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Comparative Cytology of Two Isolates of Cauliflower Mosaic Virus

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SUMMARY

The cytology of Cabb-S and Cabb-D/H isolates of cauliflower mosaic virus (CaMV) was compared by electron microscopy. In turnips (*Brassica rapa* L. cv. 'Just Right') the two strains induced similar symptoms. In the Cabb-S-infected cells the viroplasms contain large amounts of electron-dense material and small amounts of virions, in contrast to the Cabb-D/H-infected cells where there are large amounts of virions and less electron-dense material. The viroplasms could be detected 3 days after inoculation with the Cabb-S isolate but only after 6 days in the leaves infected with the Cabb-D/H isolate. Another type of inclusion, intensely stained using Bernhard's EDTA technique, was detected in the cells infected with either isolate about 10 days after inoculation. The significance of these cytoplasmic areas, which coexist with well-developed viroplasms, is discussed.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the type member of the caulimovirus group. In the host cells it induces inclusions consisting of a dense matrix (Fujisawa *et al.*, 1967; Rubio-Huertos *et al.*, 1968; Shalla *et al.*, 1980) embedding virions. The typical inclusion bodies, which in appearance resemble structures associated with some animal viruses (Stoltz, 1973), are called 'viroplasms'. These inclusions are essentially proteinaceous and are thought by some authors to be the factories for virion assembly (Martelli & Castellano, 1971). The DNA sequence of the Cabb-S isolate was reported by Franck *et al.* (1980), who suggested six putative coding regions. One of them, coding region IV, probably codes for the capsid protein (Franck *et al.*, 1980), and coding region VI is a good candidate for the viroplasmic protein (Al Ani *et al.*, 1980; Howell *et al.*, 1981; Covey & Hull, 1981). The DNA of the Cabb-D/H isolate was recently sequenced by E. Balàzs *et al.* (unpublished results). Its DNA sequence differs from Cabb-S by about 5% and has the same number of open reading frames. Thus, in view of the sequence differences between the two isolates we thought it would be interesting to see if there were also differences in the cytology of infection. In this paper, we present a study of the development of the viroplasms contained in turnips infected independently with the two CaMV isolates. We also describe the presence in infected cells of inclusions which differ from the classical viroplasms.

METHODS

Virus isolates. The two isolates were propagated in young turnip plants (*Brassica rapa* L. cv. 'Just Right') grown under the same growth chamber conditions (21 °C, 2000 lux for 16 h/day). Cabb-S isolate (Lebeurier *et al.*, 1978) was a gift from G. G. Conti (Istituto de

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Science Botaniche, Universita di Milano, Italy). Cabb-D/H isolate was isolated by J. Horvàth in Budapest, Hungary from *Brassica oleracea* L. var. Botrytis Alef. cv. 'Hoshima helios' (Horvàth *et al.*, 1980). The two isolates induce the same type of symptoms in turnips, namely severe stunting, mosaic and leaf distortions.

Virus purification. Virus was purified according to the method of Hull *et al.* (1976).

Purification of viroplasms. Two sets of six 5-week-old turnip plants were inoculated with 10 μg/ml of virus of each isolate (Cabb-S or Cabb-D/H). To compare the inclusion bodies induced by the two isolates, 10 g of inoculated or systemically infected leaves were taken from each set of plants at different times. Samples were taken 5 days and 9 days after infection for the inoculated leaves, and 13 days and 21 days after infection for the systemically infected leaves. The extraction procedure was as described by Al Ani *et al.* (1980).

SDS–polyacrylamide gel electrophoresis (*SDS–PAGE*). Twenty μl of each sample (see above) were treated with an equal volume of protein disruption buffer (125 mM-tris–HCl pH 6·8, 4% SDS, 4% 2-mercaptoethanol, 30% glycerol, 0·05% bromophenol blue) and subjected to electrophoresis on a 10% SDS–polyacrylamide slab gel using a 5% spacer gel. Gels were stained with Coomassie Brilliant Blue R250 in a methanol:acetic acid:water (5:1:5) solution and destained with several changes of methanol:acetic acid:water (2:3:60) solution.

