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Host regulation of the cauliflower mosaic virus multiplication cycle

(minichromosome/gene regulation/reverse transcription/*Brassica* susceptibility)

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ABSTRACT **The DNA genome of cauliflower mosaic virus (CaMV) replicates in the cytoplasm of infected plant cells by reverse transcription of an RNA template. Viral RNA is generated in the nucleus by transcription of an episomal minichromosome containing supercoiled DNA. We have assessed the relative activities of the nuclear and cytoplasmic phases of the CaMV multiplication cycle by monitoring unencapsidated viral DNA forms and polyadenylylated RNAs in different organs of one host plant and in different host species. Systemically infected leaves of a highly susceptible host, turnip (*Brassica rapa*), contained abundant 35S RNA and 19S RNA transcripts and unencapsidated reverse transcription products but relatively little supercoiled DNA. In contrast, supercoiled DNA accumulated in roots and other tissues of turnip plants but without significant amounts of steady-state viral RNA. Infected but asymptomatic leaves of a less susceptible CaMV host, kohlrabi (*Brassica oleracea*), contained supercoiled DNA almost exclusively but negligible viral RNA and DNA products of reverse transcription. An allotetraploid species, rape (*Brassica napus*), exhibited infection characteristics and minichromosome expression levels intermediate between the other two species from which it was derived. We conclude that expression of the CaMV minichromosome is a key phase of the virus multiplication cycle, which is regulated differentially in organs of a highly susceptible host species. Furthermore, this regulation exhibits genetic variation among different *Brassica* species and controls host susceptibility to CaMV infection.**

Cauliflower mosaic virus (CaMV) has a double-stranded DNA genome generated by reverse transcription of an RNA intermediate (for reviews, see refs. 1–3). Virion DNA contains site-specific discontinuities resulting from the replicative process. At a relatively early stage in the CaMV multiplication cycle, the discontinuities are repaired, and a supercoiled (SC) molecular form is synthesized, probably in the nucleus where it is found. Association of the SC DNA with histone-like proteins to produce a minichromosome precedes transcription directed by host RNA polymerase II (4). Two major RNAs are transcribed from the minichromosome (5–9). 35S RNA is the template for reverse transcription and a possible polycistronic mRNA. 19S RNA is the mRNA for the CaMV gene VI protein. Synthesis of these RNAs is controlled by viral transcription promoters that have characteristics typical of other eukaryotic promoters recognized by RNA polymerase II. Isolated CaMV promoter fragments have been used to drive the expression of many different foreign genes in a variety of nonhost transgenic plants in a manner often referred to as constitutive (for review, see ref. 10).

Transcription of the CaMV genome in the nucleus precedes replication in the cytoplasm. Reverse transcription generates a complex population of DNA forms that accumulate as unencapsidated molecules (11–21). Many of these molecular forms have been characterized; they can be purified from infected tissue by phenol extraction in the absence of virion DNA because CaMV virions are very stable in the presence of phenol (13). A variety of CaMV DNA forms accumulate in infected turnip leaves, including single-stranded DNAs, a variety of hairpin molecules, and linear double-stranded DNA forms with single-strand extensions of the minus strand (19, 21), structures consistent with their being generated by reverse transcription. The SC DNA of the CaMV minichromosome is present as a relatively minor component of the unencapsidated DNA fraction from leaves actively engaged in virus multiplication. However, the SC DNA form accumulates in callus derived from infected leaf tissue grown *in vitro* in which there is relatively little virus replication, implying that not all host plant tissues can support the complete CaMV multiplication cycle (22). We report here an investigation of the composition of viral RNA and DNA forms isolated from different plant organs of a highly susceptible CaMV host, *Brassica rapa* (turnip) and from less susceptible *Brassica* species. We show that the patterns of CaMV transcription and reverse transcription are not the same in all organs of one species or in different host species. Our results suggest that host regulation of the CaMV multiplication cycle is imposed upon the viral minichromosome, possibly through specific transcription factors that exhibit species variation, influencing host susceptibility to infection.

