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A second cauliflower mosaic virus gene product influences the structure of the viral inclusion body

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We have used electron microscopy of thin sections and experiments on isolated viroplasms to compare the properties of four strains of cauliflower mosaic virus (CaMV), three of which were partially or completely deleted in open reading frame (ORF) II. Our results confirm that this gene is required for aphid transmissibility and show that the product of ORF II influences the firmness with which virions are held within the viroplasm. Analysis of the proteins in the viroplasms showed that a mutant with a partial deletion in ORF II produced a protein smaller than the normal ORF product. This smaller protein was non-functional with respect both to aphid transmissibility and properties of the viroplasms.
Key words: cauliflower mosaic virus/inclusion body/gene/electron microscopy/ELISA

Introduction

Viroplasms in plants infected by cauliflower mosaic virus (CaMV) are the major site of virus accumulation (Martelli and Castellano, 1971; Conti *et al.*, 1972; Shalla *et al.*, 1980; Xiong *et al.*, 1982a). The major viroplasm protein (P66) is coded by the gene VI of the viral DNA (Xiong *et al.*, 1982b). The sequence of this major protein has been deduced from the viral DNA sequence of three strains (Franck *et al.*, 1980; Gardner *et al.*, 1981; Balàzs *et al.*, 1982). Variations have been described in the size of the viroplasms, in the ratio of virion to viroplasmic protein (Shalla *et al.*, 1980) and in the intensity of staining with uranyl acetate-lead citrate (Xiong *et al.*, 1982a) but these differences have not been related to a specific gene product.

Open reading frame (ORF) II was described by Woolston *et al.* (1983) and Armour *et al.* (1983) as coding for a product required for aphid transmission of CaMV. Here we show that the firmness with which virions are held within the viroplasm is influenced by the presence, absence or partial expression of ORF II product. Relationships between the presence of ORF II and the variation in the structure of the viroplasms and virus transmission by aphids are discussed.

Results

Electron microscopy of viroplasms

Viroplasms in thin sections of fragments of turnip leaves have been examined by electron microscopy for the following four strains of CaMV: strain S (Hohn *et al.*, 1980); strain CM4.184 (Howarth *et al.*, 1981); S.CM4, a hybrid that contains a part of ORF III, ORFs IV, V, VI of strain S and ORFs VII, I, remnants of ORF II and the other part of ORF III of strain CM4.184 (the new ORF III is functional: see Materials and methods); the mutant S.ΔII derived from strain S by deletion of the central part of ORF II (Figure 3). The proportion of viral particles to matrix protein in the viroplasms was higher in strains CM4.184 and S.CM4 (Figure 1b and c) than in strain S (Figure 1a) and mutant S.ΔII (Figure 1d, e and f). Within the viroplasms induced by strain S and the S.ΔII mutant, virions were mainly concentrated in clear areas. The viroplasms present in S.ΔII infected cells (Figure 1d, e and f) deserve special attention since virus particles were seen packed in semi-crystalline arrays outside the viroplasmic matrix. As far as we are aware this is the first situation in which particles of any CaMV strain or mutant have been observed in the cytoplasm in such an arrangement.

