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Changes in Turnip Leaf Messenger RNA Populations During Systemic Infection by Severe and Mild Strains of Cauliflower Mosaic Virus

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Host gene expression in turnip leaves systemically infected with two strains of cauliflower mosaic virus (CaMV) was examined by 2-D polyacrylamide gel analysis of polypeptides synthesized by *in vitro* translation of cellular mRNAs. We observed a greater number of changes in the level of specific translatable mRNAs in response to infection by a severe CaMV strain, Cabb B-JI (16 changes), which induces leaf chlorosis (yellowing), than to a mild strain, Bari 1 (11 changes), which does not. Six changes were common to infections by both CaMV strains. By using northern blot analysis, we identified one of the mRNAs that decreased markedly in level during Cabb B-JI infection as that encoding the precursor to the small subunit of ribulose bisphosphate carboxylase (*rbc*S). In contrast, the level of the *rbc*S mRNA was usually only slightly reduced in Bari 1-infected leaves. We also compared the translatable mRNA population of senescing and nonsenescing leaves to assess the degree of similarity to infection-induced chlorosis. Of the seven major changes in cellular mRNAs occurring during senescence, only three were common to those seen during infection by the severe CaMV strain; one of these changes was to the *rbc*S. We conclude that plants respond at the molecular level in different ways to systemic infection by mild and severe strains of the same virus and that changes in gene expression occurring during infection resulting in leaf chlorosis are not simply due to premature induction of senescence.

Additional keywords: *Brassica campestris*, symptoms, 2-D gel electrophoresis.

Plant viruses produce a wide variety of symptoms in their hosts that develop either at the site of virus entry, usually the leaves, or as a systemic infection progresses. The visible manifestation of a virus disease at the whole plant level is reflected by changes at the molecular level. However, the molecular basis of the interaction between host plant and virus that determines symptom character is not yet understood. Most molecular studies of host responses to virus infection have centered on phenomena such as hypersensitivity (Fritig *et al.* 1987), in which plant resistance to the virus is exhibited. Moreover, the most obvious consequences of the hypersensitive response are observed close to the site of infection where a necrotic lesion develops, although a general resistance can be transduced throughout the plant (Zaitlin and Hull 1987). One of the molecular responses that has been studied in some detail is the production of pathogenesis-related proteins (Bol and van Kan 1988). However, these proteins appear to be synthesized as a generalized response to a number of different pathogens and also to some nonpathogenic stimuli. Moreover, the production of pathogenesis-related proteins is not generally associated with a systemic virus infection and the symptoms that result from it (Bol and van Kan 1988).

The character of systemic symptoms of virus infection in plants is determined by the expression of both host and virus genes. Our strategy to understand symptom expression and host responses at the molecular level has been to characterize aspects of the interaction of cauliflower mosaic virus (CaMV) with host plants. CaMV is particularly amenable to the study of host-virus interactions because many naturally occurring strains exist that differ in symptom expression (Hull 1980; Shepherd 1981). CaMV has a DNA genome that is directly infectious to plants and can be manipulated *in vitro* (Howell *et al.* 1980) to study the contribution of specific DNA sequences to a particular symptom phenotype (Schoelz *et al.* 1986). The analysis of host gene expression in CaMV-infected plants at the level of the translatable mRNA population is also facilitated because the majority of virus-encoded polypeptides, with the exception of the gene VI inclusion body protein (Al Ani *et al.* 1980; Odell and Howell 1980; Covey and Hull 1981) and some relatively minor products (Plant *et al.* 1985), are not synthesized during *in vitro* translation reactions. This is most likely because of the apparent use of a polycistronic mRNA for viral gene expression *in vivo* (Gronenborn 1987) that does not translate *in vitro* (Gordon *et al.* 1988). Consequently, most of the polypeptides observed from translations of whole cell RNA are derived from host-encoded mRNA and not viral mRNA.

We have examined changes in host gene expression that occur in response to two different strains of CaMV (Stratford *et al.* 1988) that produce either severe chlorotic symptoms (strain Cabb B-JI) or mild nonchlorotic symptoms (strain Bari 1) in systemically infected turnip leaves. Comparison of the *in vitro* translation products of mRNA extracted from healthy and systemically infected turnip leaves using 2-D PAGE revealed that increases and decreases in the level of different translatable mRNAs were caused by CaMV infection. We found that several changes in host gene expression measured by this means and observed in response to one CaMV strain were not detected

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in response to infection with the other strain and vice versa. These changes were also dissimilar to those observed during senescence-induced chlorosis.

