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Point Mutations in Cauliflower Mosaic Virus Gene VI Confer Host-Specific Symptom Changes

Steve Daubert and Geoffrey Routh

Department of Plant Pathology, University of California, Davis 95616 U.S.A.
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Mutants of cauliflower mosaic virus (CaMV) strain D4 have been characterized with regard to host-specific phenotypes that resulted from specific changes in the viral DNA sequence. Both the mutant and the wild-type viruses infect a brassicaceous host, *Brassica campestris*, systemically, giving indistinguishable symptoms. However, in the solanaceous host *Datura stramonium*, which was systemically infectible by the wild-type virus, mutants induced necrotic local lesions at 21° C and above, and a veinal necrosis at lower temperatures. The mutants differed from the parental D4 strain by having single base changes in gene VI. The necrotic phenotype could be selected during serial passage of D4 in *B. campestris* or created by site-directed mutagenesis within the gene VI coding region. Full-length 62-kDa gene VI gene products were detected in extracts of plants infected with the mutant strains, as were two smaller proteins derived from the same coding region. The relationship of the host-specific phenotypes of the mutants to the detected gene VI-encoded proteins is discussed in the context of the natural variation found in this gene.

The 8-kilobase genome of cauliflower mosaic virus (CaMV) has been extensively characterized and manipulated (Bonneville *et al.* 1988). Comparative sequence analysis of various CaMV isolates reveals that gene VI contains the least conserved section of that genome. Gene VI encodes the most prominent viral-coded protein found in cells infected with CaMV. Although its function in infected plants is unknown, Bonneville *et al.* (1989) have suggested that the product of this gene acts in protoplasts to transactivate the translation of the polycistronic viral mRNA.

The response of *Datura stramonium* L. differentiates strains of CaMV into two groups: those isolates that can systemically infect this host (for example, Schoelz *et al.* 1986b) and a larger group that produces local lesions only at the site of inoculation (Lung and Pirone 1972). Depending upon environmental conditions and viral strains, this response can vary from bright chlorotic vein-clearing mosaics and stunting in the first group to pinpoint necrotic lesions limited to the inoculation site in the second. Many gradations of the response fill in the continuum between these extremes.

The CaMV genome encodes six genes, and with the exception of gene VI, all reported modifications to those genes are either silent or inactivating (for example, Daubert *et al.* 1983; Dixon *et al.* 1983). Viable strains carrying alterations in gene VI have been reported, including insertional mutations (Daubert *et al.* 1983) or substitutions of gene VI segments produced in the construction of chimeric forms of the gene (Daubert *et al.* 1984; Schoelz *et al.* 1986a). These alterations to the gene sequence result in obvious alterations in symptomatology. In this study, we continue the analysis of gene VI and its mediation of symptom expression with the description of point mutations that alter the virulence phenotype of strains of CaMV infecting *D. stramonium*.

Current address of G. Routh: Department of Plant Pathology, University of California, Riverside 92521 U.S.A.

MATERIALS AND METHODS

Inoculated plant species were the solanaceous host *D. stramonium* and the brassicaceous host *Brassica campestris* L. (turnip; cultivar Just Right).

Viral strains. Parental viral strains, from which the recombinants were derived, were CM-1841 and D4 (Daubert *et al.* 1984). Recombinant strains were constructed by replacing, in CM-1841, a DNA segment carrying 97% of the coding region of gene VI (the last 504 amino acid codons of a total of 520) and a part of the intergenic region (described in Daubert *et al.* 1984). The subcloned gene section spans viral genomic coordinates 5822 (from the *Sst*I site) to 126 (a *Bst*EII site; see Fig. 1). In chimera H7 (Daubert *et al.* 1984), the source of the replaced segment was D4. In chimera GR, the source of the gene VI segment was strain D4-GR.

