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Isolation of Recombinant Viruses between Cauliflower Mosaic Virus and a Viral Gene in Transgenic Plants under Conditions of Moderate Selection Pressure

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We demonstrate that recombinant viruses formed between a wild-type virus and a viral transgene can be isolated from transgenic plants under conditions of moderate to weak selection pressure. We inoculated cauliflower mosaic virus (CaMV) strain W260 to transgenic *Nicotiana bigelovii* plants that expressed a copy of CaMV gene VI derived from CaMV strain D4, a gene that determines systemic infection of solanaceous species, including *N. bigelovii*. Because W260 infects nontransformed *N. bigelovii* systemically, a recombinant virus formed between W260 and the D4 transgene would be expected to have little selective advantage over the wild-type W260 virus. W260 was inoculated to approximately 100 plants each of nontransformed and transgenic *N. bigelovii* and it systemically infected nearly all of the plants. An analysis of viral DNA recovered from 23 transgenic plants infected with W260 revealed that 20 infections resulted from the systemic movement of the wild-type W260 virus, while a recombinant between W260 and the D4 transgene was detected in three of the infections. To determine the percentage of recovery of recombinant viruses under strong selection pressure, we inoculated approximately 100 nontransformed and 100 D4 gene VI transgenic plants with CaMV strain CM1841, a virus that is unable to infect nontransformed *N. bigelovii*. CM1841 infected 36% of the transgenic plants systemically, but none of the nontransformed controls. An analysis of 24 infected plants showed that a recombination event occurred in every plant, demonstrating that under strong selection conditions, the recovery of CaMV recombinants from transgenic plants can be very high.

INTRODUCTION

Recombination between closely related plant virus strains has been thoroughly documented for a number of virus groups, including caulimoviruses (Howell *et al.*, 1981; Lebeurier *et al.*, 1982; Chenault and Melcher, 1994), bromoviruses (Bujarski and Kaesberg, 1986; Nagy and Bujarski, 1993), and carmoviruses (Cascone *et al.*, 1990, 1993). In addition, intramolecular and intermolecular recombination events leading to the formation of defective interfering RNAs have been observed for many plant viruses (reviewed in Simon and Bujarski, 1994). In each case, recombination has been shown to involve a copy choice mechanism, in which a viral replicase switches templates during RNA synthesis (Simon and Bujarski, 1994) or a viral reverse transcriptase switches templates during reverse transcription (Dixon *et al.*, 1986; Grimsley *et al.*, 1986; Vaden and Melcher, 1990). As data on recombination between viruses has accumulated, it has recently become possible to consider the potential for recombination between the viral genome and viral sequences present in transgenic plants. A great number of transgenic plants have been developed that are resistant to viral infection because they express entire viral genes or portions of viral genes (Beachy *et al.*, 1990; Scholthof *et al.*, 1993; Wilson, 1993). One question that is still unresolved is, what is the capacity for genetic exchange between plant viruses and transgenic plants that contain viral genes?

Until recently, there was no evidence that plant viruses were capable of recombination with their transgenic hosts. Since 1991, however, four studies have demonstrated that plant viruses can acquire an assortment of viral genes from transgenic plants. Lommel and Xiong (1991) showed that a red clover necrotic mosaic virus (RCNMV) isolate defective in cell-to-cell movement recombined with a copy of its cell-to-cell movement gene present in transgenic *Nicotiana benthamiana* plants. Cauliflower mosaic virus was shown to acquire a copy of its gene VI, a gene identified as a translational transactivator, from either transgenic *Brassica napus* (Gal *et al.*, 1992) or transgenic *Nicotiana bigelovii* (Schoelz and Wintermantel, 1993). Greene and Allison (1994) demonstrated that a cowpea chlorotic mottle virus (CCMV) isolate that contained a defective coat protein gene could acquire a functional copy through recombination with transgenic *N. benthamiana* plants that contained CCMV coat protein sequences.

One feature common to these studies is that strong selection pressure was used for the isolation of the recombinant from the transgenic plants. Strong selection

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pressure can be defined as the inability of a virus to infect a host systemically unless a recombination event occurs. In three of the studies, deletion mutants that were defective for systemic infection were inoculated to the transgenic plants (Lommel and Xiong, 1991; Gal *et al.*, 1992; Greene and Allison, 1994). The only viruses capable of systemic infection were those formed by recombination between the mutant virus and the transgene. In one study, which documented recombination between CaMV strain CM1841 and transgenic *N. bigelovii*, the selective pressure was conferred by a difference in the host range determinants present in the viral inoculum and the host range determinants present within the viral transgene (Schoelz and Wintermantel, 1993). CM1841 causes systemic infections in a wide range of crucifers, but cannot infect nontransformed *N. bigelovii* plants systemically. This virus was inoculated to transgenic *N. bigelovii* that expressed the gene VI product of CaMV strain D4, a CaMV gene that determines systemic infection of solanaceous species including *N. bigelovii* (Schoelz *et al.*, 1986a). Because sequences within the CaMV transgene specified systemic infection of *N. bigelovii*, a recombinant that acquired the transgene also acquired the ability to systemically infect *N. bigelovii*, and therefore gained a selective advantage over CM1841.

