Multiple Domains Within the *Cauliflower mosaic virus* Gene VI Product Interact with the Full-Length Protein

Yongzhong Li and Scott M. Leisner

Department of Biological Sciences, The University of Toledo, 2801 West Bancroft Street, Toledo, OH 43606, U.S.A.

Submitted 7 February 2002. Accepted 10 June 2002.

The *Cauliflower mosaic virus* (CaMV) gene VI product (P6) is a multifunctional protein essential for viral propagation. It is likely that at least some of these functions require P6 self-association. The work described here was performed to confirm that P6 self-associates and to identify domains involved in this interaction. Yeast two-hybrid analyses indicated that full-length P6 self-associates and that this interaction is specific. Additional analyses indicated that at least four independent domains bind to full-length P6. When a central domain (termed domain D3) was removed, these interactions were abolished. However, this deleted P6 was able to bind to the full-length wild-type protein and to isolated domain D3. Viruses lacking domain D3 were incapable of producing a systemic infection. Isolated domain D3 was capable of binding to at least two of the other domains but was unable to self-associate. This suggests that domain D3 facilitates P6 self-association by binding to the other domains but not itself. The presence of multiple domains involved in P6 self-association may help explain the ability of this protein to form the intracellular inclusions characteristic of caulimoviruses.

Additional keywords: viroplasm, translational transactivation.

The formation of inclusion bodies, or viroplasms, is a common feature of many plant viruses (Martelli and Russo 1977). The inclusion bodies formed by *Cauliflower mosaic virus* (CaMV) probably play an important role in infection, because they are likely sites of genome replication and virion assembly (Himmelbach et al. 1996; Mazzolini et al. 1989; Rothnie et al. 1994). CaMV inclusion bodies are mainly composed of the gene VI product (P6) (Covey and Hull 1981). CaMV gene VI conditions additional viral activities, such as chlorotic symptom formation and the degree to which a virus-infected plant is stunted (Anderson et al. 1991; Stratford and Covey 1989). Expression of P6 in transgenic plants causes the formation of viruslike symptoms (Baughman et al. 1988). Gene VI is also a host range determinant, since it controls whether CaMV isolates can infect solanaceous plants (Schoelz and Shepherd 1988; Schoelz et al. 1986) or particular *Arabidopsis thaliana* ecotypes (Agama et al. 2002). Finally, gene VI influences virus systemic spread (Qiu et al. 1997; Schoelz et al. 1991; Wintermantel et al. 1993).

To accomplish these diverse tasks, P6 must be a multifunctional protein. P6 mediates translation of the multiple open reading frames from the polycistronic 35S RNA by the process

of translational transactivation (TAV) (Bonneville et al. 1989). P6 possibly mediates TAV through ribosome reinitiation (Park et al. 2001), thereby probably regulating the levels of viral proteins (Anderson et al. 1991; Kobayashi et al. 1998). P6 from certain CaMV isolates can also act as an avirulence gene product in certain *Nicotiana* species (Cole et al. 2001; Palanichelvam et al. 2000), indicating that it likely interacts with host factors. However, it is unknown if the many tasks accomplished by P6 require self-association.

Data from several previous studies suggest that P6 self-associates. Transgenic plants expressing P6 alone form inclusion body-like structures (Cecchini et al. 1997). *Escherichia coli* expressing P6 also form inclusion bodies (Himmelbach et al. 1996). However, overexpression of proteins in *E. coli* often leads to the formation of aggregates that are not always reflective of biological function, so the significance of the bacterial P6 inclusion bodies is unclear. Based on interference data in protoplasts, a region spanning P6 amino acids 307 to 378 was implicated in protein-protein interactions, possibly self-association (DeTapia et al. 1993). Finally, experiments with chimeras suggest that the amino-terminal third and the middle third of the gene VI product may interact to influence light-dependent infection (Wintermantel et al. 1993). Taken together, these data suggest that the gene VI product self-associates. To investigate this possibility directly, we employed a LexA-based yeast two-hybrid system (Gyuris et al. 1993). The gene VI product was found to self-associate using this system. Furthermore, the self-association of the gene VI product appears complex, involving at least four independent domains.

RESULTS

CaMV gene VI product self-association can be detected by yeast two-hybrid analysis.

To directly determine if P6 self-associates, full-length CM1841 gene VI clones were generated by polymerase chain reaction (PCR) (Fig. 1) and inserted, in frame, with either the gene segment encoding the B42 activation domain in the pJG4-5 plasmid or the LexA DNA-binding domain in the pEG202 plasmid. As expected, the full-length gene VI product was found to self-associate; yeast expressing both the P6-LexA and P6-B42 fusions promoted leucine-independent growth and showed β -galactosidase activity (Fig. 2). In contrast, P6 did not interact with either LexA or the B42 domain alone. Likewise, P6 did not interact with *Arabidopsis thaliana* eIFiso4E (Wittman et al. 1997) or human actin (Nieset et al. 1997).

