Temporal and Spatial Appearance of Recombinant Viruses Formed Between Cauliflower Mosaic Virus (CaMV) and CaMV Sequences Present in Transgenic *Nicotiana bigelovii*

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Accepted 10 December 1997.

Cauliflower mosaic virus (CaMV) strain CM1841 is able to recombine with a CaMV transgene sequence present in *Nicotiana bigelovii*. In the present study we have characterized the temporal and spatial appearance of recombinant viruses formed between CM1841 and the transgene within individual transgenic plants. CM1841 infections were initiated by mechanical inoculation and by agroinoculation to nontransformed *N. bigelovii* and transgenic *N. bigelovii* that expressed the gene VI product of CaMV strain D4. In agroinoculated transgenic plants, inoculated leaf tissue turned necrotic around the point of agroinoculation, while chlorotic lesions appeared in the leaves inoculated with CM1841 virions. The first systemic symptoms in both agroinoculated and mechanically inoculated transgenic *N. bigelovii* consisted of necrotic patches. The predominant type of virus recovered from the inoculated and first systemically infected leaves was the wild-type CM1841 rather than a recombinant. As the infection progressed in the transgenic plants, symptoms changed from necrosis in the lower leaves to a chlorotic mosaic in the upper leaves. This shift in symptom type was associated with the recovery of recombinant viruses, indicating that the recombinants predominated only in later stages of pathogenesis.

The evolution of plant viruses proceeds by natural recombination mechanisms that include template switching by viral polymerases during viral replication (Lai 1992; Simon and Bujarski 1994). Recombination between plant viruses has been inferred from an analysis of the nucleotide sequences of cauliflower mosaic virus (CaMV) (Dixon et al. 1986; Chenault and Melcher 1994), tobamoviruses (Angenent et al. 1989), potyviruses (Revers et al. 1996), barley yellow dwarf virus (Gibbs and Cooper 1995; Miller et al. 1997), and barley stripe mosaic virus (Edwards et al. 1992). In addition, RNA-RNA recombination has been demonstrated for several RNA plant viruses, including brome mosaic virus (Bujarski and Kaesberg

1986), cowpea chlorotic mottle virus (Allison et al. 1990), carmoviruses (Cascone et al. 1990), alfalfa mosaic virus (van der Kuyl et al. 1991), tombusviruses (White and Morris 1994), and cucumber mosaic virus (Fraile et al. 1997). In DNA plant viruses, recombination between closely related strains has been well documented for cauliflower mosaic virus (Howell et al. 1981; Lebeurier et al. 1982; Choe et al. 1985; Grimsley et al. 1986; Vaden and Melcher 1990).

An increasing body of evidence shows that recombination can also occur between replicating viruses and viral transgenes, and, in a few cases, endogenous plant sequences. An isolate of potato leafroll virus may have acquired a sequence derived from chloroplast RNA (Mayo and Jolly 1991), while sequences of the banana streak badnavirus have been found to be integrated into the banana genome (LaFleur et al. 1996). Plant viruses are able to acquire several types of viral transgenes, including the cell-to-cell-movement protein of red clover necrotic mosaic virus (Lommel and Xiong 1991), the coat protein of cowpea chlorotic mottle virus (Greene and Allison 1994, 1996), and the gene VI product of CaMV (Gal et al. 1992; Schoelz and Wintermantel 1993).

It has been shown previously that CaMV can acquire a copy of a CaMV transgene present in *Nicotiana bigelovii* through a copy choice mechanism of recombination. The transgene consisted of CaMV gene VI of strain D4, which specifies systemic infection of solanaceous hosts including *N. bigelovii* (Daubert et al. 1984; Schoelz et al. 1986). Recombinants between CaMV strain CM1841 and the transgene were recovered from 36% of the transgenic *N. bigelovii* plants (Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996). The detection of recombinants was facilitated by the fact that the recombinant formed between CM1841 and the transgene had a distinct selective advantage over the wild type CM1841. CM1841 cannot infect *N. bigelovii* systemically after mechanical inoculation, while a recombinant that acquired the D4 gene VI sequence from the transgenic plants also acquired the ability to infect several *Nicotiana* spp. (Schoelz and Wintermantel 1993).

