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# Expression of Cauliflower Mosaic Virus (CaMV) Gene VI in Transgenic *Nicotiana bigelovii* Complements a Strain of CaMV Defective in Long-Distance Movement in Nontransformed *N. bigelovii*

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**Transgenic *Nicotiana bigelovii* var. *multivalvus* plants that express the gene VI-encoded product of strain D4 of cauliflower mosaic virus (CaMV) allow long-distance transport of CaMV strain H31, a virus that is unable to move systemically in nontransformed *N. bigelovii*. Previous work showed that the gene VI-encoded product of strain D4 determines the ability of recombinant viruses to infect *N. bigelovii* systemically. To test whether long-distance spread of H31 resulted from complementation or recombination with the "trans-gene," transformed and normal *N. bigelovii* plants were inoculated with CaMV strain H31 recovered from noninoculated leaves of transgenic plants. Transgenic *N. bigelovii* developed systemic symptoms 3 to 4 wk after inoculation with passaged CaMV strain H31, while the nontransformed *N. bigelovii* remained symptomless. These experiments show that complementation by gene VI of D4 in the transgenic plant conferred on H31 the ability to move systemically in plants that do not normally allow systemic infection by this strain of CaMV.**

Cauliflower mosaic virus (CaMV) systemically infects a wide range of crucifers. A few strains of CaMV are also able to infect solanaceous hosts. Two strains of CaMV, D4 and W260, systemically infect the solanaceous host *Nicotiana bigelovii* Wats. (Schoelz *et al.* 1986a; Schoelz and Shepherd 1988), inducing chlorotic primary lesions on *N. bigelovii* 7 to 10 days after inoculation. Systemic symptoms of D4 appear 3 to 5 days before those of W260, and D4 can spread systemically under a wider range of environmental conditions (Schoelz and Shepherd 1988). The W260 and D4 sequences that determine the systemic spread have been identified by constructing recombinant viruses with CM1841, a CaMV strain that does not induce any visible response in *N. bigelovii*. Long-distance transport of D4 in *N. bigelovii* is determined by gene VI, which encodes a 62-kDa inclusion body protein (Schoelz *et al.* 1986b). In contrast, long-distance transport of W260 is determined by gene I (movement protein) and gene IV (coat protein), in addition to gene VI (Schoelz and Shepherd 1988; S. G. Qiu and J. E. Schoelz, unpublished). The common feature between D4 and W260 is the involvement of gene VI in long-range movement.

Several studies have shown that CaMV gene VI also has a significant role in determining symptoms and that the function of gene VI may be to transactivate expression of genes on the polycistronic 35S RNA (Bonneville *et al.* 1989; Gowda *et al.* 1989). The CaMV gene VI-encoded protein, a major component of the amorphous inclusion bodies in infected cells (Odell and Howell 1980; Covey and Hull 1981), is implicated in chlorosis in turnips (*Brassica rapa* L. cv. Just Right) (Daubert *et al.* 1984), although the large intergenic region may also affect the extent of chlorosis (Stratford and Covey 1989). Gene VI expression in transgenic nonhost plants often results in viruslike symptoms (Baughman *et al.* 1988; Takahashi *et al.* 1989; Kiernan *et al.* 1989; Balazs 1990; Goldberg *et al.* 1991), while transgenic hosts of the caulimovirus group that express gene VI may remain symptomless (Goldberg *et al.* 1991).

In this study, we investigate whether expression of gene VI from CaMV strain D4 in transgenic *N. bigelovii* influences the reaction of *N. bigelovii* to CaMV infection. We report for the first time the transformation and regeneration of *N. bigelovii*, and we present evidence that these transgenic plants complement long-distance movement of CaMV isolate H31, a virus that cannot establish systemic infection in nontransformed *N. bigelovii*.

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## MATERIALS AND METHODS

**General.** Construction of recombinant virus H31 (Fig. 1) from CaMV strains W260 (Gracia and Shepherd 1985; Schoelz and Shepherd 1988) and CM1841 (Howarth *et al.* 1981) was described in Schoelz and Shepherd (1988). Genes I–V of H31 are derived from CM1841, whereas gene VI and the large intergenic region are from W260. Plasmid DNA constructions were grown in strain 71/18 of *Escherichia coli* (Migula) Castellani and Chalmers (Messing *et al.* 1977). Strain C58C1::pGV3850 of *Agrobacterium tumefaciens* (Smith and Townsend) Conn (Zambryski *et al.* 1983) was used for plant transformation. Bacteria were cultured in 2YT medium (Sambrook *et al.* 1989). The plant transformation vector pJS65 (Kiernan *et al.* 1989) was maintained in culture with 10 μg/ml each of tetracycline and kanamycin. Restriction enzymes from New England Biolabs (Beverly, MA) and Promega (Madison, WI) were used as directed.