Multiple regions of P6 interact with the full-length protein.

To identify the portions of P6 mediating self-association, several fragments of gene VI were generated by PCR (Fig. 1)

Corresponding author: Scott Leisner; Telephone: 419-530-1549; Fax: 419-530-7737; E-mail: sleisne@uoft02.utoledo.edu.

and inserted into the yeast two-hybrid vectors. The polypeptides encoded by these fragments were tested for interaction with the full-length P6 by yeast two-hybrid analysis. Deletions from the carboxyl-terminal end indicated that the amino-terminal 110 amino acids were capable of binding to full-length P6 (Fig. 2). To determine if additional regions of P6 were capable of interacting with the full-length protein, several internal fragments, as well as the carboxyl-terminal 106 amino acids, were tested. The carboxyl-terminal end of P6 (amino acids 414 to 520) bound to the full-length protein. Likewise, a fragment from amino acid 156 to 253 interacted with full-length P6, as did a fragment from 249-379. Hence, it appears that there are at least 4 regions within P6 that bind to the full-length protein. These four regions were termed: domains D1 (amino acids 1 to 110), D2 (amino acids 156 to 253), D3 (amino acids 249 to 379), and D4 (amino acids 414 to 520).

Domain D3 plays an important role in P6 self-association.

To clarify the relationships between these domains, an internal deletion mutation was made. Restriction enzyme digestion was used to remove the domain D3 coding sequences from gene VI. This deleted gene VI product (called FL-ΔN) was then tested for binding to itself, the wild type protein, and the four domains identified above by yeast two-hybrid analysis. The FL-ΔN protein interacted with the full-length gene VI product (Fig. 2). However, FL-ΔN was incapable of self-association, nor did it interact with domains D1, D2, or D4 (Fig. 3). These data suggested that domain D3 is important for interactions with domains D1, D2, or D4.

If domain D3 is critical for interactions with the other domains, then it should interact with the FL-ΔN protein. To address this hypothesis, domain D3 was tested in the yeast two-hybrid system. This fragment was found to interact with FL-ΔN (Fig. 3). The domain D3 fragment was also tested against each of the 4 domains individually in yeast two-hybrid analyses (Fig. 4). Domain D3 interacted with domains D1 and D4 but not with itself. Leucine-independent growth was not observed when domain D2 was tested for its ability to interact with domain D3. However, this combination showed approximately fourfold higher β-galactosidase activity than the controls. Thus, domain D2 may bind to domain D3, but this interaction may be too inefficient to permit leucine-independent growth.

The data above clearly implicate domain D3 as playing an important role in P6 self-association. This makes one question whether the other domains interact with one another. To address these possibilities, we tested the various domains for their ability to bind each other. Our data indicate that domains D1 and D4 bind to only domain D3 but not to the other domains (Fig. 4). With the possible exception of domain D3, domain D2 did not bind to any of the other P6 regions as well.

Domain D3 is required for CaMV systemic infection.

Although domain D3 appears to be important for P6 self-association in the yeast two-hybrid system, it was critical to determine if this region was also required for virus infection. To address this question, a CM1841 viral genome lacking P6 domain D3 was generated in the pCaMV10 (Gardner et al. 1981) vector and inoculated to turnip plants. Turnips were also inoculated with wild-type viral DNA. By 32 days postinoculation virtually all of the young leaves on all turnips inoculated with the wild-type virus DNA were covered with symptoms. None of the leaves on turnips inoculated with the deletion mutant virus showed symptoms, even up to 65 days postinoculation. PCR analyses confirmed that CaMV DNA was present in both inoculated and upper noninoculated leaves from plants infected with the wild-type virus (a representative PCR result is shown in Figure 5). However, CaMV DNA was not detected in either

inoculated or upper noninoculated leaves of turnips infected with the domain D3 deletion mutant.

DISCUSSION

CaMV forms cytoplasmic inclusion bodies during the course of a viral infection (Covey and Hull 1981; Hull and Covey 1985; Rothnie et al. 1994). CaMV inclusion bodies have been proposed to be specialized structures involved in translation of viral proteins (Hohn and Fütterer, 1997). Evidence for this comes from studies with CaMV vectors expressing the human *interferon* gene. In plants infected with this recombinant virus, interferon accumulated in inclusion bodies, suggesting that translation of CaMV 35S RNA occurs in these structures (deZoeten et al. 1989). The fact that reverse transcriptase activity is also present within inclusion bodies suggests that they may be the site of CaMV genome replication as well (Mazzolini et al. 1989). In most cases, virtually all virus particles in infected cells are located within inclusion bodies, with small amounts being located in cytoplasm near these structures or within plasmodesmata (Shepherd 1979). Since P6 (the major inclusion body protein of CaMV) has been shown to interact with the CaMV capsid protein and this interaction has been suggested to assist in virion assembly (Himmelbach et al. 1996), it is possible that inclusion bodies may also be sites for building virus particles. These data taken in toto suggest that CaMV inclusion bodies are active structures in which many viral activities occur.