MATERIALS AND METHODS

Virus propagation. CaMV strains Cabb B-JI and Bari 1 were propagated in turnip plants (*Brassica campestris* L. 'Just Right') that were grown with a 16-hr photoperiod either under greenhouse conditions at 18–22° C or in a growth cabinet at 20° C. Plants were inoculated at the two-leaf stage (10–14 days old) either with sap of infected plants or with infectious cloned CaMV DNAs, excised from their vectors by digestion with *Sal*I (Delseny and Hull 1983; Stratford *et al.* 1988). Leaves from infected and healthy plants were harvested for analysis 20–40 days postinoculation.

DNA analysis. Apical leaves from plants systemically infected with CaMV strains Cabb B-JI or Bari 1 were harvested at a number of time points postinoculation. Dot blots were prepared and probed as described by Maule *et al.* (1983) and quantified, in the linear phase of the dose-response curve of the X-ray film used, with purified viral DNA standards by using a Joyce-Loebl Chromoscan 3 densitometer. Each DNA sample to be quantified was probed with DNA of the appropriate CaMV strain.

RNA analysis. Total cellular RNA was extracted from turnip leaves and used to direct *in vitro* translation in samples of messenger-dependent rabbit reticulocyte lysate as described by Covey and Hull (1981). Leaves of the equivalent age were harvested from healthy and systemically infected plants 51 days postinoculation. To examine changes in the level of translatable mRNAs during leaf senescence, RNA was extracted from excised leaves that had been maintained either with water or with a 1 mg/L of solution of the cytokinin benzylaminopurine (BAP) to delay the onset of senescence (Osborne 1967; Woolhouse 1974). Poly(A)$^+$ RNA was prepared and northern blotted as described by Covey *et al.* (1981). Blots were probed by using a cDNA clone of the precursor to the small subunit of ribulose bisphosphate carboxylase (*rbc*S) isolated from *Nicotiana sylvestris* (kindly provided by A. G. Prescott and J. Fleck) and labeled with ^{32}P by nick translation.

Protein analysis. Polypeptides labeled with ^{35}S-methionine were separated by 2-D gel electrophoresis essentially as described by O'Farrell (1975) but with the following modifications. The first-dimension gels were run with the reverse polarity. Second, *in vitro* translation samples were prepared for electrophoresis by addition of an equal volume of lysis buffer (5 mM $MgCl_2$, 20 mM Tris-Cl, pH 7.6, 1% Nonidet P40), followed by three cycles of freeze-thawing in liquid nitrogen, and then treated with 0.5 μg/ml of pancreatic ribonuclease A at 4° C for 30 min. To each sample, urea was then added to a final concentration of 10 M and an equal volume of O'Farrell "solution A." The second-dimension sodium dodecyl sulfate gel consisted of a linear gradient of polyacrylamide (7.5–20%) bisacrylamide (0.15–0.075%) prepared and run, and radioactive polypeptides detected by fluorography as described by Covey and Hull (1981).

Chlorophyll analysis. Samples of healthy and systemically infected leaves were homogenized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and assayed for total chlorophyll spectrophotometrically as described by Arnon (1949).

RESULTS

Appearance of symptoms. Infection of turnip plants with CaMV strain Cabb B-JI, a relatively severe strain of CaMV, led to the production of vein-clearing symptoms 10–14 days postinoculation (Fig. 1B). The first leaf to exhibit vein clearing was the fourth or fifth to emerge from the meristem after the inoculated leaf. Vein-clearing symptoms then appeared on the younger and all subsequently formed leaves of the plant 2–3 days later. Generalized chlorosis (yellowing) of the leaf tissue developed 10–14 days after the first appearance of vein clearing (Fig. 1D). In addition to the leaf chlorosis, turnip plants infected with strain Cabb B-JI were severely stunted in comparison with healthy turnip plants of the same age.

