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# Mutational Analysis of Cauliflower Mosaic Virus Gene VI: Changes in Host Range, Symptoms, and Discovery of Transactivation-Positive, Noninfectious Mutants

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**Gene VI of cauliflower mosaic virus (CaMV) has been shown to be a determinant of host specificity of the virus as well as a factor influencing symptom development in infected plants. In addition, it plays a crucial role in viral gene expression through a process of posttranscriptional transactivation. In the present study, linker-insertion mutations within gene VI of a cloned, recombinant cauliflower mosaic virus genome were constructed and tested for infectivity, symptom development on solanaceous plants, and the ability to transactivate viral gene expression. Certain mutations in the first third of the gene resulted in changes in symptoms shown by test plants. Another mutation, also in the first third of the gene, blocked infectivity in the *Nicotiana* species tested and systemic movement in *Datura stramonium*. The mutants were also tested in protoplasts for the ability to transactivate virus gene expression. Infectious mutants were invariably positive for transactivation and mutants negative for transactivation were noninfectious. Interestingly, two mutants positive for transactivation were noninfectious, suggesting a second function for gene VI in the infection process. These results further suggest a role for gene VI, as yet not fully understood, in systemic movement of the virus in infected plants.**

*Additional keywords*: inclusion body, linker-insertion mutagenesis, systemic movement.

Cauliflower mosaic virus (CaMV) is a DNA plant virus with a circular, double-stranded genome of approximately 8 kilobase pairs (kbp) (Shepherd et al. 1970) containing six major open reading frames (ORFs) (Franck et al. 1981; Gardner et al. 1981; Howarth et al. 1981; Balzas et al. 1982). Gene VI, which codes for the 62-kilodalton (kDa) inclusion body protein (P62), has received attention as a factor which influences symptom development and host specificity of the virus (Daubert et al. 1984; Schoelz et al. 1986a; Schoelz and Shepherd 1988; Stratford and Covey 1989; Daubert and Routh 1990; Qui and Schoelz 1992; Wintermantel et al. 1993). In a study of the Cabbage-B strain of CaMV, it was shown that mutations within gene VI that did not inactivate the virus-caused symptoms that were markedly different from the wild-type (Daubert et al. 1983). The ability of CaMV to infect certain solanaceous hosts has been mapped to gene VI through the construction of recombinant viral genomes (Daubert et al. 1984; Schoelz et al. 1986a; Schoelz and Shepherd 1988).

Experiments using caulimovirus-based reporter gene constructs and plant protoplast systems have shown that P62 is also required for the expression of the closely spaced genes on the 35S RNA, a process termed posttranscriptional transactivation (Bonneville et al. 1989; Gowda et al. 1989; Scholthof et al. 1992a). Two virus-related transcripts are produced in infected cells, the 35S RNA (Guilley et al. 1982; Covey and Hull 1981), which is a more than full-length RNA copy of the genome, and the 19S RNA, which is a monocistronic mRNA spanning ORF VI (Covey and Hull 1981; Odell and Howell 1980). The 35S RNA serves as a template for virus replication via reverse transcription (Hull and Covey 1983; Pfeiffer and Hohn 1983) and, based on experiments with CaMV and evidence derived from a closely related caulimovirus, figwort mosaic virus (FMV), it is also a polycistronic mRNA for the expression of genes I to V and possibly gene VI (Dixon and Hohn 1984; Fütterer and Hohn 1991; Scholthof et al. 1992a).

To learn more about the role of gene VI in symptom development and host range, and its relationship to transactivation, several gene VI mutants were constructed and examined in whole plant and protoplast systems. A recombinant clone of cauliflower mosaic virus capable of infecting solanaceous hosts was constructed in such a way that gene VI could be easily subcloned, mutated, and returned to the remainder of the genome. Mutagenesis of gene VI was performed by linker-insertion.

In the present experiments, particular mutations in the N-terminal third of gene VI led to changes in host specificity and symptom development, supporting earlier findings. With one exception, the infectious mutants were located in the N-terminal third of gene VI, indicating a higher degree of plasticity in this region of P62 compared to the central and C-terminal thirds of the protein. All infectious mutants were positive for transactivation, while those mutants negative for

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transactivation were noninfectious. Two mutants were found to be transactivation positive but noninfectious. These observations show that transactivation is required for infectivity, and further, suggest that P62 performs a function in addition to transactivation that is necessary to establish infection.