P6 plays additional roles in viral infection as well, such as translational transactivation (TAV) and host range determination (Bonneville et al. 1989; Schoelz et al. 1986; Schoelz and Shepherd 1988). However, it is unclear whether P6 accomplishes these functions as a monomeric or multimeric protein. To begin to address these questions, the self-association of P6 was examined. We found the yeast two-hybrid system to be a useful tool for studying P6 self-association. Furthermore, this interaction was specific, since P6 did not bind to just any protein.

P6 self-association was found to be complex. At least four domains were identified that bound to full-length P6; the amino- and carboxyl-terminal ends (domains D1 and D4, respectively) and two internal regions (domains D2 and D3). The presence of four domains involved in P6 self-association may explain how the protein is able to form inclusion bodies; a P6 molecule may bind to another and still have binding sites free for attachment to additional molecules. This would permit association of the P6 molecules into larger complexes and could result in the formation of large cytoplasmic inclusion bodies.

Fig. 1. Gene VI product (P6) fragments examined in this study. Top construction is the full-length P6 protein, which is composed of 520 amino acids. The various fragments of P6 are shown below the full-length protein. The amino acids column indicates the amino acids present within the polypeptide. The primers column indicates the polymerase chain reaction (PCR) oligonucleotides that were used to generate DNA fragments encoding the various P6 regions. NA = not applicable, construction was generated using extant restriction enzyme sites rather than PCR. Note that the bottom construction is an internal deletion mutant of P6.

Based on computational analysis, each of the four domains appears to be mainly α -helical. It is unclear if these domains possess a type of repeating structure or are part of a common motif. However, these domains do bind to the full-length protein in isolation. This suggests that these regions are, in fact, domains, i.e., independently folding regions of protein secondary structure. Several of these domains are located within portions of P6 that have been implicated in P6 function.

Domain D1 is situated within the portion of P6 involved in virus-host interactions (Agama et al. 2002; Schoelz and Shepherd 1988; Wintermantel et al. 1993). Perhaps by domain D1 binding to domain D3, P6 recognition determinants are either exposed or masked from host resistance pathways, such as the hypersensitive response (Cole et al. 2001; Palanichelvam et al. 2000). Through this route, the domain D1-D3 interaction could influence virus-host interactions.

At this stage, it is unclear to which portion of P6 domain D2 binds. Its inability to bind to FL- Δ N suggests that the structure of this deleted form of P6 may be altered to the extent that D2

cannot bind. Alternatively, the location where D2 associates may be partially or completely removed by the FL- Δ N deletion so that binding does not occur. Our data may provide support for the latter possibility, since domain D2 may inefficiently bind to domain D3. It will be critical to clarify this since D2 contains the minimal TAV domain (De Tapia et al. 1993).

Domain D3 appears to be important for P6 self-association; if it is removed, the deleted form of the protein (FL- Δ N) is incapable of self-association. FL- Δ N is also incapable of binding to domains D1, D2, or D4. In contrast, FL- Δ N can bind to wild-type P6, as well as to isolated domain D3. This suggests that at least some portion of FL- Δ N must be in its normal conformation. Domain D3 alone can bind directly to domains D1 and D4 as well as to the full-length protein. This domain may also bind in an inefficient manner to domain D2. However, domain D3 does not appear to be capable of binding to itself. Taken together, it is likely that domain D3 mediates P6 self-association by binding to one of the other domains. Binding of domain D1 to D3 is consistent with viral chimera data suggesting that such an interaction

Fig. 2. Interaction of gene VI product (P6) regions with the full-length protein. **A**, Schematic diagram of the constructions tested for leucine-independent growth and β -galactosidase activity. Black box, LexA DNA-binding domain (from pEG202); hatched box, B42 transcription activation domain (from pJG4-5); shaded box, *A. thaliana* eiFiso4E; checkerboard, human actin. The unshaded box indicates P6 and its fragments, with the numbers inside the box indicating the P6 amino acids present. Full-length P6 consists of 520 amino acids. Numbers to the left of each pair of constructions correspond to the β -galactosidase assay data shown in B and the yeast colonies shown in C. **B**, β -galactosidase activity of yeast transformants expressing constructions shown in A. The bar graph indicates average β -galactosidase units (on abscissa) for three experiments along with the standard deviation. Numbers at the bottom (ordinate) correspond to the transformants grown on media with and without leucine in C. **C**, Growth of yeast transformants on media with (left) and without (right) leucine. The streaks are numbered to correspond to the constructions shown in A and the β -galactosidase activities shown in B. **D**, The key for the plates in C.

may be important in conditioning light-dependent infection in *Nicotiana* species (Wintermantel et al. 1993). Finally, domain D3 contains a region that is highly conserved among caulimoviruses (De Tapia et al. 1993; Sanger et al. 1991), suggesting that it plays an important role in P6 function.