Electron microscopy. Ten plants were inoculated with 1 ml CaMV (10 μg/ml) and samples were taken at 3, 6 and 10 days from the inoculated leaves and at 10, 14 and 21 days from systemically infected leaves. The leaf tissues were fixed with 5% glutaraldehyde in 0·1 M-phosphate buffer pH 7·4, post-fixed with 1% osmium tetroxide in the same buffer, ethanol-dehydrated and embedded in an Araldite–epon mixture. The thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A electron microscope. Thin sections of samples fixed with glutaraldehyde only were used for Bernhard's EDTA technique for preferential staining of ribonucleoproteins (RNP) (Bernhard, 1969).

RESULTS

Ultrastructure

The viroplasms seen in thin sections of samples from Cabb-S- and Cabb-D/H-infected leaves were approximately the same size, but differed in their development and composition. Small groups of particles embedded in a discrete matrix were found 3 days after inoculation in the Cabb-S-inoculated leaves (Fig. 1 *a*, *b*) while no particles were detected at this stage in the Cabb-D/H-inoculated leaves. At 6 days (Fig. 1 *c*) disseminated viroplasm bodies were abundant in the Cabb-S-inoculated leaves but were still scarce in the Cabb-D/H-inoculated leaves. At 10 days, larger viroplasm bodies were present in both sets of inoculated leaves; however, their structure clearly differed. This difference was also found at later stages (e.g. 21 days). Viroplasms of Cabb-S-infected cells were very electron-dense and contained relatively few virions (Fig. 1 *d* to *f*), in contrast with viroplasms of Cabb-D/H-infected cells (Fig. 2 *a*, *b*) which were very rich in virions and were less electron-dense. At the 10-day stage with both isolates, viroplasm bodies were not restricted to directly inoculated cells but were abundant in newly formed leaves where they showed the same characteristics as in inoculated leaves.

During the period of rapid virus replication (i.e. from 10 days onwards), small masses of matrix material apparently devoid of virions occurred, often in great quantity, around the typical viroplasm inclusions. These masses were clearly connected at their periphery to free ribosomes (Fig. 1 *e*); they were also seen in close contact with ribosomes on the endoplasmic reticulum and in particular with those covering the external membrane of the perinuclear cisterna. These formations together with typical viroplasm bodies were often seen in