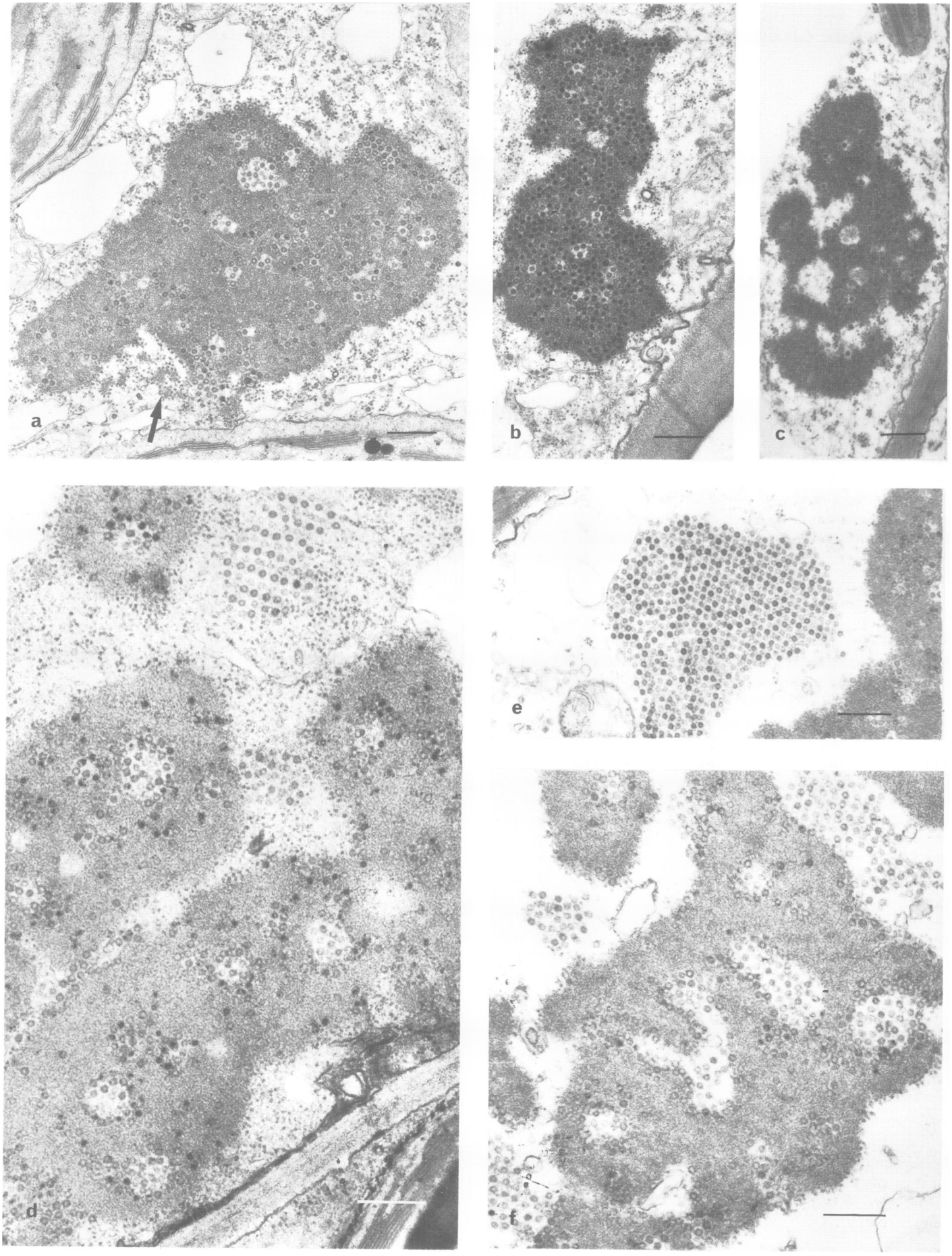
Immunochemical studies

In an attempt to determine whether there were differences between the strains in the firmness with which virions were held within the viroplasms, we compared the quantity of viral material liberated under two conditions: low speed centrifugation and urea plus Triton X-100 treatment. Relative amounts of virus released were measured serologically using a strain S antiserum. Table I shows the results of these experiments.

After centrifugation of the crude extract from plants infected with strain S no virus could be detected in the supernatant (experiments 1 and 2, first column). Thus we assume that the virions are all sedimented within the viroplasms. When the crude extract of strain S was treated with urea plus Triton X-100, virus was liberated from viroplasms and became accessible to the antibodies (experiments 1 and 2, first column). On the other hand, when the untreated crude extract of the CM4.184-infected plants was centrifuged the same quantity of virus was detected in the supernatant as in the non-centrifuged extract (experiment 1, second column). Treatment with urea plus Triton X-100 did not significantly increase the amount of virus detected (experiment 1, second column). We conclude that much of the virus in tissue infected with CM4.184 is not firmly bound within the viroplasms. The same observation (experiment 1, third column) was made with the crude extracts from leaves infected with the hybrid strain S.CM4 which contains the gene VI of the strain S but like CM4.184 lacks ORF II (see Figure 1b and c). Experiments performed with the deletion mutant S.ΔII which contains the gene VI of strain S and a deletion localized in the central part of ORF II, gave similar results (experiment 2, second column).