MATERIALS AND METHODS

Plants and Virus. The following plant species representing three major groups of *Brassica* (23) were used to propagate CaMV: *Brassica rapa*, L. (genome descriptor: aa) subspecies *rapifera* (turnip) cv. Just Right; *Brassica oleracea*, L. (genome descriptor: cc) subspecies *gongylodes* (kohlrabi) cv. Purple Vienna; *Brassica napus* (genome descriptor: aacc) subspecies *oleifera* (spring rape) cv. Brutor. Plants were mechanically inoculated with CaMV isolate Cabb B-JI at the 3- to 4-leaf stage and grown under greenhouse conditions at 18–22°C in a 16-hr photoperiod. Plant parts were harvested 25 days postinoculation. Leaves of 3–10 cm were harvested from plants of each species and taken at equivalent stages of development. Leaves from infected turnip plants 25 days postinoculation were processed for callus culture, exactly as described by Rollo and Covey (22). Stem samples were taken from plants grown in rosette form and included the stem-proximal parts of the leaf petioles. Root samples were taken from the main tap root.

Nucleic Acid Analysis. Total CaMV DNA was determined by dot-blot analysis, as described by Rollo and Covey (22). Cellular nucleic acid was isolated by phenol/chloroform extraction from various plant tissues as described by Hull and Covey (13). This procedure effects isolation of total host nucleic and unencapsidated viral DNA and RNA. CaMV

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Abbreviations: CaMV, cauliflower mosaic virus; SC, supercoiled DNA; hp, hairpin.

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virions are stable in phenol, and so virion DNA does not occur in this fraction. Polyadenylylated RNA was separated from the DNA and other cellular RNAs as described by Plant *et al.* (24). Viral unencapsidated DNA forms were analyzed by blot hybridization after two-dimensional agarose gel electrophoresis (19, 21). Cellular nucleic acid samples of 10 or 20 μg were loaded into a corner circular well of 1.1% agarose slab gels. Electrophoresis at 1.25 V/cm for 20 hr was in 25 mM Tris·phosphoric acid buffer, pH 7.6, in the first dimension and in the second dimension, at 90° orientation to the first, in alkaline medium (30 mM NaOH/2 mM EDTA). Gels were depurinated, alkali treated, and neutralized before Southern blotting. Gels for comparison contained equivalent amounts of cellular total DNA, and exposure of autoradiograms was regulated by comparing the intensity of standard quantities of size-marker DNA, unless otherwise stated in the text. Gel electrophoresis of equal amounts (2 μg) of cellular total polyadenylylated RNA and Northern blotting of CaMV-specific polyadenylylated RNA was done as described by Covey *et al.* (11). Southern and Northern (RNA) blots were probed for CaMV-specific sequences by hybridization with ^{32}P-labeled full-length cloned CaMV virion DNA.

RESULTS

The Composition of CaMV Replication Cycle Intermediates Differs in Organs of Infected Turnip Plants. The structural composition of unencapsidated CaMV DNA forms isolated from different organs of infected turnip plants was analyzed by two-dimensional agarose gel electrophoresis and Southern blotting. This technique resolved DNAs from leaves (Fig. 1*A*) that we have previously identified (19, 21) as open circular and SC forms and DNA products of reverse transcription, including genome-length and subgenomic linear forms, complete hairpin DNAs of various sizes (hp1), and molecules partially single- and partially double-stranded comigrating with nested sets of hairpin forms produced by interrupted reverse transcription (hp2 and hp3). Leaves showing systemic vein-clearing symptoms at 25 days postinoculation contained relatively little SC DNA, a component of the CaMV minichromosome but did contain abundant DNA forms generated by reverse transcription (Fig. 1*A*).