In the present study we have attempted to determine when viruses formed through recombination between CaMV and CaMV transgenes can first be detected within the CM1841 population in infected plants. In order to study the temporal and spatial appearance of recombinant viruses during patho-

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genesis, we mechanically inoculated and agroinoculated CaMV strain CM1841 to nontransformed and transgenic *N. bigelovii*. In transgenic plants, a shift in symptom type from necrosis in the lower leaves to a chlorotic mosaic in the upper leaves was associated with the recovery of recombinant viruses and indicated that recombinants predominated only in later stages of viral pathogenesis.

RESULTS

A shift in symptom type from necrosis to chlorosis is correlated with the appearance of recombinant viruses in transgenic *N. bigelovii* plants after agroinoculation.

To determine when recombinant viruses could first be detected in the CM1841 population, we initially chose to examine infections that arose through agroinfection of CM1841. The agroinfection technique was preferred over mechanical inoculation because initial experiments showed that agroinoculated plants developed systemic infections at a consistently higher rate than mechanically inoculated plants. All of the transgenic plants that were agroinoculated developed systemic infections while, in contrast, in this study 19% of the transgenic plants mechanically inoculated with CM1841 virions developed systemic infections. In agroinoculated transgenic leaf tissue, localized necrotic lesions appeared within 20 to 30 days around the sites of inoculation. (Fig. 1A). Local lesions were a consequence of viral infection rather than the result of inoculation injury or due to the effect of *Agrobacterium tumefaciens*. Leaves of both transgenic and nontransformed *N. bigelovii* mock-agroinoculated with water or *A. tumefaciens* lacking the CM1841 plasmid remained entirely symptomless. Symptoms in the first systemically infected leaves of transgenic *N. bigelovii* appeared 30 to 35 days post inoculation (dpi) and consisted of extensive areas of necrosis (Fig. 1B). Necrosis in these leaves was initiated as scattered lesions often intermixed with chlorotic spots. Within a few days, the necrotic lesions grew and coalesced until nearly the entire leaf became necrotized. The severity of necrosis depended on environmental conditions: the most severe necrosis symptoms developed under conditions of low temperature (18 to 20°C), low light intensity (100 to 180 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$), and short day length (9- to 10-h days). Necrotic symptoms continued to develop on the leaves adjacent to and directly above the first leaf that showed systemic symptoms. However, as the infection progressed up to the youngest leaves at the top of the plant, there was a distinct shift in the type of symptom from necrosis to a chlorotic mosaic. A pronounced chlorotic mosaic developed in the top systemically infected leaves around 45 dpi (Fig. 1C), occasionally intermixed with small areas of necrosis.

To identify virus genotypes present in transgenic *N. bigelovii* following agroinoculation of CM1841, we first isolated virions and treated the virion preparation with DNase to degrade unencapsidated DNA before isolating viral DNA from

virions, as described by Gardner and Shepherd (1980). Virion DNA was purified from different portions of the plants and subsequently cleaved with *EcoRI*, and restriction enzyme patterns were revealed after gel electrophoresis and Southern blotting. Restriction enzyme mapping of virion DNA with *EcoRI* indicated that viruses recovered from transgenic plants could be divided into three groups: wild type (the virus originally inoculated), recombinant, or a mixture of wild type and recombinant. Wild-type viruses could be distinguished from recombinants due to *EcoRI* site polymorphisms within gene VI. An *EcoRI* digest of a CM1841 wild-type viral DNA recovered from transgenic plants generates a 459-bp DNA band, due to an *EcoRI* site at nucleotide position 6105. On the other hand, viruses that arose through recombination with transgene sequences were distinguished by a shorter DNA fragment of 397 bp, caused by an *EcoRI* site at nucleotide position 6043 (Fig. 2), a site present only in the transgene. Previous studies have demonstrated that the *EcoRI* site polymorphisms between CM1841 and the transgene are a reliable marker for recombination (Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996). Mixtures of recombinant and wild-type viruses recovered from a single plant could be identified through the presence of both the 459-bp and 397-bp DNA bands.