**Transformation of *N. bigelovii*.** Plasmid pJS65 (Fig. 2), described previously (Kiernan *et al.* 1989), was mobilized into *A. tumefaciens* C58C1::pGV3850 by triparental mating (Ditta *et al.* 1980). Transformation of *N. bigelovii* was essentially as described by Horsch *et al.* (1985), with modifications as described by Goldberg (1989). Surface-sterilized leaf disks from 6-wk-old *N. bigelovii* plants were immersed for 20–60 sec in an overnight culture of *A. tumefaciens* containing pJS65 and blotted onto sterile filter paper. The disks were placed on Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2 mg/L of N6 (2-isopentenyl) adenine and 0.175 mg/L of indole-3-acetic acid for 2 days and then transferred to the same medium with the addition of 500 mg/L of mefoxin and 100 mg/L of kanamycin. When shoots formed, the plantlets were transferred to GA7 boxes (Magenta Corporation, Chicago, IL) containing rooting medium (half-strength Murashige and Skoog medium without phytohormones) and 500 mg/L of mefoxin. After roots formed, the plants were transferred to sterile potting soil and maintained in the greenhouse. Transformants were identified by screening leaf tissue of the resultant plants for kanamycin resistance on "shooting" medium containing 100 μg/ml of kanamycin. Transformed plants formed callus and/or shoots; nontransformed plant tissue did not.

**Northern blot analysis.** Total RNA was purified from 1.0 g of tissue according to the procedure of Chomczynski and Sacchi (1987). The RNA was electrophoresed through a 1.2% denaturing agarose gel, transferred to Genescreen membranes (New England Nuclear, Boston, MA) as described by Sambrook *et al.* (1989), hybridized with a $^{32}$P-labeled probe, and washed according to the procedure of Jones *et al.* (1990). The probe, a 2,203-base pair *Sac*I-*Hgi*AI CaMV DNA fragment cloned into pUC13, was labeled using a random-primed DNA labeling kit (U.S. Biochemical Corporation, Cleveland, OH).

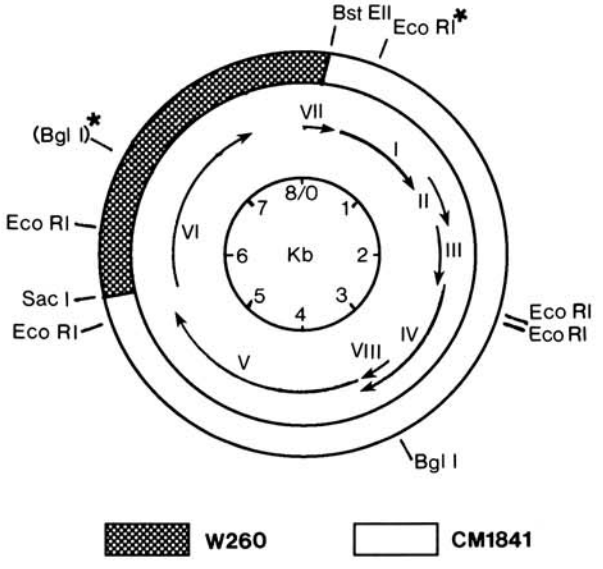


**Fig. 1.** Genome structure of recombinant cauliflower mosaic virus strain H31. H31 was constructed by exchanging *Sac*I-*Bst*EII DNA segments between cauliflower mosaic virus strains W260 and CM1841. Genes I–V are derived from CM1841, and gene VI and the large intergenic region are derived from W260. Restriction enzyme sites important for the analysis of H31 are indicated on the map. The *Bgl*I and *Eco*RI sites indicated by asterisks are present in the CM1841 genome but absent from W260. The positions of the open coding regions are indicated with arrows.