In nucleic acid preparations isolated from stem tissue at 25 days postinoculation, an increased level of SC DNA relative to that in leaves was seen, and all viral reverse transcription products were less abundant (Fig. 1*B*). In the unencapsidated DNA preparation taken from roots at the same time (25 days postinoculation), even greater levels of SC DNA were detected, compared with those in leaves or stems but with negligible reverse transcription products (compare Fig. 1*C* with *A* and *B*). Callus derived from turnip leaf discs taken from CaMV-infected plants at 25 days postinoculation and then cultured *in vitro* for 1 mo also contained very little of the DNA forms considered to be products of replication by reverse transcription but did contain considerable SC DNA (Fig. 1*D*). Furthermore, the SC DNA comprised both genome-length molecules and a range of subgenomic forms of various sizes. The smallest SC DNA we detected from callus tissue was ≈1 kilobase (kb) (observed after extended autoradiogram exposure; data not shown).

Differences in CaMV Transcripts Suggest Organ-Specific Expression in Infected Turnip Plants. Because the CaMV replication cycle appeared to be interrupted in some turnip organs and tissues, as indicated by the paucity of DNA forms generated by reverse transcription, we wished to determine whether this effect was at the level of transcript accumulation. Steady-state levels of CaMV transcripts in turnip organs were measured by Northern hybridization analysis of cellular total polyadenylylated RNA (Fig. 2). Leaves contained significant amounts of the two major CaMV species, 35S RNA and 19S RNA, together with a characteristic background of heterogeneous-sized molecules (Fig. 2, lane A). The RNA isolated from stems was qualitatively similar to that from leaves, although there was less of it (Fig. 2, lane B). However, negligible CaMV-specific polyadenylylated RNA was found in roots (Fig. 2, lane C) or in callus (Fig. 2, lane D), despite the abundance of CaMV SC DNA compared with leaves (see Fig. 1). On long exposure of autoradiograms, very low levels of largely heterogeneous CaMV RNA were found in RNA preparations from infected roots and callus (data not shown).

CaMV Replication Cycle Intermediates Also Differ in *Brassica* Species Less Susceptible to Infection. From a survey of a range of *Brassica* species we have determined that host response to CaMV infection falls into three broad categories (unpublished work). *B. rapa* (aa) variants exhibit very severe symptoms of leaf chlorosis and plant stunting (28) and yield a relatively high titer of total CaMV DNA (≈400 ng of total CaMV DNA per g of tissue at 20 days postinoculation). In contrast, *B. oleracea* (cc) accumulates relatively little virus (≈2 ng of total CaMV DNA per g of tissue at 20 days postinoculation) and exhibits very mild symptoms or no symptoms at all depending upon the subspecies. The allotetraploid species *B. napus* (aacc) shows an intermediate response to CaMV infection.

Gel separations of the CaMV DNA forms isolated from leaves of infected rape (*B. napus*, subspecies *oleifera*) and kohlrabi (*B. oleracea*, subspecies *gongylodes*) are shown in Fig. 3. Rape leaves contained much enhanced SC DNA but greatly reduced levels of reverse transcription products (observed only on longer exposure of the autoradiogram; data not shown) compared with turnip leaves (compare Fig. 3*A* with Fig. 1*A*). Kohlrabi leaves exhibited even greater levels of SC DNA, together with open circular and linear forms and no detectable products considered to be generated by reverse transcription (Fig. 3*B*), even after long exposure of the autoradiogram (data not shown). The open circular DNA forms resolved in the denaturing dimension of gel electrophoresis into their single-stranded linear and circular subcomponents (Fig. 3). These forms were always discrete spots in kohlrabi unencapsidated DNA preparations (Fig. 3*B*) and heterogeneous in rape (Fig. 3*A*). SC DNA was sometimes resolved into two subcomponents in the denaturing dimension (see Figs. 1*A* and 3 *A* and *B*), possibly due to the presence of ribonucleotides. The patterns of DNA forms in stems and roots of rape plants showed the same tendency towards increased SC DNA as turnip stems and roots; the DNA forms in kohlrabi plants were similar in all organs tested with SC DNA predominating (data not shown).

The steady-state levels of CaMV polyadenylylated transcripts were also compared by Northern blot hybridization (Fig. 4). Both 35S RNA (sometimes resolved as two closely migrating RNA species of ≈8 kb) and 19S RNA were seen in preparations from rape leaves, although the level of 35S RNA relative to 19S RNA (Fig. 4, lane B) was significantly lower than in turnip leaves (Fig. 4, lane A). CaMV-specific polyadenylylated RNA was not detectable in kohlrabi leaves (Fig. 4, lane C) at this level of autoradiogram exposure, even though kohlrabi leaves contained abundant SC DNA.