In contrast, vein-clearing symptoms on turnip plants infected with the mild CaMV strain Bari 1 did not develop until 20–25 days postinoculation (Fig. 1C). The fifth or sixth leaf to emerge from the meristem after the inoculated leaf was the first to show these symptoms. Turnip plants infected with Bari 1 not only exhibited a delayed onset of vein clearing relative to a Cabb B-JI infection, but the rate at which the vein-clearing symptoms spread progressively to the younger leaves was also slower. The appearance of vein-clearing symptoms in Bari 1-infected plants was followed, after 10–12 days, by development of dark green islands of leaf tissue rather than chlorosis. This pattern of dark green islands is not readily reproduced by black and white photography (Fig. 1E), although it is very obvious on the actual leaves. Turnips infected with Bari 1 were much less severely stunted than those infected with Cabb B-JI.

The visible differences in leaf symptoms produced by the two CaMV strains were reflected in the amount of leaf chlorophyll present. The level of total chlorophyll in Cabb B-JI-infected plants showing severe chlorosis was 0.88 ± 0.04 mg/g fresh weight compared with 2.12 ± 0.32 mg/g fresh weight in healthy leaves. However, the chlorophyll level in the Bari 1-infected leaves showing dark green islands was 2.55 ± 0.22 mg/g fresh weight, which was 1.2-fold of that in the healthy controls.

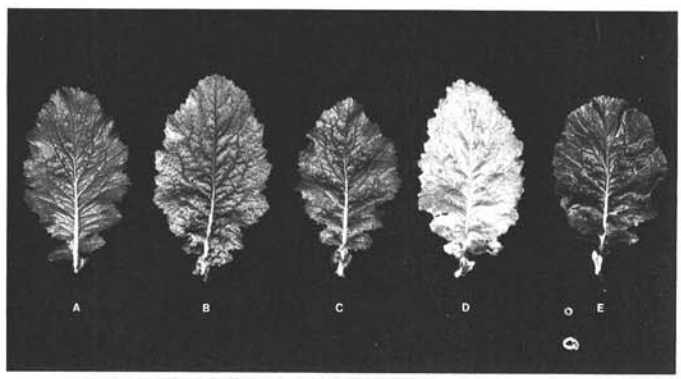

Fig. 1. Turnip leaves showing various systemic symptoms of CaMV infection compared with a noninfected leaf (A). Leaves showing early signs of vein clearing due to strain Cabb B-JI infection 14 days postinfection (B) and to Bari 1 infection 25 days postinfection (C) are illustrated. Leaf D exhibits chlorotic symptoms observed 25 days postinfection with Cabb B-JI, and leaf E has dark green regions produced by strain Bari 1 35 days postinfection.

Viral DNA levels. We compared the accumulation of viral DNA in turnip leaves, infected with the two CaMV strains, with the development of symptoms to determine the degree of correlation. Samples of apical leaves were harvested, and viral DNA was determined quantitatively by dot-blot analysis (Fig. 2). Viral DNA was first detected in Cabb B-JI-infected leaves approximately 10 days postinoculation, concomitant with the first appearance of vein-clearing symptoms. The level of viral DNA then rapidly increased over the next 5 days (Fig. 2).

In leaves infected with Bari 1, viral DNA was not detected until 20 days postinoculation, again concomitant with vein-clearing symptoms. The rate of accumulation of Bari 1 DNA was much lower than that of Cabb B-JI. Furthermore, the level of Bari 1 viral DNA never reached that of Cabb B-JI at any time during the period under study. We also determined the amount of viral DNA within the dark green islands of Bari 1-infected leaves by dot blotting, and it was found not to differ significantly from the remainder of the tissue (data not shown).

Analysis of mRNAs in CaMV-infected plants by *in vitro* translation. We analyzed one aspect of the changing pattern of host gene expression occurring during infection by studying the translatable mRNA population. Leaves of equivalent age from systemically infected and healthy plants grown under identical conditions were harvested and total cellular RNA samples prepared. Polypeptides produced by *in vitro* translation of such samples in a rabbit reticulocyte lysate were separated by PAGE and detected by fluorography. In initial experiments, translation products were analyzed on 1-D gels, although this revealed few differences between infected and noninfected plants. Instead, we chose to analyze the products with O'Farrell 2-D gels, which provided much enhanced resolution. In Figure 3 we have indicated changes in the level of specific translation products observed consistently between experiments. Three different RNA samples from Cabb B-JI-infected plants and two from Bari 1-infected plants were prepared. A similar number of RNA samples were also prepared from healthy plants of equivalent developmental age, and a minimum of