**Immunodetection of gene VI-encoded protein.** Polyclonal antibodies raised against a synthetic peptide (Cambridge Research Biochemicals, Valley Stream, NY) that was composed of 10 amino acids at the carboxyl-terminus of the CaMV gene VI-encoded protein (P62) were elicited in New Zealand white rabbits. Proteins were extracted from transgenic *N. bigelovii* by grinding 0.5 g of leaf tissue in 1.5 ml of sample buffer (0.5 ml Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol, 1% 2-mercaptoethanol, 0.25 mg/ml bromophenol blue). Samples were boiled for 5 min and centrifuged for 5 min at 14,000 × *g* to remove cellular debris. Samples (20 μl; equivalent to 6.6 μg of leaf tissue) were loaded onto 12.5% polyacrylamide gels, electrophoresed at 200 V for approximately 50 min, and transferred electrophoretically to PVDF membranes (Schleicher and Schuell, Keene, NH) (Towbin *et al.* 1979). The filter was blocked (1% nonfat dry milk in phosphate-buffered saline) for 45 min, incubated with antiserum raised against the gene VI-encoded peptide (1:1,000) for 90 min, and then washed three times with phosphate-buffered saline. The blots were blocked and incubated with goat anti-rabbit IgG alkaline phosphatase (Promega) (1:7,500 dilution) for 90 min before washing as described above. The reaction was visualized using the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. To measure virus concentration in infected plants, an enzyme-linked immunosorbent assay (ELISA) was performed as described in Anderson *et al.* (1991).

**Complementation studies.** Transgenic *N. bigelovii* and nontransformed controls were inoculated with W260 or H31 as described previously (Schoelz *et al.* 1986b). Viral DNA was isolated from infected plants according to the procedure of Gardner and Shepherd (1980).

## RESULTS

**Transformation of *N. bigelovii*.** Plants regenerated from leaf disks ($R_0$) were screened for kanamycin resistance and accumulation of the CaMV gene VI-encoded protein. Regenerated plants that expressed the gene VI-encoded product had a normal appearance. One of these, JS65.4, was selfed and seed was collected for further analysis. The highest amounts of gene VI mRNA and protein in $R_1$ progeny of transgenic JS65.4 plants were approximately 1% of that observed in turnips infected with CaMV (Fig. 3, A and B). In contrast to the $R_0$ regenerated plants, transgenic $R_1$ plants expressing the gene VI-encoded protein grew normally for approximately 6 to 7 wk and then developed a mild chlorotic mottle, distinguishing them from the nonexpressers. The $R_1$ and selected $R_2$ progenies of JS65.4 were used for complementation studies with CaMV.

**Complementation of recombinant CaMV strain H31.** CaMV strain H31, a recombinant virus of the caulimovirus group (Fig. 1) chosen for this complementation study, contains genes I–V from CM1841, a virus that does not induce any response in *N. bigelovii*, and gene VI and the large intergenic region from W260, a virus that systemically infects *N. bigelovii*. Previous work has shown that H31

infections of nontransformed *N. bigelovii* are limited to the inoculated leaf. H31 induced a few chlorotic lesions on inoculated leaves of the same plants but no systemic symptoms. An ELISA of noninoculated *N. bigelovii* leaves showed that no H31 was present in those leaves, eliminating the possibility of a symptomless systemic infection (Schoelz and Shepherd 1988).

To show complementation, 18 transgenic $R_1$ *N. bigelovii* plants that had segregated for expression or nonexpression of the CaMV strain D4 gene VI-encoded protein (JS65.4) were inoculated with H31. Five of these plants developed a severe systemic mottle (Fig. 4; Table 1) and virus was recovered from noninoculated leaves of these plants, demonstrating long-distance movement of H31. The remaining 13 $R_1$ plants did not develop viral symptoms or the characteristic mottle associated with gene VI expression, indicating that they did not express the gene VI-encoded product. Nontransformed *N. bigelovii* also did not develop systemic symptoms when inoculated with H31, although chlorotic lesions did appear on inoculated leaves of three of five plants. All five of the W260-inoculated control plants developed a systemic mosaic. Systemic symptoms of H31 in transgenic *N. bigelovii* were delayed by 5 days, relative to the W260 infection. As the H31 systemic infection progressed in JS65.4, the leaves developed a bleached appearance, a symptom quite different from infections by either the W260 or D4 strain of CaMV. H31 concentration in noninoculated leaves was determined in a subsequent test by ELISA. The average virus concentration in upper noninoculated leaves of four plants was 3.6 μg/g of leaf tissue. This amount is comparable to values published previously for D4 and W260 infections of *N. bigelovii* (Schoelz and Shepherd 1988), but it is approximately 10 times less than the amount of H31 detected in turnips (E. J. Anderson, A. T. Trese, O. P. Sehgal, and J. E. Schoelz, unpublished).