Based upon these studies, it has been suggested that recombinant viruses may not become established in transgenic plants unless the transgene confers a significant selective advantage over the wild-type virus (Schoelz and Wintermantel, 1993; Falk and Bruening, 1994). We now demonstrate, however, that recombinant viruses can be isolated from transgenic plants even when the initial virus can systemically infect the host, conditions that can be described as moderate to weak selection pressure.

MATERIALS AND METHODS

Cauliflower mosaic virus strains, chimeric viruses, and transgenic plants

The cauliflower mosaic virus strains CM1841, W260, and D4 and the chimeric virus H31 have been described previously (Lung and Pirone, 1972; Gracia and Shepherd, 1985; Schoelz *et al.*, 1986b; Schoelz and Shepherd, 1988). Each of these viruses has been cloned in infectious form into bacterial plasmid vectors (Howarth *et al.*, 1981; Schoelz *et al.*, 1986b; Schoelz and Shepherd, 1988) and infections have been established in turnips from the cloned DNAs to eliminate natural variants in the populations. The wild-type viruses and chimeric virus H31 have been stored in lyophilized turnip leaf tissue at 4° for use as inoculum in experiments.

Transgenic *N. bigelovii* that express a chimeric gene VI product have been described previously (Schoelz *et al.*, 1991). The gene VI coding region is derived primarily from CaMV strain D4 (Fig. 1) and contains the sequences of D4 required for systemic infection of solanaceous species, including *N. bigelovii* (Schoelz *et al.*, 1986; Schoelz and Wintermantel, 1993). All transgenic *N. bigelovii* used in these studies were hemizygous for gene VI, and were obtained from a cross between homozygous transgenic plants and nontransformed *N. bigelovii*.

Virus inoculum was prepared from infected turnips as described in Schoelz *et al.* (1986a) or as purified virions (Hull *et al.*, 1976). The nontransformed *N. bigelovii* and transgenic *N. bigelovii* were inoculated approximately 5 weeks after seeds were sown. The inoculated plants were maintained for 42 to 56 days either in growth chambers (Percival Scientific Inc., Boone, IA; Conviron, Asheville, NC) with a 10-hr photoperiod at 18° at a light intensity between 200 and 350 $\mu E/m^2/s^1$ or in the greenhouse during the months of October through April.

Recovery of virion DNA from transgenic and nontransformed plants

Virion DNA was recovered from transgenic plants by (1) passage through turnips and subsequent purification from turnips or (2) directly from transgenic plants by use of PCR. For passaging, turnips (*Brassica rapa* cv Just Right) were inoculated with extracts from systemically infected wild-type or transgenic *N. bigelovii* leaves. The infection was allowed to develop, and virion DNA was purified from the turnips approximately 5 weeks after inoculation by the procedure of Gardner and Shepherd (1980).

A second procedure for purification of virion DNA involved the use of PCR. All equipment in contact with samples was either disposable or was autoclaved prior to use, and all solutions were sterilized to avoid contamination by viral DNA. One gram of *N. bigelovii* tissue exhibiting CaMV symptoms was collected and pulverized by grinding in liquid nitrogen with a mortar and pestle. Pulverized tissue was suspended in 4 ml DNase I buffer (100 m*M* Tris–Cl, pH 7.5; 5 m*M* magnesium chloride) and centrifuged at 3000 *g* for 5 min. The supernatant was filtered through 1 layer of Miracloth (Calbiochem, La Jolla, CA) into a new centrifuge tube, and the pellet was discarded. DNase I was added to a concentration of 10 μg/ml, and the solution was incubated at 37° for 30 min to degrade chromosomal DNA. The DNase I was inactivated by the addition of 5 m*M* EDTA. To release viral DNA from the virion, SDS and proteinase K were added to concentrations of 1% and 100 μg/ml, respectively, followed by incubation at 37° for 30 min. The solution was subsequently extracted with phenol and the DNA precipitated with ethanol. To remove additional impurities in the DNA, pellets were resuspended in 0.5 ml TE, pH 7.5, mixed with Magic DNA clean-up resin (Promega Corp., Madison, WI), passaged through a Promega minicolumn, and eluted with 100 μl of TE.

To amplify the virion DNA by PCR (Saiki *et al.*, 1985;

Mullis and Faloona, 1987), the DNA recovered from transgenic plants was combined with reaction components to final concentrations of 200 μM each of dATP, dGTP, dCTP, and dTTP, 1 μM for each oligonucleotide primer (Fig. 1A), 1 unit Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA), and 1X Vent polymerase buffer. The nucleotide sequence of the primer BW18 was 5′-ACTTCACGTAGCAATGATCTTACA-3′ (CM1841 coordinates, 4877 to 4900; Gardner *et al.*, 1981) and the nucleotide sequence of the primer BW19 was 5-ATTCATGGCTCTGATACCAAT-3′ (CM1841 coordinates, 18 to 8029). The solution was adjusted to a volume of 100 μl with dH_2O, and an equal volume of light mineral oil was layered over the reaction mix. Cycles consisted of denaturation at 94°, annealing at 53°, and polymerization at 72°. Upon completion of PCR, DNA was extracted with chloroform and precipitated with ethanol. Viral DNA and the PCR-amplified viral DNA fragment were screened for complementation or recombination by digestion with the restriction enzyme *Eco*RI.