Recovery and Southern analysis of viral DNA from the agroinoculated leaves of transgenic *N. bigelovii* revealed only the presence of wild-type CM1841 in four out of five plants analyzed (Fig. 3A, Table 1). One inoculated leaf sample indicated the presence of a mixture of wild-type and recombinant viruses (Table 1). Southern blots of viral DNA from the first systemically infected leaves indicated a mixed virus population in a small percentage of plants (Fig. 3B), but the majority of samples contained wild-type virus only (Table 1). Analysis of viral DNA from the top leaves indicated the presence of recombinants in addition to the wild type in all transgenic plants, with approximately one half of the sampled areas containing only recombinants (Fig. 3B, Table 1). The appearance of sporadic necrosis in some of the top leaves was usually correlated with the recovery of a mixture of recombinant and wild-type viruses while, in most cases, top leaves showing only mosaic contained exclusively recombinant viruses. Therefore, these results indicated that recombinants between the agroinoculated CM1841 virus and the transgene present in *N. bigelovii* appeared late in the course of pathogenesis and a shift in symptom type was correlated with the appearance of recombinant viruses.

CaMV strain CM1841 can systemically infect nontransformed *N. bigelovii* following agroinoculation.

CaMV strain CM1841 causes systemic infection in a wide range of crucifers, but cannot systemically infect solanaceous hosts, including nontransformed *N. bigelovii*, following mechanical inoculation of virions (Schoelz et al. 1986). In contrast, we found that approximately 60% of agroinoculated,

Fig. 1. Symptoms in nontransformed and transgenic *Nicotiana bigelovii* mechanically inoculated and agroinoculated with CM1841. **A, B, and C**, Transgenic *N. bigelovii* leaves agroinoculated with CM1841 at 35, 45, and 60 days post inoculation (dpi), respectively. **D, E, and F**, Nontransformed *N. bigelovii* leaves agroinoculated with CM1841 at 35, 45, and 60 dpi, respectively. **G, H, and I**, Transgenic *N. bigelovii* leaves mechanically inoculated with CM1841 virions at 35, 45, and 60 dpi, respectively.

nontransformed *N. bigelovii* plants became systemically infected around 45 dpi (15 out of 22 inoculated). Leaves of *N. bigelovii* agroinoculated with CM1841 developed chlorotic lesions around the point of inoculation (Fig. 1D). Systemic symptoms were mild and consisted of chlorotic spots in the first systemically infected leaves (Fig. 1E) and a chlorotic mottle in the top leaves (Fig. 1F). In contrast to results with transgenic *N. bigelovii*, the nontransformed *N. bigelovii* never developed any necrosis upon agroinoculation with CM1841. Although agroinoculated CM1841 caused only mild systemic symptoms in nontransformed *N. bigelovii*, the presence of virus in these plants was confirmed by the isolation of viral DNA from inoculated, first systemically infected, and top leaves. In each type of tissue, only the 459-bp *EcoRI* band was detected (Fig. 3A and B, Table 1), an indication that only the wild-type CM1841 was present in the plants.

Although CM1841 moved systemically in nontransformed *N. bigelovii* following agroinoculation, in most cases the level of virus in systemically infected leaves was very low. No virus could be detected in six nontransgenic *N. bigelovii* plants by enzyme-linked immunosorbent assay (ELISA), even though the plants exhibited very mild symptoms (Table 2). In contrast, nontransformed *N. bigelovii* mechanically inoculated with CaMV strain D4, a virus with a wide solanaceous host range and the source of the gene VI transgene, displayed a systemic mosaic with leaf distortion and relatively high virus titers (Table 2). In addition, an ELISA of the top leaves of transgenic *N. bigelovii* plants agroinoculated with CM1841 indicated that the CaMV concentration in these leaves was equivalent to the amount of CaMV strain D4 present in nontransformed *N. bigelovii* (Table 2), indicating that acquisition of the D4 gene VI transgene by CM1841 from transgenic plants allowed that recombinant virus to replicate and spread to the same level as D4 wild-type virus.

