# Unusual Accumulations of Cauliflower Mosaic Virus in Local Lesions, Dark Green Leaf Tissue, and Roots of Infected Plants

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Received 30 October 1995. Accepted 19 February 1996.

Macroscopic in situ nucleic acid hybridization was used to detect cauliflower mosaic virus (CaMV) in infected plant organs to investigate the relationship between virus distribution and pathogenesis. More foci of infection were detected by direct hybridization to inoculated host leaves than was suggested from local lesion symptoms. Older lesions had a complex structure with a focused ring of signal and a diffuse outer halo around a hollow center lacking virus. Virus that had systemically invaded expanded leaves and caused vein clearing was concentrated close to the vein borders and veinlets; less virus was in the interveinal tissues. In contrast, virus in younger systemically infected leaves accumulated in interveinal green islands rather than in the chlorotic vein borders, suggesting a different type of host-virus interaction from those in which green islands are virus free. Hybridization directly to cut surfaces of thicker organs showed that CaMV was present in most parts of the vegetative plant in varying concentrations but predominantly in the vascular tissue of petioles, stem, hypocotyl, and root. We also discovered a significant accumulation of virus in a relatively undifferentiated zone of the hypocotyl immediately below the stem apex which could act as a reservoir of virus supplying new leaves emerging from the apex.

Disease development following a compatible interaction between a plant virus and its host is the consequence of systemic entry and multiplication of virus in different organs and tissues of the plant. The mechanisms of virus invasion of plant organs and tissues are complex and involve host and viral genetic determinants controlling cell-to-cell and long-distance movement modulated by host developmental and environmental factors (Hull 1991). Virus accumulation in certain tissues can be important in acquisition and transmission by invertebrate vectors or for other mechanisms of inter-plant movement such as seed transmission. However, the relationship between post-invasion virus accumulation in specific organs and tissues, and disease development and symptom character, is still poorly understood for most host-virus combinations. Well-known examples where a particular symptom character is related to heterogeneous virus distribution are mosaics which develop from infections by tobacco mosaic virus (TMV) (Atkinson and Matthews 1970) and

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other RNA viruses (Loebenstein et al. 1977; Suzuki et al. 1989). The dark green islands of these mosaics contain little or no virus, in contrast with the surrounding chlorotic tissue in which virus is concentrated. Development of dark green tissue with enhanced chlorophyll is a common feature of plant infections by viruses and other pathogens and is of interest since it may be related to cell apoptotic mechanisms (Johal et al. 1995). Some isolates of CaMV induce development of dark green leaf tissue (Stratford and Covey 1988), but little is known about the relationship between patterns of leaf coloration and CaMV accumulation. We have also found that CaMV in roots and shoot tissue progresses to a postreplicative phase more rapidly than in leaves (Covey et al. 1990). Both of these features suggest important CaMV functions are related to particular organ- and tissue-specific interactions between host and virus. For these reasons, we wish to understand more about the location of CaMV in infected plants.
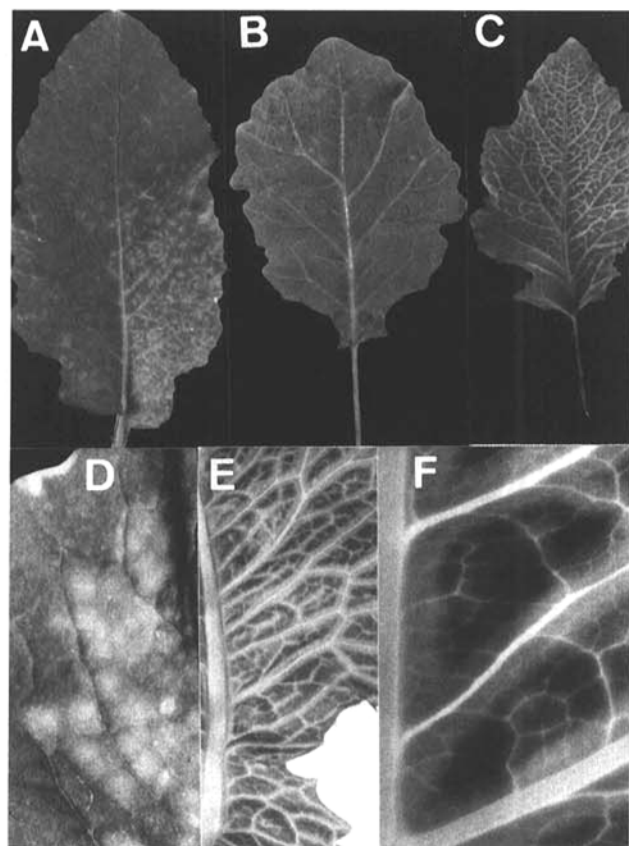
The method of plant skeleton in situ nucleic acid hybridization, in which organs or whole plants are fixed rapidly and treated with SDS-proteinase K before direct probing, was originally developed for studying CaMV infection at the site of inoculation (Melcher et al. 1981, 1992) and modified to study long-distance movement of CaMV in infected plants (Leisner and Howell 1992; Leisner et al. 1992, 1993). This method has the advantage of being able to locate virus in whole organs in contrast to the more time-consuming conventional microscopic in situ hybridization which is especially suitable for focusing on smaller groups of cells. In this paper, we have modified the skeleton hybridization method to obtain a higher resolution picture of CaMV distribution in infected organs at the whole plant level to gain further insight into CaMV-host interactions. We show that local lesions induced by CaMV have a more complex structure than is suggested from the appearance of symptoms and find an unusual distribution of virus in tissues of leaves and roots.