For competition studies between W260 and W260R, viral DNA was purified from 5–8 g of systemically infected nontransformed *N. bigelovii* leaves as in Gardner and Shepherd (1980). The *Eco*RI-cleaved viral DNA was separated on a 1.5% agarose gel, transferred to nitrocellulose, and probed with ^{32}P-labeled DNA as described in Maniatis *et al.* (1982). The probe consisted of equal proportions of the 459-bp *Eco*RI fragment of CM1841 and the 397-bp *Eco*RI fragment of D4.

Identification of recombination junctions

Recombination junctions were identified by sequencing of cloned viral DNA according to the dideoxy chain termination method of Sanger *et al.* (1977) using oligonucleotide primers complementary to viral sequences. Viral DNA was recovered from transgenic plants by PCR amplification as described above. The 3172-bp viral DNA fragment was cloned into the bacterial plasmid vector pGEM-7Zf(+) (Promega Corp., Madison, WI) at unique *Xba*I and *Cla*I restriction enzyme sites located in the viral DNA near the ends of the amplified fragment (Fig. 1A).

General molecular techniques

Techniques for transformation of *Escherichia coli* strain JM101, purification of cloned DNA from bacteria, restriction enzyme digests, ligation, and electrophoresis were from Maniatis *et al.* (1982). Restriction enzymes and T4 DNA ligase were purchased from Promega Corp. (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotide primers for PCR and DNA sequencing were synthesized by the DNA Core Laboratory of the University of Missouri at Columbia. Enzymes and solutions for DNA sequencing were purchased in the Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH). Radioisotopes for DNA sequencing were purchased from New England Nuclear (Boston, MA). Chemicals were purchased from Sigma Chemicals (St. Louis, MO), Boehringer-Mannheim Biochemicals (Indianapolis, IN), and Fisher Scientific (Pittsburgh, PA).

RESULTS

Comparison of viral DNA populations isolated directly from transgenic *N. bigelovii* to populations recovered after passaging through turnips

In a previous study designed to identify recombinants between CaMV and viral transgene sequences, viruses were passaged from transgenic *N. bigelovii* to turnips before purification of viral DNA (Schoelz and Wintermantel, 1993). Passaging through turnips was considered necessary because not only are CaMV concentrations much lower in solanaceous species than in turnips, but the viral DNA isolated directly from solanaceous species could not be cleaved consistently with restriction enzymes. A potential disadvantage of passaging virus through turnips is that this extra step might promote the selection of either complemented or recombinant viruses. To verify that the virus population recovered after passaging through turnips was representative of the virus present in transgenic *N. bigelovii*, the polymerase chain reaction was used to amplify viral DNA directly from small amounts of transgenic *N. bigelovii* tissue.

To ensure that the gene VI transgene sequences in *N. bigelovii* would not be amplified, oligonucleotide primers for PCR were selected that flanked the CaMV sequences present within the transgenic plants (Fig. 1A). In addition, transgenic plant samples were treated with deoxyribonuclease (DNase) to degrade chromosomal DNA prior to the isolation of the viral DNA from virions. Previous studies have shown that viral DNA contained within the virion is protected from DNase digestion (Gardner and Shepherd, 1980). Although a smear of amplified material was observed in samples of uninfected transgenic plants, no viral DNA bands were amplified, demonstrating that these procedures were sufficient to prevent the amplification of viral transgene sequences. In contrast, a 3172-bp segment of viral DNA, which contained the entire transgene VI transcriptional unit, was amplified by PCR from transgenic plants that developed systemic symptoms. (Fig. 1B, lanes 1 and 2).

Regardless of the technique used to isolate viral DNA from transgenic plants, restriction enzyme mapping with *Eco*RI revealed that viruses recovered from the transgenic plants could be divided into three groups: wild-type (the virus originally inoculated), recombinant, or a mixture of wild-type and recombinant. A typical analysis of the *Eco*RI sites of viruses recovered from the transgenic plants is illustrated in Fig. 1. Wild-type viruses could be distinguished from recombinants by the presence of an *Eco*RI site within gene VI at nucleotide position 6105 (Fig. 1A). An *Eco*RI digest of a wild-type viral

DNA recovered from the transgenic plants by either PCR amplification or passage through turnips results in the appearance of a 459-bp DNA band (Fig. 1B, lane 4, and Fig. 1C, lane 2), in addition to other bands. Viruses that arose through recombination with transgene sequences were distinguished by the presence of an *Eco*RI site within gene VI at nucleotide position 6043 (Fig. 1A), a site present only in the transgene. Nucleotide sequencing of gene VI of recombinant viruses recovered from the transgenic plants has demonstrated that this *Eco*RI site is a reliable marker for recombination (Schoelz and Wintermantel, 1993). An *Eco*RI digest of a recombinant virus results in the appearance of a 397-bp DNA band (Fig. 1B, lane 6, and Fig. 1C, lane 4). Mixtures of recombinant and wild-type viruses recovered from a single plant could be identified through the presence of both the 459- and 397-bp DNA bands (Fig. 1B, lane 5, and Fig. 1C, lane 3).