Electrophoresis of the proteins contained in the viroplasms induced by the four isolates

The proteins associated with inclusion bodies were analyzed by PAGE under denaturating conditions. The major viroplasm protein P66 (Xiong *et al.*, 1982b) was present in every preparation from infected plants (Figure 2A, B, arrow a).


a
b
c
d
e
f

The ORF II product, described by Woolston *et al.* (1983), migrating with a mol. wt. of 18 kd (P18), was associated with viroplasms from strain S-infected plants (Figure 2A, lane 5, B, lane 2, arrow b) but was absent in the S.CM4 and CM4.184 viroplasm preparations (Figure 2A, lanes 3 and 6) and in healthy plants (Figure 2A, lane 2).

With the S.ΔII mutant, a protein, absent from other preparations, migrating slightly more rapidly (~16 kd) than P18, was observed (Figure 2B, lane 3, arrow b'). This is consistent with the expression of the new shortened ORF II which, from sequence data, would be able to be translated (see Figure 3 legend). In a set of experiments in which all conditions of viroplasm preparations were identical, the protein of ~16 kd was sometimes absent. In such experiments a product migrating more rapidly (~12 kd) (Figure 2A, lane 4, arrow c) could be observed. This indicates that the ORF II product of the mutant S.ΔII may be unstable. This property could be associated with the physiological state of different batches of infected plants.

Aphid transmissibility

The isolate CM4.184 is not aphid transmissible (Hull, 1980). In two experiments aphids were fed on plants infected with strain S and then transferred to test plants; 19/26 test plants became infected in the first experiment and 26/28 in the second. No transmission of the S.ΔII mutant was observed when the same number of test plants was used.

Discussion

Our results demonstrate that the main matrix protein of CaMV, the gene VI product, is not the only element involved in determining the properties of viroplasms. The ORF II product also determines some of their properties. Polyacrylamide gel analysis of viroplasmic proteins revealed that the ORF II product (P18) (Woolston *et al.*, 1983) is absent in viroplasm preparations extracted from plants infected with viral DNA in which ORF II is completely deleted. In the strain where ORF II has the potentiality to encode a 15-kd protein (S.ΔII mutant) an unstable protein was detected (Figure 2A, lane 4; B, lane 3). The same observation has been described for defective interfering (D.I.) particles of poliovirus: when 1200 nucleotides near the 5' OH end were deleted, the translation product was degraded rapidly to acid solubility (Cole and Baltimore, 1973). We are currently attempting to obtain definitive evidence that the P18 product and the unstable 16-kd protein are ORF II products.

Work in several laboratories has established that the product of ORF II plays a role in the aphid transmissibility of CaMV. Our results define a part of ORF II which is essential for aphid transmission of CaMV. How much of the rest of ORF II is necessary remains to be determined. We show that the product of ORF II also influences the firmness with which virions are held within the viroplasm. Our results do not allow us to establish whether it is a complex of P18 and the major viroplasm protein (P66) which is necessary to retain virions firmly bound in the viroplasm or whether P18 interacts directly with virions. The exact function of ORF II remains to be elucidated.

Materials and methods

CaMV DNA mutants used

Clones used were pCaBT37 (pBR322-SalI-CaMV) (Hohn *et al.*, 1980) named S; pCaDavis20 (pBR327-SalI-CM4.184), a gift from R.Shepherd, called CM4.184; the hybrid DNA clone pCaT86 named S.CM4 obtained from isolated restriction fragments of the parents (Figure 3). S.CM4 was constructed as follows: the large *Bam*HI-*Bst*EII fragment of clone S (clockwise from nucleotide 1926, *Bam*HI site, to nucleotide 126, *Bst*EII site) was obtained after partial digestion in the presence of ethidium bromide (Shortle and Nathans, 1978) with *Bam*HI and complete digestion with *Bst*EII restriction enzymes; the small *Bst*EII-*Bam*HI fragment of clone CM4.184 (nucleotide 126 to nucleotide 1505) was obtained by complete digestion with the enzymes. The fragments were separated in low melting agarose and ligated in gel slices as described by Dixon *et al.* (1983). The hybrid S.CM4: 7603 bp (8024 bp of strain S minus 421 bp of strain CM4.184; Howarth *et al.*, 1981) possesses a new ORF III with a length of 389 nucleotides: the reading frame begins with an ATG situated in position 1409 and ends with a terminator codon at the new position 1798. The nucleotides located between 1409 and 1505 (a *Bam*HI restriction site) originate from the genome of CM4.184, the remainder from clone S (1926–2219). In the amino acid sequence coded by the nucleotides 1409–1505, the only change is an alanine (strain S) replaced by a serine (strain CM4.184) in position 23. Recipient bacterial cells were DH1 prepared as described by Morrison (1979). Cloning of CaMV DNA was as described by Hohn *et al.* (1980).