## RESULTS

### Construction, infectivity, disease phenotype, and host range of the gene VI mutants.

The wild-type CaMV clone used in these experiments, pCaMV H103, contained unique *Eco*RV and *Xho*I sites at the 5′ and 3′ ends of gene VI, respectively. The gene VI sequences were subcloned into pIBI 20 (International Biotechnologies Inc.), subjected to insertion mutagenesis, and then used to reconstruct full-length clones (see Materials and Methods for a more detailed description). H103, like the closely related D4, systemically infected turnip, *Datura stramonium, Nicotiana edwardsonii,* and *N. bigelovii.* Inoculated turnips displayed veinal necrosis similar to, but less severe than, that seen with CaMV strain D4. Additionally, inoculated turnips were frequently symptomless, and this may be a result of the virus adaptation to solanaceous hosts. In *D. stramonium,* H103 caused chlorotic local lesions on inoculated leaves followed by marked vein clearing and severe distortion on the upper leaves. The *Nicotiana* spp. used responded to inoculation with chlorotic local lesions followed by chlorotic lesions, vein clearing, stunting, mild mosaic, and occasional distortion of the upper leaves.

A total of 14 mutations in 11 different locations within gene VI of pCaMV H103 were constructed and sequenced; the locations and amino acid changes generated by the mutations are shown in Table 1. Six of the 14 mutants were infectious and, with one exception, the infectious mutants contained changes located within the N-terminal third of gene VI (Fig. 1). The mutants H109, H111, and H119 showed no changes from H103 with respect to symptoms and host range. The mutations that resulted in changes in host range or disease phenotype (H108, H116, and H118) were located in the N-terminal third of gene VI, a region previously shown to be involved in host-range determination (Schoelz et al. 1986a). H111 was the only infectious mutant with changes located outside the N-terminal third of the protein. The most dramatic change due to mutation was seen with H108, which failed to infect *Nicotiana bigelovii* or *N. edwardsonii* and caused only local lesions in *Datura stramonium.* This effect is of interest because the mutations in H109 and H119, located only five codons upstream, did not affect the host range of the virus or alter the disease phenotype. These results suggest that this region of P62 is critical in controlling the host response to infection or systemic movement of the virus. The local lesions caused by H108 on *D. stramonium* were identical in appearance to those caused by H103, H109, and H119. That H108 failed to move into the upper leaves of *D. stramonium* was shown by ELISA (Table 2).

The mutants H116 and H118 were markedly attenuated in *D. stramonium* (Fig. 2), but displayed symptoms similar to the wild type in *N. bigelovii* and *N. edwardsonii.* When H116 was inoculated to *D. stramonium,* approximately half of the plants failed to show symptoms on the upper leaves. ELISA tests showed that the virus was not present in the symptomless upper leaves (Table 2). Likewise, systemic movement of H118 was apparent only in three-fourths of the inoculated plants. H103, by contrast, moved to the upper leaves in every plant inoculated (Table 2).

H116-inoculated *D. stramonium* initially showed chlorotic local lesions identical to those caused by H103. Whereas H103 caused marked vein clearing, stunting, and distortion of the uppermost leaves, H116 caused only a mild vein clearing with no distortion of leaves or reduction in their size. The mutant H118 caused symptoms similar to H116, but these were slightly more severe. Some distortions of the upper leaves were evident in H118 infection and vein clearing was more pronounced, though still not to the extent observed with H103 (Fig. 2).

**Table 1.** Structure of insertion mutations in CaMV P62

| Mutant | aa/nt[a] position | Restriction site | Amino acid changes From | To |
|---|---|---|---|---|
| H114 | 16/5822 | *Sac*I | EL | AGSR |
| H115 | 24/5848 | *Hin*dIII | LA | LGTSLA |
| H109 | 50/5921 | *Xmn*I | AV | ADPV |
| H119 | 50/5921 | *Bam*HI[b] | AV | ADPSTV |
| H108 | 55/5940 | *Bal*I | A | GYP |
| H116 | 91/6040 | *Eco*RI | NS | NWVPNS |
| H118 | 91/6040 | *Kpn*I[b] | NS | NWRDPANS |
| H106 | 181/6318 | *Pvu*II | A | GIP |
| H117 | 239/6490 | *Bgl*II | IS | IRIRIS |
| H112 | 345/6808 | *Sca*I | ST | SGSEFT |
| H110 | 412/7007 | *Xmn*I | I | RIL |
| H107 | 413/7013 | *Hinc*II | RQ | RRIQ |
| H113 | 413/7013 | *Hinc*II | RQ | RRYQ |
| H111 | 510/7302 | *Xmn*I | D | GDP |