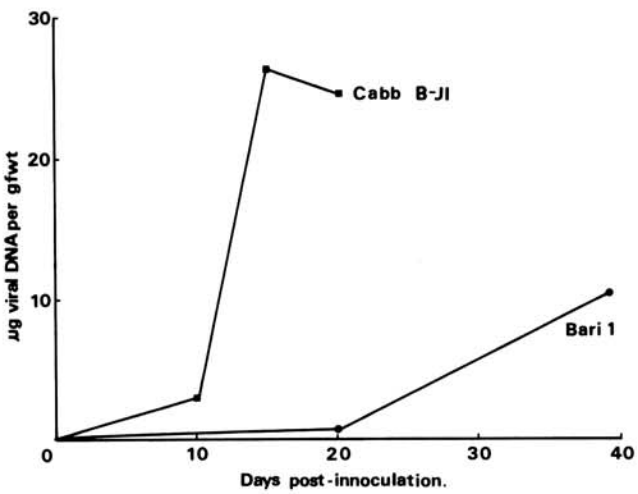

Fig. 2. Accumulation of viral DNA per gram fresh weight of tissue during the early stages of systemic infections in leaves containing the severe (Cabb B-JI) and mild (Bari 1) CaMV strains.

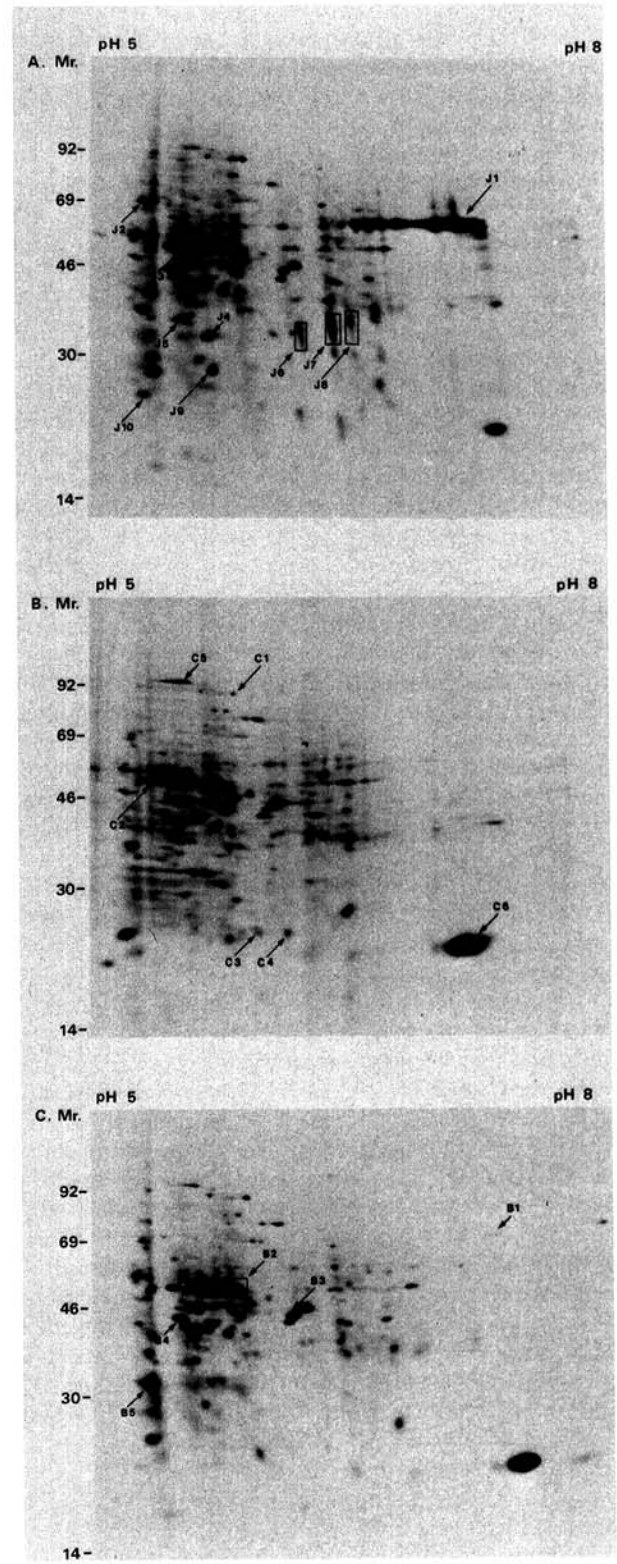

Fig. 3. *In vitro* translation products of total RNA from (**A**) Cabb B-JI-infected, (**B**) healthy, and (**C**) Bari 1-infected turnip leaves separated by 2-D PAGE. The levels of radioactive polypeptides products found to change consistently between experiments are indicated with arrows. Those changes resulting specifically from infection either by CaMV strain Cabb B-JI (panel A, arrows labeled J1–J10) or by strain Bari 1 (panel C, arrows labeled B1–B5) are shown. The levels of some polypeptides changed in response to infection by both virus strains; these are indicated in panel B (arrows labeled C1–C6) showing translation products of RNA from noninfected leaves. The polarity of the first dimension and molecular weight markers ($M_r \times 10^{-3}$) for the second dimension are shown.

two gels were run from each RNA preparation. The changes marked occurred in all experiments, and those specific to Cabb B-JI infections are labeled J1–J10, those specific to Bari 1 are marked B1–B5, and changes common to both are labeled C1–C6.

In the translation products of RNA extracted from Cabb B-JI-infected turnips, the most prominent infection-specific polypeptide was J1 (Fig.3A), previously identified as the product of CaMV gene VI, the inclusion body protein P62 (Covey and Hull 1981; Stratford *et al.* 1988). A number of novel polypeptides were synthesized as a result of infection by Cabb B-JI and not produced in samples from healthy turnip leaves (Fig. 3A, groups J6, J7, and J8); these boxed areas in Figure 3A actually contain a number of individual spots not resolved in the photograph. In addition to *de novo* products, increased levels (Fig. 3A, labeled J2, J4, J5, and J9) and decreased levels (Fig. 3A, labeled J3 and J10) of some polypeptides resulted specifically from infection with Cabb B-JI.