Domain D3 may be involved in TAV. A deleted form of P6 lacking the carboxyl-terminal end of the minimal TAV domain prevented translational transactivation mediated by the wild-type protein when both forms were present within protoplasts (De Tapia et al. 1993). These data suggest that P6 self-association is involved in translational transactivation. Intriguingly, deletion of amino acids 307 to 378 (which is contained within domain D3) abolished the inhibition of translational transactivation.

In addition to its potential role in P6 self-association, domain D3 is required for development of systemic viral infections. It is likely that the inability of the mutant virus to systemically infect plants may be due to a lack of viral viability, since CaMV DNA was not detected in inoculated leaves. This lack of infectivity for viruses lacking domain D3 may be due to a disruption of P6 structure, an inability to self-associate, or for some other reason and warrants additional investigation.

It is important to note that domain D3 contains both of the RNA-binding domains of P6 (De Tapia et al. 1993). However, the ability of domain D3 to bind domains D1 and D4 strongly suggests that these are protein-protein interactions and are not mediated via an RNA molecule.

Domain D3 also contains the portion of P6 that interacts with eukaryotic initiation factor 3 subunit g (eIF3g) and large

ribosomal subunit protein L24 (Park et al. 2001). These interactions have been shown to be important for P6-mediated ribosome reinitiation. It is interesting that the region of P6 that interacts with eIF3g and L24 also interacts with other parts of P6. This may indicate that binding of one P6 molecule to another may facilitate the association of one of the P6s with eIF3g/L24. It has been suggested previously that P6 can act as a chaperone protein (Himmelbach et al. 1996).

Domain D4 is in the region of P6 that, when deleted, results in a reduction in the rate of virus movement (Turner et al. 1996). Mutations in this region have also been reported to influence virus host range (Daubert and Routh 1990). As with domain D1, binding of domain D4 to P6 via domain D3 may influence virus-host interactions.

In summary, the CaMV gene VI product specifically self-associates, and this binding is complex, involving at least four domains. One of these, domain D3, appears important for binding to domains D1 and D4 (and possibly domain D2) but not to itself. In the future, it will be important to determine the role of the various P6 self-interaction domains in the many activities mediated by this essential viral protein.

MATERIALS AND METHODS

Construction of clones for the two-hybrid assays.

All enzymes were purchased from Promega Corporation (Madison, WI, U.S.A.) and were used in accordance with the manufacturer's specifications. S. H. Howell (Iowa State Uni-

Fig. 3. Interaction of gene VI product (P6) regions with the domain D3 deletion mutant. **A**, Schematic diagram of the constructions tested for leucine-independent growth and β -galactosidase activity. Black box, LexA DNA-binding domain (from pEG202); hatched box, B42 transcription activation domain (from pJG4-5). The unshaded box indicates the P6 fragments, with the numbers inside the box indicating the P6 amino acids present. Note that P6 amino acids 1 to 110 are domain D1; 156 to 253, domain D2; 249 to 379, domain D3; and 414 to 520, domain D4. Numbers to the left of each pair of constructions correspond to the β -galactosidase assay data shown in B and the yeast colonies shown in C. **B**, β -galactosidase activity of yeast transformants expressing constructions shown in A. Bar graph indicates average β -galactosidase units (on abscissa) for three experiments, along with the standard deviation. Numbers at the bottom (ordinate) correspond to the transformants grown on media with and without leucine in C. **C**, Growth of yeast transformants on media with (left) and without (right) leucine. The streaks are numbered to correspond to the constructions shown in A and the β -galactosidase activities shown in B. **D**, The key for the plates in C.

versity, Ames, IA, U.S.A.) provided the cloned genome for CaMV isolate CM1841 (in plasmid pCaMV10) (Gardner et al. 1981). Plasmids pEG202 and pJG4-5, as well as yeast strain EGY48 harboring pSH18-34, were a gift from R. Brent (Molecular Sciences Institute, Berkeley, CA, U.S.A.). The human *actin* gene was a gift from M. Wheelock and K. Johnson (University of Nebraska Medical Center, Omaha, NE, U.S.A.). The *Arabidopsis thaliana* *eIFiso4E* gene was a gift from J.-F. Laliberte (Centre de recherche en virologie, Institut Armand-Frappier, Quebec, Canada).

The primer sequences used to amplify the CaMV gene VI fragments are given in Table 1 and were synthesized by DNA International (Lake Oswego, OR, U.S.A.). The primers were designed to contain restriction enzyme sites (usually *Eco*RI, *Bam*HI, or *Xho*I) based on the multiple cloning sites in yeast plasmids pEG202 and pJG4-5 (Ausubel et al. 1993; Gyuris et al. 1993). The restriction enzyme site engineered into the forward primer permitted in frame fusion of the gene VI fragment with either the *LexA* DNA-binding domain encoding region of pEG202 or the DNA sequence coding for the B42 activation domain in pJG4-5. Reverse primers were designed to contain a termination codon. The portions of P6 encoded

by the various PCR-generated fragments are shown diagrammatically in Figure 1.