[a] Nucleotide numbers represent the first nucleotide in the restriction site used in making inserts. Amino acid number shown is last CaMV-encoded amino acid before insert. Map positions based on DNA sequence of CaMV-10 gene VI beginning with ATG at nt position 5774 (Gardner et al. 1981).

[b] Restriction sites were created by insertion mutagenesis in previous experiments and are not present in native geneVI. Thus, H119 was made by modification of the *Bam*HI site present in H109.

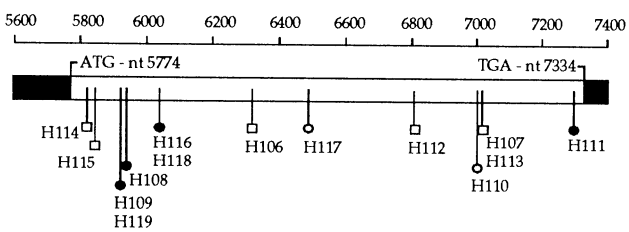


**Fig. 1.** Schematic diagram of the gene VI mutants showing location of insertions. Nucleotide numbering is based on CaMV 1841 (Gardner et al. 1981). Infectious mutants (●), noninfectious mutants (○,□) transactivation positive mutants (●,○), and transactivation negative mutants (□). Infectivity of the mutants based on inoculation of turnip with cloned CaMV DNA digested with *Sal*I.

### Transactivation potentials of the gene VI mutants.

The results of four separate transactivation experiments are shown in Table 3. Mutants which tested negative for transactivation were tested at least four times in protoplast assays. Mutations that destroyed transactivation were located in the extreme N-terminal region, the central region, and the C-terminal one-third of P62 (Fig. 1). The infectious mutants

were invariably positive for transactivation and, conversely, mutants that tested negative for transactivation were, without exception, noninfectious. However, two noninfectious mutants, H110 and H117, were found to be transactivation positive, indicating that the gene VI product has a second function in the infection process.

In addition to the standard tests to determine infectivity (see Materials and Methods), H110 and H117 were subjected to further tests to substantiate their noninfectious phenotypes. Twenty turnip seedlings were inoculated with 40 μg each of *Sal*I cleaved pCaMV H110 and H117, and were tested for the presence of the virus by ELISA weekly for 90 days. Both the inoculated and upper leaves of these plants tested negative for CaMV. To investigate the possibility that a fortuitous mutation in genes I through V or in the intergenic regions was responsible for lack of infectivity, the *Eco*RV to *Xho*I fragments containing gene VI of pCaMV H110 and H117 were replaced with the wild-type *Eco*RV to *Xho*I fragment from p2036. These clones, designated pCaMV H310 and H317, were both infectious when inoculated to turnip.

Two frameshift mutants of gene VI were constructed and tested in the protoplast assays. Both of these mutants were negative for transactivation, indicating that P62, and not the 19S transcript, is active in transactivation. The mutant H114f contained a frameshift in the extreme N-terminal region of P62, and, based on DNA sequence data, would have allowed the translation of 16 codons before translation was terminated. The frame change in H112f allows for the translation of the first 346 codons of the 521 codons of gene VI before translation is prematurely halted.

**Table 2.** Responses of *Datura stramonium* to inoculation with CaMV gene VI mutants

| | Symptoms | | | | | |
|---|---|---|---|---|---|---|
| | | Systemic | | ELISA values($A_{450}$)[c] | | |
| Mutant[a] | Local[b] | Mild | Severe | Mild | Severe | No Symptoms |
| H103 (23) | 23 | 6 | 17 | 0.118 | 0.340 | ... |
| H109 (20) | 20 | 2 | 18 | 0.270 | 0.439 | ... |
| H119 (16) | 16 | 3 | 13 | ND[d] | 0.503 | ... |
| H108 (17) | 17 | 0 | 0 | ... | ... | 0.013 |
| H116 (18) | 18 | 8 | 0 | 0.081 | ... | 0.006 |
| H118 (24) | 24 | 17 | 0 | 0.087 | ... | ND |
| H111 (15) | 15 | 2 | 13 | 0.053 | 0.310 | ... |

[a] Numbers in parentheses represent the number of plants inoculated with the mutant virus.
[b] Chlorotic local lesions on inoculated leaves.
[c] Average from three plants, when possible, displaying the symptom type indicated. Upper (systemic) leaves were tested 35 days after inoculation. Mean ELISA value for healthy samples was 0.008.
[d] ND: Not determined.