## RESULTS

### Accumulation of CaMV DNA at the site of inoculation.

One of our objectives was to understand the relationship between CaMV accumulation and symptom character and to detect possible heterogeneity in virus distribution as suggested from symptoms of CaMV-infected leaves (Fig. 1) such as in turnip, with a severe response (Fig. 1A, D–F), and in kohlrabi, with a less severe response (Fig. 1B, C) to infection. CaMV was de-

tected by direct hybridization first in inoculated turnip leaves at 16 days postinoculation (dpi). We usually inoculate the tip of the second leaf of seedlings at the 2-leaf stage and most of the hybridization was associated with this part of the leaf with virtually no background signal to other parts of the inoculated leaf or to a noninfected control leaf (Fig. 2A). The high resolution afforded by the ^{33}P-labeled probe revealed many distinct infection foci of different sizes (Fig. 2A). Considerably more detail than has been previously observed with CaMV was found on enlargement of the autoradiogram (Fig. 2B), whereas a much more diffuse signal was obtained using a more usual ^{32}P-labeled probe (Fig. 2C, D). Local lesions were resolved by hybridization as more complex structures than was suggested from the appearance of the chlorotic symptoms (compare Fig. 1D with 2B). Some lesions had a peripheral ring of intense signal and an outer halo with the center of the lesion apparently containing little or no virus (Fig. 2B, arrowed). There were also many very small foci of infection, and the signal was associated with the midrib and some major veins, sometimes discontinuously. At 7 dpi, when local lesion symptoms are usually first observed, the hy-

bridization signal was more uniform across the lesion in turnip with many small foci also present (Fig. 2E). Although the lesions in kohlrabi were much less distinct than in turnip (see Fig. 1), the hybridization signal was very similar in both hosts (compare Fig. 2E with F).

## Distribution of CaMV in systemically infected leaves.

The first stages of systemic symptom development in CaMV infections are characterized by vein clearing in part of a leaf (Maule et al. 1989; Leisner et al. 1992) in a proximal (leaf base) quarter or half of the third or fourth leaf following inoculation of the second leaf (see Fig. 1A). Direct hybridization to such leaves produced signal which coincided with the zone of chlorotic vein clearing when both [32]P- and ^{33}P-labeled probes were used (Fig. 3). However, the higher energy isotope produced a less detailed picture (Fig. 3A) than the lower energy probe (Fig. 3B) when the leaves were each exposed to give maximum resolution. On longer exposure of samples such as that shown in Figure 3A, the ^{32}P-labeled probe was found to be much more prone to overexposure artefacts giving an apparently uniform hybridization signal over the leaf (not shown). The ^{33}P-labeled probe, in contrast, afforded significantly enhanced resolution providing much detail upon enlargement (Fig. 3C). This showed that the hybridization signal was associated with vein borders, as can be seen at the lefthand boundary of the signal (Fig. 3C) which coincides with the edge of the symptomatic zone of the partially infected leaf (see Fig. 1A). Hybridization also extended into the interveinal tissues of the symptomatic leaf sector but with some interveinal islands lacking signal (Fig. 3C). There appeared to be little signal associated specifically with the midrib or other veinal tissue (Fig. 3B,C).