DISCUSSION

We have found that the relative levels of unencapsidated nucleic acid forms of the CaMV multiplication cycle vary significantly in different tissues of host turnip plants and in different host species. Tissues active in virus replication contained abundant viral transcripts and reverse transcription products but relatively little SC DNA. In contrast, tissues producing little or no virus accumulated high levels of SC DNA but were relatively inactive in generating RNA transcripts and, thereby, replication

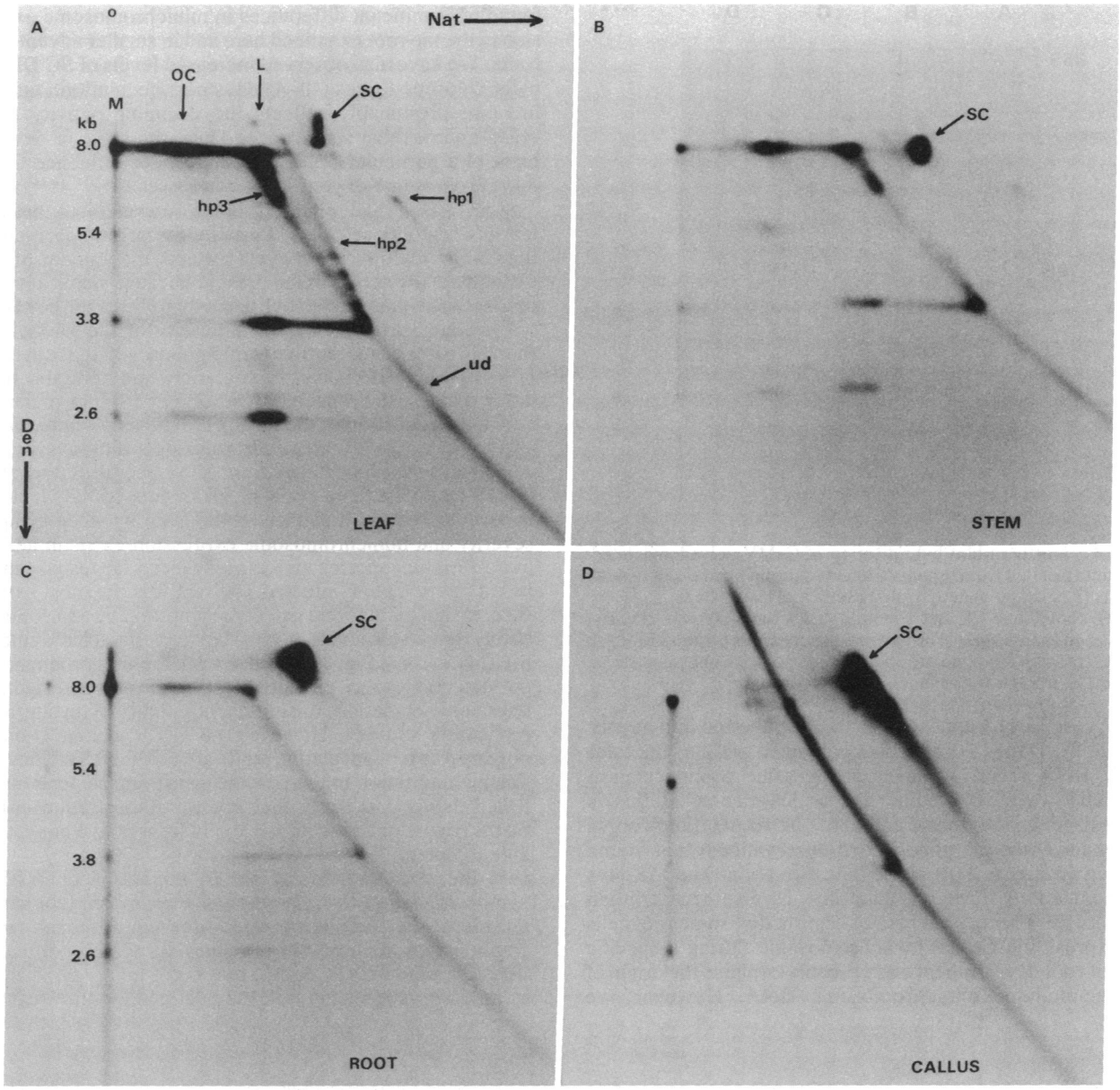

FIG. 1. Two-dimensional agarose gel electrophoresis of CaMV intracellular unencapsidated DNA forms from different organs of infected turnip plants. DNA samples were applied to a single well shown as o for leaf (*A*) and in equivalent positions for stem (*B*), root (*C*), and 1-mo-old callus cultured from CaMV-infected turnip leaves (*D*). Electrophoresis of equivalent amounts of DNA in the first dimension was in a nondenaturing buffer (Nat) and in the second in a denaturing alkaline medium (Den). Size markers (M) were included in the second dimension. After electrophoresis, DNAs were blotted to nitrocellulose and probed with radioactive cloned full-length CaMV virion DNA. Double-stranded DNA molecules with similar relative mobilities in both dimensions migrate along the unit diagonal (ud). Open circular (OC) DNAs migrate in the nondenaturing dimension more slowly than full-length linears (L), but supercoiled DNA (SC) migrates more rapidly. The SC DNA from leaves sometimes resolves as two components, whereas that from callus also has heterogeneous subgenomic form as well. Various types of hairpin DNAs generated by reverse transcription migrate in the line of spots labeled hp1 and others in the chevrons hp2 and hp3. *B* has been slightly under-exposed to enhance detail.