To ensure that no mutations had occurred in the CM1841 genome that might influence host range, the top leaves of a

single, nontransformed *N. bigelovii* plant agroinoculated with CM1841 were used as a source of virus to inoculate turnips. The virus subsequently recovered from those infected turnips was designated CM1841B. The CM1841B virus caused mild stunting and extensive vein clearing in turnips, symptoms characteristic of CM1841. In addition, viral DNA isolated from CM1841B produced DNA fragments identical to that of CM1841 viral DNA following digestion with the restriction enzymes *EcoRI*, *EcoRV*, and *BglII* (data not shown). To demonstrate that the host specificity of CM1841B had not been altered, nontransformed *N. bigelovii* plants were mechanically inoculated with virions of CM1841B, CM1841, and D4. Both CM1841B and CM1841 failed to systemically infect any of the six *N. bigelovii* plants, while all plants inoculated with D4 developed strong systemic symptoms (Table 2). A subsequent ELISA conducted on samples from each plant confirmed infection by D4, and that CM1841 and CM1841B were unable to infect *N. bigelovii* systemically (Table 2). This study demonstrated that systemic infection of nontransformed *N. bigelovii* by agroinoculated CM1841 was not due to viral mutations, but instead resulted from the agroinoculation process itself.

A shift in symptom type from necrosis to chlorosis is correlated with the appearance of recombinant viruses in transgenic *N. bigelovii* plants after mechanical inoculation.

The presence of CM1841 viral DNA in upper, noninoculated leaves of both nontransformed and transgenic *N. bigelovii* plants might be a consequence of the agroinoculation procedure. However, earlier studies had shown that the D4 gene VI transgene facilitated the long-distance movement of a CaMV chimera defective for systemic infection of *N. bigelovii* (Schoelz et al. 1991; Wintermantel and Schoelz 1996). To determine whether the D4 gene VI transgene could facilitate the systemic movement of CM1841, we mechanically inoculated CM1841 virions to transgenic *N. bigelovii* plants and, as a

Fig. 2. Partial *EcoRI* maps of the cauliflower mosaic virus (CaMV) gene VI sequence present in transgenic *Nicotiana bigelovii*, the CM1841 virus, and a putative recombinant virus formed between CM1841 and the transgene. Transgenic plants contained an *XbaI*-*ClaI* DNA segment of CaMV and produced an mRNA from the CaMV 19S promoter and polyadenylation signals. An *SacI*-*HgiAI* 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. An *EcoRI* site in the D4 sequence at nucleotide position 6043 results in a 397-bp *EcoRI* DNA fragment in recombinant viruses, while an *EcoRI* site in CM1841 at nucleotide position 6105 results in a 459-bp DNA fragment in the wild-type CM1841.

control, nontransformed *N. bigelovii*. In nontransformed *N. bigelovii* plants, symptoms appeared only in inoculated leaves as local chlorotic lesions (data not shown), which is in accordance with previous observations that CM1841 cannot infect nontransformed *N. bigelovii* systemically following the inoculation of virions (Schoelz et al. 1986). In contrast, about 19% of transgenic *N. bigelovii* plants inoculated with CM1841 virions developed systemic symptoms (seven out of 36 inoculated plants), in approximate agreement with the 36% value reported by Wintermantel and Schoelz (1996).

The symptom development of the CaMV infection in transgenic plants mechanically inoculated with CM1841 was similar to the infection induced by agroinoculation of CM1841. The only difference in symptom type between virion- and agroinoculated plants occurred in the inoculated leaves: local lesions in virion-inoculated, transgenic *N. bigelovii* were chlorotic, rather than necrotic (Fig. 1G). However, strong necrotic symptoms appeared in the first leaves that were systemically infected (Fig. 1H), followed by a pronounced chlorotic mosaic in the top of the plant (Fig. 1I)—the same reaction seen in corresponding agroinoculated plants. Furthermore, the amount of virus in the top of transgenic plants mechanically inoculated with CM1841 was similar to that found in plants inoculated with D4 or agroinoculated with CM1841 (Table 2).

Most importantly, the shift from necrosis to mosaic symptoms was correlated with a shift from recovery of the wild

type to recovery of the recombinant virus. For example, the inoculated leaves of the five virion-inoculated plants analyzed contained only wild-type CM1841 viral DNA (Table 3). In the first systemically infected leaves, three out of four plants contained recombinant viruses, while in the top leaves all five plants analyzed contained recombinants and none contained the wild-type virus alone (Table 3). These results demonstrated that transgenic *N. bigelovii* plants that express the D4 gene VI transgene could facilitate the systemic movement of CM1841, and that during the course of the systemic infection the CM1841 wild-type virus was gradually outcompeted by the recombinant virus.