Turnip leaves showing full systemic symptoms of CaMV infection emerge from the plant apex three to four leaves after the inoculated leaf, and these are usually more stunted and distorted than those showing partial symptoms. Depending on the infecting CaMV isolate, such leaves develop chlorotic vein banding to a lesser or greater extent with the interveinal tissue retaining or accumulating chlorophyll (see Fig. 1E, F). Direct hybridization to such leaves produced signal covering the whole area of the leaf, but with more intense regions in the interveinal tissues and little signal associated with the midrib and veins (Fig. 4A). Kohlrabi leaves show only mild symptom expression with most CaMV isolates, but distinct vein banding is observed on infection with the severe CaMV isolate Aust (see Fig. 1C). When subjected to direct hybridization, kohlrabi leaves showed a relatively strong signal in the interveinal regions with distinct bands lacking hybridization associated with, but significantly wider than, the lesser veins. However, hybridization was sometimes observed to vascular strands on the upper surface of the petiole and proximal part of the midrib (Fig. 4B). Occasionally, we observed a variable number of small intense spots of hybridization to the leaf lamina and margin of systemically infected leaves (see Fig. 4A). We attributed this to damage during handling of infected tissue since we observed no such signal to uninfected leaves, which always produced a very low background with our method (see Figs. 2A, 3B).

In general, we found a reduced signal associated with some systemically infected leaves, and, in particular, those showing distortion or stunting due to infection. We suspected that the reduction in signal was due to thickening of the outer layers of the leaf impairing access of the probe to tissues below. This was



**Fig. 1.** Symptoms of cauliflower mosaic virus (CaMV) infection in leaves of turnip (**A, D-F**) and kohlrabi (**B, C**). Kohlrabi infected with a severe CaMV isolate (Aust) develops indistinct local lesions (B), whereas turnip inoculated with a less severe isolate (Cabb B-JI) produces large chlorotic local lesions at 15 dpi (shown enlarged in D). The first leaf to show systemic symptoms in turnip develops vein clearing in a proximal sector (A). Younger leaves show full systemic symptoms with chlorotic vein banding and dark (green) interveinal areas as in infections with CaMV isolate CM4-184 (E,F). Vein banding is also produced in kohlrabi leaves by the severe isolate Aust but without obvious changes in chlorophyll content in the interveinal regions (C).

supported by the observation of more intense spots of signal associated with apparently damaged portions of the leaves (see Fig. 4). To test this, we removed the epidermis covering veins and interveinal tissue from part of a turnip leaf showing vein banding with dark green interveinal areas, and subjected it to direct hybridization (Fig. 5). This produced an enhanced signal on that part of the leaf, including veins and interveinal regions, from which the epidermis had been removed compared with the surrounding tissue (Fig. 5A). On enlargement, a more intense signal was found associated with the green interveinal tissue than with the veins or vein borders (Fig. 5B) reinforcing the earlier conclusion deduced from the intact leaf (Fig. 4). However, when the epidermis was removed from the fine veins of the symptomatic part a leaf showing partial systemic symptoms (as in Fig. 1C), signal was clearly associated with the veinlets (Fig. 5C) not observed with whole tissue (Fig. 3C). Veinlets in

full systemically infected leaves, however, did not show such a strong hybridization signal following epidermis removal (Fig. 5B) as the partially infected leaf. Heterogeneous concentrations of virus were also found in CaMV-infected rape leaves in the interveinal regions after removal of the epidermis which coincided with darker green tissue (Fig. 5D). The removed epidermis itself also contained CaMV (Fig. 5E). Neither the removed epidermis nor the tissue revealed beneath showed any background signal when performed on noninfected tissue (data not shown).

### Detection of CaMV in turnip sections.

From the preceding experiments, we conclude that the efficiency of detection of viral nucleic acid in organs of infected plants depends in part on the ability of the probe to gain access to the contents of cells deeper in the tissue. Detection of virus in
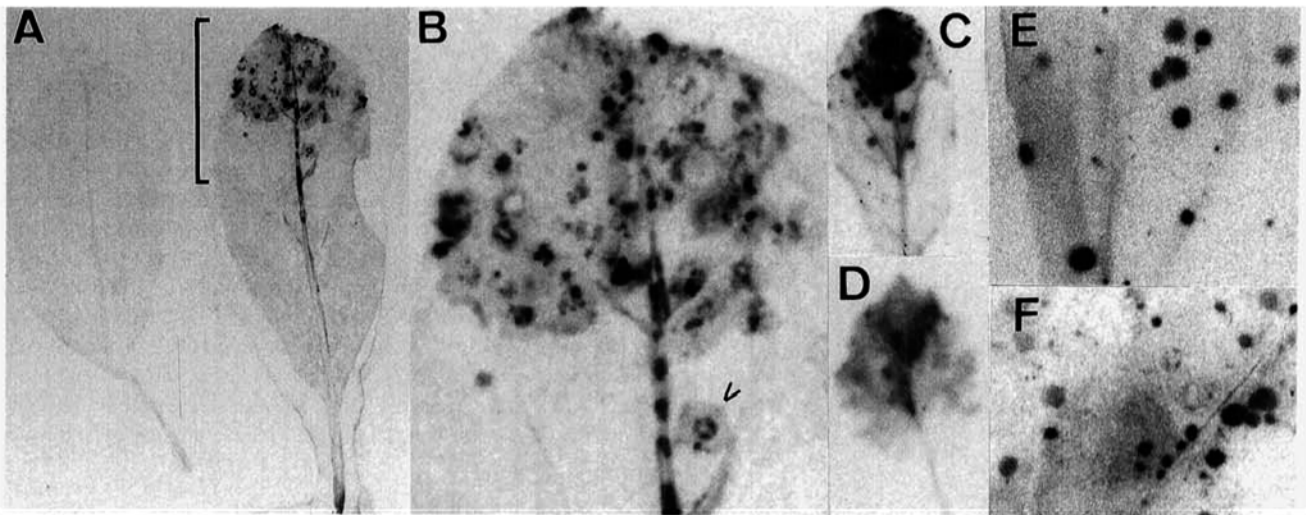


