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Induction of Malate Synthase Gene Expression in Senescent and Detached Organs of Cucumber

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Expression of the malate synthase (MS) gene is activated in cotyledons of cucumber seedlings during postgerminative growth and then repressed as the cotyledons become photosynthetic. MS gene expression is subsequently reactivated in the cotyledons as they senesce a few weeks later. In situ hybridization revealed that MS RNA is distributed throughout the organ during postgerminative growth and senescence, showing that the same cells express the gene at different stages of development. MS RNA also appears in senescing leaves and petals of cucumber plants. In addition, we found that MS RNA appears in mature expanded leaves and roots when they are removed from the plant and incubated in darkness for several days, thus providing a potential experimental system for the manipulation of MS gene expression. Leaves from transgenic *Nicotiana plumbaginifolia* containing the cucumber MS promoter fused to the β-glucuronidase (GUS) reporter gene accumulated GUS activity when detached, demonstrating an activation of transcription from the MS promoter following leaf excision. These results are discussed in terms of the metabolic regulation of MS gene expression.

INTRODUCTION

The glyoxylate cycle was first described in Pseudomonas (Kornberg and Madsen, 1957), where it provides a means of growth on acetate as the sole carbon source. Two enzymes, malate synthase (MS) and isocitrate lyase (ICL), allow the decarboxylation steps of the citric acid cycle to be bypassed, thereby achieving the net conversion of 2 mol of acetate into one of succinate. This pathway has subsequently been shown to allow growth on acetate or other two-carbon molecules in a range of microorganisms, including other bacteria (Kornberg, 1966; Hillier and Charnetzky, 1981), yeasts (Zwart et al., 1983), filamentous fungi (Sjogren and Romano, 1967; Armitt et al., 1976; King and Casselton, 1977), and single-celled algae (Syrett et al., 1963; Graves and Becker, 1974). In several of these cases, it has been shown that MS and ICL synthesis is induced by growth on acetate and repressed by glucose (Kornberg, 1966; Sjogren and Romano, 1967; McCullough and John, 1972).

The glyoxylate cycle was shown to operate in higher plants by Kornberg and Beevers (1957) in endosperm tissue of germinating castor oil seeds. The activity of the glyoxylate cycle and the control of synthesis of MS and ICL have since been extensively investigated in storage tissues of seeds of many higher plants (Weir et al., 1980; Comai et al., 1989; Turley and Trelease, 1990). These enzymes are synthesized during postgerminative growth when lipid is undergoing rapid mobilization through lipase and β-oxidation activities to produce acetyl coenzyme A. The glyoxylate cycle then mediates the conversion of acetyl coenzyme A to four-carbon acids for subsequent conversion to carbohydrates, particularly sucrose. After this phase of postgerminative growth, the developing seedling becomes photosynthetic, glyoxylate cycle enzyme synthesis stops, and the enzyme activity decreases to undetectable levels.

Recently, Gut and Matile (1988) made the important observation that MS and ICL activities appear in leaves of barley after excision and incubation in the dark for a few days. Subsequently, De Bellis et al. (1990) have made similar observations for leaves of rice and leaf beet and cotyledons of pumpkin. MS and ICL have also been shown to appear in senescent leaves of rice and wheat and in leaves of rice or wheat plants placed into darkness for 11 and 7 days, respectively (Pistelli et al., 1991). De Bellis and Nishimura (1991) have subsequently demonstrated the presence of MS and ICL in senescent pumpkin cotyledons. In some of these cases, lipid breakdown and β-oxidation have been demonstrated, indicating that similar metabolic pathways may operate both in detached or senescent organs and during postgerminative growth.

There are several reasons for believing that MS and ICL synthesis in plants may also be under metabolic control, as previously demonstrated in microorganisms. Most convincing are the studies of Kudielker and Theimer (1983a, 1983b). Using suspension cultures of anise, these workers have shown that

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when sucrose is removed from the medium, MS and ICL activities are induced within 24 hr and that the induction is enhanced by the addition of acetate. The addition of sucrose represses enzyme activity, whether or not acetate is present. Consistent with this model of metabolic regulation is the observation that when cucumber seedlings are germinated and grown in darkness, photosynthate is not produced, and MS and ICL synthesis continues in the cotyledons for several days beyond the time when synthesis stops in light-grown seedlings (Weir et al., 1980; Smith and Leaver, 1986). The finding of Gut and Matile (1988) that MS and ICL activities increase in detached leaves, coincident with a decline in photosynthetic activity and an activation of lipid breakdown, is also consistent with a metabolic control of enzyme synthesis.