The mutation carried by strain 58 was produced by site-directed mutagenesis (Kunkel *et al.* 1987) using plasmids carrying the M13 origin of single-stranded DNA replication (Vieira and Messing 1987). Mutagenesis was conducted on the subcloned *Sst*I-to-*Bst*EII DNA segment shown in Figure 1. The synthetic primer for the strain 58 mutation is shown in Figure 2, line 58. At the site of the mismatch, a G residue replaces a C residue creating an *Hgi*AI site (dotted underline in Fig. 2). This site facilitated initial screening of mutagenized subclones prior to confirmatory sequencing of the mutations. The segment carrying this site-directed mutation was used in the construction of strain 58, a chimera analogous to H7. The *Hgi*AI site facilitated confirmation of the retention of the mutation in virion genomic DNA recovered from a second-passage infection.

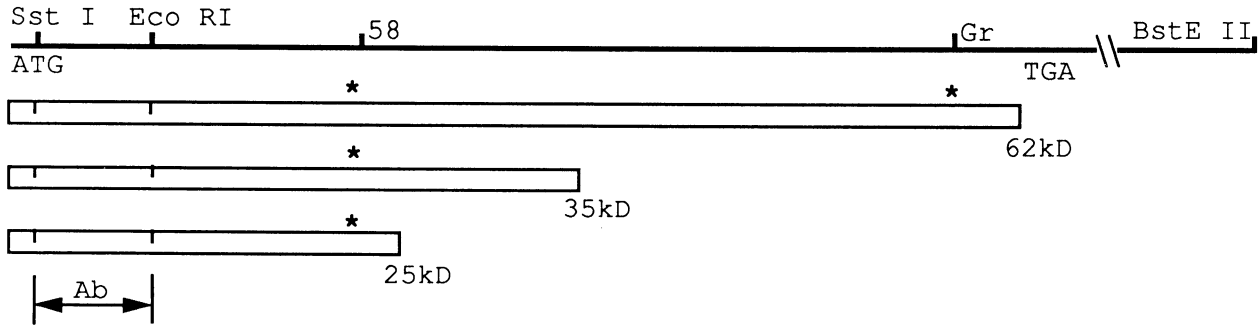

Fig. 1. Gene VI diagram of (top line) the cauliflower mosaic virus DNA segment from the *Sst*I site (at genomic coordinate 5822) to *Bst*EII (at 126) used in chimera construction. Relative to the ATG codon at gene position 1 the *Sst*I site is at position 49; mutation 58 is at 563; mutation GR is at 1462; and the TGA codon is at 1561. The gene product is 520 amino acids long; mutation 58 alters amino acid 121, and mutation GR alters amino acid 488. The binding domain of the antibody (Ab) used in western blot analysis is delineated by the *Sst*I site at position 49 and an *Eco*RI site at 273. The full-length 62-kDa protein product and the smaller proteins visualized in Figure 4 are diagramed in the lower three lines. The asterisk designates the positions at which the mutations alter the protein sequence.

Strain D4-GR was derived by the acclimation of strain D4 to turnip. Transfer of strain D4 soon after symptoms appeared on turnip (3–4 wk) produced systemic, chlorotic, mosaic symptoms in the solanaceous host *D. stramonium* grown at 18° C. Transfers from turnips that had borne the infection for longer periods gave rise to altered symptomatology in *D. stramonium*, that is, mosaic symptoms including scattered necrotic spots. Strain D4 infections in turnips 10 wk after inoculation, or in turnips in which the virus had been propagated through serial transfers for at least 10 wk, appeared to contain "adapted" virus. The adapted D4 strain, when inoculated to *D. stramonium*, produced necrotic localized lesions.

DNA sequence determinations, including those of the coding regions of gene VI from strains D4 and GR, were made by the dideoxy chain termination method of Sänger *et al.* (1977). The sequence of gene VI from strain D4, completed during the course of this study, from nucleotide positions 1 through 1560 (coding region) as well as the following 342 nucleotides (35S promoter region) has been submitted to GenBank as accession number M23620.

Viral genomes were maintained as cloned DNAs, then excised from their cloning vectors with *Sal*I and used to inoculate turnips as described in Daubert *et al.* (1984). After 3 wk, sap from infected turnip leaves was used to inoculate *D. stramonium* seedlings. Plants were maintained at 18° C, with 10 hr of light per day at 160 $\mu E \cdot cm^{-2} \cdot s^{-1}$. Effects of growth conditions were also assessed at 21° C with 12 hr of light and at 24° C with 14 hr of light.