**Fig. 2.** In situ hybridization probing for cauliflower mosaic virus (CaMV) (isolate Cabb B-JI) in inoculated leaves of turnip (**A-C,E**) and kohlrabi (**D, F**). Signal can be seen in the tip of the infected leaf at 15 dpi (A, right) but not in a noninfected leaf (A, left). The leaf tip bracketed in A is shown enlarged in B. Turnip (C,E) and kohlrabi leaves (D,F) at 7 dpi hybridized with probes labelled with $^{32}$P (C,D) and $^{33}$P (E,F).
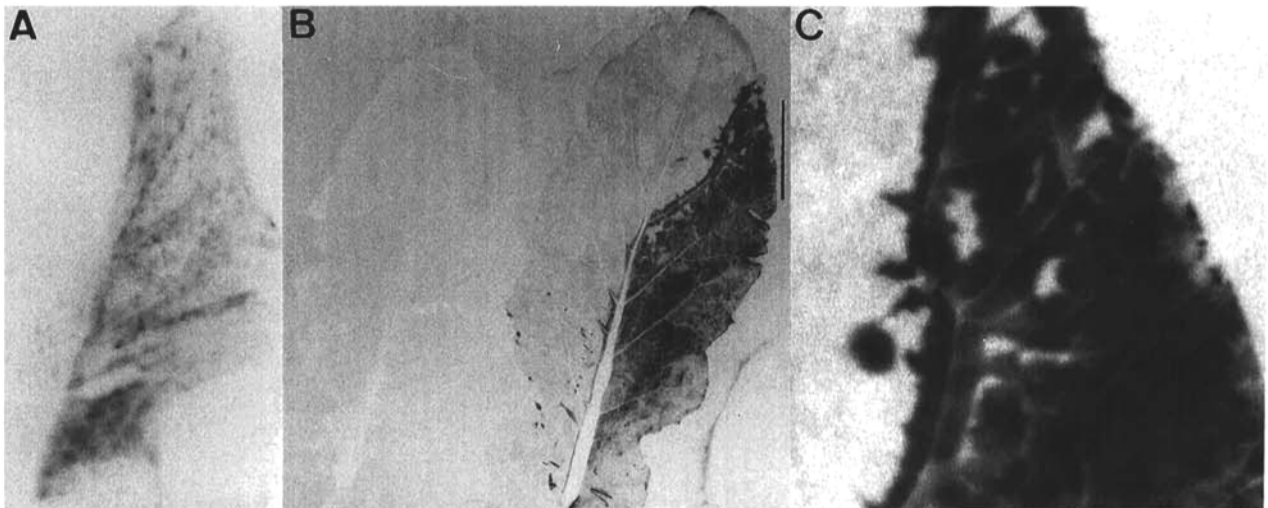


**Fig. 3.** In situ hybridization to turnip leaves showing partial systemic symptoms of infection by cauliflower mosaic virus (CaMV) isolate Cabb B-JI. Hybridization with **A,** $^{32}$P-labeled probe; **B,** $^{33}$P-labeled probe to noninfected (left) and infected (right) leaves. **C,** Enlargement of B (right) shown by the bar.