In previous work, we isolated a cucumber MS cDNA and investigated the appearance of MS gene transcripts, protein, and enzyme activity during germination and growth of cucumber seedlings (Smith and Leaver, 1986). MS gene products are below the level of detection in dry seeds, but appear 2 days after imbibition, reach a peak after 3 or 4 days, and then decline to an undetectable level after 7 days of growth in the light as the seedlings acquire photosynthetic competence. Similarly, developing and mature leaves lack MS. More recent studies have shown that glyoxylate cycle enzymes are synthesized in senescing cotyledons of pumpkin (De Bellis and Nishimura, 1991) and senescing leaves of rice and wheat (Pistelli et al., 1991). We have therefore investigated the expression of the MS gene in senescent and detached organs to learn more about the control of enzyme synthesis. We found that MS gene transcription was activated during senescence and in detached organs, and we discuss these results in terms of the metabolic regulation of gene expression.

RESULTS

MS Gene Expression in Cucumber Cotyledons during Plant Growth

Cotyledons of plants that exhibit epigeal germination provide an excellent experimental system in which to investigate metabolic and developmental changes taking place from an early phase of heterotrophic growth through phototrophic growth to senescence. These changes take place in the same organ in the absence of cell division (Becker et al., 1978) and include transitions of the microbody population from glyoxysomes to peroxisomes and back to glyoxysomes (De Bellis and Nishimura, 1991). We therefore investigated MS gene expression in cotyledons at intervals throughout the 31 days following germination, by which time the cotyledons were yellow. Total RNA was isolated at intervals, and amounts equivalent to one tenth of a cotyledon were fractionated by gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with the MS cDNA probe. The result shown in Figure 1 confirms previous observations of MS RNA accumulation during the first few days of postgerminative growth followed by a rapid decline (Smith and Leaver, 1986). However, we observed MS RNA accumulating again after ~26 days of growth (Figure 1).

To determine which cells or regions of the cotyledon contain MS RNA, thin sections of cotyledons at 3 and 31 days were prepared for hybridization with sense and antisense MS riboprobes. The organization of the tissue, shown by staining in Figures 2A and 2D, is significantly different during senescence, with palisade cells poorly defined and most cells highly vacuolated. Hybridization with the antisense probe, presented in Figures 2B and 2E, showed that MS RNA is distributed throughout the cotyledon tissue in both 3- and 31-day-old cotyledons, indicating that the same cells express the MS gene at both stages of development.

The abundance of MS protein in extracts prepared from single cotyledons at day 3 and at intervals from 23 to 31 days postimbibition is shown in Figure 3. Protein gel blot analysis using a specific antibody (Smith and Leaver, 1986) clearly showed that MS protein accumulates at day 3 as expected and that it reappears after day 26. Attempts to measure MS enzyme activity in extracts of senescent tissue have not been successful, presumably because of the presence of inhibitory factors, as proposed by others (De Bellis et al., 1990).

MS Gene Expression in Senescent Leaves and Petals

Leaves at three different stages of development were removed from a flowering cucumber plant for isolation of RNA and chlorophyll. One leaf contained 1.25 mg of chlorophyll per gram fresh weight of tissue, which is the maximum contained in leaves from such plants. Another leaf at a later stage of development contained 0.75 mg of chlorophyll per gram fresh weight of tissue, whereas the third was clearly senescent and contained only 0.38 mg of chlorophyll per gram fresh weight of

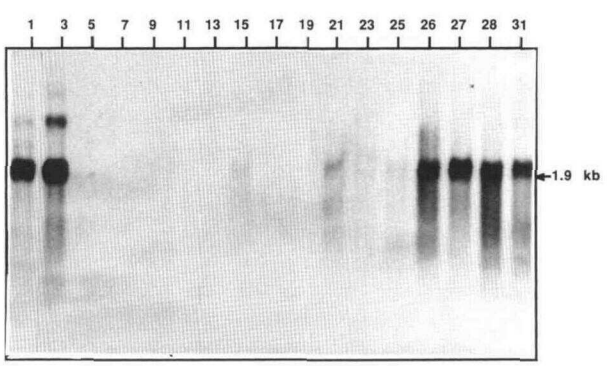

Figure 1. RNA Gel Blot Analysis of MS during Cotyledon Development.

Numbers above lanes represent days of development after seed imbibition. One tenth of total RNA extracted from a single cotyledon at each stage of development was loaded per lane.