To show that the five $R_1$ plants which supported a systemic infection following H31 inoculation expressed the D4 gene VI-encoded protein, they were selfed and seed was collected. The $R_2$ progeny from each of these plants were propagated for approximately 6 wk before testing for expression of the gene VI-encoded product. Immunoblot analysis revealed that each of the five $R_1$ lines segregated for the presence of gene VI, indicating that the five $R_1$ plants must have been heterozygous and providing evidence that expression of gene VI in transgenic plants is associated with long-distance movement of H31 (Table 2).

**Analysis of CaMV H31 passaged through transgenic *N. bigelovii*.** To rule out the possibility that the altered long-

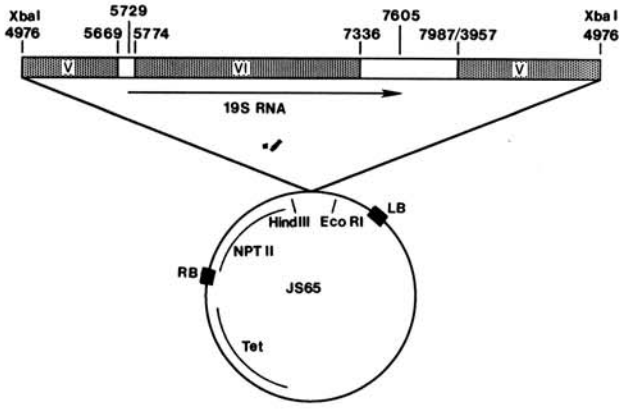


**Fig. 2.** Diagram of pJS65. The *Xba*I fragment of cauliflower mosaic virus, which contains the 19S promoter, CaMV strain D4 gene VI open reading frame, and polyadenylation signal, was cloned into a modified pGA472 (An *et al.* 1985) plant transformation vector to create pJS65 (Kiernan *et al.* 1989). The location of the 19S RNA is indicated by an arrow. The boxed areas indicate the right (RB) and left (LB) borders of the T-DNA. The positions of the kanamycin (*nptII*) and tetracycline (*tet*) resistance genes are indicated.

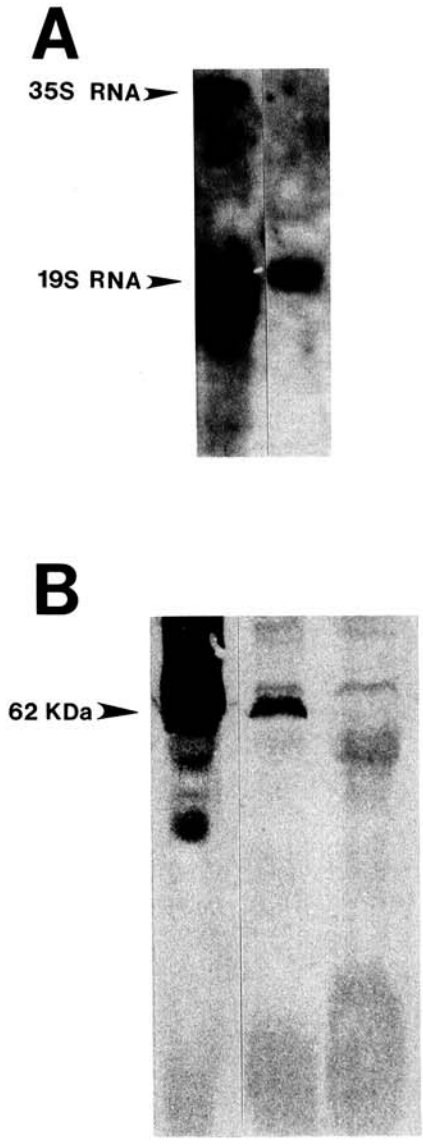


**Fig. 3.** Analysis of gene VI expression in the transgenic *Nicotiana bigelovii* line JS65.4. **A**, Northern blot analysis of cauliflower mosaic virus (CaMV) strain D4 gene VI (19S RNA) expression. Lane 1 contains RNA from a turnip leaf infected with CaMV (total RNA loaded = 0.1 μg), and lane 2 contains RNA from an uninoculated $R_1$ plant of *N. bigelovii* line JS65.4 (total RNA loaded = 20 μg). **B**, Western blot analysis of CaMV strain D4 gene VI expression. The 62-kDa gene VI-encoded product is indicated by an arrow. Lane 1 contains total protein from a turnip leaf infected with CaMV (1.6 μg of tissue); lane 2, total protein from *N. bigelovii* line JS65.4 (6.6 μg of tissue); and lane 3, total protein from nontransformed *N. bigelovii* (6.6 μg of tissue).