DISCUSSION

In this study we were interested in determining when recombinant viruses formed between CaMV viral DNA and CaMV transgenes could first be detected within the CM1841 population present in infected plants. An additional objective was to learn more about the competition that occurs between wild-type and recombinant viruses, because the competitiveness of the recombinant virus will ultimately determine whether it is able to persist in nature. Mixtures of recombinant virus and wild-type virus had been noted in a previous paper (Wintermantel and Schoelz 1996), but it was not clear if the mixtures represented a stable population or a population in transition from wild type to recombinant. The data obtained

Fig. 3. Cauliflower mosaic virus (CaMV) genotypes recovered from nontransformed and transgenic *Nicotiana bigelovii* after agroinoculation with strain CM1841. Viral DNA was recovered from 1 g of leaf tissue, digested with *Eco*RI, and subjected to Southern blot analysis with a probe that consisted of an equal mixture of the 397-bp *Eco*RI fragment of CaMV strain D4 and the 459-bp *Eco*RI fragment of CaMV strain CM1841. Arrows indicate the *Eco*RI polymorphism that exists between the wild-type CM1841 virus and the recombinant virus. The 459-bp band indicates presence of a wild-type (CM1841) virus; the 397-bp band indicates presence of a recombinant virus in a given leaf sample. **A**, Recovery of CaMV viral DNA from an inoculated *N. bigelovii* leaf. **B**, Recovery of CaMV viral DNA from the first *N. bigelovii* leaf on the plant that developed systemic symptoms and from the top of the plant.

by agroinoculation of CM1841 was valuable because it showed clearly that mixtures are transitory. The two viruses could coexist in the same leaf, but the recombinant virus would gradually replace the wild-type virus as the infection progressed.

CaMV strain CM1841 was delivered to nontransformed and transgenic *N. bigelovii* plants by either agroinoculation or mechanical inoculation of virions. Regardless of the technique, recombinant viruses were usually detected and predominated in transgenic *N. bigelovii* at a rather late phase of systemic infection, between 45 and 60 days after inoculation. Recombinants were detected in the inoculated leaves in only a few cases. Although recombination between CaMV isolates has been examined in great depth, no study included an analysis of the inoculated leaves for the presence of recombinants. In several of the papers, recombination was assumed to occur in the inoculated leaf because the mutants would not be expected to be capable of replication (see for example Howell et al. 1981; Lebourier et al. 1982; Grimsley et al. 1986). Furthermore, it has been shown that different variants of CaMV can be isolated from the same primary lesion (Riederer et al. 1992), an important prerequisite if recombination were to occur in the inoculated leaf. In contrast to CaMV, several studies have examined the formation of recombinant RNA viruses in inoculated leaves and protoplasts. Recombinants between cucumber necrosis and tomato bushy stunt viruses were detected in protoplast extracts (White and Morris 1994), as were recombinants between turnip crinkle virus and its satellites (Carpenter and Simon 1996). Similarly, recombinants between alfalfa mosaic virus RNA3 deletion mutants were found in inoculated tobacco leaves (van der Kuyl et al. 1991). In contrast, recombination between wild-type and mutant RNA3 of BMV was not apparent at 3 dpi in systemically infected leaves and was assumed to occur between 3 and 10 dpi (Bujarski and Kaesberg 1986).

It remains to be seen whether a greater time frame is required to detect recombinants between viruses and transgenes than between two distinct viruses. There are no direct comparisons in the literature yet. However, the lengthy time frame that may be required for the detection of recombinants is one factor that should be considered in studies designed to measure recombination rates between viruses and viral transgenes. For example, the measurement of recombination rates between viruses and transgenes in plant protoplasts over a 24 to 48 h period may underestimate the potential for recombination. The detection of recombinants is likely to vary, depending on the plant virus studied, the host, and the nature of the selection pressure.

