Infectious and Non-infectious Mutants of Cauliflower Mosaic Virus DNA

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SUMMARY

Mutants of cauliflower mosaic virus (CaMV), generated in vitro by modification of recombinant DNA plasmids containing the viral genome, either retained the ability to induce disease symptoms on turnip plants, produced less severe symptoms or failed to induce symptoms. Wild-type symptoms were produced by a variant CaMV DNA of the Cabbage S isolate that had 4 bp in open reading frame (ORF) III replaced with a 16 bp sequence. Less severe symptoms, due to a delay in symptom appearance relative to inoculation with wild-type DNA, were induced by a mutant with a frameshift mutation in ORF II (pSA103). CaMV DNA, recovered from plants infected with pSA103, contained a second mutation which restored the original translation reading frame. Nucleic acid hybridization to 'squishes' of leaf tissue from plants that had been inoculated with mutant DNAs that included DNAs modified in each of the six major ORFs of CaMV DNA revealed that only those plants that appeared diseased had detectable CaMV nucleic acid in uninoculated leaves. Replicated CaMV DNA was also not detected in non-encapsidated and virion DNA fractions from inoculated leaves of non-diseased plants.

Mutants of cauliflower mosaic virus (CaMV) DNA have been created by in vitro mutagenesis of CaMV DNA cloned in bacterial plasmids (Daubert et al., 1983; Dixon et al., 1983; Howell et al., 1981; Dixon & Hohn, 1984; Woolston et al., 1983; Givord et al., 1984; Armour et al., 1983) and by random chemical mutagenesis of virions (Tomlinson & Shepherd, 1978). Three effects of mutations on the ability of CaMV DNAs to induce disease symptoms in host plants have been described: no alteration of disease symptoms, reduced symptom severity and complete prevention of symptom appearance. We, by in vitro mutagenesis, have created mutants of CaMV DNA that illustrate each of the three effects. We report a novel, completely infectious CaMV DNA modified in open reading frame (ORF) III, the basis for reduced symptom severity of the previously described pSA103 (Armour et al., 1983), and experiments on a possible cause of the lack of symptoms in plants inoculated with several mutants of CaMV DNA.

One effect of mutation of CaMV DNA, a reduction in the severity of disease symptoms, could be due to a lowered virus titre in the infected plants, altered viral products that are less harmful to the host, or to a delayed start to the infection. Two mutations in the C-terminal portion of the ORF VI product, the major protein of virus-induced inclusion bodies, give rise to lower viral titres and reduce symptom severity (Daubert et al., 1983). Experiments in which segments of
DNA were exchanged between different isolates implicated the ORF VI polypeptide in the determination of symptom severity (Daubert et al., 1984). A delay in symptom appearance was noted for DNAs with mutations in ORF II, ORF IV and ORF VII (Armour et al., 1983; Dixon et al., 1983, 1986). The DNAs mutated in ORF VII only became infectious after a second mutation had occurred in the plant restoring an altered initiation codon (Dixon et al., 1986). Only if the ORF VII termination codon used remains close to the ORF I initiation codon to ORF VII mutations fail to interfere with symptom production (Dixon & Hohn, 1984). Efficient translation of CaMV ORFs I to V may require a close spacing of termination and initiation codons (Dixon & Hohn, 1984). These observations resemble those with synthetic templates in mammalian cells (Kozak, 1984) and suggest that some of the CaMV polypeptides may be translated from a polycistronic messenger RNA (K. Sieg & B. Gronenborn, unpublished; cited in Dixon & Hohn, 1984).

