Glyoxysomal Malate Synthase of Cucumber: Molecular Cloning of a cDNA and Regulation of Enzyme Synthesis during Germination

Steven M. Smith* and Christopher J. Leaver
Department of Botany, University of Edinburgh, The King’s Buildings, Mayfield Road, Edinburgh EH9 3JH, Scotland

ABSTRACT
A cDNA clone for the glyoxysomal enzyme malate synthase was isolated from a cDNA library made with polyadenylated RNA from the cotyledons of germinating Cucumis sativus L. This cloned DNA sequence was used as a probe to characterize changes in the amounts of malate synthase gene transcripts in cotyledons of cucumber seeds grown both in the light and in the dark. Malate synthase gene transcripts increase in amount to a peak at day 3 or day 4, and thereafter decline. In the light, this rate of decline is significantly greater than in the dark. Measurement of the changes in the amounts of malate synthase by assaying enzyme activity directly, and by immunological reaction with a specific antiserum indicate that the developmentally regulated synthesis of malate synthase in germinating cucumber is brought about primarily by changes in the amount of malate synthase gene transcripts, rather than through a control of translation. Similarly, the effect of light on the amount of malate synthase correlates precisely with its effect on the abundance of malate synthase gene transcripts.

The cotyledons of germinating seeds of cucumber (Cucumis sativus L.) provide us with an excellent opportunity to study and to understand the genetic and environmental controls of gene expression during the growth and differentiation of a plant organ. Within a period of only a few days of germination, the cotyledon develops from a protein and lipid-storing heterotrophic organ, into a photosynthetic one. The changes associated with this transition occur in the absence of cell division, are dependent upon illumination, and can be modified by the exogenous application of plant growth substances (3, 8, 26, 27). This tissue is amenable to biochemical analysis (2, 3). Studies of gene structure and function are facilitated by the relatively low nuclear DNA content of C. sativus (about 10⁹ base pairs per haploid genome) (5), by its susceptibility to genetic transformation by Agrobacterium tumefaciens (7), and by its ability to regenerate from cultured leaf explants (15).

Previous work from this laboratory has described the changes which take place in the profiles of storage proteins, lipids, ribosomal RNAs, messenger RNAs, and in some enzymes of the glyoxysomes and plastids (2, 3, 26, 27). The synthesis of glyoxysomal malate synthase (EC 4.1.3.2) and isocitrate lyase (EC 4.1.3.1) is of particular importance because these enzymes are crucial for, and unique to, the glyoxylate cycle which catalyzes the net conversion of acetate (from lipid hydrolysis) into succinate (9).

Our aim is to understand the mechanisms by which the developmentally-regulated synthesis of MS² and ICL is achieved. Both enzymes appear in the cotyledons after 2 d of germination and reach a peak of activity on d 3 or 4. During this time, the lipid reserves are mobilized. After this peak of enzyme activity, both MS and ICL decline rapidly until they are undetectable by d 5 or 6 of germination, when photosynthetic competence has been achieved, in light-grown seedlings. If the seedlings are germinated in the absence of light, however, MS and ICL remain in the cotyledons beyond d 5 and 6, and chloroplasts do not develop (26, 27). These changes in the activities of MS and ICL are mirrored by changes in the amounts of their messenger RNAs which are translatable in vitro (27). The aim of our current research is to clone DNA sequences encoding these enzymes so that we may begin to analyze the structures of their genes in relation to the control of gene expression. We anticipate that these studies will also contribute to work aimed at understanding how these enzymes are imported into the glyoxysomes during microbody biogenesis (10, 12, 14). Here we report the cloning of cDNA encoding MS, and show that the synthesis of MS in germinating seeds is controlled either by the rate of transcription of MS genes or by the rate of turnover of their transcripts.

MATERIALS AND METHODS

Growth of Plant Material. Seeds of Cucumis sativus L. var. Long Green Ridge were germinated as described previously (2). Harvesting of cotyledons and preparation of tissue homogenates for enzyme and protein analyses were also as described (3).

cDNA Cloning and Sequencing. Polyadenylated RNA was prepared from cotyledons which had been germinated for 60 h, as described previously (27). Complementary DNA was synthesized for cloning using established procedures (16) which included the use of avian myeloblastosis virus reverse transcriptase, DNA polymerase (Klenow fragment) from Escherichia coli, and S1 nuclease. The 3' ends of the cDNA were tagged with deoxycytidine residues and cloned into pBR322 which had been tagged with deoxyguanidine residues at the Pst 1 endonuclease site (16). Colony hybridizations using 32P-labeled cDNA probes were carried out as described previously (22). Fragments of cloned cDNA were subcloned into coliphage M13mp18 and M13mp19 (28) for sequencing by means of the dideoxynucleotide chain-terminating procedure (21, 22).