One unique aspect of this study was that a change in symptom type was correlated with the appearance of recombinant viruses in CM1841-infected transgenic *N. bigelovii*. As the infection progressed, symptoms changed from necrosis in the lower leaves to a chlorotic mosaic in the top leaves, the site where the majority of recombinant viruses were detected. The necrotic symptom may represent an unusual synergism between two variations of the same gene product. Necrosis was not a result of increased gene VI product levels per se, because systemic infection of transgenic plants by the D4 strain or the CM1841 recombinant virus resulted in mosaic rather than necrosis. Furthermore, necrosis was not dependent upon the expression of the CM1841 gene VI product alone, since nontransformed *N. bigelovii* that had been agroinoculated with CM1841 did not develop necrosis. Since both the D4 and CM1841 gene VI products are simultaneously required for the necrosis symptom, one possible explanation may be that the CM1841 gene VI product may be responsible for the necrotic symptoms, but that it may be unable to attain a high enough concentration in nontransformed *N. bigelovii* to trigger necrosis. The role of the D4 gene VI product in transgenic *N. bigelovii* may be to facilitate the movement of CM1841, which thus increases the level of the CM1841 gene VI product, rais-

Table 1. Recovery of wild-type and recombinant cauliflower mosaic virus (CaMV) following agroinoculation of CaMV strain CM1841

Type of virus recovered	Transgenic <i>Nicotiana bigelovii</i> that express gene VI of CaMV strain D4			Nontransformed <i>N. bigelovii</i>		
	Inoculated leaf (35 dpi) ^a	First systemically infected leaf (45 dpi)	Top of plant (60 dpi)	Inoculated leaf (35 dpi)	First systemically infected leaf (45 dpi)	Top of plant (60 dpi)
Wild type	4 ^b	13	0	9	11	15
Mixture ^c	1	3	11	0	0	0
Recombinant	0	0	10	0	0	0

^a Days post inoculation.

^b Number of samples that contained this viral type.

^c Mixture of wild-type and recombinant viruses.

Table 2. Virus concentrations of CM1841, CM1841B, and D4 in non-transformed and transgenic *Nicotiana bigelovii*

Inoculum	<i>N. bigelovii</i>	
	Nontransformed	Transgenic
CM1841 agroinoculated	0 (6) ^a	9.75 ± 7.7 ^b (4)
CM1841 virions	0 (6)	12.87 ± 4.65 (4)
CM1841B virions	0 (6)	ND ^c
D4 virions	11.17 ± 5.37 (11)	ND

^a Number of plants analyzed 60 days after inoculation.

^b Micrograms of virus per g of leaf tissue.

^c Not determined.

Table 3. Recovery of wild type and recombinant cauliflower mosaic virus (CaMV) following mechanical inoculation of CaMV strain CM1841

Type of virus recovered	Inoculated leaf (35 dpi) ^a	First systemically infected leaf (45 dpi)	Top of plant (60 dpi)
Wild type	5 ^b	1	0
Mixture ^c	0	2	2
Recombinant	0	1	3

^a Days post inoculation.

^b Number of samples that contained this viral type.

^c Mixture of wild-type and recombinant viruses.

ing its concentration above the threshold required for development of necrosis.

Previous studies have emphasized that CM1841 is unable to infect *N. bigelovii* systemically, possibly because of an active defense response targeted against the CM1841 gene VI product (Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996). The present study revealed that the resistance of *N. bigelovii* could be overcome by two different mechanisms: one mechanism involves the agroinoculation procedure, while a second is related to the function of the CaMV transgene.

In the first mechanism, the agroinoculation procedure itself circumvented the defenses of *N. bigelovii*. Although CM1841 could not move systemically after mechanical inoculations, it could infect nontransformed *N. bigelovii* systemically following agroinoculation. Systemic movement of the CM1841 virus in agroinoculated, nontransformed plants was not a result of mutations in the virus, but instead a consequence of the agroinoculation process itself. A similar phenomenon has been noted in *Arabidopsis thaliana*. The resistance of *A. thaliana* ecotype Blanes-14 to CaMV infection was found to be overcome by agroinoculation (Callaway et al. 1996). Two theories, which may not be mutually exclusive, may explain how agroinoculation of CM1841 broke the resistance of *N. bigelovii*. One possibility may be that leaf cells surrounding agroinoculation sites contain sufficient amounts of T-DNA to serve as a constant source of viral inoculum for prolonged periods, because agroinoculated leaves remain on the plant up to 70 to 75 days after inoculation. The constant supply of virus from these leaves may enable systemic movement of CM1841 throughout the infected plant. A second theory concerns the survival of *A. tumefaciens* in agroinoculated leaves. In a study by Mogilner et al. (1993), several plant species, including tomato, avocado, and grapefruit, were agroinoculated with *A. tumefaciens* strain LBA4404 that carried a dimeric cDNA of citrus exocortis viroid (CEVd). Large amounts of *A. tumefaciens* containing the CEVd genome were detected in extracts of agroinoculated tissues up to 3 months after inoculation (Mogilner et al. 1993).