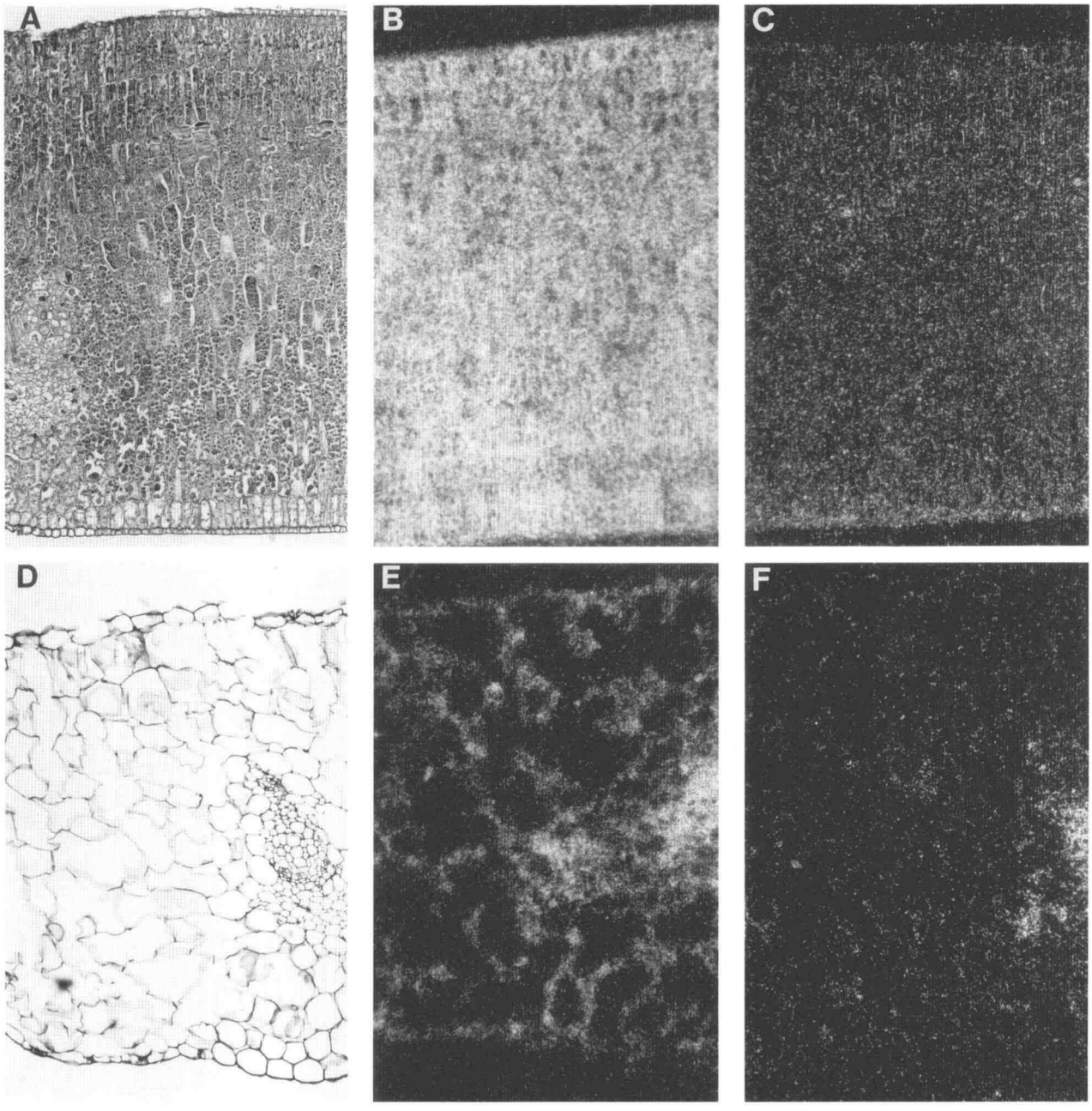

Figure 2. In Situ Hybridization of MS RNA in Cotyledons of Cucumber.

Serial sections of a 3-day-old cotyledon and a 31-day-old cotyledon are shown. Dark-field illumination is used in those sections hybridized with RNA. ×80.

(A) Cross-section of a 3-day-old cotyledon stained with Fast Green and Safranin O.
(B) Cross-section of a 3-day-old cotyledon hybridized with antisense MS RNA.
(C) Cross-section of a 31-day-old cotyledon hybridized with sense MS RNA.
(D) Cross-section of a 31-day-old cotyledon stained with Fast Green and Safranin O.
(E) Cross-section of a 31-day-old cotyledon hybridized with antisense MS RNA.
(F) Cross-section of a 31-day-old cotyledon hybridized with sense MS RNA.

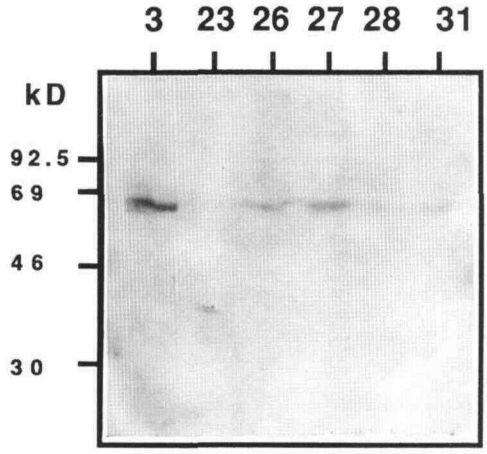

Figure 3. Protein Gel Blot Showing Pattern of MS in Cotyledons.