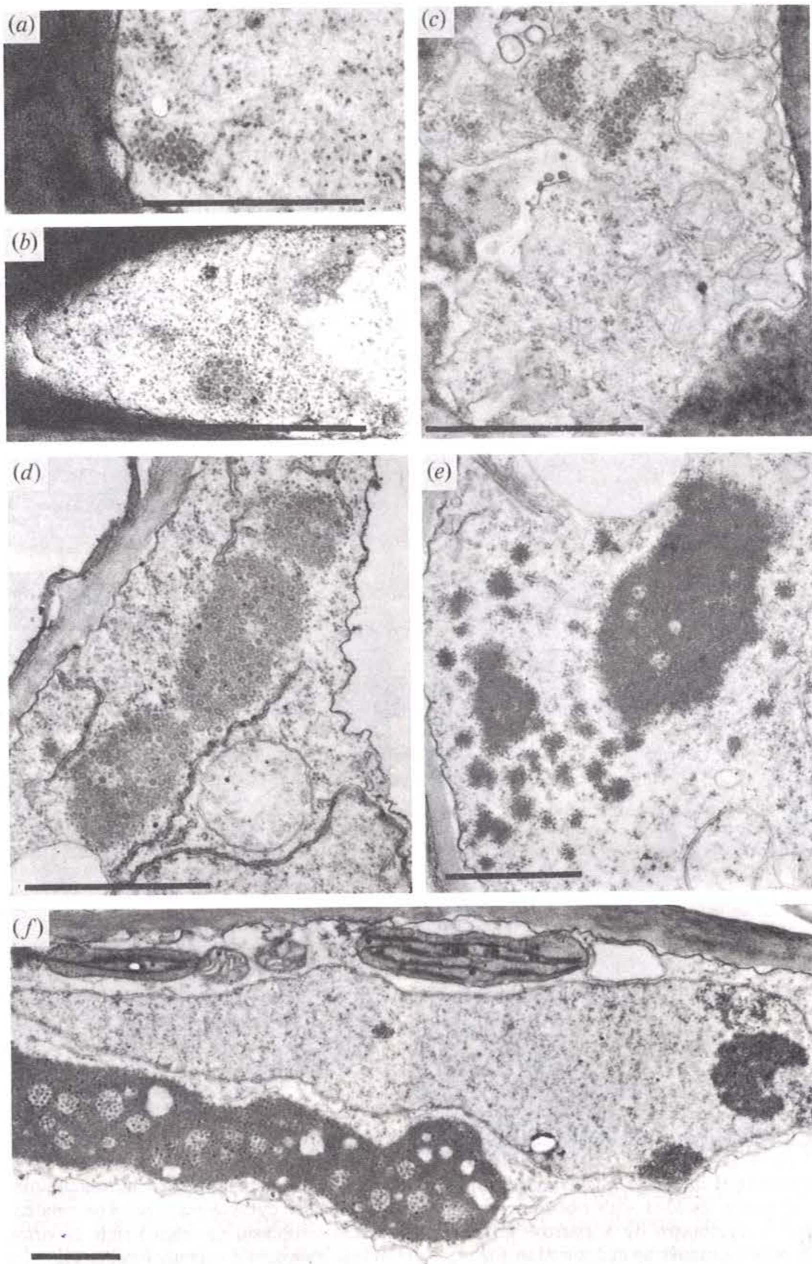

Fig. 1. Development of Cabb-S in infected leaves. (*a*, *b*) Small isolated aggregates of particles embedded in a discrete matrix 3 days after inoculation. (*c*) Individual cells containing an increasing number of aggregates 6 days after infection. (*d*) Progressive enlargement of aggregates forming typical viroplasms 10 days post-infection. Note the relationship between the matrix substance and ribosomes either free or fixed on endoplasmic reticulum cisternae (*a*). (*e*) Cabb-S infection 21 days after inoculation, showing typical viroplasm containing particles scattered in a dense matrix, surrounded by numerous small masses of matrix substance devoid of virions and studded with ribosomes. (*f*) Cabb-S infection 21 days after inoculation, showing typical viroplasm containing an abundant dense matrix substance which encloses virus particles, mainly concentrated in clear areas. All bar markers represent 1 μm.