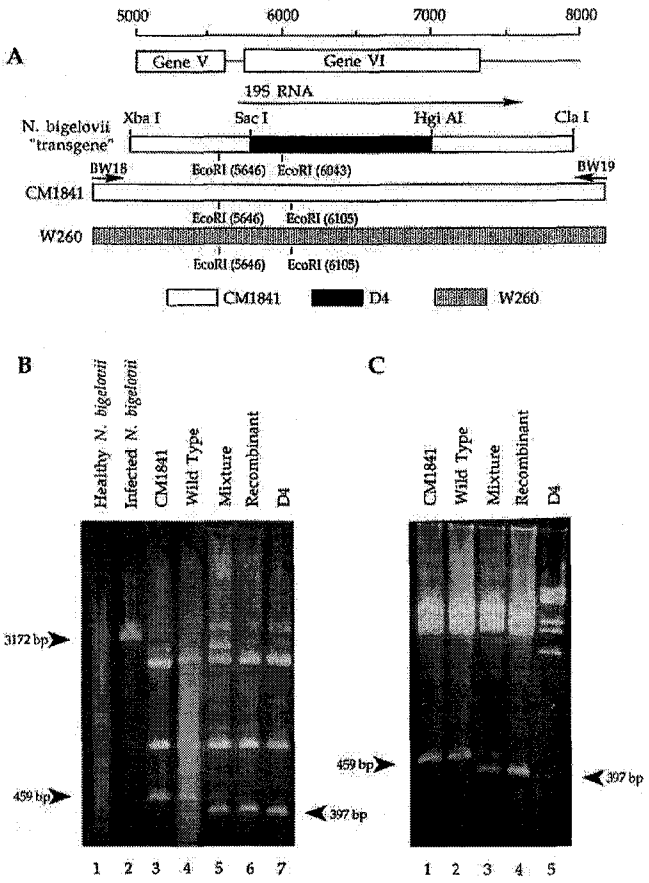

FIG. 1. Identification of wild type, recombinant, and mixed populations of viruses isolated from transgenic *N. bigelovii* through PCR or by passaging through turnips. (A) Partial *Eco*RI maps of CM1841, W260, and CaMV transgene sequences. Transgenic plants contained a *XbaI–ClaI* DNA segment of CaMV and produced a mRNA from the CaMV 19S promoter and polyadenylation signals. A *SacI–HgiA*I DNA segment of the transgene was derived from CaMV strain D4. This segment contained the essential D4 sequences necessary for systemic infection of solanaceous species. Flanking regions of the transgene were derived from strain CM1841. The primers BW18 and BW19 used for PCR are indicated by arrows flanking the *XbaI* and *ClaI* sites. (B) Direct isolation of virion DNA from transgenic plants by PCR. Viruses were isolated from systemically infected transgenic *N. bigelovii,* and viral DNA was amplified by PCR. Samples in lanes 3–7 were cleaved with *Eco*RI. The DNA fragments were separated on a 1.5% (w/v) agarose gel and visualized by staining with ethidium bromide. The arrows indicate the *Eco*RI polymorphism between the transgene and the viral inoculum. The *Eco*RI restriction enzyme patterns of wild-type viral DNA, recombinant viral DNA, and a mixture of recombinant and wild-type DNA recovered from transgenic plants were compared to cloned DNA of CM1841 and D4 that had been amplified by PCR. (C) Indirect isolation of virion DNA after passage through turnips. The viruses present in systemically infected transgenic *N. bigelovii* were passaged to turnips and viral DNAs purified from infected turnip leaves. Viral DNAs were cleaved with *Eco*RI and analyzed as in Fig. 1B. The CM1841 digest consists of viral DNA that had not been passaged through the transgenic plants. The D4 digest consists of the full-length D4 virus cloned into pBR322.

TABLE 1

Comparison of Viruses Recovered Directly from Transgenic *N. bigelovii* by PCR and by Viral DNA Isolation after Passaging through Turnips

Virus	Number of comparisons	Transgenic *N. bigelovii*	Turnip
W260	5	Wild type	Wild type
	1	Mixture	Mixture
CM1841	2	Recombinant	Recombinant
H31[a]	3	Wild type	Wild type
	1	Recombinant	Recombinant

[a] H31 is a chimeric virus composed of W260 and CM1841 sequences. H31 sequences derived from W260 are illustrated in Fig. 3.

To verify that the virus passaged through turnips was representative of the population present in systemically infected leaves of transgenic plants, we compared the *Eco*RI restriction enzyme maps of viral DNA after PCR amplification from transgenic plants to viral DNA obtained after passaging through turnips. We made twelve comparisons using three different CaMV viruses, and in each case the results obtained with one technique were confirmed with the other (Table 1). This study demonstrated that passaging through turnips and the direct isolation technique were equally valid for analyzing viruses recovered from transgenic plants.

Recovery of recombinant viruses from transgenic plants under conditions of strong and moderate selection pressure

We have previously demonstrated that CM1841 was able to recombine with CaMV sequences present in transgenic *N. bigelovii,* but at that time did not assess the percentage of recovery of recombinant viruses from transgenic plants. To determine the percentage of recovery of recombinants in the presence of strong selection pressure, CM1841 was inoculated to approximately 100 plants each of nontransformed and transgenic *N. bigelovii.* None of the nontransformed *N. bigelovii* became systemically infected with CM1841, which is in agreement

TABLE 2

Isolation of Recombinant Viruses from Transgenic *N. bigelovii* Plants

Virus	Number of tests	Number of plants with systemic symptoms[a]		Number of infections analyzed[b]			
		Wild-type *N. bigelovii*	Transgenic *N. bigelovii*	W	R	M	Total analyzed
W260	12	101/102	104/106	20	1	2	23
H31	13	0/114	79/116	29	2	1	32
CM1841	13	0/102	38/106	0	22	2	24

[a] The total number of plants which developed systemic symptoms/the number of plants inoculated.