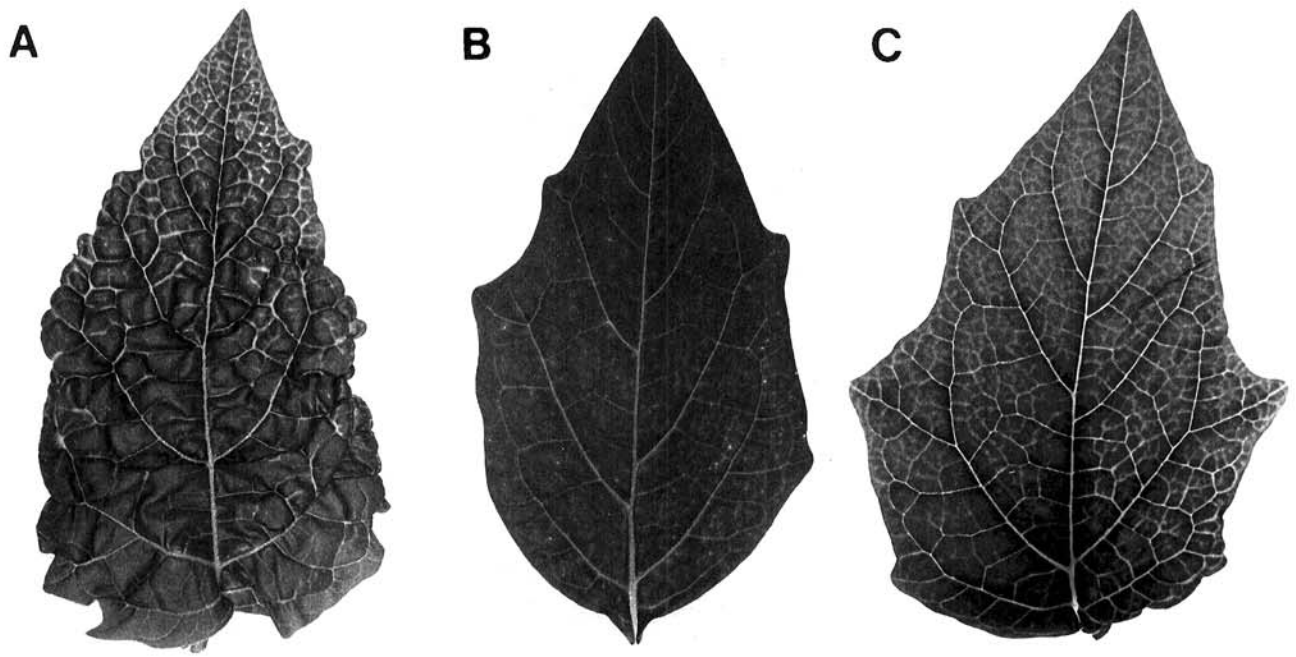


**Fig. 2.** Symptoms shown on upper leaves by *Datura stramonium* 40 days after inoculation with mutant and wild-type virus. **A,** H103 (wild type); **B,** H116; C. H118. Severe leaf distortions are seen with H103; slight leaf distortions are evident in lower third of leaf inoculated with H118. H116 caused only vein-clearing and chlorotic spots.

## DISCUSSION

A large body of evidence has accumulated showing gene VI to be a determinant of the host range of CaMV and a factor in pathogenesis. How gene VI exerts this effect in infected plants remains unknown. The discovery of a molecular function for P62, that of posttranscriptional transactivation, has yet to provide additional clues to the details of the interaction between gene VI and the host plant. The demonstration that certain gene VI mutants retain the ability to transactivate viral gene expression in protoplasts but are noninfectious at the whole plant level may indicate that transactivation and interaction(s) with the host plant in respect to pathogenesis are separate functions of P62.