thicker organs such as major veins, petioles, stems, major roots, or storage organs like the swollen hypocotyl of turnip, is likely to be much less efficient with intact organs. Therefore, we cut the thicker organs into sections of greater than 1 mm thickness and hybridized directly to the cut surfaces with a $^{33}$P-labeled probe (Fig. 6). Although the uninfected control section showed a diffuse and variable signal of nonspecific probe binding (Fig. 6A), the sections of infected turnip organs showed a much more specific and high-resolution hybridization pattern (Fig. 6B–D). Infection with CaMV isolate Cabb B-JI distorts growth of the swollen hypocotyl by reducing its diameter but with little effect on its length. Hybridization to longitudinal sections of turnip plants with the rosette of leaves cut off at the petiole bases, at about 35 dpi, showed significant accumulation of virus in a region just below the crown of the plant where root and stem join, and in major vascular strands of the hypocotyl (Fig. 6B left). A similar pattern of distribution in the younger infected root, before enlargement of the hypocotyl had taken place, was apparent but with less detail discernible (Fig. 6B right). The apical meristem is located just above that portion of the hypocotyl where virus accumulates, but this region had an especially low level of virus (Fig. 6B left). Hybridization directly to transverse sections of hypocotyl, infected with a less severe CaMV isolate (XJ) not causing root distortion, showed fairly uniform distribution of CaMV across the widest part of the swollen hypocotyl with a ring of more intense hybridization associated with cambial vascular tissue (Fig. 6D). With sections taken closer to the top of the hypocotyl, intense spots of hybridization were associated with vascular tissue and it was also concentrated in other tissues (Fig. 6C). A section through the base of the turnip rosette including stem and petiolar tissue again showed accumulation of virus particularly in the vascular tissue, but it was also present in most other tissues (Fig. 6C).

## DISCUSSION

Using an improved resolution macroscopic in situ hybridization method, we have gained new insight into CaMV distribution during infection of host brassicas. In a previous study of CaMV infection of turnip using skeleton hybridization, Leisner et al. (1992) observed many foci of infection on the inoculated leaf, but the relatively low resolution afforded with a $^{32}$P-labeled
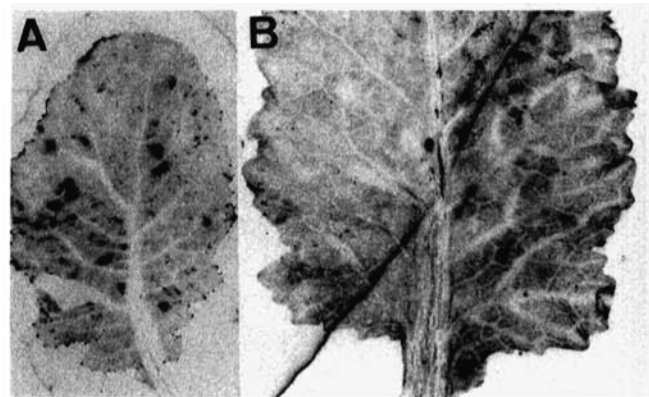
DNA probe precluded a detailed analysis of local lesion morphology. At the site of inoculation, we detected by hybridization more foci of infection than was suggested from the number of chlorotic lesions with many of the additional foci being of relatively small size (Fig. 2). Furthermore, although local lesions in kohlrabi, with a mild response to CaMV, were symptomatically relatively indistinct, the size and number of infection foci were similar to those found in turnip which has a more severe response producing large chlorotic lesions. This suggests that the difference in symptomatic response in the two hosts is not related to virus titer at the inoculation site. Young lesions (6 dpi) in turnip showed a relatively uniform distribution of virus, whereas, in older lesions asymmetric distribution of CaMV was observed, as reported previously (Melcher et al 1981), but with much more detail. Indeed, the centers of some lesions had virtually no virus, with a ring of virus accumulation and a more diffuse halo beyond the ring. Thus, the structure of the lesion revealed by hybridization was different from its symptom morphology and more complex. We were also able to observe small secondary foci in both the lesion ring and outer halo (Fig. 2B, arrowed). The absence of virus in the lesion center suggests that it is degraded as the infection front radiates outward. The presence of a diffuse halo beyond the ring indicates that the initial virus front actually moves outward faster than the lesion ring. The complex lesion structure observed later in infection compared with the more uniform earlier structure suggests that a host response might be involved in limiting virus to certain cells around the lesion. Some of the small foci of infection were closely associated with larger lesions and might have arisen as secondary infections—possibly after emergence of a new virus genotype. Variant genotypes arise readily because of the mode of CaMV replication involving reverse transcription; we have previously shown that an infected plant contains a mixture of
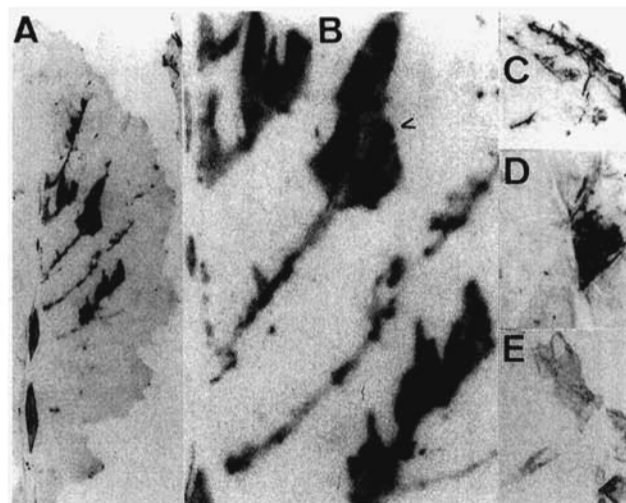