[b] Number of infected plants analyzed in which the systemic infection resulted from: (W) the wild-type virus; (R) recombinant with the transgene; (M) a mixture of wild-type and recombinant viruses.

with our previous findings (Schoelz and Shepherd, 1988; Schoelz *et al.*, 1991). In contrast, 36% of the transgenic plants developed systemic symptoms (Table 2). To characterize the virus responsible for those infections, we isolated virus from systemically infected leaves of 24 transgenic plants and cleaved the viral DNA with *Eco*RI. The *Eco*RI digests revealed that only the recombinant virus was present in 22 of the infected plants, while a mixed population of wild-type and recombinant viruses was isolated from the remaining two plants.

To determine whether recombinant viruses could be recovered from transgenic plants under conditions of lower selection pressure, we examined the infections of transgenic *N. bigelovii* caused by CaMV strain W260 and the chimeric virus H31, a virus in which gene VI is derived from W260 and genes I–V from CM1841. W260 systemically infects nontransformed *N. bigelovii* (Schoelz and Shepherd, 1988). Although H31 cannot infect nontransformed *N. bigelovii* systemically, earlier studies with limited sample numbers had indicated that the transgenic *N. bigelovii* complemented this virus for systemic infection (Schoelz *et al.*, 1991; Schoelz and Wintermantel, 1993). Hence, a recombinant virus formed between either W260 or H31 and the transgene would presumably have little selective advantage over the initial viral inoculum.

Both W260 and H31 were inoculated to approximately 100 nontransformed and 100 transgenic *N. bigelovii* plants. W260 infected almost all of the plants systemically. To characterize the virus responsible for the infections, viral DNA was recovered from systemically infected leaves of 23 transgenic plants and cleaved with *Eco*RI. The *Eco*RI restriction enzyme patterns revealed that 20 plants developed infections resulting from the systemic movement of the wild-type W260 virus, while a recombinant virus was detected in the three remaining plants (Table 2). Two of these three infections consisted of a mixed population of wild-type W260 and a recombinant (Table 2; Fig. 2, lane 2). In one plant, only the recombinant virus was recovered from systemically infected leaves (Table 2; Fig. 2, lane 3), indicating that the recombination event had occurred early after the initial infection and that the wild-type W260 had been excluded from the upper leaves by the recombinant. In contrast to W260, the chimeric virus H31 was unable to infect any of the nontransformed *N. bigelovii* and only 68% of the transgenic plants systemically. An analysis of the *Eco*RI patterns of viral DNA recovered from 32 transgenic plants revealed that 29 infections were caused by the wild-type H31, two infections were caused by the recombinant form of H31, and one infection consisted of a mixed population of recombinant and wild-type viruses (Table 2).

To prove conclusively that W260 and H31 had recombined with the transgene, we characterized the recombination junctions of viral DNA recovered from infections in which only the recombinant was detected (Table 2). The viral DNAs of the W260 and one of the H31 putative recombinants were each cloned into the plasmid vector pUC18 and the recombination junctions identified by nucleotide sequencing of portions of gene VI. The recombination junctions of the W260 recombinant virus were localized to short stretches of homologous sequences that contained the transgene transcript initiation and termination sites (Fig. 3), further evidence that the mechanism of recombination involves template switches between the 35S RNA and the transgene mRNA during reverse transcription (Gal *et al.*, 1992; Schoelz and Wintermantel, 1993). The W260 recombinant virus arose from a template switch during reverse transcription from the 35S RNA to the transgene mRNA that occurred within 67 nucleotides of the 3′ end of the transgene mRNA. A second crossover, from the transgene mRNA back to the 35S RNA, was identified within 73 nucleotides of the 5′ end of the transgene mRNA. In essence, the entire gene VI coding sequence of W260 had been replaced with the corresponding sequence of the transgene.

In the H31 recombinant virus, the recombination junction between the 35S RNA and the 3′ end of the transgene mRNA was identical to that of the W260 recombinant (Fig. 3). The location of the second crossover could not be identified because the nucleotide sequence

of both the transgene and H31 in this region is derived from CaMV strain CM1841. The junction delimiting CM1841 sequences from D4 sequences, however, was present in the H31 recombinant (Fig. 3). This sequence information, coupled with the *Eco*RI restriction enzyme pattern of the H31 recombinant and the location of the 3′ recombination junction, indicated that the entire D4 sequence present in the transgene had been incorporated into the H31 recombinant virus.

Comparison of the competitiveness of the wild-type W260 virus versus the recombinant W260 virus

The observation that a recombinant virus could predominate over the wild-type W260 virus or the chimeric virus H31 might reflect differences in the aggressiveness of the recombinant relative to the initial inoculum. For example, the host specificity information present in the transgene, which is derived from CaMV strain D4, may confer a selective advantage to the recombinant virus over viruses in which gene VI is derived from W260. Although both D4 and W260 are capable of infecting *N. bigelovii* systemically, D4 systemic symptoms usually appear at 18–20 days postinoculation, 4–5 days before those of W260 (Schoelz and Shepherd, 1988).