Gene VI-encoded products were analyzed by electrophoresis through 40-cm polyacrylamide gels and immunoblotting (Young *et al.* 1987). Gene VI gene product preparations consisted of infected tissue ground immediately after harvest in 5 volumes of 5% sodium dodecyl sulfate (SDS), 2 mM 2-mercaptoethanol, 5% glycerol, 100 mM Tris-HCl, pH 6.8.

RESULTS

Phenotypes. At 21° C, CM-1841 produced no symptoms of infection on *D. stramonium*, while D4 produced a systemic mosaic. On turnip, CM-1841 produced a systemic mosaic, while D4 produced systemic necrotic flecking (death of secondary leaf veins). The symptoms of adapted strain D4-GR were the same as those of the parent strain on turnip. However, at all temperatures tested, D4-GR produced necrotic lesions limited to the inoculation site on *D. stramonium*. All the recombinant strains produced similar mosaic symptoms on turnip, which were much milder than the bright pattern produced by CM-1841.

A

GluLeuAsnSerThr
D4 GAGCTTAACAGCACAG
58 GAGCTTAAGAGCACAG$_{-370}$
GluLeuLysSerThr$_{-123}$

B

IleGlnAspAlaSer
D4 ATTCAAGATGCCTCT
GR ATTCAAAATGCCTCT$_{-1470}$
IleGlnAsnAlaSer$_{-490}$

Fig. 2. Sequence context of gene VI mutations, relative to the D4 parent sequence, which is given above in each case. **A**, Mutation 58, which creates an *Hgi*AI site (dotted underline). The sequence is from nucleotide 355 to 370, relative to adenosine 1 in the initial ATG codon of gene VI; the Glu residue is at position 119 in the inferred amino acid sequence. **B**, Mutation GR from nucleotide 1456, amino acid 486.

On *D. stramonium*, strain H7 produced a systemic mosaic that at 21° C included necrotic spots. In contrast, the infections produced by strains GR and 58 had no chlorotic mosaic character, inducing only necrotic symptoms under the same conditions.

Phenotypic variation with conditions. The interaction of these strains of CaMV with *D. stramonium* varied widely with conditions of temperature and day length. *D. stramonium* is a summer annual (Munz 1973) growing to 1 meter in height with dark green foliage if grown under 14 hr of full sunlight per day with daily temperature maxima of about 40° C. In contrast, at 18° C with 10 hr of artificial light per day, the pale green plants flower at 10 cm in height. As noted in previous experiments with chimeric forms of CaMV (Schoelz *et al.* 1986a), this host shows

greatest susceptibility under these suboptimal conditions of temperature and day length.

The response of *D. stramonium* to the chimeric strains was similarly temperature dependent (Fig. 3). The host plant showed the most pronounced symptoms at a lower temperature, where parental chimera H7 induced a systemic vein chlorosis (referred to in Fig. 3 as mosaic). Strain 58 infection at 21° C was limited to necrotic lesions at the site of inoculation only (referred to in Fig. 3 as restricted). At 18° C this necrotic symptom "escaped" from local restriction, inducing a limited vein necrosis (necrotic). At 18° C strain 58 had a less severe effect on this host than did strain GR: necrotic leaves infected with strain 58 senesced early and were shed, leading to eventual and complete recovery of the host, while plants infected with strain GR became stunted and did not recover. The symptom induced by strain 58 at 18° C is illustrated in Figure 3 of Daubert (1988). The spreading of the necrosis produced by strain GR along the veins could be found to a limited extent at 21° C. At 21° C, the H7 infection induced necrotic spots superimposed on the mosaic (limited necrosis), and at 24° C, it was restricted to necrotic lesions on the inoculated leaf and sometimes one leaf above. Strain D4 itself is similarly restricted at 24° C, inducing light green localized lesions.

Strain GR. This chimeric strain carries the *Sst*I-to-*Bst*EII fragment (Fig. 1) from adapted strain D4-GR. In this construct the viral gene VI coding section corresponding to the span from amino acid 16 to the end of the protein is derived from D4-GR; all other coding frames are derived from CM-1841. Thus, the chimera is identical in design to H7, which produces chlorotic mosaic symptoms in *D. stramonium*. However, strain GR produces necrotic symptoms on *D. stramonium* as does adapted strain D4-GR. The causative mutation in the DNA segment bearing gene VI was identified by sequence comparison of the segment derived from strain GR with that from H7. The comparison revealed a single base difference, which was in the carboxy-terminal protein coding region of the gene at nucleotide position 1462 (Fig. 2B).