Infection with pSA103 was delayed relative to infection following inoculation with wild-type DNA (Armour et al., 1983). Nucleotide sequencing across the deletion in pSA103 (Fig. 1) revealed a 77 bp deletion in ORF II of pSA103. The deletion results in a frameshift and termination of translation at a UAG, 6 codons 3' to the deletion and 140 nucleotides 5' of the ORF III AUG initiation codon. Plants inoculated with virus from pSA103-infected plants became diseased at about the same time as did those inoculated with wild-type virus. Nucleotide sequencing of the region around the deletion in DNA extracted from pSA103-infected plants (Fig. 1) revealed that one of the six contiguous adenylate residues at 1582 to 1587 (numbering of Franck et al., 1980) that precede the pSA103 deletion had been deleted. The extra 1 bp deletion about four codons 5' of the 77 bp deletion restored the original reading frame, allowing ORF II translation to end one nucleotide away from the ORF III AUG. The delayed appearance of symptoms when plants were inoculated with pSA103 DNA (Armour et al., 1983) may thus have been due to the time required for a suppressor mutation to appear.

A second effect, in which mutants of CaMV DNA nonetheless induce wild-type symptoms, is produced by mutations that alter the putative ORF VII protein, by some DNAs modified in the large intergenic region (Dixon & Hohn, 1984; Daubert et al., 1983), and by some DNAs mutated in ORF II (Woolston et al., 1983; Dixon et al., 1983; Daubert et al., 1983; Givord et al., 1984). The ORF II product is needed for virus transmission by aphids but not for mechanical transmission (Woolston et al., 1983; Armour et al., 1983). It is reasonable to expect that limited regions of other viral protein products can be modified without affecting symptom production. Previously described plasmid pUM41 (Table 1; Choe et al., 1985) was digested with SmaI and ligated in the presence of an 8 bp EcoRI linker to give pUM130. The DNA sequence of the region altered in pUM130 predicts the substitution of Pro–Glu–Phe–Arg–Ala for Thr at amino acid position 72 of the 129 amino acid-long ORF III polypeptide. The symptoms appearing on
Fig. 2. Autoradiographic images of squish hybridization using $^{32}\text{P}$-labelled CaMV DNA as probe. Row 1, purified CaMV spotted at spot densities of 210 (a), 76 (b), 40 (c), 13 (d), 5.2 (e) and 4.1 (f) ng/cm$^2$. Row 2, DNA isolated from CaMV at spot densities of 130 (a), 45 (b), 21 (c), 6.0 (d), 2.6 (e) and 1.6 (f) ng/cm$^2$. Rows 3 and 4, squishes of healthy turnip leaves (a, b, c); squishes of CaMV-infected turnip leaves (d, e, f). Squishes were performed by a modification of the method of Flavell et al. (1983). Leaves, still attached to the plant, were apposed to filter paper (Whatman No. 1) and crushed with a wooden dowel of diameter 0.2 cm ("squished"). Known amounts (0.2 to 50 ng) of virus or CaMV DNA were applied to the filter paper in spots 1 cm apart. The paper was then immersed in 1.5 M-NaCl, 0.5 M-NaOH rapidly to prevent the tissue browning and incubated for 15 min at room temperature. The paper was then soaked in 3.0 M-NaCl, 0.5 M-Tris-HCl pH 7.1 for 15 min followed by a 5 min incubation in 2 × SSC at room temperature. Hybridization with $^{32}\text{P}$-labelled CaMV DNA was as previously described (Melcher et al., 1981). Exposure was at $-70^\circ\text{C}$ for 18 h for rows, 1, 2 and 3 and for 65 h for row 4.
Fig. 3. CaMV and pBR322 DNA in non-encapsidated and virion DNA fractions of turnip leaves. Leaves were inoculated 2 weeks prior to DNA preparation. Non-encapsidated DNA was prepared from inoculated leaves by phenol : chloroform extraction as described by Lebeurier et al. (1982). DNA encapsidated in virions is not recovered by this procedure. Virion DNA was prepared from similarly treated leaves by differential centrifugation and proteinase K–SDS digestion (Gardner & Shepherd, 1980). Digested and undigested samples (10 µg of non-encapsidated DNA or virion DNA fractions from 0.7 g leaf tissue) were electrophoresed in 1% agarose, blotted to nitrocellulose and probed by hybridization with 32P-labelled CaMV DNA (a, c) or 32P-labelled pBR322 (b). (a) Non-encapsidated DNA from leaves inoculated with SalI-linearized pCMS31 nicked by DNase I (lane 1) or not treated (lane 2). (b) Non-encapsidated DNA from leaves inoculated with SalI-linearized pBR322 and digested with EcoRI (lane 1), SalI (lane 2) or not treated (lane 3). (c) DNA in virion fractions of leaves inoculated with SalI-digested plasmids pCMS31 (lane 1) or pBR322 (lane 2). Fractions from leaves inoculated with SalI-digested mutant DNA plasmids (Table 1, virion DNA '−') gave results indistinguishable from lane 2.