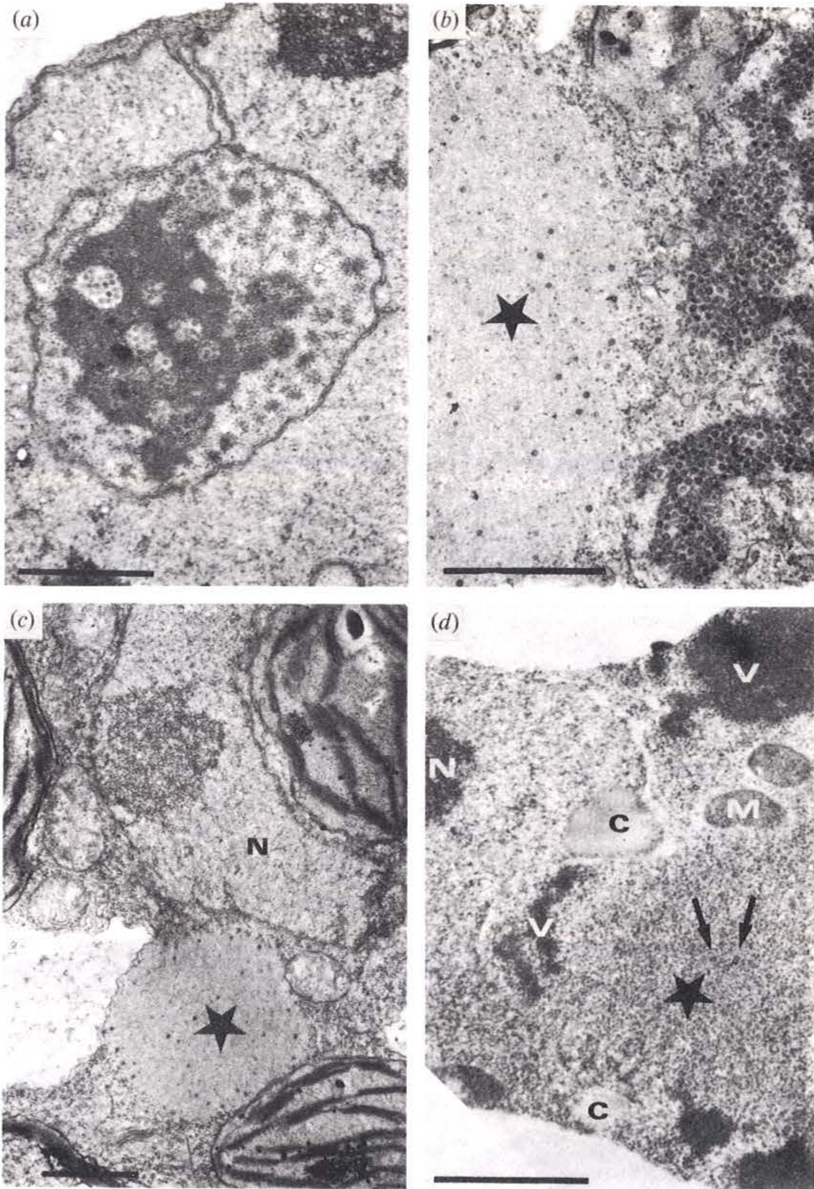

Fig. 2. Cabb-D/H infection 21 days after inoculation. (*a*) Viroplasm surrounded by minute masses of matrix substance studded with ribosomes in a large intranuclear cytoplasmic inclusion related to the remainder of cytoplasm by a narrow pedicle. (*b*) Typical viroplasm (at right) rich in virions and relatively poor in matrix as compared to Fig. 1 (*f*). The star indicates a vaguely fibrillar area devoid of organelles, containing a few scattered virus particles. (*c*) Low power appearance of the cytoplasmic area shown in (*b*) (indicated by star) appearing as a well-outlined inclusion in the cell; N = nucleus. (*d*) Similar cytoplasmic area containing few virions (arrows) in close contact with an indented nucleus (left), positively stained with Bernhard's EDTA technique (preferential staining for RNP) which reveals a granulo-fibrillar structure in this inclusion. Note intensely stained viroplasms (V), nucleolus (N), bleached chromatin (C) and mitochondria (M). All bar markers represent 1 μm.

cytoplasmic areas invaginated into the nucleus (Fig. 2*a*). The frequency of such invaginations did not depend on the strain of virus.

Further cytoplasmic alterations occurred in infected cells from 10 days onwards. They consisted of more or less spherical weakly electron-dense cytoplasmic areas, without