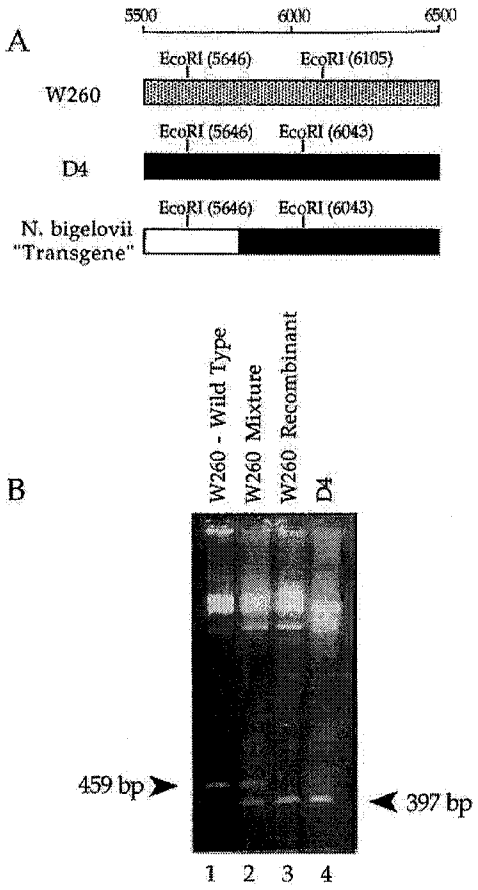

FIG. 2. *Eco*RI restriction enzyme digest of W260 viral DNAs recovered from transgenic *N. bigelovii*. (A) Partial *Eco*RI maps of W260, D4, and CaMV sequences present in transgenic *N. bigelovii*. W260, CM1841, and D4 sequences are indicated by stippling, open boxes, and filled boxes, respectively. Numbers within parentheses indicate nucleotide positions. (B) *Eco*RI restriction enzyme analysis of the W260 viral DNAs recovered from transgenic *N. bigelovii*. Lane 1, W260 inoculum. Lanes 2 and 3, W260 viral DNAs after passage through transgenic *N. bigelovii* and subsequent passage through turnip. Lane 4, D4 plasmid DNA. The DNA fragments were separated on a 1.5% (w/v) agarose gel and visualized by staining with ethidium bromide. The arrows indicate the *Eco*RI fragment polymorphism between D4 and W260 sequences. The 459-bp DNA fragment is indicative of W260 sequences within gene VI while the 397-bp DNA segment is indicative of D4 sequences within gene VI.

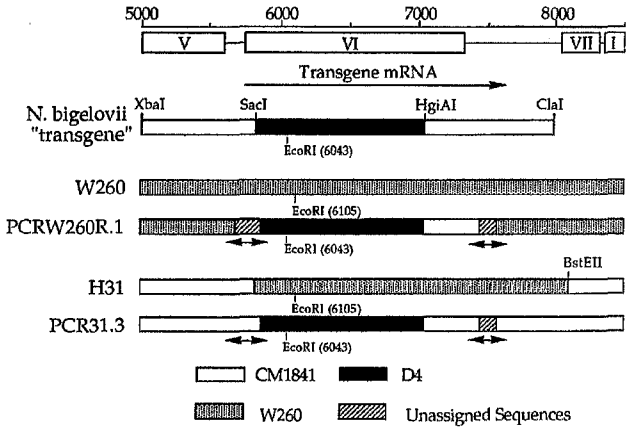

FIG. 3. Location of recombination junctions between CaMV and transgene sequences. The CaMV transgene sequences present in *N. bigelovii* are the same as in Fig. 1A. Virion DNAs were recovered from transgenic plants by PCR using primers BW18 and BW19, and a *XbaI–ClaI* DNA segment was cloned into pGEM-7Zf(+). The double arrows indicate the regions that were sequenced to identify the recombination junctions. The unassigned sequences cannot be identified as being derived from the transgene or the CaMV viral inoculum because of a lack of sequence polymorphisms.

To determine whether the W260 recombinant virus, designated W260R, was more competitive than the wild-type W260, the two viruses were inoculated separately and in varying ratios to nontransformed *N. bigelovii*. To eliminate any potential carryover of wild-type W260 virus in the W260R population, the complete W260R genome was first cloned at its unique *SalI* site into pUC18 and then the cloned W260R virus was inoculated to turnips. The W260 and W260R inoculum was subsequently prepared from infected turnip leaves and purified virions were inoculated to *N. bigelovii* at a concentration of 75 μg/ml. The competitiveness of the two viruses was examined by mixing W260 and W260R virions at ratios of 1:1, 5:1, and 1:5, respectively, at a total concentration of 75 μg/ml.