DNA fragments larger than 500 bp were amplified with *Pfu* DNA polymerase. The other fragments were amplified with *Taq* DNA polymerase. The standard PCR amplification program was 35 cycles of 92°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Following amplification, the PCR reactions were phenol extracted and the DNAs were concentrated by ethanol precipitation (Ausubel et al. 1993). DNA fragments were digested with the appropriate enzymes (Table 1) and inserted into the similarly digested and phosphatased (with calf intestinal phosphatase) pEG202 and pJG4-5 plasmids using T4 DNA ligase. Ligation mixtures were then introduced into *E. coli* strain DH5 α by calcium chloride transformation (Ausubel et al. 1993).

The FL- Δ N gene VI in pJG4-5 was made by digestion of pCa6-3 with *Nco*I and religation of the plasmid. Plasmid pCa6-3 consists of pJG4-5 harboring a full-length gene VI in the *Xho*I site. The two *Nco*I sites in pCa6-3 are within gene VI and are in frame with one another. Digestion and religation of this plasmid generates a gene VI lacking the region encoding P6 amino acids 250 to 379 (domain D3). FL- Δ N gene VI was then excised from the pCa6-3 plasmid background by *Xho*I digestion

Fig. 4. Interactions of the four gene VI product (P6) domains with one another. **A**, Schematic diagram of the constructions tested for leucine-independent growth and β -galactosidase activity. Black box, LexA DNA-binding domain (from pEG202); hatched box, B42 transcription activation domain (from pJG4-5). The unshaded box indicates P6 fragments, with the numbers inside the box indicating the P6 amino acids present. Note that P6 amino acids 1 to 110 are domain D1; 156 to 253, domain D2; 249 to 379, domain D3; and 414 to 520, domain D4. Numbers to the left of each pair of constructions correspond to the β -galactosidase assay data shown in B and the yeast colonies shown in C. **B**, β -galactosidase activity of yeast transformants expressing constructions shown in A. Bar graph indicates average β -galactosidase units (on abscissa) for three experiments, along with the standard deviation. Numbers at the bottom (ordinate) correspond to the transformants grown on media with and without leucine in C. **C**, Growth of yeast transformants on media with (left) and without (right) leucine. The streaks are numbered to correspond to the constructions shown in A and the β -galactosidase activities shown in B. **D**, The key for the plates in C.

and was ligated into similarly cleaved pEG202 that was treated with calf intestinal phosphatase.

Domain D3 was excised from pCa6-3 by *Nco*I digestion and ligated into that site within pEG202. The resulting plasmid was then digested with *Bam*HI, filled in with DNA polymerase I Klenow fragment, and religated to put the gene VI fragment in frame with the LexA gene of pEG202. In this case, the termination codon was supplied by the pEG202 plasmid.

Yeast transformation and analysis of interactions.

The recombinant plasmids were amplified in *E. coli* and were subsequently introduced into *Saccharomyces cerevisiae* EGY48 harboring the pSH18-34 β -galactosidase reporter plasmid using a lithium acetate yeast transformation procedure (Ausubel et al. 1993; Gyuris et al. 1993). First, the pEG202 plasmids harboring either full-length gene VI or its various

fragments were introduced into yeast strain EGY48 containing pSH18-34 and were selected on synthetic defined (SD)/-Ura-His medium. Individual colonies were used to establish yeast lines harboring recombinant pEG202 plasmids. Recombinant pJG4-5 plasmids were then introduced into these lines. The transformants were grown at 30°C on yeast plates containing either SD Base/Gal/Raf/-Ura-His-Trp or SD Base/Gal/Raf/-Ura-His-Trp-Leu (Clontech Laboratories, Palo Alto, CA, U.S.A.). Vector pJG4-5 without a gene VI insert was used as a negative control for each set of yeast transformations.

To measure reporter gene activity, β -galactosidase assays were performed (Ausubel et al. 1993). Briefly, a 5 ml yeast culture was grown in the appropriate liquid medium at 30°C to an optical density at 600 nm (OD₆₀₀) value (determined using a Beckman DU640 spectrophotometer) between 0.5 and 1.0, which was recorded. A 1-ml sample was taken from this culture, and the cells were harvested by centrifugation and suspended in 0.8 ml of Z-buffer (10.0 mM KCl, 1.0 mM MgSO₄, 0.27% β -mercaptoethanol, and 100 mM sodium phosphate buffer, pH 7.0). The cells were lysed by the addition of 2 drops each of chloroform and 0.1% sodium dodecyl sulfate (SDS) and were mixed for 15 s. The samples were preincubated for 5 min at 30°C, and then 0.2 ml of 13 mM O-nitrophenyl β -D-galactopyranoside (Sigma Chemical Company, St. Louis) was added. The samples were incubated until they developed a yellow color, and the time to develop this color was noted. The reactions were stopped by adding 0.5 ml of 1 M Na₂CO₃, centrifuged for 10 min to remove cell debris and the pellets were discarded. The OD₄₂₀ of the reactions was determined using a Beckman DU640 spectrophotometer. Units of β -galactosidase were determined by multiplying the OD₄₂₀ value by 1,000 and dividing this number by the product of the OD₆₀₀ value and the number of minutes it took for the reactions to turn yellow.