Fig. 5. Effect of removal of the epidermis on the hybridization signal in leaves. A, Turnip leaf with full systemic symptoms of infection including stunting. The regions with epidermis removed are those with the enhanced signal shown enlarged in B. The heterogeneous distribution of hybridization is more intense in the interveinal dark green islands and less intense over the veins (arrowed). C, Removal of epidermis from the veinlets of the symptomatic portion of a leaf with partial systemic symptoms (as in Fig. 3) shows the presence of virus in the vascular tissues. D, Epidermis removed from interveinal region of a CaMV-infected rape leaf showing heterogeneous virus distribution. E, Hybridization signal associated with the removed epidermis.



Fig. 4. In situ hybridization to A, turnip and, B, kohlrabi leaves using a $^{33}$P-labeled probe. Note the hybridization to the interveinal regions in the turnip leaf and the absence of signal to the vein borders at the bottom right of the kohlrabi leaf coincident with vein banding symptoms (not shown).

variants with differing pathogenic properties (Al-Kaff and Covey 1994). Since most of the minor infection foci are presumably secondary to the larger lesions, they are not likely to become established systemically in most cases.

The appearance of the local lesions reported here show some similarities to those observed by Chapman et al. (1992) in infection of *Nicotiana clevelandii* with potato virus X (PVX) carrying a GUS reporter gene, suggesting a similar course of infection in two very different host-virus combinations. However, Tésci et al. (1994) observed homogeneous distribution of cucumber mosaic virus in local lesions even though the lesions had an outer ring of starch with a hollow center. We also observed that veins in inoculated leaves infected with CaMV showed discontinuous lines of hybridization suggesting nonuniform distribution of virus in the vascular tissue. This might reflect inefficient detection of virus although similar discontinuity of virus was apparent in veins of leaves infected with PVX expressing GUS (Chapman et al. 1992) and in tobacco leaves infected with cherry leaf roll virus determined by tissue print hybridization (Más and Pallás 1995), suggesting this reflected the true distribution of virus.

In turnip leaves showing partial symptoms, we found that virus was most closely associated with the vein border tissue, in the symptomatic zone, with some interveinal tissue apparently lacking virus. Virus was also found in the veinlets of these leaves, revealed following removal of the epidermis (Fig. 5C). In their study of long-distance movement of CaMV in turnip plants, Leisner et al. (1992) also found heterogeneous accumulation of CaMV in leaves showing partial symptoms with spots of more intense hybridization next to the minor veins which they interpreted as virus exiting from the veins. We also observed these spots using a $^{32}$P-labeled DNA probe (Fig. 3A) and the lower energy $^{33}$P-labeled probe (Fig. 3C), but, the latter probe also showed more hybridization to the vein border tissue in general than the $^{32}$P-labeled probe which could have exaggerated these foci of hybridization due to the higher energy radiation producing localized overexposure. Even so, both studies suggest that virus is exiting from the veins in the leaves showing partial symptoms. Such leaves were likely to have been relatively well

formed when virus first entered them through the vascular tissue. Virus entry into the adjacent vein border tissue would have preceded invasion of the interveinal tissue producing the hybridization pattern observed in Figure 3. Thus, most of the virus replication in these leaves occurred after the major phase of leaf expansion was completed.