	1841	58	GR	H7	D4
18°	R	N	N	M	M
21°		R	N	LN	M
24°			R	R	R

Fig. 3. Effect of environmental conditions on symptomatology of strains of cauliflower mosaic virus in *Datura stramonium*. Strains CM-1841 and D4 and the chimeras and mutants derived from them (described in the text) are given above their respective columns, listed in increasing order of aggressiveness. Growing temperatures are given at left: these conditions were 18° C with 10 hr of light per day, 21° C with 12 hr of light per day, or 24° C with 14 hr of light per day (described in the text). The resulting symptoms (R, restricted; N, necrotic; LN, limited necrosis; and M, mosaic) are described in the text. The restricted lesions of strain D4 were chlorotic, as opposed to the necrosis seen in the other examples. Restricted lesions of CM-1841 were few, small, and sometimes undetectable.

Strain 58. A phenotype similar to that of the GR mutation was produced by strain 58, which carries a site-directed mutation in the amino-terminal coding region of gene VI. We selected this site for mutagenesis due to its location within a hypervariable domain in gene VI (discussed below) in order to assess whether or not alterations in the region would have an effect on viral infection.

Gene VI-encoded product. Mutations 58 and GR (Fig. 2) give rise to single amino acid substitutions in the open reading frame of gene VI. Though an effect on electrophoretic mobility in denaturing gel analysis might not be expected from single amino acid substitutions, effects upon mobility were evident on analysis of the full-length gene VI-encoded protein (Fig. 4). Well-resolved truncated gene VI-encoded products of approximately 35 and 25 kDa were also consistently observed, migrating as sharp zones (Fig. 4B). An effect of the mutation at amino acid position 121 on the mobility of the 35-kDa product was also evident. In Figure 4A, lane 4, is shown a >70 kDa form of the full-length gene VI-encoded product. This zone is often absent from this analysis.

In *D. stramonium* at 18° C, infection with strain GR progressed sufficiently to allow the analysis of extracts for the gene VI-encoded product from that host. The mobility of the gene VI-encoded protein derived from strain GR in this host was again greater than that of the gene VI-encoded product from strain H7 grown in *D. stramonium* (data not shown); their relative mobilities were identical to those seen in *B. campestris*. The gene VI-encoded product from strain 58 in *D. stramonium* was not visualized.

DISCUSSION

We observed a spontaneous mutation that arose during the propagation of strain D4 of CaMV in turnip. The mutant phenotype was only expressed upon the infection of *D. stramonium*. The DNA segment bearing gene VI derived from this mutant conferred the necrotic phenotype upon an otherwise identical, chimeric viral construct, confirming that the viral gene VI DNA segment contained the causative mutation. The mutant phenotype was also inducible by mutation at a second site in gene VI. At 21° C both of these mutants induce necrotic initial lesions and show little or no spread, while unmutated parental strain H7 is systemically infectious, producing chlorotic initial lesions followed by a systemic mosaic containing variable necrotic spots. The altered strains differ from strain H7 by single base changes in gene VI. The differential response of *D. stramonium* to H7 and to the mutants indicates the specificity of the host-pathogen interaction initiated ultimately by the sequence of gene VI.

Strain GR was a spontaneous mutation that was apparently selected during acclimation of strain D4 to *B. campestris*. The GR mutation arose near the carboxy-terminal end of gene VI; the site-directed mutation producing strain 58 was near the amino-terminal end of the gene. Thus, both amino-terminus-proximal and -distal domains of this protein appear to be determinants of this host response. The lack of any effect of the mutations in

the brassicaceous host relative to parental strain H7 rules out the possibility that the lesions simply result in general dysfunction of the gene product.