Generation and analysis of CaMV genomes lacking domain D3.

Gene VI contains two *Nco*I restriction enzyme sites that are in frame with one another on either side of domain D3. Hence, cleavage with *Nco*I deletes domain D3. A *Hpa*I-*Bsu*36I fragment containing the 3' end of gene V, all of gene VI, the large intergenic region, and most of gene VII was excised from plasmid pCaMV10 (Gardner et al. 1981). This fragment was filled in with DNA polymerase I Klenow fragment and was inserted into the filled-in *Nru*I and *Bam*HI restriction enzyme sites of pBR322. This plasmid was digested with *Nco*I to delete the domain D3 coding sequences and was religated to generate the plasmid pCaMV10-NH-BB-N. This plasmid was digested with

Fig. 5. Polymerase chain reaction (PCR) detection of wild-type and domain D3 deletion mutant virus in infected plants. Plant tissue was harvested 32 days postinoculation. W = PCR product generated from turnip tissue infected with wild-type virus, M = from tissue infected with the domain D3 deletion mutant virus, N = from uninfected plants, I = tissue from inoculated leaves, U = tissue from upper non-inoculated leaves, and L = 100-bp ladder (New England Biolabs, Beverly, MA, U.S.A.). Arrow indicates the position of the 500-bp gene II PCR product.

Table 1. Polymerase chain reaction primers used to generate gene VI fragments

Primer ^a	Sequence ^b	Position ^c	Amino Acid ^d
FL-1F	ACTGAGAggATCcGACCTCC	5750	1
L-1F	CTGGAGctcGAGAAATCAG	5744	1
DS-1F	GTGCCAGGGatCcTTTTACG	6074	106
MT-1F	CGATGAaTtcGGTTGTAC	6229	156
KGL-1F	CCCCAAAGgGATCcCCTTTG	6484	243
HG-1F	AGAAGAggATCcTCGTCACC	6999	414
ECB-2R	GTGGGATccGAATtCACTG	6101	110
DT-2R ^e	TCGCAAAGCTCccGGGCTTG	6407	203
LGK-2R	CCTAGATCcTcGAGtTAGAG	6549	253
HG-2R	GAGTcTCGaGCTaCACCATG	7018	417
FL-2R	TGCGTCATCCCTcgaGTCAG	7350	520

^a Primer name with suffix ending in 1F = forward primer; suffix ending in 2R = reverse primer.

^b Sequence of primer from 5' to 3' end; nucleotides mutated from the CaMV sequence (Gardner et al. 1981) are given in small letters, and restriction enzyme sites are underlined.

^c Location of 5' nucleotide on the CM1841 sequence (Gardner et al. 1981).

^d First amino acid encoded by forward primer and last amino acid encoded by reverse primer.

^e This primer uses *Sma*I rather than the standard restriction enzyme sites and the stop codon is provided by pEG202.

SacI and *SpeI* to release a fragment containing most of gene VI harboring the *NcoI* deletion as well as the intergenic region and the 5' portion of gene VII. This fragment was inserted into the *SacI* and *SpeI* sites of pCaMV10 to generate the plasmid pCaMV10-*NcoI*-DE. Hence, this plasmid contains a full-length CaMV genome lacking the domain D3 coding sequence.

For inoculations, the viral genomes from pCaMV10 and pCaMV10-*NcoI*-DE were excised from their pBR322 vectors by *SalI* digestion. Ten µg of pCaMV10 or pCaMV10-*NcoI*-DE was used per plant. The plasmid DNA was then dissolved in 50 µl of 2× SSC, Celite (J. T. Baker, Phillipsburg, NJ, U.S.A.) was added, and the resulting mixture rubbed onto leaves 4, 5, and 6 of each of five turnip plants at the eight-leaf stage. By 32 days postinoculation, the inoculated and upper noninoculated leaves were harvested and tested for the presence of virus by polymerase chain reaction (PCR) as described by Callaway and associates (1996). Briefly, the tissue was ground in extraction buffer (250 mM NaCl, 0.25% SDS, 30 mM EDTA, and 100 mM Tris, pH 8) at the rate of 1 ml of buffer per g of tissue. Ten µl of a proteinase K solution (10 mg per ml of water) per ml of tissue extract was added, and the mixtures were incubated at 55°C for 60 min. An equal volume of sterile distilled water was added to the extracts, and they were incubated at 95 to 100°C for 8 to 10 min. Following this incubation, the extracts were cooled to room temperature, centrifuged at 13,000 × *g* for 5 min, and the supernatants were used in PCR reactions.