Protein Synthesis in Vitro. Polyadenylated RNA was translated in a cell-free extract of wheat germ as described previously.

---

1 Supported by the Agricultural and Food Research Council.

2 Abbreviations: MS, malate synthase; ICL, isocitrate lyase.
Specific mRNAs were selected by hybridization to recombinant plasmids as described previously (4). Translation products were immunoprecipitated (27) and isolated using protein A-Sepharose (6). Polypeptides labeled with [35S]methionine were fractionated by electrophoresis through 16% (w/v) polyacrylamide gels in the presence of SDS (13) and visualized by autoradiography.

**Enzyme Assay.** Malate synthase was assayed as described previously (3). One enzyme unit corresponds to the conversion of 1 nmol of substrate into product/min.

**Western Blot Analyses.** Polypeptides fractionated by PAGE in the presence of SDS were transferred to nitrocellulose sheets by electroblotting for immunoreaction with malate synthase antiserum (24). The nitrocellulose sheets were then incubated in the presence of 5% (w/v) BSA for 5 h at 37°C before being incubated with antiserum for 16 h at room temperature. The antibodies remaining after subsequent washing of the filters were visualized by incubation with 125I-labeled protein A (from *Staphylococcus aureus*) and autoradiography.

**Northern Blot Analyses.** RNA was fractionated by electrophoresis through agarose gels containing 6% (w/v) formaldehyde, transferred to nitrocellulose by blotting and hybridized in the presence of 50% (v/v) formamide (16). The probe used was pMS730 which was labeled by nick-translation (20) in the presence of α-[32P]dCTP (Amersham).

**Densitometry.** Autoradiographs were scanned in a Quick Scan R and D densitometer (Helena Laboratories). The results were quantified by measuring both peak heights and peak areas, each of which yielded similar results.

**RESULTS**

**Isolation of a cDNA Clone Encoding Malate Synthase.** Cotyledons of *C. sativus* contain maximal amounts of MS and ICL mRNA activity, as determined by in vitro translation, after 48 to 72 h germination (27). Therefore, polyadenylated RNA isolated from cotyledons after 60 h germination (60 h RNA) was copied into DNA and cloned in *E. coli* using pBR322 (13). Approximately 1500 such clones were analyzed by means of colony hybridizations, using probes made from the following polyadenylated RNA preparations which contained different amounts of MS and ICL mRNA activity. The clones were first screened by hybridizing with 32P-labeled cDNA made from RNA of green leaves. The 800 clones hybridizing most strongly (not shown) were analyzed no further since MS and ICL mRNAs are not detected in leaves (SM Smith, unpublished observations). The remaining 700 clones were subsequently hybridized with 32P-labeled cDNA made from 60 h RNA. Of the 60 clones hybridizing to this probe, 12 also hybridized to a probe enriched for MS and ICL mRNA activities by size fractionation (4, 27) of the same 60 h RNA preparation (data not shown). These 12 clones were analyzed further by hybrid-selected-translation experiments (4), and the polypeptide products tested for reactivity with antisera specific for MS and ICL.

Several clones were found to encode polypeptides of between 55,000 and 65,000 mol wt, one of which was specifically immunoprecipitated by the MS antisera. The hybrid-selected-translation product of clone 730 (lane 3) is immunoprecipitated by the MS antisera (lane 1) but not by the ICL antisera (lane 2) (Fig. 1). Lanes 5 and 6 show the immunoprecipitation of the products of translation of total 60 h RNA (lane 4) with the MS and ICL antisera respectively. Controls which employed other plasmid DNAs instead of that from clone 730 failed to select any RNA which could be translated in vitro to produce MS-related polypeptides (not shown). Thus, clone 730 encodes a polypeptide which comigrates with MS synthesized in vitro (some distortion of bands in Fig. 1, lanes 1 and 4, is due to interference by unlabeled immunoglobulin heavy chain and BSA), and is immunologically related to MS. In addition to the result obtained with the antisera raised against *C. sativus* MS (Fig. 1), the polypeptide encoded by clone 730 is immunoprecipitated by an antisera raised against castor bean (*Ricinus communis*) MS (not shown). Our conclusion that clone 730 encodes MS is also supported by Northern blot hybridization experiments (see below).