products. From this we conclude that transcription of the minichromosome is a key stage in the CaMV multiplication cycle that is differentially regulated by the host plant. Moreover, this regulation at the level of transcription is correlated with symptom severity in three CaMV host plant species representative of the *Brassicae*.

Unexpectedly, we observed an increase in amount of SC DNA in tissues containing relatively little viral RNA. One explanation for this is that transcription is accompanied by turnover of the SC DNA. One of the earliest phases of the CaMV multiplication cycle is presumed to be conversion of the discontinuous virion DNA into SC DNA (2). Our results suggest that, even in tissues supporting only minimal virus replication, sufficient virion DNA appears to be produced to give rise to the SC form, which accumulates because it is not being transcribed. In support of this hypothesis, callus derived from infected leaves, originally containing much virion DNA (22), accumulated SC DNA, although in a transcriptionally inactive form. The callus SC DNA differed from that in roots in that it comprised both full-length and a range of heterogeneous subgenomic SC forms. The origin of these forms is unknown, although similar SC DNAs have been isolated from turnip leaves (25) and were present in low abundance in leaves of infected rape and kohlrabi plants (Fig. 3).

We presume that virus particles enter roots of infected plants as a fortuitous consequence of systemic spread where

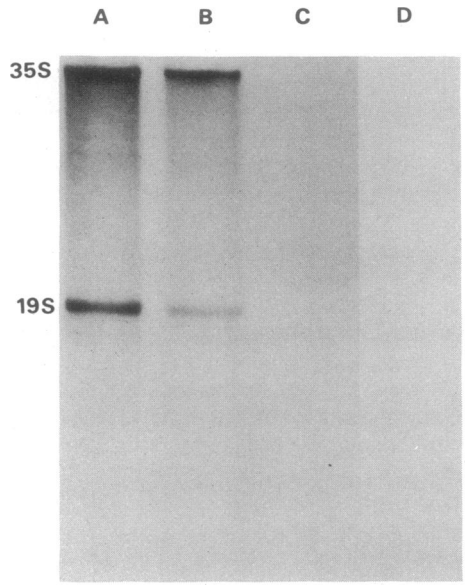

FIG. 2. Northern blot hybridization of CaMV polyadenylylated transcripts isolated from organs of infected turnip plants. Equivalent amounts (2 μg) of polyadenylylated RNA from leaves (lane A), stems (lane B), roots (lane C), and 1 mo-old callus (lane D) were electrophoresed on agarose gels. The two major transcripts are the 35S RNA reverse transcription template and possible polycistronic messenger and the 19S mRNA for gene VI.