Two µl of plant tissue extract was added to a PCR mixture to make up a 100-µl reaction. The PCR mixture contained a final concentration of 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 0.1% triton X-100, 200 µM of all four deoxynucleotide triphosphates, 0.5 U Taq DNA polymerase, 2 × 10⁻⁷ M of the G1IF (5'-GAATGAATTCAGCATTACGG-3') and G1IR (5'-TAAGTTTACGCAATTCAGCC-3') primers (synthesized by DNA International Inc., Lake Oswego, OR, U.S.A.). The PCR program consisted of a five-cycle preamplification step of 1 min at 95°C, 1 min at 35°C, and 1 min at 72°C, with a 1-min 95°C dwell at the beginning of the program and a 10-min elongation step at 72°C at the end. Finally, a 30-cycle amplification program of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C with a 10-min 72°C dwell at the end was performed. The amplifications were performed in a TempTronic/Amplitrone Programmable Thermal Cycler (Barnstead-Thermolyne, Dubuque, IA, U.S.A.). A 500-bp fragment spanning gene II is generated using the G1IF/G1IR primer pair. PCR products were separated by electrophoresis through a 1.4% agarose gel in 1× Tris-borate-EDTA (Ausubel et al. 1993). Each set of reactions was tested at least twice for each plant.

ACKNOWLEDGMENTS

The authors thank S. Goldman, L. Shemshedini (both at The University of Toledo), and The University of Toledo Plant Science Research Center, for their assistance. The authors also thank S. H. Howell (Iowa State University) for the cloned genome of CaMV isolate CM1841; R. Brent (Molecular Sciences Institute) for the yeast strains and plasmids for the two-hybrid system; M. Wheelock and K. Johnson (University of Nebraska Medical Center) for the human *actin* gene; and J.-F. Laliberte (Centre de recherche en virologie, Institut Armand-Frappier) for the *Arabidopsis thaliana* *eIFiso4E* gene. Finally, the authors thank D. Sturtz and M. Hapiak for their assistance. This work was supported in part by USDA grant number 96-3503-3284, the University of Toledo deARCE Memorial Endowment Fund In Support Of Medical Research and Development, and the Ohio Plant Biotechnology Consortium that was administered through the Ohio Agricultural Research and Development Center.

LITERATURE CITED

Agama, K. A., Beach, S., Schoelz, J., and Leisner, S. M. 2002. The 5' third of *Cauliflower mosaic virus* gene VI conditions resistance break-