distance spread was due to selection of a spontaneous mutant strain or gene conversion, H31 virus from uninoculated leaves of two of the five systemically infected JS65.4 plants was inoculated to turnips. H31 viral DNA was isolated from infected turnips and compared with W260, CM1841, and the original H31 inoculum by restriction enzyme analysis. The two H31 isolates that were passaged through transgenic *N. bigelovii* and the original H31 inoculum from turnips possessed similar DNA patterns following digestion of the viral DNAs with *Eco*RI or *Bgl*I (Fig. 5, A and B, respectively) and *Eco*RV, *Pvu*II, and *Acc*I (data not shown).

However, it was still possible that a point mutation had occurred within gene VI DNA other than at sites for these five restriction enzymes. Even minor changes in gene VI can significantly alter the ability of CaMV to move systemically and the type of symptoms that develop on host plants (Daubert and Routh 1990). To confirm that gene VI of H31 remained unchanged during passage through

**Fig. 4.** Systemic symptoms of cauliflower mosaic virus strain H31 infection in transgenic *Nicotiana bigelovii* line JS65.4. Systemic symptoms appeared approximately 22 days after inoculation with H31. As infection progressed, the noninoculated leaves developed a bleached appearance as illustrated.

**Table 1.** Reaction of transgenic and nontransformed *Nicotiana bigelovii* to inoculation with selected strains of cauliflower mosaic virus (CaMV)

| Virus | Host | No. of plants systemically infected |
|---|---|---|
| W260 | *N. bigelovii* | 5/5[a] |
| H31 | *N. bigelovii* | 0/5 |
| H31 | *N. bigelovii* JS65.4[b] | 5/18 |

[a] The numerator indicates the number of plants that developed systemic symptoms.
[b] *N. bigelovii* transformed with pJS65 (gene VI of CaMV D4), $R_1$ generation.

**Table 2.** Expression of cauliflower mosaic virus (CaMV) D4 gene VI-encoded protein in $R_2$ progeny of five $R_1$ transgenic *Nicotiana bigelovii*

| Selfed $R_1$ plant | No. of $R_2$ plants expressing the 62-kDa protein |
|---|---|
| *N. bigelovii* JS65.4 plant 2 | 8/18[a] |
| *N. bigelovii* JS65.4 plant 3 | 14/18 |
| *N. bigelovii* JS65.4 plant 4 | 12/18 |
| *N. bigelovii* JS65.4 plant 5 | 8/18 |
| *N. bigelovii* JS65.4 plant 6 | 14/18 |

[a] The numerator indicates the number of transgenic *N. bigelovii* plants that expressed the CaMV gene VI-encoded protein.