CaMV virion DNA appears also to be converted into largely inactive SC DNA. Turnip roots contained ≈7% of the total CaMV DNA found in leaves at the same postinoculation time, and we must assume that at least some of this is synthesized in the roots. In fact, our recent time-course studies have shown (unpublished observations) that young roots go through a short phase (between about 9 and 15 days postinoculation) during which time reverse transcription products were seen. This result suggests that some root cells can express the CaMV minichromosome during a specific stage of root development and probably explains the origin of the accumulated minichromosome DNA. However, we found no significant differences in minichromosome expression in the tap root examined here and in smaller adventitious roots. We have also observed increased levels of SC DNA in older chlorotic leaves (40–45 days postinoculation), and this increase presumably reflects the declining activity of an organ approaching senescence. Thus, the stage of development of a particular organ also appears to influence CaMV minichromosome expression.

Stem tissue had characteristics intermediate between leaves and roots or callus. Possibly the organ specificity of the CaMV multiplication cycle reflects the amount of photosynthetically active tissue present because stems have less of this tissue than leaves but more than the other cell types.

We were surprised by the similarity of the DNA forms found in roots of susceptible turnip plants with those in leaves of less susceptible hosts, such as rape and kohlrabi. In the latter species, the regulation was apparently not organ specific but a reflection of the host genotype as a whole. In rape and kohlrabi, the low virus titers and symptom severity were correlated with host regulation of a specific phase of the CaMV multiplication cycle in all organs tested, including leaves. Moreover, a similar relationship between symptom severity and minichromosome expression exists in all 20 or more *Brassica* species and subspecies so far tested (unpublished data). CaMV-infected kohlrabi was the most extreme case, in that plants were usually asymptomatic and contained barely detectable levels of virus but had appreciable amounts of SC DNA (see Fig. 3). Reverse transcription products were not detected in any part of the kohlrabi plants tested at 25 days postinoculation, raising the possibility of an alternative replication strategy. However, no DNA accumulation was observed after inoculation with a CaMV DNA clone containing a mutation in the reverse transcriptase gene (unpublished data). It is likely that reverse transcription in these plants occurs at a time or at a level that we have not yet been able to detect.

In the allotetraploid species *B. napus* (aacc), originally synthesized from *B. rapa* (aa) and *B. oleracea* (cc) parents, an intermediate condition was observed. Although reverse transcription replication products were more difficult to detect in rape than in turnip, they were more abundant than in kohlrabi, suggesting that the host genetic trait regulating