Intensity of the autoradiographic spots due to standard virus (row 1) and DNA (row 2) preparations was dependent on the density (amount/area) of virus or DNA spotted. Fig. 2, row 3(a, b, c) shows that squishes of leaf tissue from uninfected plants did not bind probe CaMV DNA. Longer exposures (Fig. 2, row 4, a, b, c) also failed to show any binding above background to such squishes. In contrast, squishes from leaves of turnip plants infected with CaMV (rows 3 and 4, a, b, c) were easily detected on autoradiographs. The approximately 100 squishes of infected leaves performed to date all gave readily detectable images and none of a similar number of squishes of healthy leaves has given a detectable autoradiographic image. Detection was sequence-specific since no spots were detected when squishes from CaMV-infected leaves were probed with unrelated 32P-labelled DNAs (bacterial plasmids pBR322 or pACYC177). The autoradiographic intensities of the positive squishes were between that generated by a spot density of 6 ng CaMV DNA/cm² and that produced by 40 ng DNA/cm² which suggests that most of the CaMV DNA present in the leaves was being bound to the nitrocellulose.
## Short communication

### Table 1. CaMV plasmid mutants

| Plasmid* | Insertion | Deletion | Net change (bp) | Infec-
| Mutant | At bp | Sequence | From† | To† | tivity‡ | Squish
| CaMV plasmid mutants | | | | | | hybridization
| | | | | | | Non-
| ORF I | | | | | | encapsi-
| pUM124 (S) - - None 886 1032 -147 | | | | | | dated DNA† | Virion
| pLW214 (C) 1285 8 GGAATTCC | None | | - +8 | - | NT | NT |
| ORF II | | | | | | DNA‡ |
| pSA103 (N) - - None 1598 1674 | | | | | | |
| ORF III | | | | | | |
| pCMS34 (N) - - None | 1927 2148 | -222 | - | - | - | - |
| pUM41 (S) 2040 8 GCCCGGGC 2041 2044 +4 | | | | | | |
| pUM130 (S) 2040 16 GCCCGGAA TTCCGGGC | 2041 2044 +12 | + | + | NT | NT |
| ORF IV | | | | | | |
| pUM13 (S) 3096 8 GCCCGGGG | None | | +8 | - | - | NT |
| pUM37 (S) - - None | 2579 3517 | -939 | - | - | NT | NT |
| pCS101 -4250 (S) | 3034 8 GCCCGGGG | 3035 3150 | -108 | - | - | - |
| pDLS19 (N) - - None | 3233 3427 | -195 | - | - | - | - |
| ORF V | | | | | | |
| pUM133 (S) - - None | 3906 5271 | -1366 | - | - | NT | NT |
| pHLM3 (C) 5504 8 CGGTACCG | 5505 5527 | -15 | - | - | - | - |
| ORF VI | | | | | | |
| pUM11 (S) 6326 2 CG | None | | +2 | - | - | NT |
| pUM24 (S) 6298 16 (GCCCGGGGC)$_2$ | 6299 6338 | -24 | - | - | - | - |
| pUM45 (S) 5915 16 (GCCCGGGGC)$_2$ | 5916 6648 | -717 | - | - | NT | NT |
| Parent | | | | | | |
| Ca37 (S) 4837 | pBR322 | None | - | - | + | + | + |
| pCMS31 (N) 4837 | pBR322 | None | - | - | + | + | + |
| pLW414 (C) 4837 | pBR322 | None | - | - | + | + | + |
| Ca4 (S) 5391 | pBR322 | None | - | - | + | + | NT | NT |
| pLW303X (W) XhoI | pACYC177 | None | - | - | + | NT | NT | NT |