The faint component labeled B1 in the translation products of RNA extracted from Bari 1-infected turnips (Fig 3C), but not in those from healthy turnips (Fig. 3B), was the Bari 1-encoded inclusion body polypeptide that migrates with an apparent molecular weight of 70 K (Stratford *et al.* 1988) and is less abundant than the equivalent Cabb B-JI product (Fig. 3A). A novel polypeptide (B5), not present in healthy turnip plants, was synthesized in response to infection by Bari 1 (Fig. 3C), and increased levels of other polypeptides were also observed (group B2, and B3, and B4). In total, a change in the level of 11 major translatable mRNA species (B1–B5, C1–C6) was observed in Bari 1-infected leaves compared with the noninfected controls. Some of the changes in levels of translatable mRNAs that occurred in response to infection by Bari 1 were found to be similar to those in Cabb B-JI-infected leaves (Fig. 3B).

Changes in the level of the *rbc*S mRNA. The most abundant *in vitro* translation product of RNA from healthy turnip plants was a 22 K M_r polypeptide (labeled C6 in Fig. 3B). The level of this product was much lower in turnip leaves infected by strain Cabb B-JI than in noninfected leaves (compare Fig. 3B with 3A). Because of its size and abundance, we suspected that this polypeptide was the precursor to the small subunit of *rbc*S. To test this hypothesis, we incubated the products of the *in vitro* translation reaction with a chloroplast preparation that subsequently processed the P22 precursor to the mature P14 form of the *rbc*S by using the methods of Highfield and Ellis (1978). Chloroplasts isolated from Cabb B-JI-infected turnip plants as well as healthy turnips were able to process the *rbc*S precursor in this way (data not shown).