Leaves emerging from the apex after a systemic infection had been established, and subsequently developing full systemic symptoms, probably received virus to most cells before the major phase of leaf expansion had begun. This could explain why virus distribution in them was different from that in the partially expanded leaves. Although the veins and vein border tissue contained CaMV as revealed following epidermal removal (see Fig. 5B), most virus was concentrated in the interveinal tissue. The interveinal tissue developed as dark green islands in turnip, and to a lesser extent in rape, but remained mostly asymptomatic in kohlrabi. The finding of accumulations of CaMV in dark green islands contrasts with infections as with TMV where such tissue apparently lacks virus (Atkinson and Matthews 1970). We have also found that chlorotic vein borders often had much reduced levels of CaMV as in kohlrabi (Fig. 4) and turnip (Fig. 5). One possible explanation for this is that the younger leaves, which already contained virus, underwent a transition from photosynthetic sink to source and tissues adjacent to the veins lost metabolites at a greater rate than interveinal cells and, thus, had a lower capacity to support virus replication. In contrast, the interveinal cells more distant from the veins could be less affected by metabolite loss, as shown by their accumulation of chlorophyll and virus. This situation pertains when turnip plants are inoculated as seedlings allowing virus to spread to the apex and leaf primordia at an early stage. When plants are inoculated at a later stage, access to more mature leaves can be inhibited as the leaves undergo sink to source transition (Leisner et al. 1992). An alternative explanation is that the dark green islands of tissue continue to support virus replication through an active mechanism preventing cell apoptosis (Johal et al. 1995). The vein-clearing symptoms (narrow chlorotic vein borders) of a partially infected leaf (Fig. 1A) in which virus accumulates in the vein borders are subtly different from the wide chlorotic vein
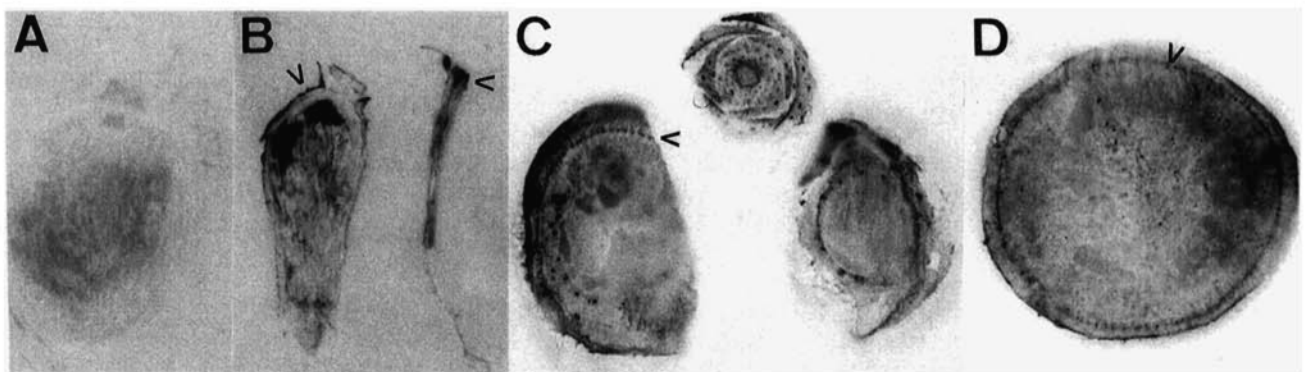


**Fig. 6.** Direct hybridization to the cut surfaces of thicker organs from cauliflower mosaic virus (CaMV)-infected turnip plants. Probing of longitudinal sections through turnip crown and hypocotyl of, **A,** uninfected and, **B,** infected plants. In B, hybridization is concentrated in a zone below the crown (arrowed) and in vascular strands below this in plants 35 dpi (B, left) and 15 dpi (B, right). In sections of turnip plants infected with CaMV isolate XJ, which does not produce severe distortion, hybridization to transverse sections through the crown of the plant (C, center) shows signal associated with vascular tissues in the petiole bases. In transverse sections just below the crown (C, right) and in the upper part of the hypocotyl (C, left) hybridization is again concentrated in the vascular tissue (arrowed in C, left) which is not symmetrically arranged, but a more regular arrangement is seen in a transverse section of the bulk of the swollen hypocotyl, **D,** with signal associated with cambial vascular tissue (arrowed) and more heterogeneously across the whole section.

borders (Fig. 1E) of the younger leaves with reduced virus levels relative to the adjacent interveinal tissue. We conclude that these differences in symptom character and virus distribution are related to the stage of leaf development at which virus first entered the leaves.

Hybridization signals in leaves were enhanced following removal of the epidermis. However, the accumulation of virus in the interveinal green islands was apparent in whole and stripped leaves (compare Figs. 4 with 5). The pattern of virus distribution in inoculated and partially infected leaves was also similar after removing the epidermis (data not shown). Hybridization to whole leaves also suggested that veinal tissue contained relatively low levels of virus. Our interpretation of this is that tissues covering the vascular strands and accessible by the probe contain relatively little virus, except where the vascular strands were close to the surface as in the midrib of kohlrabi leaves (Fig. 4B). Where the vascular strands were not close to the surface, it was necessary to gain access to them to assay virus. Thus, virus detection was enhanced by removal of the epidermis from both the smaller (Fig. 5C) and medium-sized veins (Fig. 5A). Where virus was in more deep-seated vascular tissue, as in the petioles, its presence was revealed in transverse sections (Fig. 6C). Leisner et al. (1992), using skeleton hybridization, obtained a strong signal to infected veinal tissue but the presence of high background to veins in noninfected tissue makes it difficult to draw conclusions about virus concentration in the veins of their samples. We have also confirmed the presence of virus along the length of the midrib and medium-sized veins by extracting DNA from them, and by tissue-print hybridization of transverse sections of turnip leaves (Al-Kaff and Covey, unpublished data). We conclude that the thicker veins do contain more virus than is suggested in Figure 4A but this is in the more deeply seated vascular strands than in the upper layers of cells which are relatively free of virus.