The sequence of the GR mutation in gene VI is similar to the sequence determined by M. Volovitch, N. Modjtahedi, Y. Chouik, and P. Yot (personal communication) for CaMV strain Cabbage-B in Paris (Fig. 5). Their maintenance program for this virus was by passage in *B. perviridis*. Another isolate of the same virus had been concurrently maintained in England at the John Innes Institute, Norwich, by passage in *B. campestris* (Hull 1980). The John Innes isolate has the same Asp-Ala-Ser amino acid sequence at position 488–490 (Fig. 4 in Stratford *et al.* 1988) as have other strains of CaMV for which the sequence is known. These include CM-1841, D/H, S, Bari 1 (Gardner *et al.* 1981; Balazs *et al.* 1982; Franck *et al.* 1980; Stratford *et al.* 1988) as well as D4, but not Xinjiang (Fang *et al.* 1985), which is the most divergent among CaMV isolates in this hypervariable region. However, the sequence of the strain propagated in France contains the same Asn-Ala-Ser variation as that described here from strain GR (Fig. 5).

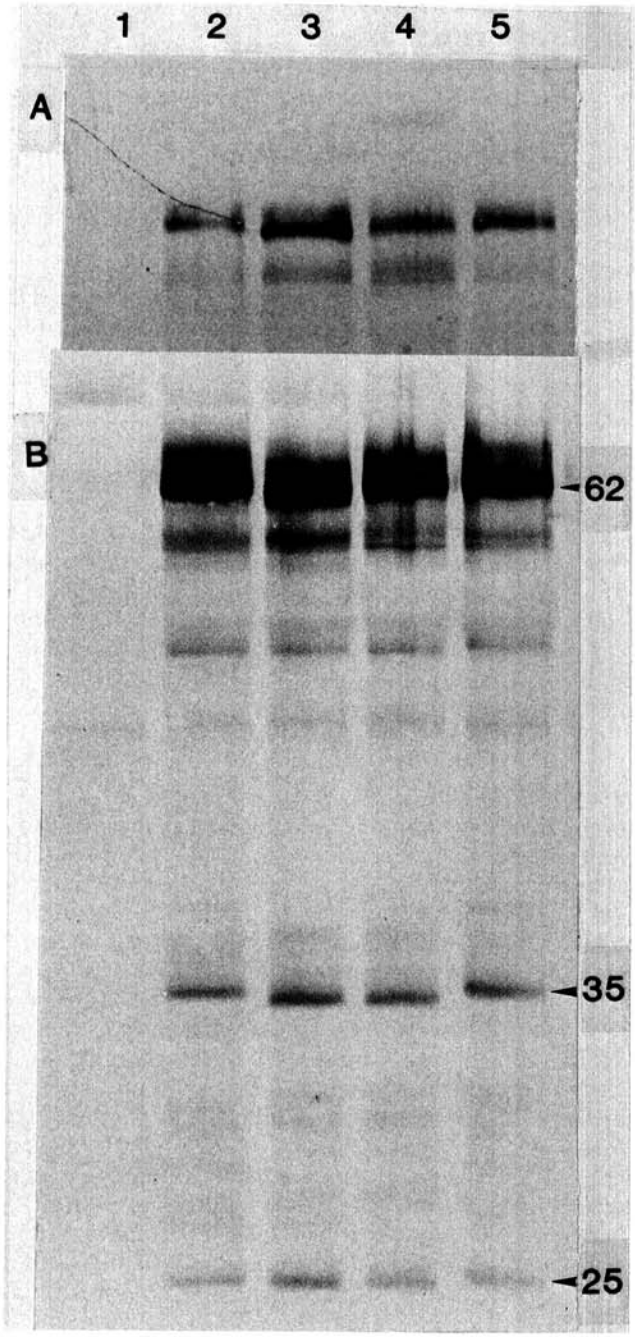

Fig. 4. Western blot analysis of proteins separated on 40-cm polyacrylamide gels, as described in the text, showing electrophoretic mobilities of the gene VI-encoded products produced by strain 58 (lanes 2 and 5), strain GR (lane 3), and strain H7 (lane 4). Lane 1 shows analysis of the mock-inoculated plant. **A**, Gel section showing the relationships between the full-length products, which have apparent molecular masses of approximately 62 kDa. **B**, Gel section overloaded for the full-length products showing the minor products, which have apparent molecular masses of approximately 35 and 25 kDa.