This was further investigated by northern blotting poly(A)$^+$ RNA prepared from healthy and Cabb B-JI-infected turnip plants and probing it with a cDNA clone of the *rbc*S from tobacco (Fig. 4A). An RNA species of about 0.9 kb was observed, which corresponds well to the size of the *rbc*S precursor mRNA from other plants (Bedbrook *et al.* 1980). The level of this host mRNA in leaves infected by Cabb B-JI (Fig. 4A, II) was about 5% of that in healthy leaves (Fig. 4A, I). This corresponds reasonably well with the reduction in the level observed for the putative *rbc*S translation product (Fig. 3B, spot C6). The reduction in the level of the equivalent translation product of RNA from Bari 1-infected leaves (Fig. 3C) compared with the healthy control (Fig. 3B) was much less marked than that observed with Cabb B-JI (Fig. 3A). This was reflected in the relative level of the *rbc*S mRNA in Bari 1-infected plants determined by northern blotting (Fig. 4B). Generally, the level of the *rbc*S mRNA was found to be more variable in Bari 1-infected plants than in healthy or Cabb B-JI-infected plants. In some experiments, there was no difference observed between Bari 1 and healthy plants with respect to the *rbc*S mRNA level, whereas in Cabb B-JI-infected plants it was always lower. We have also found that Cabb B-JI, but not Bari 1, infection causes a reduction in both large and small subunit levels of *rbc* protein in leaves (data not shown).

Changes in host mRNAs during senescence. Because Cabb B-JI infection of turnips caused generalized chlorosis of leaf tissue, a reduction in chlorophyll, and a characteristic change in the mRNA population, we wished to determine whether any similar mRNA changes occurred during development of chlorosis resulting from leaf senescence. Healthy turnip leaves were excised from near the apex of plants and maintained by immersion of the petioles in water or a solution containing BAP. Over a subsequent period of 2–3 wk, leaves that were maintained with only water became chlorotic, whereas leaves supplemented with BAP remained green. Total RNA samples were prepared from these leaves and compared by *in vitro* translation and 2-D gel electrophoresis (Fig. 5).

Several polypeptide products were found to decrease in leaves maintained only with water (Fig. 5A, spots S1, S2, S3, and S7) compared with leaves that had been supplemented with BAP (Fig 5B). Although the decreased level of polypeptide S2 is not particularly clear in Figure 5 due to the proximity of other spots, it was clearly visible on the original autoradiograph. Increased levels of some products were also observed (Fig. 5A, spots S4, S5, and S6). Three changes in the level of translation products were found to be common to Cabb B-JI infection and senescence, and in each

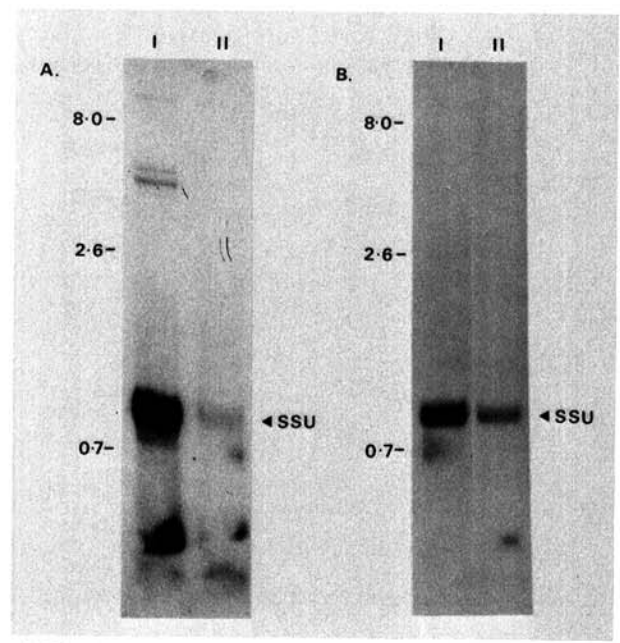

Fig. 4. Northern blot hybridization to detect the mRNA encoding the precursor to *rbc*S (labeled SSU) by using a cDNA probe from tobacco. **A**, poly(A)$^+$ RNA from healthy (I) and Cabb B-JI-infected (II) turnip leaves. **B**, poly(A)$^+$ RNA from noninfected (I) and Bari 1-infected (II) plants.

case this was a reduction in the level of a pre-existing mRNA. These are to the senescence-specific product S2 (Fig. 5A), which is equivalent to the Cabb B-JI product J3 (Fig. 3A); senescence product S1 (Fig. 5A), which is reduced following infection by both CaMV strains (Fig. 3B, spot C5); and senescence product S7 (Fig. 5A), also reduced following infection by both CaMV strains, but to different degrees, and believed to be caused by the the *rbc*S mRNA (Fig. 3B, spot C6).