Construction of a deletion mutant within the ORF II of strain S

The deletion mutant named S.ΔII (Figure 3) was constructed from pCaBT37 as follows: pCaIT159, carrying a 10-bp insert with a *Sma*I restriction site in position 1537 (Dixon *et al.*, 1983) was cleaved at the *Xho*I site. It was then ligated with a purified *Sal*I restriction fragment carrying a kanamycin (Km) resistance marker (NPTI) derived from LT98 Km^R (Dixon *et al.*, 1983). This fragment was flanked by polylinkers which include *Sal*I restriction sites (used to isolate the fragment) and *Sma*I restriction sites.

After transformation of *Escherichia coli* cells (DH1 strain), tetracycline-and kanamycin-resistant clones were isolated. The plasmid was purified and digested by *Sma*I, religated and used to transform *E. coli* cells. Tetracycline-resistant and kanamycin-sensitive clones were selected and subjected to restriction analysis. The resulting strain has a 105-bp deletion from nucleotide 1537 to nucleotide 1643 and a 12-bp insert (Figure 3). The truncated ORF II has its original start and stop codons in phase.

Multiplication of CaMV strains in turnip plants

The different CaMV clones described above were inoculated independently to six turnip plants (*Brassica rapa* L., c.v. Just Right) each at a concentration of 10 μg of cloned DNA/ml in sterile water. Three weeks after inoculation, 1 g of newly formed infected leaves was collected from each batch of plants and ground in a mortar with 5 ml of sterile water and inoculated to 10 healthy plants. Inoculated plants were maintained under greenhouse conditions as described by Lebeurier *et al.* (1980).

Characterization of viral DNA

The restriction enzyme patterns of viral DNA of each strain isolated from turnips were verified as described by Gardner and Shepherd (1980). The migration of viral DNA extracted from infected plants is shown in Figure 4. Each strain produced the expected pattern of DNA fragments obtained after cleavage by *Eco*RI restriction enzyme. The amount of DNA extracted from plants infected with the hybrid S.CM4 was nearly the same as those extracted from plants inoculated with the other strains. This confirms that the ORF III is functional (Lebeurier *et al.*, 1982).

Purification and analysis of viroplasms

Three weeks after inoculation of plants, viroplasms were isolated and analyzed according to the techniques described by Xiong *et al.* (1982b).

Fig. 1. Electron microscopic observation of viroplasms. Viroplasms of the four strains were observed 21 days after inoculation. **(a)** Intracytoplasmic viroplasm inclusion induced by strain S. Virions are mainly concentrated in clear areas within the viroplasms. The matrix is relatively poor in scattered viral particles. Note small masses of matrix substance devoid of virions and studded with ribosomes at the periphery of the viroplasm (arrow) (magnification: 25 000 x). **(b,c)** Viroplasms induced by the strain CM4.184 and S.CM4 hybrid. Viroplasms contain a higher proportion of virus particles than strain S **(a)**. **(d,e,f)** Viroplasms induced by the S.ΔII mutant strain. As in **(a)** virions are concentrated in clear areas and only a few are scattered in the matrix substance **(d** and **f)**. Clear lacunae with accumulated virions seem to enlarge and open on the periphery of the viroplasm **(f)**. Note numerous virions located outside the viroplasm, many are present as semi-crystalline arrays (these are most obvious in **(d)** and **(e)** (magnification: **d** = 36 000, **e** = 26 000, **f** = 30 000 x). All bars represent 400 nm. Note: the difference in depth of staining seen in this plate between viroplasms of different strains was not a consistent feature.