- age in *Arabidopsis* ecotype Tsu-0. *Phytopathology* 92:190-196.
- Anderson, E. J., Qiu, S. G., and Schoelz, J. E. 1991. Genetic analysis of determinants of disease severity and virus concentration in cauliflower mosaic virus. *Virology* 181:647-655.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1993. *Current Protocols in Molecular Biology*. Greene Publishing Associates and John Wiley & Sons, INC, Cambridge, MA, U.S.A..
- Baughman, G. A., Jacobs, J. D., and Howell, S. H. 1988. Cauliflower mosaic virus gene VI produces a symptomatic phenotype in transgenic tobacco plants. *Proc. Natl. Acad. Sci. U.S.A.* 85:733-737.
- Bonneville, J. M., Sanfacon, H., Futterer, J., and Hohn, T. 1989. Post-transcriptional trans-activation in Cauliflower mosaic virus. *Cell* 59:1135-1143.
- Callaway, A., Liu, W., Adrianov, V., Stenzler, L., Zhao, J., Wettlaufer, S., Jayakumar, P., and Howell, S. H. 1996. Characterization of cauliflower mosaic virus (CaMV) in virus-resistant ecotypes of *Arabidopsis*. *Mol. Plant-Microbe Interact.* 9:810-818.
- Cecchini, E., Gong, Z., Geri, C., Covey, S. N., and Milner, J. J. 1997. Transgenic *Arabidopsis* lines expressing gene VI from cauliflower mosaic virus variants exhibit a range of symptom-like phenotypes and accumulate inclusion bodies. *Mol. Plant-Microbe Interact.* 10:1094-1101.
- Cole, A. B., Király, L., Ross, K., and Schoelz, J. E. 2001. Uncoupling resistance from cell death in the hypersensitive response of *Nicotiana* species to *Cauliflower mosaic virus* infection. *Mol. Plant-Microbe Interact.* 14:31-41.
- Covey, S., and Hull, R. 1981. Transcription of cauliflower mosaic virus DNA. Detection of transcripts, properties, and location of the gene encoding the virus inclusion body protein. *Virology* 111:463-474.
- Daubert, S., and Routh, G. 1990. Point mutations in cauliflower mosaic virus gene VI confer host-specific symptom changes. *Mol. Plant-Microbe Interact.* 3:341-345.
- deZoeten, G. A., Penswick, J. R., Horisberger, M. A., Ahl, P., Schultze, M., Hohn, T. 1989. The expression, localization, and effect of a human interferon in plants. *Virology* 172:213-222.
- De Tapia, M., Himmelbach, A., and Hohn, T. 1993. Molecular dissection of the cauliflower mosaic virus translation transactivator. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3305-3314.
- Gardner, R. C., Howarth, A. J., Hahn, P., Brown-Luedi, M., Shepherd, R. J., and Messing, J. 1981. The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. *Nucleic Acids Res.* 9:2871-2888.
- Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* 75:791-803.
- Himmelbach, A., Chapdelaine, Y., and Hohn, T. 1996. Interaction between cauliflower mosaic virus inclusion body protein and capsid protein: Implications for viral assembly. *Virology* 217:147-157.
- Hohn, T., and Futterer, J. 1997. The proteins and functions of plant pararetroviruses: Knowns and unknowns. *Crit. Rev. Plant Sci.* 16:133-161.
- Hull, R., and Covey, S. N. 1985. Cauliflower mosaic virus: Pathways of infection. *BioEssays* 3:160-163.
- Kobayashi, K., Tsuge, S., Nakayashiki, H., Mise, K., and Furusawa, I. 1998. Requirement of cauliflower mosaic virus open reading frame VI product for viral gene expression and multiplication in turnip protoplasts. *Microbiol. Immunol.* 42:377-386.
- Martelli, G. P., and Russo, M. 1977. Plant virus inclusion bodies. *Adv. Virus Res.* 21:175-266.
- Mazzolini, L., Dabos, P., Constantini, S., and Yot, P. 1989. Further evidence that viroplasm is the site of cauliflower mosaic virus genome replication by reverse transcription during viral infection. *J. Gen. Virol.* 70:3439-3449.
- Nieset, J. E., Redfield, A. R., Jin, F., Knudsen, K. A., Johnson, K. R., and Wheelock, M. J. 1997. Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. *J. Cell Sci.* 110:1013-1022.
- Palanichelvam, K., Cole, A. B., Shababi, M., and Schoelz, J. E. 2000. Agroinfiltration of *Cauliflower mosaic virus* gene VI elicits hypersensitive response in *Nicotiana* species. *Mol. Plant-Microbe Interact.* 13:1275-1279.
- Park, H.-S., Himmelbach, A., Browning, K. S., Hohn, T., and Ryabova, L. A. 2001. A plant viral reinitiation factor interacts with the host translation machinery. *Cell* 106:723-733.
- Qiu, S. G., Wintermantel, W. M., Sha, Y., and Schoelz, J. E. (1997). Light-dependent systemic infection of solanaceous species by cauliflower mosaic virus can be conditioned by a viral gene encoding an aphid transmission factor. *Virology* 227:180-188.
- Rothnie, H. R., Chapdelaine, Y., and Hohn, T. 1994. Pararetroviruses and retroviruses: A comparative review of viral structure and gene expression strategies. *Adv. Virus Res.* 44:1-67.

- Sanger, M., Daubert, S., and Goodman, R. M. 1991. The regions of sequence variation in caulimovirus gene VI. *Virology* 182:830-834.
- Schoelz, J. E., and Shepherd, R. J. 1988. Host range control of cauliflower mosaic virus. *Virology* 162:30-37.
- Schoelz, J., Shepherd, R. J., and Daubert, S. 1986. Region VI of cauliflower mosaic virus encodes a host range determinant. *Mol. Cell. Biol.* 6:2632-2637.
- Schoelz, J. E., Goldberg, K.-B., and Kiernan, J. 1991. Expression of cauliflower mosaic virus (CaMV) gene VI in transgenic *Nicotiana biglovii* complements a strain of CaMV defective in long-distance movement in nontransformed *N. biglovii*. *Mol. Plant-Microbe Interact.* 4:350-355.
- Shepherd, R. J. 1979. DNA plant viruses. *Ann. Rev. Plant Physiol.* 30:405-23.
- Stratford, R., and Covey, S. 1989. Segregation of cauliflower mosaic virus symptom genetic determinants. *Virology* 172:451-459.
- Turner, D. S., McCallum, D. G., and Covey, S. N. 1996. Roles of the 35S promoter and multiple overlapping domains in the pathogenicity of the pararetrovirus cauliflower mosaic virus. *J. Virol.* 70:5414-5421.
- Wintermantel, W. M., Anderson, E. J., and Schoelz, J. E. 1993. Identification of domains within gene VI of cauliflower mosaic virus that influence systemic infection of *Nicotiana biglovii* in a light-dependent manner. *Virology* 196:789-798.
- Wittman, S., Chatel, H., Fortin, M. G., and Laliberte, J. F. 1997. Interaction of the viral protein genome linked of turnip mosaic virus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two hybrid system. *Virology* 234:84-92.