The mutations fall in "hypervariable" domains of the gene. These domains are defined by comparative inspection of the various known gene VI sequences, which shows the division of the gene into sections. The main conserved domain is bifurcated by a pair of variable domains (to be described elsewhere: M. Sanger, R. Goodman, and S. Daubert, unpublished). Though the variable domains have diverged to an extent reminiscent of protein regions where structure is not constrained by function (such as the fibrinopeptide domain in fibrinogen [Kimura and Ohta 1974]), the biological consequences of mutations GR and 58 show the opposite in the case of gene VI. These mutations, both within the variable regions, show that those domains are integral to function, in at least one host species, and may play a part in the host-virus interaction.

The gene VI-encoded products produced by mutant strains GR and 58 and unmutated strain H7 differed significantly in their SDS-gel electrophoretic mobilities. Whether the smaller gene VI-encoded proteins seen in Figure 4 are autonomously functional or merely prominent breakdown products is not known. Since the antibody used in these visualizations is specific for the extreme amino-terminus of the gene VI-encoded product (Fig. 1), the small proteins must be derived from that end of the gene. The 58 mutation lies 121 amino acids from the terminus of the gene VI- encoded protein. Thus it falls within the span of both of the smaller amino-terminal proteins (Fig. 1), and its relative retardation of both of their mobilities is evident in Figure 4. The GR mutation is located at the opposite end of gene VI from the region at which the antibody binds to the gene product. The increase in the mobility of the full-length product from strain GR, in comparison with that of strain H7 (Fig. 4A), is not reflected

Viral Strain	Peptide sequence
GR	I Q N A S T D S
Cabbage-B (France)	V Q N A S A D S
Cabbage-B John Innes	I Q D A S A D S

Fig. 5. Single letter-coded sequence of amino acids 486 to 493 comparing the region of the GR mutation with the corresponding regions from two isolates of cauliflower mosaic virus strain Cabbage-B, which were propagated separately in France and England (see text).

in the mobilities of the truncated products: the 35- and 25-kDa gene VI-encoded products from strain GR appeared identical in mobility to those of H7. This was expected since the GR mutation is located at the carboxy-terminus of the protein, a domain that is lost from the smaller products visualized by this antibody (Fig. 1). The incremental decrease in mobility in the smaller gene VI-encoded products carrying the strain 58 mutation is not obviously reflected in the mobility of the full-length protein bearing the mutation; its mobility is difficult to distinguish from that of the full-length strain H7 product (Fig. 4A).

The 62-kDa product from strain GR, which carries one less negatively charged amino acid residue than the other strains, migrates faster than those of strains 58 and H7. The 35-kDa product encoded by gene VI from strain 58, carrying one more positive charge than the others, migrates relatively more slowly (Fig. 4). Thus, the relative mobilities of these proteins are not easily correlated with the sum of the charges in their altered amino acid sequences.

Posttranslational modification of the gene VI-encoded product could be used to explain the altered electrophoretic mobilities. The mutations lie at Asn residues in the motif Asn-X-Ser/Thr-X, where X is any amino acid but proline. This motif constitutes the site of posttranslational N-linked glycosylation (Kornfeld and Kornfeld 1985). Mutant 58 loses the Asn residue, while mutant GR has gained a potential glycosylation-accepting Asn. However, if we were to postulate that these Asn residues are modified with carbohydrate moieties, we would be constrained in interpreting Figure 4 as showing that mobilities of the gene VI-encoded products in SDS-gel electrophoresis increase as a consequence of the modification. Though the CaMV gene VI-encoded product behaves anomalously in electrophoretic analysis (for example, see Stratford *et al.* 1988), we know of no precedent for increased mobility as a consequence of glycosylation.

Future experiments designed to characterize such putative modifications must first overcome problems of the purification of this insoluble protein. The fine structure in the banding pattern seen in Figure 4 is degraded upon manipulation or incubation of the total SDS-soluble protein extracts used here. This technical problem hinders experimentation on the structure and/or functional potential of the gene VI-encoded product, including questions as to the selective pressures in its interaction with *Brassica* that apparently drive the conversion of Asp 488 to Asn.

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