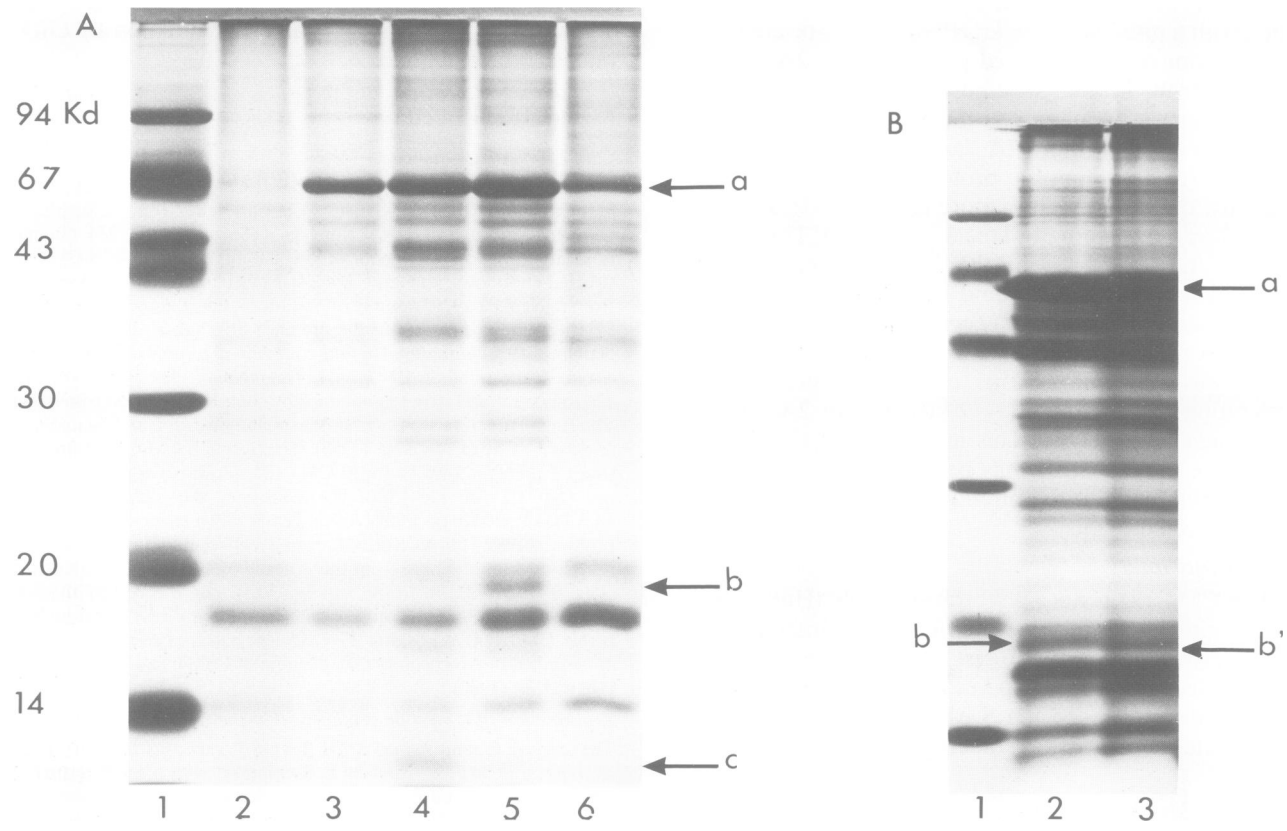


Fig. 2. Electrophoresis of viroplasm preparations (Vp) in 12.6% SDS-PAGE. 20 μl of Vp (1 ml/5 g leaves), pre-treated by boiling for 2 min in 125 mM Tris HCl, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol was loaded in each slot. **(A) Slot 1:** mol. wt. standards in kd. **Slot 2:** healthy plants. Plants infected with **slot 3:** S.CM4, **slot 4:** S.ΔII, **slot 5:** strain S, **slot 6:** CM4.184. **(B) Slot 1:** mol. wt. standards in kd identical to **A, slot 1. Slot 2:** strain S, **slot 3:**. S.ΔII. Arrows indicate location of P66 (a), P18 (b), pP15 (b') and P12 (c).