The infections induced by W260 and W260R were indistinguishable until systemic symptoms developed. There was no difference in the temporal appearance of primary lesions induced by W260 and W260R. Both viruses induced chlorotic primary lesions in *N. bigelovii* leaves at 7–8 days postinoculation. There was also no difference in the specific infectivity of the two virion preparations. W260 induced an average of 82.9 ± 33.7 pri-

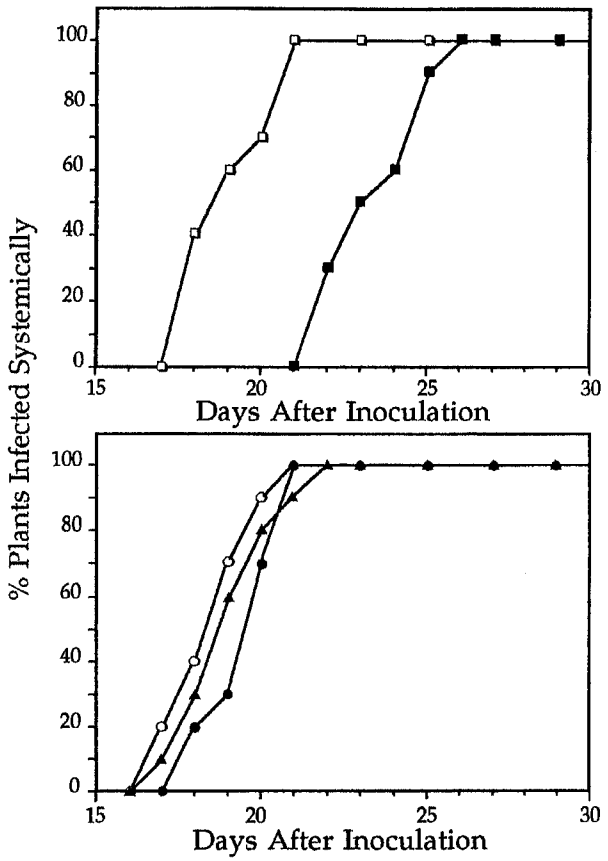

FIG. 4. Appearance of CaMV systemic symptoms in *N. bigelovii* after inoculation with W260, W260R, or varying mixtures of the two viruses. Ten *N. bigelovii* plants were inoculated with W260 (■), W260R (□), a 5:1 mixture of W260 vs W260R (●), a 1:1 mixture of W260 vs W260R (▲), or a 1:5 mixture of W260 vs W260R (○).

mary chlorotic lesions per leaf (n = 10), while W260R induced 89.0 ± 33.2 primary lesions per leaf (n = 10). Although primary lesions of W260 and W260R were initiated at the same time, there was a striking difference in the appearance of systemic symptoms between W260 and W260R. The systemic symptoms of W260R began 4 days prior to those of W260 (Fig. 4), indicating that W260R was more aggressive than W260. Furthermore, the *N. bigelovii* plants inoculated with mixtures of W260 and W260R developed systemic symptoms at the same rate as those inoculated with W260R alone, suggesting that W260R could predominate in mixed infections even when a minor component of the initial virus population.

To identify the virus responsible for the systemic infection in plants inoculated with mixtures of W260 and W260R, viral DNA was purified from each of *N. bigelovii* plants, cleaved with *Eco*RI, and the restriction enzyme patterns were revealed after gel electrophoresis and southern blotting. The restriction enzyme digests indicated that W260R was more competitive than the wild-type W260. Only W260R was recovered from each of the 10 plants inoculated at ratios of 5:1 or 1:1 (W260R vs W260). Of the plants inoculated at a ratio of 1:5 (W260R vs W260), W260R alone was recovered from nine plants, while W260 alone was recovered from one plant (data not shown). A second experiment in which viruses were inoculated at approximately 40 μg/ml yielded the same results.

DISCUSSION

We have demonstrated that virulent recombinant viruses formed between a virus and a viral transgene can be isolated from transgenic plants under conditions of moderate selection pressure. A recombinant formed between the W260 strain of CaMV and a transgene derived from gene VI of the D4 strain of CaMV was detected in three of 23 transgenic *N. bigelovii* plants exhibiting symptoms. In one plant the recombinant was the only virus detected in systemically infected leaves. Chimeric virus H31 was also capable of recombination with the transgenic *N. bigelovii* plants. Results obtained with H31 represent a different situation from W260 because H31 is unable to infect nontransformed *N. bigelovii* and was previously shown to be complemented for systemic infection in transgenic *N. bigelovii* plants (Schoelz *et al.*, 1991). Surprisingly, the level of selection pressure for a defective virus such as H31 can be equivalent to that of a wild-type virus such as W260. An analysis of 32 transgenic plants infected with H31 revealed that 29 infections were caused by the unaltered H31, while the recombinant form of H31 was detected in three of the plants. The chimeric virus H31 was important because it provided an essential confirmation that recombinant viruses could be recovered from transgenic plants under conditions of moderate selection pressure.

Because recombination between CaMV and transgenic plants has been documented only recently, very little is known about competition between wild-type and recombinant viruses in infected transgenic plants. In contrast, previous studies have shown that two CaMV isolates inoculated to the same plant are in competition for the plant's resources, and that there can be clear winners and losers. For example, the Cabbage S isolate has a competitive advantage over other isolates such as W, D/H, and UM130 (Melcher *et al.*, 1986; Zhang and Melcher, 1989). If turnip plants are inoculated with equal amounts of any of these viruses and Cabbage S, only Cabbage S is recovered from the infected plant. An equal mixture of Cabbage S and UM130 was recovered from infected plants only when the ratio of Cabbage S virions to UM130 virions in the inoculum was 2:23. Although mixtures of two isolates can be isolated from infected plants, it is generally believed that CaMV isolates recovered from infected plants in nature consist of one predominant isolate and several populations of minor variants (Vaden and Melcher, 1990; Riederer *et al.*, 1992; Al-Kaff and Covey, 1994).