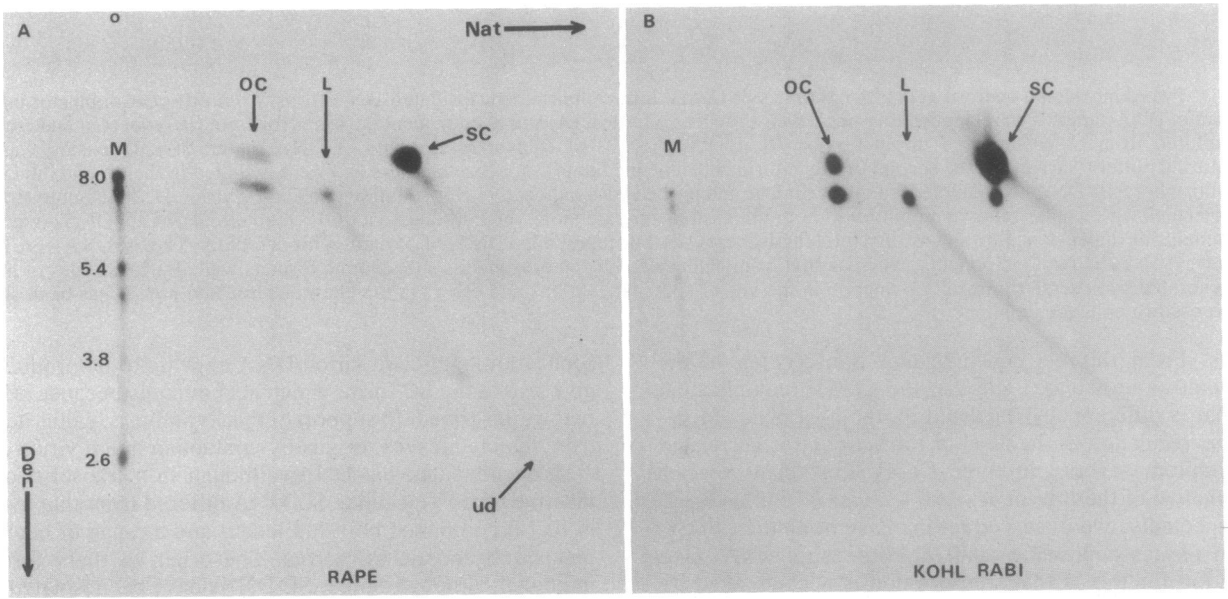

FIG. 3. Two-dimensional agarose gel electrophoresis of CaMV intracellular unencapsidated DNA forms from CaMV-infected leaves of rape (*B. napus*) (*A*) and kohlrabi (*B. oleracea*) (*B*) plants. The predominant DNA forms are open circular (OC), linear (L), and SC. The SC and OC DNAs resolve into two components in the second dimension. The autoradiogram of *B* has been slightly underexposed to enhance detail. M lane contains size markers in kb.

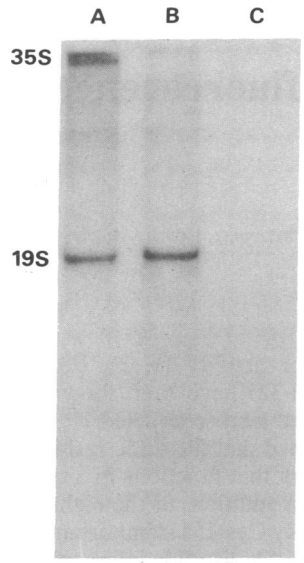

FIG. 4. Northern blot hybridization of CaMV polyadenylylated transcripts isolated from CaMV-infected leaves of turnip (lane A); rape (lane B); and kohlrabi (lane C).

the CaMV multiplication cycle was codominant in the allotetraploid.

By analogy with other regulated genes, differential expression of the CaMV minichromosome might be expected to be controlled by host trans-acting factors, although we cannot yet discount the possibility of increased turnover of viral transcripts or exclusion of the minichromosome from the nucleus. If regulation is assumed to be at the minichromosome level, then our finding of organ-specific expression in susceptible host plants is surprising because the two CaMV transcription promoters (35S RNA and 19S RNA) are generally considered to be constitutive. This feature has been exploited in gene constructs to drive high levels of expression of reporter genes in transgenic plants of a wide range of species, although there have been some reports that the 35S RNA promoter is not expressed to the same level in all tissues (10, 26). However, it is difficult to compare the activity of viral promoters as part of an episomal minichromosome in the context of infection of host plants with the situation pertaining in nonhost transgenic plants. In producing such constructs for transgenic plants, a viral DNA fragment is removed from other possible viral regulatory elements, inserted into the nuclear genome where positional effects and nonhost transcription factors are likely to influence expression in a different manner.

The roles of root- and shoot-specific elements recently identified in the enhancer of the 35S RNA promoter (27) are not immediately obvious in the context of CaMV infection of host plants. These elements must be overridden in *B. rapa* because there is little expression in roots when the root-specific element must be present in the intact viral genome. Possibly, therefore, the regulation we have observed is related specifically to host species in which the virus has evolved and adapted. The fact that these species exhibit such considerable genetic variability in their regulation of the CaMV minichromosome suggests a precise role for putative host transcription factors. Moreover, this phenomenon might represent one component defining the limit to the host range of viruses that utilize host transcription machinery in their multiplication cycle.

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