Table I. Relative amounts of virus in crude extracts of plants infected with isolates and mutants of CaMV, after various treatments

Treatments	Strains				
	S	CM4.184	S.CM4	S	S.ΔII
crude extract	1.1	1.49	1.44	0.88	1.70
low-speed centrifugation supernatant	0.00	1.44	1.43	0.05	1.58
treated[a] with urea 1 M plus Triton X-100 2.5% overnight	1.61	1.62	1.70	1.51	1.42
tested without centrifugation		experiment 1			experiment 2

[a]These treatments increased the volumes of the extracts by 20%.
The absorbance readings indicated in the table have been corrected for this.
Absorbance at 405 nm obtained in indirect double antibody sandwich ELISA method using anti-CaMV (strain S) serum. Coating was done with yolk globulins (5 μg/ml) for 2 h. The virus extracts (1:100) were incubated for 3 h. The intermediate antibody was anti-CaMV (strain S) serum diluted 1:30000 and was allowed to interact for 2 h. The enzyme conjugate, which was a goat anti-rabbit globulin diluted 1:2000, was allowed to interact for 2 h.

Electron microscopy

The techniques used were those described by Xiong *et al.* (1982a).

Antisera

Rabbits, chickens and a goat were immunized by a series of i.m. injections of CaMV S in Freund's incomplete adjuvant, as described by Van Regenmortel (1975). Antiserum was obtained from a bleeding collected 6 months after the start of immunization. Since no antigenic variants have been found among strains of CaMV (Du Plessis and Wechmar, 1980) this antiserum serves for the assay of all strains used.

Pure globulins from an immunized goat were prepared by Rivanol precipitation (Hardie and Van Regenmortel, 1977). Chicken globulins were obtained from the eggs of immunized hens (Polson *et al.*, 1980).

Enzyme conjugates were prepared with alkaline phosphatase (Sigma) by coupling globulins with enzyme at 1:1.5 (w/w) ratio using 0.06% glutaraldehyde (Clark and Adams, 1977). Bovine serum albumin (1%) was added to the conjugate, which was stored at 4°C.

Crude extract preparation

Turnip leaves were quick frozen in liquid N_2 and ground with phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 (PBS-T) (1 g tissue to 1 ml buffer) and sterilized quartz sand in a chilled mortar. The sap was pressed through a layer of Miracloth. For testing the CaMV strains and mutants, the crude extract was divided into three aliquots; one was kept untreated, another was clarified by low speed centrifugation in Eppendorf tubes (10 min at 15 000 *g*), the third was stirred overnight in the presence of urea (1 M) and Triton X-100 (2.5%). All samples were then diluted in 1:100 PBS-T.

Indirect double antibody sandwich method for enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates (Cooke M 129 B, Dynatech) were used. Wells were coated at 37°C by incubation (2 h) with yolk globulins (200 μl at 5 μg/ml) diluted in 0.05 M sodium carbonate, pH 9.6. Wells were rinsed three times with PBS-T. 250 μl of 1% bovine serum albumin in PBS-T were then deposited in the wells (incubation 2 h at 37°C). After rinsing as before, 200 μl of virus extract diluted 1:100 in PBS-T, was added for a 3 h incubation at 37°C. After rinsing, anti-CaMV (strain S) rabbit serum diluted 1:30 000 in PBS-T was allowed to react at 37°C (2 h). After further rinsing, the wells were incubated for 2 h at 37°C with a goat anti-rabbit globulin conjugate (Van Regenmortel and Burckard, 1980) diluted 1:2000. After further rinsing, the bound enzyme conjugate was detected by adding 200 μl of the substrate (*p*-nitrophenyl phosphate) at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8.