There are several possible explanations for the transactivation-positive, noninfectious mutants. For instance, H110 and H117 may be competent to exert the required translational effect, but may be deficient in the required *cis* elements (Scholthof et al. 1992b). Another possibility is that these mutations destroyed the ability of P62 to interact with other viral gene products. This could include a defect in the formation of inclusion bodies through interactions with other P62 molecules, the coat protein, or with other viral proteins present in inclusion bodies (Maule et al. 1989). Reverse transcription appears to occur within partially assembled virions (Marsh and Guilfoyle 1987) in the inclusion bodies (Modjtahedi et al. 1984) and the inclusion bodies are almost certainly the sites of virus assembly (Shepherd 1976). Therefore, a defect in P62 affecting inclusion body formation, or assembly of the virions, could disrupt replication of the viral DNA and destroy infectivity. In addition, Schoelz and Shepherd (1988) and Qui and Schoelz (1992) have shown that the protein product of gene VI must interact with another locus of CaMV located between genes I and V for systemic movement of the virus in certain hosts. It may be that the transactivation-positive, noninfectious mutants are unable to interact with this as yet unidentified locus. In addition to an interaction with other viral genes, the gene VI product may interact with a component(s) of the host cell to determine the viral host range. In the transactivation-positive, noninfectious mutants, the ability of P62 to interact effectively with the host may have been destroyed.

**Table 3.** Transactivation by the gene VI mutants in *Nicotiana edwardsonii* protoplasts[a]

| Transactivating plasmid | % Conversion to acetylated form[b] | | | |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
| S10CAT4 alone | 1.5 | 1.3 | 0.8 | 1.8 |
| H101 | 1.3 | 2.1 | 1.6 | 1.3 |
| H103 | 15.9 | 68.7 | 13.2 | 6.9 |
| H114 | 0.6 | 1.7 | ... | ... |
| H115 | 1.9 | 1.9 | ... | ... |
| H109 | 4.8 | 55.5 | 9.2 | 9.0 |
| H119 | 18.6 | 54.3 | 15.7 | 5.9 |
| H108 | 13.1 | 37.3 | 13.3 | 4.9 |
| H116 | 10.8 | 37.0 | 11.1 | 4.2 |
| H118 | 12.7 | 49.6 | 13.9 | 3.5 |
| H106 | 1.2 | 1.4 | ... | ... |
| H117 | 12.6 | 22.4 | 15.6 | 4.3 |
| H112 | 1.1 | 0.7 | ... | ... |
| H110 | 11.2 | 57.6 | 2.8 | 4.3 |
| H107 | 1.2 | 1.6 | ... | ... |
| H113 | 1.2 | 0.8 | ... | ... |
| H111 | 3.9 | 50.2 | 10.3 | 8.2 |

[a] Results of four representative experiments to determine the transactivation potentials of the different mutants. Experiments 1 and 2, all in-frame mutants tested. Experiments 3 and 4, only those mutants initially testing positive were tested. Mutants testing negative were assayed a minimum of four times and mutants testing positive were assayed a minimum of 12 times with comparable results.

[b] $2 \times 10^6$ protoplasts were electroporated with pS10CAT4 and the transactivating plasmid and then were incubated at room temperature for approximately 24 h. Percent conversion to acetylated form of chloramphenicol from assay using $2 \times 10^5$ surviving protoplasts per sample.

The infectious mutants, with one exception, and all of the mutants with phenotype or host-range differences contained changes located in the N-terminal third of gene VI. These observations indicate that this region of the protein is more tolerant of changes than the central and C-terminal regions, and further, that these changes may represent adaptations to different host plants. The DNA sequence of gene VI of the D4 strain was determined (data not shown) and was compared to the published sequence of CM1841 (Gardner et al. 1981). Comparison of the two sequences and the deduced amino acid sequences of the genes revealed a very high degree of conservation between D4 and CM1841 in the central and C-terminal thirds of the protein, indicating that changes in these regions may be detrimental to its normal functioning. In total, we found 12 nucleotide changes in these regions (approximately 1 kbp in length). Eight of these nucleotide substitutions were silent and of the four that resulted in amino acid changes, two were conservative. This is in contrast to over 30 nucleotide substitutions in the N-terminal third (approximately 500 bp) of the gene, 19 of which result in amino acid changes, only three of which were conservative (Wintermantel et al. 1993). However, the extreme N-terminal sequences of gene VI of CaMV appear to be important for transactivation and therefore, infectivity. This observation is supported by the fact that there are no nonconservative amino acid changes between CM1841 and D4 in the first 38 amino acids of P62 and the fact that both H114 and H115 (16 and 24 codons from the first ATG, respectively) were transactivation negative.