A second mechanism that negated the resistance of *N. bigelovii* involved the expression of gene VI of CaMV strain D4 in the transgenic plants, as our work indicates that the D4 transgene facilitated the systemic infection of CM1841. Although gene VI of CaMV has been described as a translational transactivator (Bonneville et al. 1989; Gowda et al. 1989), it may have a role in long-distance transport independent from gene expression. CM1841 could not move out of the initially infected leaves of nontransformed *N. bigelovii* after mechanical inoculation, while in the transgenic plants that expressed the D4 gene VI product CM1841 predominated in the first systemically infected leaves and could still be detected in the top leaves of many of the transgenic plants. The picture that emerges is one in which two competing elements determine the nature of the CaMV population in transgenic *N. bigelovii*; the D4 gene VI product facilitates the movement of the wild-type CM1841 virus, while the plant selects against viruses that have retained gene VI of CM1841. The net result is that the wild-type CM1841 virus is gradually replaced with a recombinant virus that has acquired the D4 transgene.

MATERIALS AND METHODS

Inoculation of CaMV strains CM1841 and D4 to nontransformed and transgenic plants.

Nontransformed and transgenic *N. bigelovii* were inoculated with partially purified virus prepared as described by Schoelz et al. (1986) or inoculated as purified virions (Hull et al. 1976). For agroinoculation of CM1841, the binary vector construct p306 was used (a gift from Neil Olszewski, University of Minnesota). p306 consists of a partially redundant infectious clone of CaMV strain CM1841 inserted between the T-DNA border sequences present in the disarmed *A. tumefaciens* Ti plasmid vector pOCA28. The redundant CaMV segment extends from *PvuII* (nucleotide position 6318) to *ClaiI* (nucleotide position 7987), and includes the CaMV 35S promoter and termination signals such that a complete 35S RNA could be synthesized after transfer to plant cells. Plasmid p306 was electroporated into the *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983) according to Hanahan et al. (1991). Prior to agroinoculation, *A. tumefaciens* cells were grown in 40 ml of Luria-Bertani (LB) medium containing 300 µg of streptomycin per ml and 100 µg of spectinomycin per ml for 48 h at 28°C. The suspension was centrifuged at $8,000 \times g$ for 15 min and the pellet was resuspended in 2 ml of sterile water. Agroinoculation of nontransformed and transgenic *N. bigelovii* was done by puncturing leaves with a syringe and placing a droplet of bacterial suspension on each wound site.

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). All transgenic *N. bigelovii* used in this study were hemizygous for gene VI, and were obtained from a cross between homozygous transgenic plants and nontransformed *N. bigelovii*.

Nontransformed and transgenic *N. bigelovii* were inoculated 4 to 5 weeks after seeds were sown. *N. bigelovii* seeds were soaked in 2.0% NaOCl for 15 to 20 min prior to sowing to overcome seed dormancy as described by Burk (1957). Inoculated plants were maintained for up to 75 days in growth chambers set for a 10-h day with a light intensity of 100 to 180 µE · s⁻¹ · m⁻² at 20°C.

Recovery and analysis of CaMV viral DNA.

To distinguish recombinant viruses from wild-type CM1841, CaMV virions were isolated from 1 g of tissue and viral DNA was purified as described by Gardner and Shepherd (1980). Viral DNA was subsequently cleaved with *EcoRI*, and then separated on a 2% agarose gel, transferred to nylon membrane, and probed with a ³²P-labeled DNA probe as described in Maniatis et al. (1982). The probe consisted of equal proportions of the 459-bp *EcoRI* fragment of CM1841 and the 394-bp *EcoRI* fragment of D4 (Fig. 1).

ACKNOWLEDGMENTS

We wish to thank N. Olszewski for providing the *Agrobacterium tumefaciens* clone p306. This research was supported by a grant from the Food for the 21st Century program at the University of Missouri and by U.S. Department of Agriculture/Biotechnology Risk Assessment Grant

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