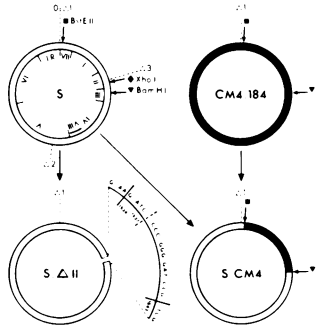


Fig. 3. Genomes of the different strains used. Viral strains S and CM4.184 correspond to the hybrid DNA molecules pCaBT37 and pCaDavis20 as described in Materials and methods. Δ1, Δ2, Δ3 represent the three gaps in strain S DNA (Volovitch *et al.*, 1978). Unshaded and black segments represent the double-stranded DNA from the S (8024 bp) and CM4.184 (7610 bp) strains, respectively. Numbers indicate the eight ORFs (Hohn *et al.*, 1982). Construction of the S.ΔII mutant. Nucleotides 1534–1642 were deleted (see Materials and methods), and a 16 bp long insert was introduced in the *Xho*I (◆) site. Note that the original reading frame is maintained. The potential translation product would have a mol. wt. of 15 kd (potential protein pP15). The partial nucleotide sequence of the insertion fragment is indicated in the blown-up form. Nucleotides 1534–1537 are common in the S.ΔII and strain S. Construction of the S.CM4 recombinant strain: a large *Bst*EII (■, position 126)-*Bam*HI (▼, position 1926) restriction fragment of S and the small one of CM4.184, *Bst*EII (■, position 126)-*Bam*HI (▼, position 1505) were joined by ligation.

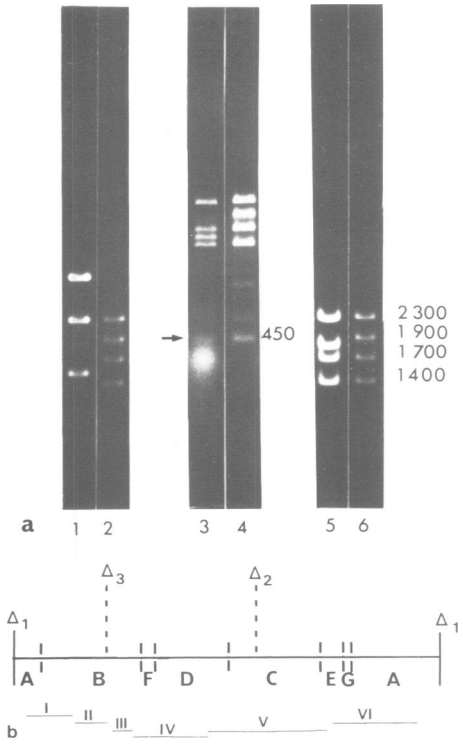


Fig. 4. (a) Electrophoretic analysis in a 1% agarose gel of DNA fragments produced by *Eco*RI cleavage of the four CaMV strains used in the experiments. 1 μg of DNA was loaded in each slot and submitted to electrophoresis at 30 V for 20 h at room temperature **(1,2,5,6)**. To visualize the short E fragment (450 bp) the migration time was shorter in slots 3 and 4 (50 V for 5 h). **Lanes 2, 4** and **6**, strain S: A (2300), B (1900), C (1700), D (1400), E (450). **Lane 1**, CM4.184: C + D (3100), A (2300), B with 421 bp deleted (1500). **Lane 3**, S.CM4: A (2300), C (1700), B with 421 bp deleted (1500), D (1400), E (450). **Lane 5**, S.ΔII: A (2300), B with 93 bp deleted (1800), C (1700), D (1400). **(b)** *Eco*RI restriction map of the strain S DNA genome. Δ1, Δ2, Δ3 indicate the location of the gaps, A (2300), B (1900), C (1700), D (1400), E (450), F and G (60), the restriction fragments, I to VI, the six ORFs (Franck *et al.*, 1980).

After 0.5 h of hydrolysis at 37°C, the tests were read with a photometer (Titertek Multiskan MC) by measuring the absorbance at 405 nm.

Aphid transmission tests

The aphids used were mature apterae of *Myzus persicae* (Sulz). They were maintained on radish *(Raphanus sativus)*. Turnip (*B. rapa* L. c.v. Just Right) served both as a source plant and as a virus indicator. Other conditions were as described by Armour *et al.* (1983).

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