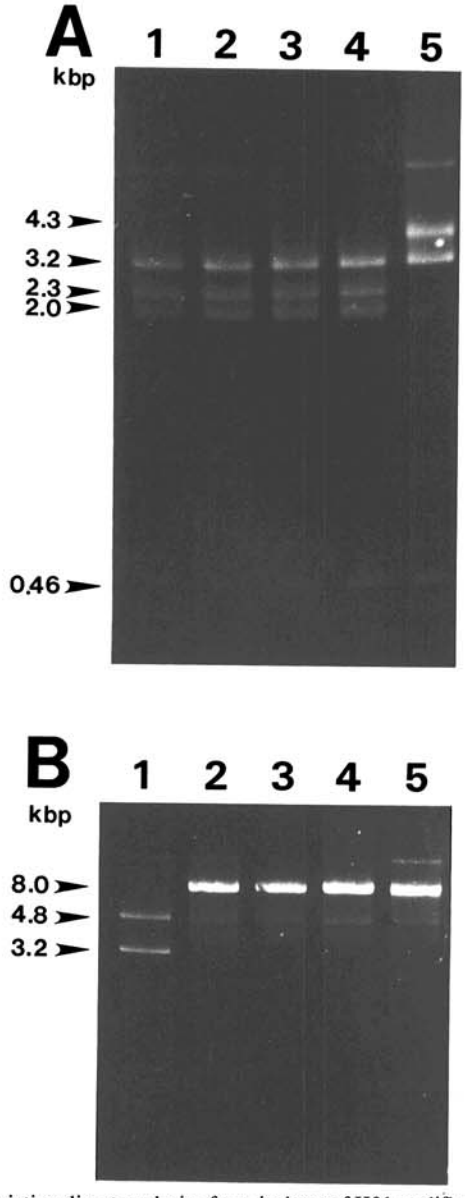


**Fig. 5.** Restriction digest analysis of two isolates of H31 cauliflower mosaic virus DNA after passage through JS65.4 transgenic *Nicotiana bigelovii*. H31 virus isolates were inoculated to turnips after passage through transgenic *N. bigelovii*. Passaged cauliflower mosaic virus strain H31 DNAs were purified from turnips and compared to W260, CM1841, and the original H31 inoculum by cleavage with the restriction enzymes *Eco*RI and *Bgl*I. The DNA fragments were separated on a 1.0% (w/v) agarose gel and stained with ethidium bromide. **A**, *Eco*RI digest. The *Eco*RI site at nucleotide position 408 is present in CM1841 and H31, but absent in W260 (Fig. 1). Lane 1 contains CM1841 DNA; lane 2, H31 DNA; lane 3, passaged H31 isolate plant 1 DNA; lane 4, passaged H31 isolate plant 2 DNA; and lane 5, W260 DNA. **B**, *Bgl*I digest. The *Bgl*I site at nucleotide position 6656 is present in CM1841 and absent in W260 and H31. Lane 1 contains CM1841 DNA; lane 2, H31 DNA; lane 3, passaged H31 isolate plant 1 DNA; lane 4, H31 passaged isolate #2 DNA; and lane 5, W260 DNA.

the transgenic JS65.4 *N. bigelovii*, the virus was reinoculated to nontransformed *N. bigelovii* and, as a control, inoculated to $R_2$ *N. bigelovii* progeny that segregated for expression of the D4 gene VI-encoded product (Table 3). Of the transformed *N. bigelovii*, six of 10 plants developed a systemic mottle and bleached leaves. The 10 nontransformed *N. bigelovii* plants remained symptomless, showing that the host specificity of H31 had not been altered following one passage through transgenic plants expressing gene VI.

## DISCUSSION

Expression of gene VI from CaMV strain D4 in transgenic *N. bigelovii* altered the plant's response to viral infection. Previous studies revealed that sequences within gene VI of CaMV strain D4 act as determinants for systemic infection of *N. bigelovii* (Schoelz *et al.* 1986b). Transgenic *N. bigelovii* expressing the D4 gene VI-encoded product allowed long-distance movement by H31, a recombinant virus of the caulimovirus group that is normally limited to the inoculated leaf of nontransformed *N. bigelovii*. Subsequent experiments showed that this change was not due to a genetic alteration of H31 in the transgenic plants.

The ability of transgenic plants to complement defective viruses has been demonstrated with a number of viruses, including tobacco mosaic virus (Deom *et al.* 1987; Osbourn *et al.* 1990; Holt and Beachy 1991), alfalfa mosaic virus (van Dun *et al.* 1988), and tomato golden mosaic virus (Hanley-Bowdoin *et al.* 1990). With each of these viruses, transgenic plants that express viral proteins complemented viruses in which a mutation had been introduced into a viral gene or in which the entire gene was deleted. The one aspect that distinguishes our system from others is that the defect of H31 is host-specific. H31 replicates and moves systemically in turnips, attaining virion and DNA concentrations that are comparable to CaMV strain W260 (E. J. Anderson, A. T. Trese, O. P. Sehgal, and J. E. Schoelz, unpublished).