DISCUSSION

In our experiments, we have correlated specific symptom characteristics produced by systemic infection of turnip leaves by mild and severe strains of CaMV with changes in the level of some translatable host mRNAs (Table 1). We observed three types of changes in host gene expression in response to CaMV infection. The level of some translatable mRNAs increased in response to infection; others were found to decrease. Some translatable mRNAs appeared that had not been detected in healthy turnip plants and thus represented *de novo* products. Among this latter group, one was the CaMV gene VI mRNA encoding the virus inclusion body protein, which exhibits gel mobility variation between the two CaMV strains (Stratford *et al.* 1988) and has been implicated as a viral symptom determinant (Daubert *et al.* 1984; Baughman *et al.* 1988). The only other virus-specified product shown to be synthesized during *in vitro* translation reactions is the reverse transcriptase enzyme, and this was only a very minor component of the translation of poly(A)$^+$ RNA (Plant *et al.* 1985). However, we cannot discount the possibility that some of the novel products were derived from translation of the CaMV 35S RNA enhanced by a host polypeptide not present in products derived from hybrid-selected viral mRNA. Even so, it is more likely that most of the novel mRNAs observed during CaMV infection are host encoded. The majority of changes in the translatable mRNA population were found to be specific to the particular CaMV strain, although six of the mRNAs that decreased in response to infection did so in response to both Cabb B-JI and Bari 1 strains (Fig 3B). By northern blot analysis of poly(A)$^+$ RNA, we identified one of the changes in host gene expression common to both infections as the mRNA encoding *rbc*S (Fig. 4).

Because infection by Cabb B-JI resulted in a decrease in the level of total chlorophyll and also a decrease in the *rbc*S

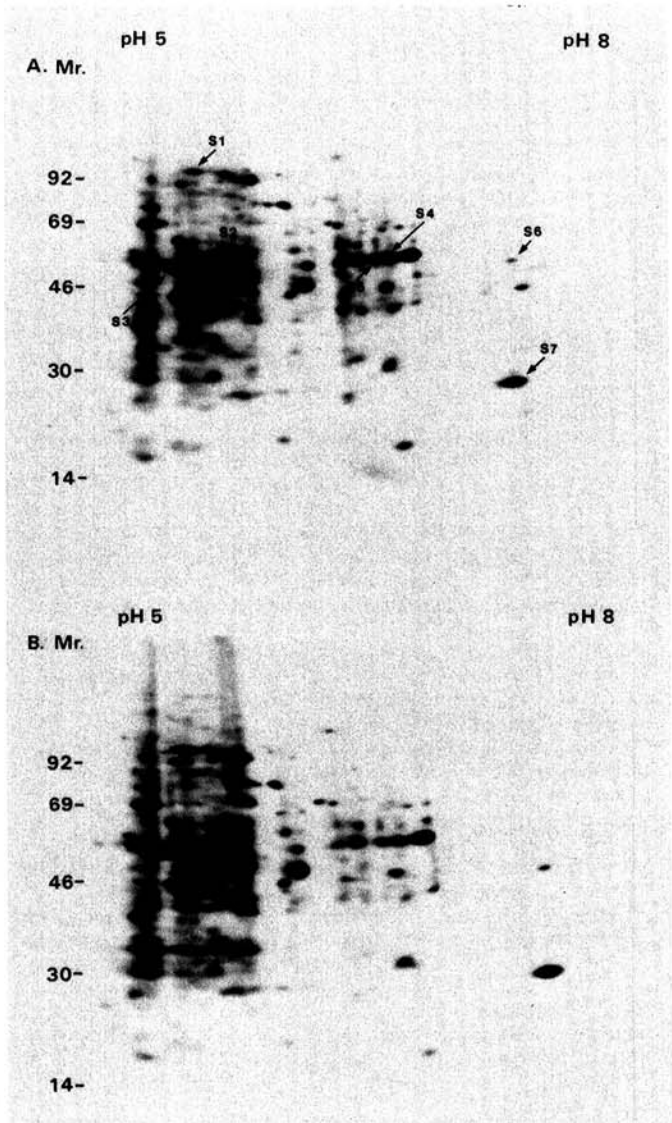

Fig. 5. *In vitro* translation products of total RNA isolated from excised healthy turnip leaves maintained with (**A**) only water to promote senescence or (**B**) with the cytokinin benzylaminopurine to delay it. Radioactive polypeptides were separated by 2-D gel electrophoresis. The polarity of the first dimension and molecular weight markers ($\times 10^{-3}$) for the second dimension are shown. Products of mRNAs whose levels change during senescence have arrows labeled S1–S7.