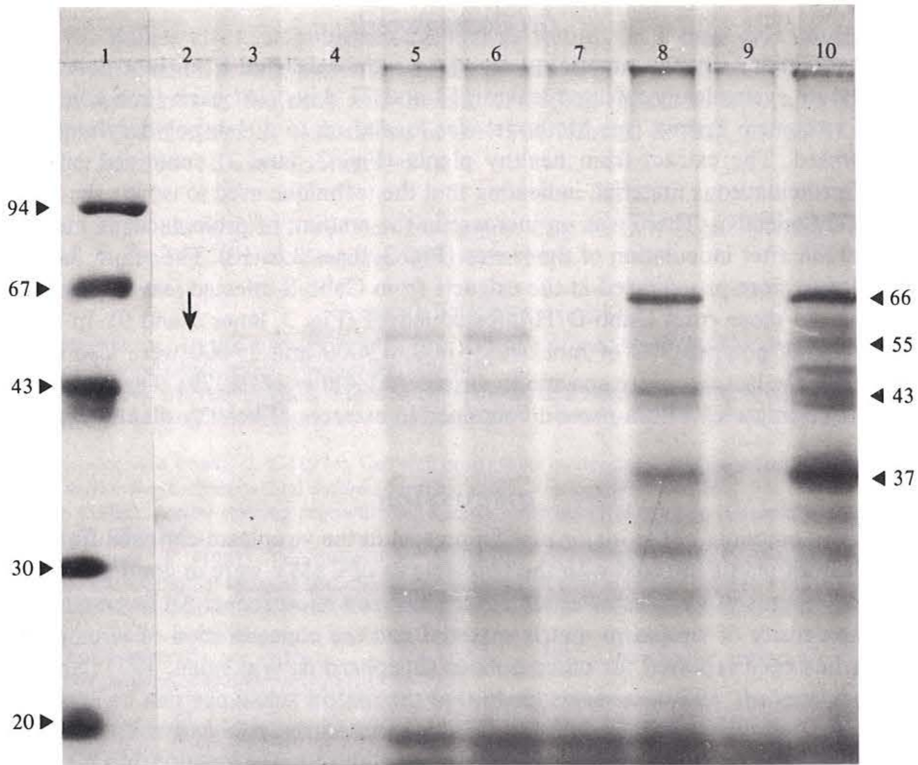

Fig. 3. Electrophoresis of purified inclusion bodies in SDS–PAGE. The gel consisted of a 5% spacer gel and a 10% running gel. Lane 1, mol. wt. markers: phosphorylase b (94000); bovine serum albumin (68000); ovalbumin (43000); carbonic anhydrase (30000); trypsin inhibitor (20100) (mol. wt. shown $\times 10^{-3}$). Lane 2, healthy plants subjected to the same extraction procedure as described for infected plants. Twenty μl samples of 2 ml suspensions were treated as described in Methods before being loaded on to the gel. Lanes 3 to 10, infected plants. Twenty μl of each sample were treated as for the healthy plant sample (see above). Lanes 3 and 5, viroplasm-enriched fraction from Cabb-D/H-inoculated leaves 5 and 9 days after infection respectively. Lanes 4 and 6, viroplasm-enriched fraction from Cabb-S-inoculated leaves 5 and 9 days after infection respectively. Lanes 7 and 9, systemically infected (Cabb-D/H) leaves 13 and 21 days after infection respectively. Lanes 8 and 10, systemically infected (Cabb-S) leaves 13 and 21 days after infection respectively. This comparison has been repeated several times and the results were always found to be the same as described in this figure.

well-defined outlines. They appeared empty of organelles and presented a poorly defined granulo-fibrillar appearance (Fig. 2*b*, *c*). These structures always contained a few scattered virus particles. Although coexisting with well-developed viroplasms, there was no obvious relationship between them. Bernhard's EDTA technique for preferential staining of RNP, which gives staining of the matrix material of the viroplasms, also produces strong staining of these cytoplasmic structures (Fig. 2*d*), revealing a fine granulo-fibrillar structure. This staining suggests a possible accumulation of RNP material in these inclusions. It is interesting to note that these particular structures were seen only in the infected cells containing viroplasms.

Nuclei were deeply indented in later stages of infection, but other ultrastructural changes, such as the mitochondrial swelling and cell wall abnormalities reported by Bassi *et al.* (1974) to be induced in CaMV-infected cells, were not seen in our material. Also, we never found the intraplastidic inclusions described by Shalla *et al.* (1980) for the CaMV-CM4 184 isolate.