Numbers above lanes represent days of development after seed imbibition. One twentieth of total protein extracted from a single cotyledon (the comrade of that used for the RNA extraction shown in Figure 1) was loaded per lane. Molecular mass markers are as indicated. The MS protein has a predicted molecular mass of 64.9 kD (Graham et al., 1989).

tissue. Results of RNA gel blot hybridization are shown in Figure 4A. MS RNA was not detected in the leaf containing the maximum amount of chlorophyll, but was present at a very low level in the leaf with an intermediate level of chlorophyll and was abundant in the senescent leaf.

RNA was next isolated from petals on 3 successive days of development. On the first day, the flowers had not opened, so the petals were contained entirely within the sepals. On the second day, the flowers had opened and the petals were fully expanded. On the third day, the petals were wilting (senescent). When equal amounts of RNA from each of these stages were hybridized with the MS cDNA probe, MS RNA was detected in the senescent petals but not in the earlier stages, as shown in Figure 4B. Our earlier observation that preparations of petal RNA from cucumber or transgenic *Nicotiana plumbaginifolia* plants contain low levels of MS gene transcripts (Graham et al., 1990) is therefore explained in terms of senescent petals in the sample.

In each of the cases described above (cotyledons, leaves, and petals), the amount of total RNA extracted from senescent tissues was less than that isolated from earlier stages. This is to be expected because RNA degradation is known to be a feature of the senescence process (Thomas and Stoddart, 1980). However, it is clear from our results that the relative accumulation of MS gene transcripts in senescence represents an active synthesis against this background of RNA degradation (see also activation of GUS gene expression below).

Experimental Induction of MS Gene Expression in Cucumber Organs

Previous experiments of Gut and Matile (1988) and De Bellis et al. (1990) have shown that glyoxylate cycle enzymes accumulate in leaves or cotyledons of some plants when these organs are detached and incubated in darkness for several days. Pistelli et al. (1991) have also shown that glyoxylate cycle enzymes appear in leaves of rice or wheat plants placed into darkness for 11 or 7 days, respectively. It is unlikely that the physiological processes taking place under these circumstances reflect those taking place during senescence. However, such experimental systems tell us something about processes that take place in cut fruit and vegetables or when plants are shaded for various reasons, and they may also provide us with important information about the factors that can control glyoxylate cycle enzyme synthesis.

To determine whether MS gene expression can be experimentally induced in cucumber, plants with eight leaves were

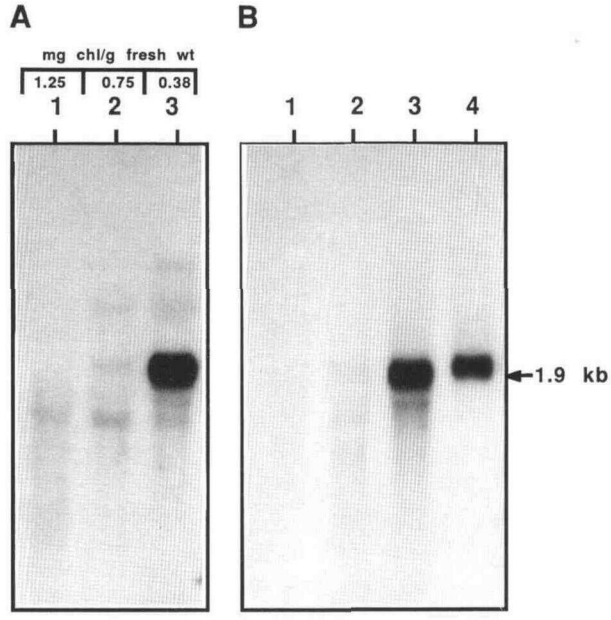

Figure 4. RNA Gel Blots Showing Appearance of MS in Leaves and Petals.

(A) Leaves: Lanes 1 to 3 represent the transition from photosynthetic to senescent leaf tissue. The stage of development is indicated by amount of chlorophyll (chl) per gram (g) fresh weight of tissue. Five micrograms of total RNA was loaded per lane.

(B) Petals: Lanes 1 to 3 represent petals from flowers at different stages of development. Lane 1, immature petal; lane 2, mature petal; lane 3, senescing petal. Five micrograms of total RNA was loaded per lane. In lane 4, 5 μg of total RNA from 3-day-old cucumber cotyledons was used as a control.

