thus, the opportunities for recombination in mixed infections of viruses may be quite different than the opportunities for recombination in transgenic plants constituitively transcribing viral RNA.

In addition, VRTPs may provide novel opportunities for interviral recombination that do not exist naturally. Most plant viruses have limited host ranges, a factor which lowers the possibility of two viruses coming into contact through mixed infections. However, resistance is often at the level of movement, and most plant viruses can replicate in the cells of non-hosts. Theoretically, if two viruses can replicate in the same cell, the opportunity for recombination exists. However, if a plant virus is to recombine with another virus in mixed infection of a non-host plant, the virus must enter and begin replicating in a cell at the same time as the RNA of the second virus is available for recombination. In contrast, transgenic plants provide a steady state level of unencapsidated viral RNA in every cell, providing a supply of potential recombination material for non-pathogens.

Perhaps the most interesting contribution of the mixed infection recombination study may be the discovery of a correlation between selection pressure for efficiency in viral movement and recombination rates. Recombinants were only recovered in mixed

infections where the inoculum was either movement defective (SR1/SR2) or showed slowed movement (AJ8/AJ11). In addition, all of the transgenic recombination studies have made use of movement defective mutants. Recombinants were never recovered in mixed infections of wild type bromoviruses, or in control transgenic plants inoculated with wild type bromoviruses. There are a number of possible explanations for this correlation, the simplest being that recombinants cannot compete with the wild type virus in terms of movement and/or replication. Regardless of the mechanism, the apparent effects of selection pressure on recombination levels led us to investigate the selection pressure for wild type capsid gene sequence in CCMV.

The experiments in Chapter Four describe the effects of selection on genetically and phenotypically distinct viral recombinants over the course of serial passaging. When passaged, both of the recombinants reverted towards wild type sequence in less than ten passages. There were additional mutations outside of the reversions, but these were compensating mutations which either permitted translation of the full length protein or stabilized a potential RNA secondary structure. Despite the high error rates associated with viral RNA-dependent RNA polymerases, there were no spurious mutations elsewhere in the sequenced area, including the untranslated regions. This indicates that there is strong selection pressure for the wild type CCMV sequence *in planta*.

The strong selection for wild type sequence and the lack of recombinant recovery in wild type infections may indicate that recombination between wild type viral pathogens and transgenically expressed viral RNA may be a minor concern. The opportunities for evolution of new pathogens via recombination increase, however, when selection pressure for movement-effecient recombinants increases. Thus, the greatest unique risk presented

by VRTPs may be the situations where transgenic plants are infected by a non-pathogenic virus capable of replicating in the cells of the transgenic plant, as suggested by Allison et al. (1996). In these cases selection pressure for a viable recombinant capable of systemic movement is quite high. There are already reports of functional hybrid viruses (Sacher et al., 1988; Ishikawa et al., 1991), including functional hybrid recombinants derived from transgenic plants (Greene, 1995). Still, it is difficult to predict how often a functional hybrid would arise in the field, because little is known about the viral components that contribute to viral movement and host specificity. However, we can fairly safely predict that as VRTPs are released, the expressed transgenes will be involved in recombination with challenging viruses.

The work of our lab has led to three specific recommendations regarding the engineering of VRTPs (Allison et al., 1996). We have recommended that 1) known or predicted replication initiation sites should be excluded from transgenes, 2) the transgene should be the smallest possible fragment of viral genome which provides resistance, and 3) transgenic plants should be chosen for the maximum resistance with the minimal amount of transgene expression. Based on the data presented in this thesis, it seems prudent to further suggest that we should have a detailed knowledge of the biological roles of the genes chosen for transformation. Additionally, further research should be done on the role of selection in the promotion of recombinant recovery.

APPENDIX

APPENDIX

Separation of brome mosaic bromovirus and cowpea chlorotic mottle bromovirus virions using differential isoelectric points and electrophoresis

Brome mosaic bromovirus (BMV) and cowpea chlorotic mottle bromovirus (CCMV) are two closely related members of the bromovirus family. Although the two viruses have markedly distinct host ranges, mixed infections are possible in the common systemic host *Nicotiana benthamiana*. Experimentally, when analyzing mixed infections of bromoviruses it is helpful to separate the BMV virions from the CCMV virions. However, the standard virion preparations used to isolate bromoviruses result in the recovery of both BMV and CCMV. BMV and CCMV can be separated by mechanically passaging the mixed viruses to virus specific hosts, but this requires an additional two weeks for viral propagation. Additionally, passaging does not allow for the examination of viral populations directly from mixed infections.