Table 1. Changes to the levels of *in vitro* translation products directed by RNA isolated from turnip leaves either senescing or exposed to CaMV infection

Condition	Spot[a]	Polypeptide	Change[c]
Infection by	J1	62 K (gene VI)	+
CaMV strain	J2	69 K	↑
Cabb B-JI	J3	55 K[b]	↓
	J4	33 K	↑
	J5	36 K	↑
	J6	30 K–33 K	+
	J7	31 K–35 K	+
	J8	31 K–36 K	+
	J9	27 K	↑
	J10	25 K	↓
Infection by	B1	70 K (gene VI)	+
CaMV strain	B2	55 K	↑
Bari 1	B3	47 K	↑
	B4	42 K	↑
	B5	32 K	↑
Changes	C1	87 K	↑
common to	C2	55 K	↓
both infections	C3	23 K	↓
	C4	23 K	↓
	C5	95 K[b]	↓
	C6	20 K[b] (*rbc*S)	↓
Changes	S1	95 K[b]	↓
during	S2	55 K[b]	↓
senescence	S3	55 K	↓
	S4	55 K	↑
	S5	55 K	↑
	S6	51 K	↑
	S7	20 K[b] (*rbc*S)	↓

[a] Products marked for identification after 2-D gel electrophoresis.
[b] Polypeptide changes common to infections and senescence.
[c] Arrows represent increase/decrease, and plus signs are *de novo* polypeptide products.

mRNA level, we wondered if Cabb B-JI infection was accelerating the normal cellular process of senescence. We examined the translatable mRNA population in excised turnip leaves, maintained for 2–3 weeks with a solution containing the cytokinin BAP, and compared it with that of turnip leaves maintained with water only (Fig. 5). Cytokinins are known to delay the onset of senescence (Osborne 1967; Woolhouse 1974). The translatable level of seven mRNAs was found to be altered in senescing leaves relative to the leaves maintained on BAP. Three of these senescence-related mRNAs (Fig. 5, spots S1, S2, and S7) that decreased in amount were found to be identical, on the basis of molecular weight and isoelectric point, to three of the mRNAs that decreased in response to infection with CaMV strain Cabb B-JI (Fig. 3, spots J3, C5, and C6). One of these changes (C6) was identified as *rbc*S.

Only three of the changes in mRNA levels resulting from infection by Cabb B-JI were observed during senescence (Table 1), and so it would seem that virus-induced chlorosis is not simply due to the induction of premature senescence. First, we have shown that other changes in gene expression occur during senescence that do not occur in Cabb B-JI-infected turnips and vice versa. Second, senescence is associated with both a decrease in chlorophyll content and the breakdown of chloroplast structure (Thomas and Stoddart 1980), and although we found that the level of chlorophyll was lower in leaves infected with Cabb B-JI, the integrity of the chloroplast was maintained (K. Plaskitt, personal communication). Furthermore, the process of senescence is invariably associated with both a decrease in chlorophyll content and a decrease in *rbc*S level (Peterson and Huffaker 1975; Wittenbach 1978). We report here that infection of turnip plants with CaMV strain Bari 1 can result in a decrease in the *rbc*S mRNA level and an increase the total level of chlorophyll. Consequently, it is possible that during infection with CaMV, these two events, which are usually concurrent in the senescence process, can become "uncoupled."

It has been reported that infection of plants with fungi can result in a decrease in the level of *rbc*S mRNA (Higgins et al 1985; Mouly *et al.* 1988). Heat shock can also cause a decrease in the *rbc*S mRNA level (Vierling and Key 1985). This suggests that the decrease in *rbc*S mRNA as a result of CaMV-infection of turnip plants is stress, rather than senescence, specific.

The CaMV strain-specific induced changes in host gene expression must originate within the 8-kb virus DNA genome. We do not yet know whether the difference in the interaction of the CaMV strains and the host plant shown here is a consequence of amino acid differences in the virus-encoded products (Stratford *et al.* 1988), the rate and level of virus DNA synthesis, or a combination of both factors. We are addressing this question by constructing hybrids between the genomes of strains Cabb B-JI and Bari 1.

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