Sections of the subterranean parts of the turnip plant showed the presence of CaMV in the vascular tissue, but there was also significant accumulation in a zone of the swollen hypocotyl just below the stem apex which, in contrast, had little or no signal associated with it (Fig. 6). The subapical zone contains relatively undifferentiated parenchyma tissue and it is not clear why virus should become concentrated in it. Little is known about virus replication in roots and its role in establishment of a systemic infection in the plant as a whole although we have previously shown that CaMV replication in turnip roots apparently ceases earlier than in leaves (Covey et al. 1990). Due to the proximity of this zone to the stem apex and its meristem, it is possible that the subapical accumulation of virus acts as a reservoir supplying virus through the vascular tissue to the newly emerging leaves. Intriguingly, we have found that a CaMV site-directed mutant with part of the enhancer to the 35S promoter removed shows an even higher accumulation in the subapical zone than wild-type virus (Turner and Covey, unpublished data). This indicates the existence of subtle organ-specific virus-host interactions whose role in CaMV pathogenesis is yet to be elucidated.

## MATERIALS AND METHODS

### Inoculation with CaMV and propagation of infected plants.

CaMV isolates used in this study, Cabb B-JI, Aust, CM4-184, and XJ (Al-Kaff and Covey 1995), were propagated in one or more of the following host species: *Brassica rapa rapifera* (turnip) cv. Just Right, *B. oleracea gongylodes* (kohlrabi) cv. Purple Vienna, *B. napus* (rape) cv. Topaz. Seedlings (10 to 15 days old) were inoculated at the 2-leaf stage (leaves 1 to 2 cm long) by gentle rubbing of the second leaf using a flattened glass rod with 1 to 2 μg of purified virion DNA in 10-μl buffer (5 mM Tris-HCl, pH 7.8, 5 mM MgCl$_2$) containing a trace of Celite abrasive. Plants were grown in a glasshouse at 20°C ± 2 in a 16-h photoperiod.

### In situ hybridization.

The method of direct in situ hybridization used to determine CaMV distribution in infected leaves was based on the plant skeleton hybridization method developed by Melcher et al. (1981) and modified by Leisner et al. (1992). In preliminary experiments using this method, we found that leaves treated with SDS-proteinase K were extremely fragile and difficult to handle. We found that omission of this treatment did not alter the hybridization patterns in our hands (data not shown) but made handling of the leaves much easier. We further enhanced the technique to obtain higher resolution by employing a DNA hybridization probe labeled with ^{33}P, an isotope which emits lower energy β-particles than [32]P. Use of hybridization bottles rather than plastic bags enabled more thorough washing producing very low backgrounds to noninfected tissue. Direct hybridization was performed as follows. Leaves were fixed in absolute ethanol for 2 to 4 h at 37°C or overnight at room temperature. Tissue and DNA was then denatured by soaking the fixed leaves in 0.5 M NaOH for 13 to 15 min at room temperature followed by two washes in water. Leaves were then neutralized with 1 M Tris-HCl for 7 to 10 min and washed briefly in 2× SSC (1× SSC is 0.3 M sodium chloride, 0.03 M trisodium citrate) and cross-linked to fix the DNA using a UV Stratalinker 2400 (Stratagene). During these manipulations, the leaves were handled carefully and arranged using a soft brush. The leaves were prehybridized in 3× SSC containing 5× Denhardt's blocking medium (1× = 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) for 1 to 2 h at 65°C in a rotary hybridization oven (Hybaid). Radioactive DNA probes were prepared by labeling cloned CaMV DNA with [32]P- or ^{33}P-dCTP (NEN). Hybridization was carried out for 16 h following which, the leaves were washed for 15 min at 65°C: in 2× SSC (3 to 4 washes), and once each in 1× SSC then 0.5× SSC. The labeled leaves were placed carefully onto blotting paper, covered with plastic film and air-dried or placed on a sequencing gel dryer at 60°C for 15 min before exposure to X-ray film at −70°C. Pieces of stem, root, and hypocotyl were cut into thick sections and then treated in exactly the same way as the leaves. Several control samples of uninfected tissue were included in each hybridization.

## ACKNOWLEDGMENTS

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