Even though BMV and CCMV have significant homology in their serologically related capsid proteins, (Lane, 1981), the two viruses have distinct isoelectric points, BMV at pH 6.8 and CCMV at pH 4.0 (Lane, 1974). Theoretically, this difference in isoelectric points can be used to separate BMV and CCMV electrophoretically. At pH's above a given virus isoelectric point the virions of that virus will run toward the positive electrode, and at pH's below the isoelectric point the virions will run toward the negative

electrode. Theoretically, BMV and CCMV will migrate in opposite directions during gel electrophoresis if the pH is maintained between the isoelectric points of the two viruses.

This theory was tested using complete virions in agarose gels as described by Ramsdell (1979). Electrophoretic mobility of BMV and CCMV virions were determined in 0.6% agarose gels made in 0.02 M dibasic sodium phosphate and 0.02 M Tris buffer titrated to pH 5.0 with citric acid. The electrophoresis buffer was also 0.02 M dibasic sodium phosphate and 0.02 M Tris buffer titrated to pH 5.0 with citric acid. The agarose gel was cast with a comb in the middle of the gel, and approximately 50 μg of virions purified from either BMV infected *N. benthamiana*, CCMV infected *N. benthamiana*, or BMV/CCMV mixed infections were loaded into each well. The gel was run at 75 watts of power for one hour and fifteen minutes, taking care to maintain the temperature of the gel and buffer at or around 4° C. The virions were visualized most efficiently by staining the viral RNA with ethidium bromide (Figure 1). Staining the agarose gel with coomassie blue stain for three hours followed by destaining with 10% acetic acid for three hours proved to be a less efficient means of detecting virions within the agarose matrix (data not shown). In all cases, the CCMV virions migrated towards the positive electrode and the BMV virions migrated towards the negative electrode (Figure 1). Following electrophoresis, the virion band was removed from the gel, crushed in an eppendorf tube, and approximately three volumes of 0.01 M sodium phosphate buffer was added. This mixture was used to inoculate BMV or CCMV host plants. Systemic BMV and CCMV infections were recovered, but the infection rate was quite low (data not shown). This indicates that intact full length viral RNA was present in the virions, and could be extracted for reverse transcription-PCR analysis.

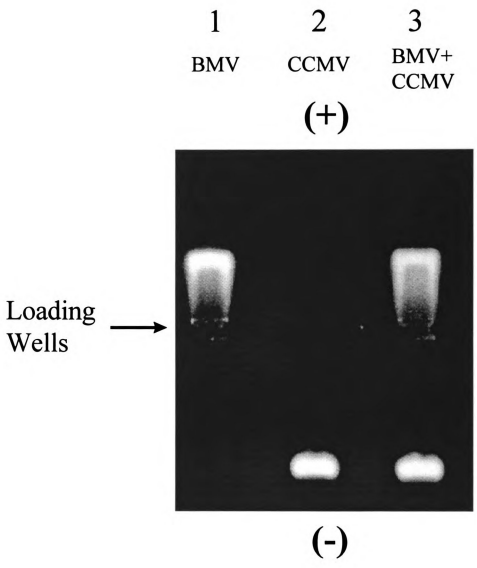

Figure 1. Agarose gel used to separate BMV and CCMV virions based on isoelectric points. Lane 1 is loaded with virions from BMV infected *N. benthamiana*, land 2 is loaded with virions from CCMV infected *N. benthamiana*, and lane 3 is loaded with virions derived from BMV/CCMV mixed infection *N. benthamiana*. The wells are located in the middle of the gel, indicated by the arrow. BMV virions migrate toward the positive electrode (+), and CCMV virions migrate toward the negative electrode (-).

This demonstrates that BMV and CCMV can be separated using electrophoresis in buffers that have a pH between the isoelectric points of the two viruses. This method could perhaps be used to separate two or more viruses in other situations where isolation procedures are insufficient to do so.

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