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 viroplasm.

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; Bälás 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.AI1 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.DII (Figure 1d, e and f). Within the viroplasms induced by strain S and the S.AI1 mutant, virions were mainly concentrated in clear areas. The viroplasms present in S.DII 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 plant 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.DII 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

Or proteins associated with inclusion bodies were analyzed by PAGE under denaturing conditions. The major viroplasm protein P66 (Xiong et al., 1982b) was present in every preparation from infected plants (Figure 2A, B, arrow a).
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.AI1 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.AI1 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.AI1 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. Polycrylamide 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.AI1 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 CaMV must be transmitted is not clear. 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. Polycrylamide 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.AI1 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.

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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.DII, 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.AI1. 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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Strains</th>
<th>S</th>
<th>CM4.184</th>
<th>S.CM4</th>
<th>S</th>
<th>S.DII</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude extract</td>
<td></td>
<td>1.1</td>
<td>1.49</td>
<td>1.44</td>
<td>0.88</td>
<td>1.70</td>
</tr>
<tr>
<td>low-speed centrifugation</td>
<td></td>
<td>0.00</td>
<td>1.44</td>
<td>1.43</td>
<td>0.05</td>
<td>1.58</td>
</tr>
<tr>
<td>treated with urea 1 M plus Triton X-100 2.5% overnight</td>
<td></td>
<td>1.61</td>
<td>1.62</td>
<td>1.70</td>
<td>1.51</td>
<td>1.42</td>
</tr>
<tr>
<td>tested without centrifugation</td>
<td>experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>experiment 2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*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 1:30,000 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).

Antiserum
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 immuno-sorbent 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 (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.

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Indirect double antibody sandwich method for enzyme-linked immuno-sorbent 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 phosphatase) at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8.
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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**References**


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