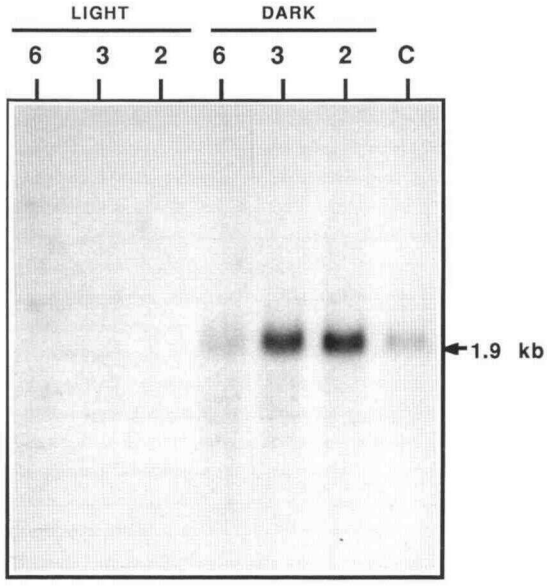

Figure 5. RNA Gel Blot Showing Appearance of MS in Leaves of Dark-Treated Plants.

RNA was isolated from individual leaves from two plants, one kept in continuous light for 7 days (light) and one kept in darkness for the same period (dark). Numbers 6, 3, and 2 represent leaf positions on the plant. Each plant has eight leaves, with the oldest leaf being number 1. The leaves chosen for analysis all contained >1 mg of chlorophyll per gram fresh weight of tissue at the start of the experiment (compare with Figure 4A). Five micrograms of total RNA was loaded per lane. One microgram of total RNA from 3-day-old cucumber cotyledons was used as a control (C).

placed into darkness for 7 days, with control plants kept in continuous light. After this time, RNA was isolated from leaves numbered 6, 3, and 2 (where 1 is the oldest) and hybridized with the MS cDNA probe. The results shown in Figure 5 indicate that MS RNA accumulation can be induced in leaves by dark treatment of plants. The accumulation of MS RNA was more pronounced in older leaves, possibly reflecting changes in leaf physiology during development. It might be that older leaves contain less carbohydrate than younger leaves or contain higher levels of enzymes involved in lipid breakdown.

The experimental induction of MS RNA was further investigated by detaching leaves at a similar stage of development from cucumber plants and incubating them in darkness for up to 9 days. When RNA was isolated from samples at 3-day intervals and hybridized with the MS cDNA probe, a dramatic increase in MS RNA was observed, as shown in Figure 6A. Similarly, roots detached from such plants and incubated in darkness accumulated MS RNA, as shown in Figure 6B. This timing of MS RNA accumulation and subsequent decline is different in detached leaves and roots, possibly reflecting different physiological or metabolic conditions within each organ type. Roots detached from seedlings only 5 days after imbibition also accumulated MS RNA with a timing similar to that of those shown in Figure 6B (data not shown). Cotyledons detached from seedlings after 14 days of growth and incubated in darkness also accumulated MS RNA (data not shown).

To determine whether MS RNA in detached organs is accompanied by a corresponding synthesis of MS protein, protein gel blot analysis was carried out on extracts of leaves detached and incubated in darkness for up to 8 days. Figure 7 shows that MS protein appears after only a 2-day incubation period and accumulates during the treatment.

Activation of MS Gene Transcription

The accumulation of MS RNA in senescing and detached organs could be due to an increase in the rate of gene transcription or to a decrease in MS RNA turnover. To investigate these two alternatives, leaves from transgenic *N. plumbaginifolia* plants containing the cucumber MS promoter linked to the β-glucuronidase (GUS) reporter gene (Graham et al., 1990) were detached and incubated in darkness for up to 12 days before assaying GUS activity. The results presented in Figure 8 showed that GUS activity appears after 6 days of incubation, but only in leaves of transformed plants, and then only

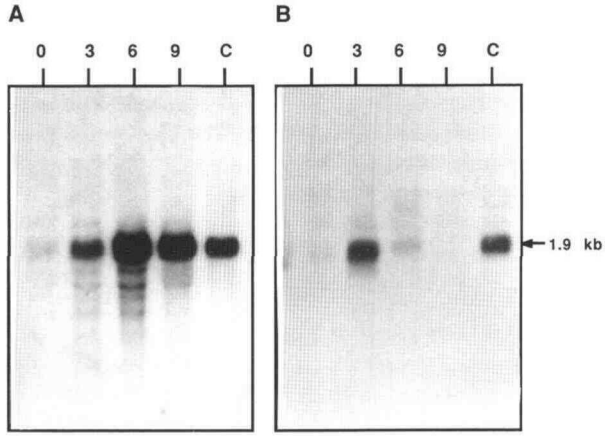

Figure 6. RNA Gel Blot Showing Appearance of MS in Detached Organs.

(A) Expanded leaves containing at least 1 mg of chlorophyll per gram fresh weight of tissue were detached from plants between 4 and 6 weeks old. Each lane represents 5 μg of total RNA from leaves 0, 3, 6, and 9 days after excision. Five micrograms of total RNA from 3-day-old cucumber cotyledons was used as a control (C).

(B) Roots were excised from the same plants. Each lane represents 5 μg of total RNA from roots 0, 3, 6, and 9 days after excision. Five micrograms of total RNA from 3-day-old cucumber cotyledons was used as a control (C).