Several of the mutants showed changes in disease phenotype and host range. The mutants H116 and H118 showed an altered disease phenotype in *D. stramonium* and also a reduced level of systemic movement. These mutants were not phenotypically different from the wild-type in the *Nicotiana* species used, but they were present at consistently lower concentrations (data not shown). H116 and H118 contained insertions of different sizes in the *Eco*RI (nt position 6040) site of gene VI, between amino acid residues 91 and 92. Daubert and Routh (1990) reported that a single amino acid change at amino acid residue 104 in CaMV strain D4 caused the virus to produce necrotic local lesions. D4 normally causes chlorotic local lesions and systemic infection in *D. stramonium* (Schoelz et al. 1986b). The proximity of the mutations in H116 and H118 to the site identified by Daubert and Routh (1990) indicates that this region of the protein may be involved in host-virus interaction.

The mutant H108 did not move systemically in *D. stramonium*, and was unable to infect either of the *Nicotiana* species used. The mutants H109 and H119, which contained changes located only five codons upstream of the mutation in H108, were able to infect all the test plants and reached high concentrations in these plants as well. Although these differences

could be due to the respective amino acids encoded by the particular mutations, it is also possible that these sites enclose the N-terminal border of a region within P62 that interacts with the host plant. These results further point to involvement of P62 in systemic movement of the virus, although its role in systemic movement is not yet clear. Alternatively, P62 may be involved in overcoming the host plant's defense response, and due to its mutation, H108 is impaired in this function.

This work suggests that P62 has a multifunctional role in the infection process. The currently available information suggests that P62 interacts with an as yet unknown host component and other viral genes in determining host range. The protein also interacts with the 35S RNA and possibly ribosomes in its role as transactivator of viral gene expression and further, with other P62 molecules, the virus capsid protein, and other viral gene products in inclusion body formation.

## MATERIALS AND METHODS

### Recombinant DNA methods.

Restriction enzymes, DNA modifying enzymes, and synthetic linkers were purchased from several sources (Boehringer-Mannheim, International Biotechnologies Inc., New England Biolabs, United States Biochemicals) and used according to the manufacturer's instructions. Cloning protocols and mutagenesis were performed as described in Maniatis et al. (1982). *Escherichia coli* strain JM101 (Messing 1979), which was used in all cloning experiments, was maintained on M9 minimal medium and cultured in LB medium containing appropriate antibiotics. DNA sequencing was performed using the dideoxy chain-termination method (Sanger et al. 1977).

### Construction of gene VI mutants of cauliflower mosaic virus.

The recombinant virus clone pCaMV H103 was constructed by exchanging genome fragments from two CaMV recombinant clones, pCaMV NRT17, and pCaMV H12 (Schoelz et al. 1986a). The in vivo recombinant virus NRT17 was made by inoculating individual *Nicotiana bigelovii* plants with virions of three solanaceous strains of CaMV; D4 (Schoelz et al. 1986b), W260, and W283 (Gracia and Shepherd 1985). After several passages through *N. bigelovii*, viral DNA was isolated from infected plants and cloned in the *Sal*I site of pUC19. Symptoms induced by NRT17 on solanaceous hosts closely resembled those caused by D4, but appeared earlier and were more severe. In turnip, NRT17 caused a mild veinal necrosis, but were frequently symptomless. The other parent of pCaMV H103, pCaMV H12, was an in vitro recombinant between CM1841 and D4 cloned at the *Sac*I site of pUC13 (Schoelz et al. 1986a). Gene VI of H12, and thus H103, is composed of sequences from both D4 and CM1841. DNA sequencing (data not shown) revealed that the putative amino acid sequence of gene VI of H103 was identical to that of gene VI of D4 except for four amino acid changes in the C-terminal region of the protein.