The observation that a recombinant virus could predominate over the wild-type W260 virus or the chimeric virus H31 was unexpected, but it can be explained by

an increase in aggressiveness conferred by the transgene. An experiment involving direct competition between W260 and the recombinant W260R clearly showed that W260R was more aggressive in *N. bigelovii* than the wild-type W260. Even when five times as much W260 was present in the inoculum as W260R, W260 was recovered from systemically infected leaves of only 1 of 10 *N. bigelovii* plants. This study demonstrates that recombination with a transgene can actually enhance the competitiveness of a virus if the transgene confers even a slight selective advantage and recombination occurs between closely related strains. It is important to note, however, that a selective advantage in one host may not mean that the virus has a selective advantage in all hosts. Significantly, mixtures of wild type and recombinant detected in the transgenic plants remained mixtures even after passage through turnips (Table 1), an indication that the recombinant may not have a selective advantage over the wild type in that host.

Complementation vs recombination

Neither H31 nor CM1841 are able to infect nontransformed *N. bigelovii* plants systemically, yet H31 was primarily complemented for systemic infection while a recombinant virus predominated in transgenic plants inoculated with CM1841. What is the difference between the two viruses? The results obtained with H31 and CM1841 suggest a distinction between viruses that are defective for systemic movement and those that contain dominant negative inhibitors. Two lines of evidence show that CM1841 gene VI is involved in conditioning the dominant negative inhibition exhibited by CM1841. First, the only nucleotide differences between H31 and CM1841 are confined to gene VI and the large intergenic region (Fig. 3), so sequences that condition dominant inhibition must be localized to these regions. Second, several studies have shown that sequences within CM1841 gene VI inhibit the ability of chimeric viruses to infect solanaceous species systemically (Schoelz *et al.*, 1986a; Qiu and Schoelz, 1992; Wintermantel *et al.*, 1993), and in some hosts CM1841 gene VI elicits a hypersensitive response (Schoelz and Shepherd, 1988; Daubert and Routh, 1990), a reaction that is consistent with its role in dominant inhibition. In contrast, the present study shows that the gene VI coding region of H31, which is derived from CaMV strain W260, clearly does not function as a dominant negative inhibitor. H31 is unable to systemically infect nontransformed *N. bigelovii* presumably because the gene VI product of W260 cannot properly interact with genes I–V of CM1841 (Qiu and Schoelz, 1992).

The mechanism of recombination between CaMV and transgenic plants is similar to the recombination mechanism between RNA viruses and transgenic plants

We have shown in this study that the mechanism of recombination between CaMV and transgenic plants involves two template switches during reverse transcription of the CaMV 35S RNA to circular, dsDNA, which is in agreement with previous studies (Gal *et al.*, 1992; Schoelz and Wintermantel, 1993). The first template switch occurs from the 5′ end of the viral RNA to the 3′ end of the transgene mRNA produced by the transgenic plants. A second switch occurs at the 5′ end of the transgene mRNA back to the viral 35S RNA. In essence, the gene VI transgene completely replaces the gene VI coding region present on the viral inoculum. Thus, CaMV recombination can occur between two RNA molecules and is mediated by the viral reverse transcriptase. This recombination mechanism is similar to that of the RNA viruses, in which the RNA polymerase switches from one template to another location on the same template, or to a completely different template (Cascone *et al.*, 1993; Simon and Bujarski, 1994).

Although similar mechanisms are utilized by RNA viruses and CaMV for recombination with viral genes in transgenic plants, the isolation frequency of recombinant viruses is much higher with CaMV. For example, a recombinant form of CCMV was recovered from 3% of transgenic *N. benthamiana* plants containing CCMV sequences under conditions of high selection pressure (Greene and Allison, 1994). In contrast, we found that CM1841 infected 36% of the transgenic *N. bigelovii* inoculated, and all of the infected plants that we examined contained a recombinant virus. The high recovery rate of CaMV recombinants may be a consequence of the replication strategy of CaMV and the high degree of homology at the 3′ ends of the transgene mRNA and the 35S RNA. During first strand DNA synthesis, the viral reverse transcriptase must switch templates from the 5′ end of the 35S RNA to the 3′ end for replication to continue. There is a 180-nucleotide terminal redundancy in the 35S RNA, and the template switch during viral replication occurs within this 180-nucleotide stretch (Mason *et al.*, 1987). The gene VI mRNA produced in transgenic *N. bigelovii* has the same 3′ end as the 35S RNA produced by the viral inoculum, and this may facilitate the template switch from the 35S RNA to the transgene mRNA. In fact, it has been suggested that a template switch between the 35S RNA and the 19S RNA (the viral gene VI mRNA) might be a common occurrence in mixed infections of CaMV (Dixon *et al.*, 1986). Further research will be directed toward determining whether CaMV is more likely to recombine with transgenic plants that express the gene VI mRNA than with transgenic plants that express other CaMV genes.

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