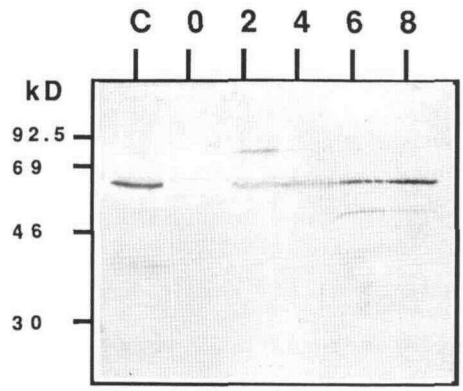

Figure 7. Protein Gel Blot Showing Appearance of MS in Detached Leaves.

Expanded leaves containing at least 1 mg of chlorophyll per gram fresh weight of tissue were detached from plants between 4 and 6 weeks old. Lane C represents one twentieth of total protein from a 3-day-old cotyledon. Remaining lanes represent one five hundredth of total protein from individual leaves 0, 2, 4, 6, and 8 days after excision. Molecular mass markers are as indicated. The MS protein has a predicted molecular mass of 64.9 kD (Graham et al., 1989).

when the MS promoter is linked in correct orientation to the GUS gene. These observations are consistent with the proposal that transcription from the MS promoter is activated because post-transcriptional processes would not be expected to act in the same way upon both the GUS and the MS mRNAs. The timing of gene expression is faster in detached leaves of cucumber than in *N. plumbaginifolia*. This difference reflects different rates at which the detached leaves break down chlorophyll and eventually degenerate.

DISCUSSION

Function of MS in Senescence

Our results support and extend those of Gut and Matile (1988), De Bellis et al. (1990), De Bellis and Nishimura (1991), and Pistelli et al. (1991) by showing that MS gene expression is activated during senescence and in detached organs of cucumber. Our preliminary results also show that ICL gene expression is activated during senescence in cucumber (I. Graham and J. McLaughlin, unpublished results). Gut and Matile (1988) have suggested that the glyoxylate cycle might operate in detached barley leaves to metabolize products of chloroplast lipid breakdown. Furthermore, De Bellis et al. (1990) have observed that β-oxidation activity develops in microbodies of senescing leaves together with MS activity. However, no experiments have been carried out to directly demonstrate glyoxylate cycle activity. Such experiments will be important to establish the function of MS and ICL in senescence.

It should be noted that MS and ICL are not always coordinately synthesized. In developing seed of oilseed rape, mRNAs encoding the two enzymes exhibit similar qualitative but different quantitative patterns of accumulation during embryogeny and postgerminative growth (Comai et al., 1989). In addition, these patterns for MS and ICL RNAs do not correspond to the relative accumulation of their proteins or enzyme activities (Ettinger and Harada, 1990). In developing cotton seeds, ICL and MS are synthesized at different times (Turley and Trelease, 1990). In yeast, MS is specifically synthesized from the DAL7 gene during growth on allantoin (Yoo and Cooper, 1989; Rodriguez et al., 1990). Under these circumstances, glyoxylate is produced from ureidoglycolic acid breakdown to urea, obviating the need for ICL activity. We should therefore consider the possibility, first postulated by Rodriguez et al. (1990), that in senescing tissues MS is involved in the metabolism of products of nucleotide breakdown as in yeast. Nucleic acid breakdown is a characteristic feature of senescing plant tissues (Thomas and Stoddart, 1980). Uricase and allantoinase are known to be present in glyoxysomes of castor bean endosperm, but the next enzyme in the pathway, allantoicase (which produces ureidoglycolic acid from allantoic acid), is not (Theimer and Beevers, 1971). This observation suggests that the production of glyoxylate by ureidoglycolate hydrolysis is unlikely to occur in these organelles. Critical experiments are

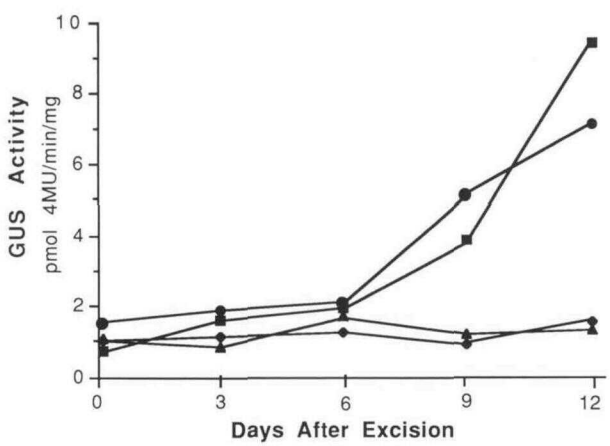

Figure 8. Fluorogenic Analysis of GUS Activity in Detached Leaves of Transgenic *N. plumbaginifolia*.