* Letters in parentheses indicate the parental CaMV isolate from which the mutant DNAs were derived: C, CM4-184; S, CabbS; N, NY8153; W, W (Choe et al., 1985). The construction of the following plasmids has been previously described: pCMS31, pSA103 (Armour et al., 1983), pLW414, pLW214 [obtained from S. Howell (Howell et al., 1980, 1981)], Ca4, Ca37 (Lebeurier et al., 1982), pUM124, pUM41, pUM13, pUM37, pUM133, pUM45, pUM11 (Choe et al., 1985). Partial digestion of pCMS31 with BamHI and re-ligation led to pCMS34. An HpaI digest of Ca37 was treated with BAL-31 exonuclease, polished and re-ligated in the presence of Sinai linker to generate pCS101-4250. Partial PstI digestion of pCMS31, followed by ligation resulted in ORF IV deletion mutant pDLS19. Ligation of BAL-31-digested, HpaI-linearized, pLW414 with KpnI linkers gave rise to pHLM3. In all cases the location of the mutation was confirmed by appropriate restriction enzyme digestion. The extent of deletions was determined by Maxam & Gilbert (1980) sequencing of the DNAs in the altered regions (Franck et al., 1980).

† Inclusive. Numbering of nucleotides according to Franck et al. (1980).

‡ Turnip leaves were inoculated with restriction enzyme-digested (SalI for all plasmids except pUM133 and Ca4 for which PstI was used and pLW303X for which XhoI was used) plasmid DNAs (see Fig. 1 legend) and harvested 2 weeks later for preparation of non-encapsidated and virion DNA fractions as described in Fig. 2. ' + ' indicates detection of hybridization (other than to linear inoculum molecules) when samples were electrophoresed, blotted and probed with CaMV DNA. '-' indicates the absence of such hybridization. Each plasmid was tested at least three times. 'NT' indicates plasmids that were not tested three times. For infectivity testing, plants were similarly inoculated. Each experiment included plants inoculated with wild-type DNA and with buffered celite, the former of which all became diseased while the latter remained healthy throughout the 30 day observation period.

§ DNA recovered from plants inoculated with this DNA had an additional 1 bp deletion. See text.

Positive squish hybridizations were obtained from plants that had obvious systemic symptoms of CaMV infection (Table 1, plants inoculated with Ca37, pCMS31, Ca4, pSA103 and pUM130) but never from plants inoculated with non-infectious mutant DNAs (Table 1). For each mutant DNA, squishes from at least three leaves from three or more plants were
analysed. The sensitivity of squish hybridization was sufficient to detect concentrations of CaMV that are 100-fold less than in wild-type CaMV-infected plants.

The failure to detect CaMV DNA in young uninoculated leaves raised the possibility that the mutant DNA replicated in, but did not spread from, inoculated leaves. Non-encapsidated and virion DNA fractions from inoculated leaves were therefore analysed. Non-encapsidated DNA fractions (Lebeurier et al., 1982) of leaves inoculated with wild-type CaMV DNA from salI-digested pCMS31 (Fig. 3a, lane 2), Ca37 or XhoI-digested pLW303X (not shown) contained CaMV DNA that migrated as a diffuse band of mobility approximately that of linear DNA. Incubation of the non-encapsidated DNA (Fig. 3a, lane 2) with traces of DNase I and MgCl2 produced CaMV DNA molecules that co-migrated with relaxed circular CaMV DNA (Fig. 3a, lane 1). The fast mobility, heterodisperse distribution and convertibility to relaxed circular DNA are properties expected of supercoiled CaMV DNA known to be present in nuclei of infected cells (Olszewski et al., 1982; Menissier et al., 1982).