Gel electrophoresis

After inoculation, viroplasm-enriched fractions were extracted from inoculated leaves (5 and 9 days) or systemically infected leaves (13 and 21 days). At each time-point the same volume of viroplasm extract (see Methods) was loaded on to a slab polyacrylamide gel and electrophoresed. The extract from healthy plants (Fig. 3, lane 2) contained only a small amount of proteinaceous material, indicating that the technique used to isolate the viroplasms was relatively selective. There was an increase in the amount of proteinaceous material with increasing time after inoculation of the leaves (Fig. 3, lanes 3 to 10). The major band of mol. wt. 66000 was more pronounced in the extracts from Cabb-S-infected leaves (Fig. 3, lanes 8 and 10) than in those from Cabb-D/H-infected leaves (Fig. 3, lanes 7 and 9). In addition to this major band, polypeptides of mol. wt. 55000, 43000 and 37000 were also detected in both extracts; the last two were specific to virions (Al Ani *et al.*, 1979). The 55000 mol. wt. polypeptide co-migrates with a protein contained in extracts of healthy plants (Fig. 3, arrow lane 2).

DISCUSSION

The relative amounts of the major protein present in the viroplasm-enriched fraction (mol. wt. 66000) from plants infected with the two isolates of CaMV were in good agreement with the relative amounts of viroplasms as seen in the electron microscope. An inverse relationship between the density of viroplasm matrix material and the concentration of virus particles in viroplasms has been reported for other isolates (Shepherd & Wakeman, 1971; Shalla *et al.*, 1980; Shockey *et al.*, 1980). It seems likely that the matrix substance can be synthesized in the absence of virions, as in late stages of infection matrix material can be found containing few virions (Fig. 1 *a*, viroplasms induced by isolate Cabb-S). The relationship between virus replication and the production of matrix material is not well understood. The formation of virions is obviously more rapid using Cabb-S isolate, which also induces more marked synthesis of matrix material; however, there appeared to be larger numbers of particles in cells infected with Cabb-D/H, the isolate which has relatively little viroplasm matrix material. The yield of virions is higher from plants infected with the Cabb-D/H isolate than from the Cabb-S isolate (C. Xiong *et al.*, unpublished results).

The matrix of viroplasms seems to be composed of proteins or glycoproteins. However, the presence of other components such as nucleic acids has also been reported (Shepherd, 1979; Kamei *et al.*, 1969). The presence of RNA in the viroplasms is suggested by light microscopy of histochemically stained leaf material (Martelli & Castellano, 1971). Bernhard's EDTA technique stained the matrix of viroplasms strongly, which suggests that RNA is present in the viroplasms in large amounts. The other cytoplasmic structures described are also intensely stained with the Bernhard EDTA technique, revealing zones of fine granular aggregates. The morphology of these structures could indicate that they are storage sites of degraded material rather than active metabolic sites. It might be that these cytoplasmic structures which contain very few virions correspond to those isolated by Shepherd *et al.* (1980) but not cytologically visualized by Shalla *et al.* (1980). If that is confirmed it is then possible that the 55000 mol. wt. polypeptide (Fig. 3, lanes 3 to 10) is the 55000 mol. wt. polypeptide extracted from cells infected with CaMV-CM4 184 isolate and characterized by Shepherd *et al.* (1980).

It is likely that the differences in the cytology of cells infected with the two isolates of CaMV are in some way related to differences in their nucleotide sequences. However, it is not possible at present to directly attribute the cytological differences to any particular differences in nucleotide sequences. It is worth noting that in addition to simple individual base changes there are oligonucleotide deletions in the Cabb-D/H isolate when compared to the Cabb-S isolate (21 base pairs in coding region V, 5 base pairs between region I and II, 3 base pairs in

region IV) (E. Balàzs *et al.*, unpublished results) as well as an 8 base pair insertion in the non-coding region, and a 6 base pair insertion at the end of coding region IV. The presence of several point mutations at the start and the end of coding region VI may also be of significance as this region is heavily transcribed in the virus (Howell & Hull, 1978; Al Ani *et al.*, 1980; Odell *et al.*, 1981; Covey & Hull, 1981).

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