pCaMV H103 was constructed in three steps. First, the 4,707 base pair (bp) *Bst*EII to *Sal*I fragment containing genes VII, I to IV, and the first 1,200 bp of gene V of pCaMV NRT17 was ligated to the *Bst*EII to *Sal*I fragment containing the remainder of gene V, gene VI, the pUC13 cloning vector, and the large intergenic region of pCaMV H12. The resulting virus clone, pCaMV H101, was recloned in pUC119 at its unique *Sal*I site and further modified by the insertion of an 8-bp *Xho*I linker in the *Eco*RV site just downstream of the 3′ end of ORF VI. Thus, pCaMV H103 contained a unique *Eco*RV site near the 5′ end of ORF VI and a unique *Xho*I site at the 3′ end of ORF VI. The *Eco*RV to *Xho*I fragment containing ORF VI was subcloned in pIBI20 (International Biotechnologies Inc.) and designated p2036. Linker-insertion mutagenesis was performed by cleaving p2036 with restriction enzymes and inserting synthetic oligonucleotide linkers. Hexameric linkers were used when restriction enzyme digestion resulted in blunt-ended DNA molecules. DNA cut with enzymes generating 5′ or 3′ overhangs was made blunt-ended with T4 DNA polymerase or Klenow fragment, and linkers of the proper size to maintain the reading frame were inserted. Plasmids containing linkers were selected by the presence of the new restriction site, *Bam*HI or *Kpn*I, and sequenced to detect unexpected frame-shifts or the insertion of multiple copies of the linker. Full-length virus clones containing mutations in gene VI were made by exchanging the *Eco*RV to *Xho*I gene VI fragment of H103 for the *Eco*RV to *Xho*I gene VI fragment of the mutant subclones.

### Infectivity and host range studies.

The gene VI mutants were inoculated to *Brassica campestris* (cv. Just Right) by rubbing 2 to 4 μg of *Sal*I cleaved plasmid DNA (in TE buffer) on Celite-dusted leaves of 10- to 14-day-old seedlings. Approximately 20 greenhouse-grown seedlings were inoculated per test and the tests were repeated three or more times for the noninfectious mutants. Since H103 and the mutants did not always cause symptoms in turnip, infectivity was determined by a modification of the double-antibody sandwich enzyme-linked immunosorbant assay (Schoelz et al. 1986a). Mutants judged noninfectious by ELISA were further tested by: (i) inoculation of *Sal*I cleaved cloned DNA to *Datura stramonium*, which would be expected to show symptoms, (ii) mechanical transmission tests from inoculated turnips to plants likely to show symptoms, (iii) concentration of inoculum (from DNA-inoculated turnips) by partial purification procedures and inoculation to test plants. Infectious mutant and wild-type viruses were purified from infected turnip as described by Schoelz et al. (1986a) and used to inoculate solanaceous test plants, *D. stramonium*, *N. bigelovii*, and *N. edwardsonii*. The amount of virus in the test plants was determined using ELISA and purified virus of known concentration. Viral DNA was routinely purified from infected plants (Gardner and Shepherd 1980) and restriction mapped to confirm that reversions or contamination with other viruses had not occurred.

### Protoplast assays for transactivation.

The gene VI mutants were tested for the ability to transactivate expression of CaMV genes in protoplasts prepared from suspension cell cultures of *N. edwardsonii* as described in Gowda et al. (1989). Assays were performed by co-electroporating 20 μg of pS10CAT4 (Gowda et al. 1989) and 50 to 100 μg (7 to 14 μg of gene VI) of cloned, full-length gene VI mutants. After electroporation, the protoplasts were incubated at room temperature under fluorescent lighting. After approximately 24 h, $2 \times 10^5$ protoplasts were collected

and assayed for CAT activity. CAT assays were performed essentially as described (Gorman et al. 1982) and quantified by excising $^{14}C$-labeled spots from the silica gel plate and reading in a scintillation counter. Counts from acetylated forms of chloramphenicol were divided by total counts to give percent conversion to the acetylated form of chloramphenicol, as described in Gowda et al. (1989). pS10CAT4 contains the CaMV 35S promotor, the large intergenic region, and gene VII followed by the chloramphenicol acetyl transferase gene (CAT) fused in-frame with CaMV gene I. pS10CAT4 does not express appreciable levels of CAT activity in the absence of the gene VI protein. Cloned DNA of pCaMV H103 was used as positive control and pCaMV H101, which was cloned at the *Sac*I site in gene VI, was used as a negative control.

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