Two models might explain the ability of gene VI of D4 to complement H31. The first model is based on the function of gene VI, which is to transactivate translation of genes on the 35S RNA (Bonneville *et al.* 1989; Gowda *et al.* 1989). In this model, the inability to move long distances may be explained on the cellular level as a defect in translation of genes I–V on the 35S RNA. The gene VI-encoded product of D4 may be able to interact properly with host components and CM1841 sequences to facilitate translation, whereas the W260 gene VI-encoded product might not be able to interact efficiently with CM1841 sequences. The defect of the W260 gene VI-encoded product would have to be very subtle for two reasons. First, H31 is capable of limited replication and cell-to-cell movement in the inoculated leaf of nontransformed *N. bigelovii*. Second, Bonneville *et al.* (1989) found that gene VI of CaMV transactivates gene expression in hosts and nonhosts of CaMV. If the defect is related to transactivation, it would mean that a higher level of viral gene expression is required for long-distance transport than for the formation of primary lesions.

An alternative model postulates that long-distance transport of CaMV is not related to the transactivating function of gene VI. In this model, long-distance transport might be governed by an interaction of gene I (movement protein), gene IV (coat protein), and gene VI. The defect of H31 might be an inability of the W260 gene VI-encoded product to interact with CM1841 proteins. Transgenic *N. bigelovii* plants that express the D4 gene VI-encoded product might be able to complement H31, because the gene VI-encoded product of D4 interacts properly with CM1841 proteins. However, it is not immediately clear how these viral proteins could interact to facilitate long-distance transport.

Expression of the CaMV gene VI-encoded product has been reported in *N. tabacum* L. (Baughman *et al.* 1988; Takahashi *et al.* 1989; Balazs 1990; Goldberg *et al.* 1991), *Datura inoxia* Christie & D. W. Hall. (Goldberg *et al.* 1991), *N. edwardsonii* (Kiernan *et al.* 1989), and now in *N. bigelovii*. The expression of gene VI in *N. tabacum* and *D. inoxia* did not change the reaction of those plants to CaMV infection (Balazs 1990; K.-B. Goldberg, unpublished). Because CaMV is unable to move systemically in either of these plants, this result is not surprising. CaMV infection might be blocked at a number of steps, and it is likely that the proper viral component must be expressed in a transgenic plant to achieve complementation of long-range movement. *N. edwardsonii* has been transformed with gene VI of D4, but has not yet been tested for complementation. Because D4 spreads systemically in *N. edwardsonii*, transgenic *N. edwardsonii* plants that express gene VI of D4 may also complement CaMV isolates defective in long-distance transport.

Previously, we reported that low levels of gene VI expression in transgenic nonhost plants result in chlorosis or a chlorotic mottle, while similar accumulations of the gene VI-encoded product in systemic hosts of the virus did not produce any observable effects (Kiernan *et al.* 1989; Goldberg *et al.* 1991). The results of this study were surprising in that expression of the gene VI-encoded product was correlated with a mild generalized chlorosis in the $R_1$ and $R_2$ progenies. We would not have predicted that this protein would induce symptoms, because previous studies showed that D4 infects *N. bigelovii* systemically (Schoelz *et al.* 1986a). One explanation for this apparent contradiction is that *N. bigelovii* might be very sensitive to the accumulation of gene VI-encoded protein. It may be significant, therefore, to note that we could not regenerate *N. bigelovii*

**Table 3.** Reaction of transgenic and nontransformed *Nicotiana bigelovii* to infection with cauliflower mosaic virus (CaMV)

| Virus | Host | No. of plants systemically infected |
|---|---|---|
| W260 | *N. bigelovii* | 9/10[a] |
| W260 | *N. bigelovii* JS65.4 plant 1[b] | 6/6 |
| H31 | *N. bigelovii* | 0/10 |
| H31 | *N. bigelovii* JS65.4 plant 1 | 7/10 |
| H31/*N. bigelovii*[c] | *N. bigelovii* | 0/10 |
| H31/*N. bigelovii* | *N. bigelovii* JS65.4 plant 1 | 6/10 |

[a] The numerator indicates the number of plants that developed systemic symptoms.
[b] *N. bigelovii* transformed with pJS65 (gene VI of CaMV D4), $R_2$ generation.
[c] H31 that had been passaged through *N. bigelovii* JS65.4 plants.

plants which expressed the gene VI-encoded product of CM1841, a CaMV strain that is unable to infect *N. bigelovii* (J. Kiernan, unpublished). The gene VI-encoded product of CM1841 might be too toxic to allow regeneration of *N. bigelovii*, even though this protein can be expressed in *N. tabacum*, *D. inoxia*, and *N. edwardsonii*.

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