Leaves were excised from transgenic plants carrying the MS promoter/GUS reporter gene fusion, as described by Graham et al. (1990). GUS activity was measured in leaves 0, 3, 6, 9, and 12 days after excision. Analysis was carried out on leaves from transformants PCOR1 (■) and PCOR3 (●), both of which have the MS promoter in the correct orientation relative to the GUS reporter gene; transformant PREVB (▲), which has the MS promoter in the reverse orientation relative to the GUS reporter gene; and untransformed *N. plumbaginifolia* (♦). 4 MU, 4-methylumbelliferone.

needed to determine whether or not allantoicase and ureidoglycolate hydrolase are synthesized during senescence and, if so, whether or not they are localized to microbodies.

Regulation of MS Gene Expression

We have demonstrated MS gene expression during postgerminative growth (Graham et al., 1990) and in a range of senescing and detached organs. In addition to the expression in leaves, roots, cotyledons, and petals shown here, we have also detected variable levels of expression in cultured callus tissue and a very low level in fruits (data not shown). Occasionally, leaves that appeared green and healthy could be shown to contain some MS RNA. Furthermore, older leaves of dark-treated plants accumulated more MS RNA than younger leaves on the same plant. These observations together show that the expression of the MS gene is not organ or tissue specific but can be activated under the appropriate conditions in many tissues. The breakdown of lipid is probably common to all of these situations, and nucleotide breakdown may also be significant. It is probably more appropriate therefore to consider expression to be physiologically or metabolically regulated rather than developmentally regulated. A range of stresses to the plant including temperature or wounding might activate MS gene expression, possibly by triggering localized lipid breakdown. Such events might explain those few cases in which we have observed MS RNA in apparently healthy leaves.

Based upon MS gene expression patterns in higher plants and cell cultures, we propose a model that is consistent with all the available data and provides us with a set of hypotheses to test. The model shown in Figure 9 is based upon the premise that sugars (probably sucrose and others) repress MS gene expression and that products of lipid breakdown (acetate or acetyl coenzyme A are candidates) induce expression. Glucose repression of isocitrate lyase has been demonstrated in endosperm and cotyledons of castor bean seeds and in cotyledons of squash seeds (Lado et al., 1968). In cucumber seeds, the effect of exogenous metabolites on synthesis of glyoxylate cycle enzymes has not been investigated, but sucrose has been shown to inhibit lipid breakdown (Slack et al., 1977). The experiments of Kudielka and Theimer (1983a, 1983b) would indicate that sucrose repression dominates over acetate induction. Thus, MS gene expression is repressed in any tissue where sucrose is being synthesized (photosynthetic tissues) or is actively imported (roots, developing petals, seeds, and fruits), or is derived from assimilatory starch (roots and leaves at night). MS gene expression is induced in those tissues where sugar concentrations are low and lipid breakdown occurs. Such is the case in seed storage tissues during early postgerminative growth and in senescing or detached organs. We found that MS gene expression is more pronounced when detached leaves or cotyledons are incubated in the dark rather than in the light (I. Graham and J. McLaughlin, unpublished

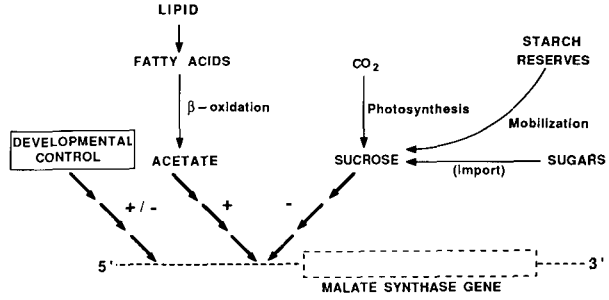

Figure 9. Model of Transcriptional Control of the MS Gene.

This model proposes that metabolic status, either in addition to or as an alternative to specific developmental signals, regulates transcription. We do not propose that acetate and sucrose interact directly with the gene or that they are necessarily the active metabolites, but rather that they characterize two different metabolic states. +, induction of transcription; −, repression of transcription.

results); under these conditions, photosynthesis is prevented, and chlorophyll and lipid breakdown is more rapid.

Our model predicts that the level of MS gene transcription responds sensitively to the relative concentrations of particular metabolites. If the ICL gene is similarly responsive, we see how the coordinate expression of both genes may be achieved (Comai et al., 1989). Where low levels of noncoordinate expression of MS and ICL are seen during seed development (Turley and Trelease, 1990), slight variations in the responsiveness of each gene may be occurring, which is to be expected if each has its own distinct promoter. In such developing seeds, sugar concentrations are finely balanced by the relative rates of import, catabolism, and conversion into food reserves. Because this model is consistent with that proposed by Sheen (1990) for the regulation of photosynthetic gene expression, it suggests that metabolic control may feature in many regulatory cascades during plant development.