No hybridization was detected when non-encapsidated DNA fractions of leaves inoculated with SalI-digested pCMS31 were probed with pBR322 DNA. However, non-encapsidated DNA fractions prepared 10 to 20 days after inoculation of turnip leaves with SalI-linearized pBR322 contained molecules that reacted with a pBR322 probe. Fig. 3(b) shows that these molecules had the same electrophoretic mobility as linear pBR322, could not be further digested with SalI, and were digested by EcoRI to a product with a mobility consistent with EcoRI digestion of SalI-linearized pBR322.

Non-encapsidated DNA fractions from leaves inoculated with SalI-digested plasmids bearing non-infectious mutant CaMV DNAs were also electophoresed, blotted and probed with either CaMV or pBR322 DNA (Table 1). Hybridization was detected to CaMV and pBR322 probes, to only the pBR322 probe, or to neither probe. The mobility of hybridizing bands was in all cases the same as that of the linear molecules present in the inocula. The preparations in which CaMV DNA sequences were found were digested with restriction endonucleases that recognize single sites distant from the initial site of linearization. When Southern blots of gels were probed with CaMV DNA, fragments expected from digestion of linear inoculum molecules were detected. The presence of the inoculum DNA even 2 weeks after inoculation interfered with the detection of possibly replicated CaMV mutant DNAs. In contrast, wild-type CaMV DNA inocula almost completely disappeared from inoculated leaves during the 2 weeks following inoculation, a result consistent with findings of E. Balazs (unpublished, cited in Lebeurier et al., 1982).

Virion DNA fractions (Gardner & Shepherd, 1980) from leaves inoculated with cloned wild-type CaMV DNA were also analysed by electrophoresis, blotting and hybridization with a CaMV probe. The pattern of bands (Fig. 3c) was indistinguishable from that obtained with DNA from purified CaMV virions. No hybridization was obtained when similar fractions from leaves inoculated with cloned mutant CaMV DNAs (Table 1) were probed. In inoculated leaves, CaMV remains localized in small circular lesions which presumably arise by radial spread of the infection from a single initially infected cell (Melcher et al., 1981). By serial dilution of virion DNA from wild-type CaMV DNA-inoculated leaves, we determined that the sensitivity of detection was sufficient to detect 1000-fold less virion DNA than was present in leaves inoculated with wild-type DNA. The sensitivity is sufficient to detect virion DNA replication in inoculated leaves of non-host plants (M. M. Hussain et al., unpublished) but not sufficient to detect CaMV virion DNA replication in the absence of spread from the initially infected cells to neighbouring cells.

For the mutants tested here, in every case where systemic spread of viral nucleic acid was found, symptoms were seen, while in every case where symptoms of CaMV infection were not seen, no viral DNA was detected in inoculated or later-emerging leaves. Thus, mutations in ORFs I, III, IV, V and VI prevented the production of symptoms by preventing or severely limiting the replication and/or spread of CaMV in turnip plants. Some of the mutations used here may be non-infectious because of reading frame shifts or because the deletions were too large for efficient DNA packaging. At least one mutant DNA for each ORF met the following criteria: the modification was entirely limited to that ORF; the modification resulted in the
preservation of the reading frame; the size was within the limits of DNA sizes (7610 to 8260 bp) known to be packaged into CaMV virions (Howarth et al., 1981; Gronenborn et al., 1981). Thus, it is likely that the protein products of ORFs I, III, IV, V and VI are essential for viral replication or spread. CaMV mutants, such as V3 (Tomlinson & Shepherd, 1978) that produce symptomless infections and ORF VI mutants that produce less severe symptoms (Daubert et al., 1983), must bear mutations that produce minor alterations in one of these protein products, such that the function of these proteins in viral replication or spread is not drastically affected.

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REFERENCES


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