METHODS

Plant Material

Cucumber seed (*Cucumis sativus* var Masterpiece) was obtained from W.K. McNair (Portobello, Edinburgh) and germinated as described previously (Becker et al., 1978). Three-day-old seedlings were transferred to individual pots and maintained under greenhouse conditions with 16 hr per day of supplementary lighting and day/night temperatures of 25 and 20°C, respectively. Transgenic *Nicotiana plumbaginifolia* plants, as described in Graham et al. (1990), were grown under the same conditions.

Maintenance of Excised Organs

The various organs were excised and placed on moist filter paper (Whatman) in Petri dishes that were then sealed with parafilm. The

dishes were incubated in permanent darkness at a constant temperature of 25°C.

Isolation of Total RNA

To maximize the yield of nucleic acids from small amounts of plant material, the method of Castresana et al. (1988) was used with minor adaptations. Plant material was ground to a fine powder with a pestle and mortar using liquid nitrogen. Frozen powder was placed in a prechilled 15-mL COREX tube, to which was added a minimum of 4 mL of grinding buffer (5 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% [w/v] sarcosyl, 2 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM Tris-Cl, pH 7.6). Each tube was then vortexed for 10 sec. For amounts of plant material greater than 1 g, 4 mL of grinding buffer per gram fresh weight of tissue was added.

Centrifugation was carried out at 5000*g* for 10 min in a rotor (model No. HB-4; Sorvall Instruments Div., Du Pont). The supernatant was removed to a fresh tube and to this was added an equal volume of phenol/chloroform (50:50 v/v). Each tube was then vortexed as above and centrifuged for 15 min at 15000*g*. The upper aqueous phase was removed to a 30-mL COREX tube, the volume was increased to 7.5 mL with sterile water, and the nucleic acids were precipitated with 2.5 volumes of ethanol (increasing the volume of the aqueous phase decreases the amount of salt that precipitates). After centrifugation for 10 min at 15000*g*, the resulting pellet was washed in 70% (v/v) ethanol, centrifuged as before, and all of the supernatant removed. The pellet was resuspended in 2.5 mL of sterile double-distilled water, and RNA was precipitated by adding 5 M lithium chloride to a final concentration of 2 M and storing the sample overnight at 4°C. Each tube was centrifuged as was done for ethanol precipitation; the pellet was washed in 70% ethanol and resuspended in 2.5 mL of water. The ethanol precipitation step was repeated to remove excess salt, and the RNA pellet was finally resuspended in water at a final concentration of 1 mg mL^{-1}.

RNA Gel Blot Analysis

Total RNA was fractionated by electrophoresis through an agarose/formaldehyde denaturing gel system and transferred to Hybond-N membranes as described in the manufacturer's protocol (Amersham International). Prehybridization and hybridization were carried out in 50% (v/v) formamide at 42°C as described in the same protocol. The pBSMS1.9 EcoRI fragment (Graham et al., 1989) was used as a hybridization probe. This fragment was radiolabeled by incorporation of α-^{32}P-dCTP by the random primer extension method of Feinberg and Vogelstein (1984).

Chlorophyll Determination

Leaf tissue was assayed for chlorophyll content following the procedure described by Arnon (1949), in which the chlorophyll was extracted by solubilization in 80% acetone.

Fluorogenic Assay of GUS Activity in Detached *N. plumbaginifolia* Leaves

Individual leaves were ground to a fine powder in liquid nitrogen, and 50 mg of this material was weighed into prechilled microcentrifuge tubes. To this was added 100 mL of β-glucuronidase (GUS) extraction buffer, and the assay was carried out exactly as described by Jefferson (1987).

In Situ Hybridization

In situ hybridization procedures were carried out essentially according to Langdale et al. (1988), using ^{35}S-labeled malate synthase (MS) RNA and paraffin-embedded sections. The 0.56-kb EcoRI cDNA from pMS730 (Smith and Leaver, 1986) was subcloned into pBluescript KS– (Stratagene), and the bacteriophage RNA polymerase promoters were used to synthesize ^{35}S-labeled sense and antisense MS RNA in vitro.

To visualize internal structure, representative sections were stained with Fast Green and Safranin O. Photography was carried out using a microscope (model Dialux 20; E. B. Leitz Inc., Rockleigh, NJ) and tungsten film. Sections of 3-day-old and senescing cotyledons were mounted on the same slides and thus were treated identically during hybridization with sense or antisense RNA.

Isolation of Protein and Protein Gel Blot Analysis

Protein preparations were carried out according to Nelson et al. (1984). To guard against protease activity, particularly in the senescing samples, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM aminocaproic acid were added to the homogenization buffer (18% w/v sucrose, 10 mM magnesium chloride, 100 mM Tris-Cl, pH 8.0, 40 mM β-mercaptoethanol, and 0.1% w/v SDS) just before use.

Following SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes (Amersham International) for immunoreaction with MS antiserum as described by Smith and Leaver (1986). Alkaline phosphatase anti-IgG–conjugated antibody was used to visualize cross-reactivity on the membrane.

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