**Physical Characterization of the Malate Synthase cDNA Clone.** The plasmid containing the MS cDNA insert is designated pMS730. When this DNA is cleaved with restriction endonuclease *Pst I*, the complete cDNA fragment of approximately 560

---

**FIG. 1.** Identification of malate synthase cDNA clone by hybrid-selected-translation. Polypeptides were synthesized in the presence of [35S]methionine in a wheat germ system programmed with polyadenylated RNA from cotyledons of cucumber seeds germinated for 60 h. The products shown here were visualized by autoradiography following fractionation by PAGE in the presence of SDS. The products shown in lanes 1, 2, and 3 were synthesized in response to RNA selected by hybridization of total polyadenylated RNA to pMS730. Those shown in lanes 4, 5, and 6 were synthesized in response to the total polyadenylated RNA. Polypeptides immunoprecipitated by an antisera raised against malate synthase are shown in lanes 1 and 5. Those immunoprecipitated by an antiserum raised against inositol lyase are shown in lanes 2 and 6. The mobilities of proteins of known mol wt are indicated on the left, and are: serum albumin (66,000), catalase (58,000), aldolase (39,000), carbonic anhydrase (29,000), trypsin inhibitor (20,000), and myoglobin (17,000). The mobilities of ICL and MS are indicated on the right.
base pairs is excised from the vector (not shown). This \textit{Pst} 1 fragment was sequenced using the dideoxynucleotide chain terminating method (21, 22) following subcloning into M13 vectors (28). Figure 2 shows the sequence and the restriction endonuclease sites which were used to generate smaller fragments for cloning into M13. The sequence at one end of the cDNA molecule is comprised of 34 adenine residues and 21 cytosine residues, corresponding to the poly A tail and 3' homopolymer tail, respectively. The opposite end of the sense (noncoding) strand of the cDNA insert terminates with 26 guanine residues. Between these homopolymer ends of the cDNA are 481 nucleotides. When translated in all three reading frames using the 'universal' genetic code, it is possible to predict three alternative C-terminal peptide sequences. These sequences are comprised of 1, 9, and 94 amino acids in turn, which leave 3' untranslated regions of 478, 452, and 198 nucleotides, respectively (Fig. 2).

We do not know if one of these sequences corresponds to the MS polypeptide. Further cloning and sequencing experiments will be necessary to confirm the identity of the MS cDNA clone, since the only polypeptide sequence available to us is derived from the N-terminus of the enzyme. The available nucleotide sequence shows that the 3' untranslated region of the progenitor mRNA is rich in adenine + uracil residues (65% for the shortest predicted sequence shown in Fig. 2). Despite this high adenine + uracil content, the hexanucleotide sequence 5' AUAADA 3' is not found, supporting the view that this sequence does not comprise part of a recognition sequence for the polyadenylation of plant mRNAs (1, 18, 23) as it does in other eukaryotes (19).

\textbf{Regulation of Malate Synthase Synthesis during Germination.}

Cucumbers were imbibed and germinated both in darkness and in the light as described previously (27). Cotyledons were collected for analysis from dry seeds, from seeds imbibed in water for 16 h at 4°C (referred to as day 0 cotyledons) and from seeds or seedlings after each successive day of germination until the 7th d. Total soluble protein was prepared from samples of cotyledons at each stage, for measurements of MS activity, and total protein was prepared by solubilization with SDS for immunological ('Western blot') analysis of MS. Total RNA was also isolated at each stage, for the analysis of the abundance of MS transcripts during germination.

Coty-ledon proteins dissolved in SDS were fractionated by electrophoresis through a 16% (w/v) polyacrylamide gel and transferred to nitrocellulose electrophoretically. MS was visualized by incubation with MS antiserum followed by 125I-labeled protein A (from \textit{S. aureus}) and autoradiography (Western blot analysis) (24). Figure 3a shows the region of the resulting autoradiograph which encompasses polypeptides of between 50,000 and 65,000 mol wt. No other polypeptides react reproducibly with the MS antiserum (not shown). This result shows how the amount of MS polypeptide changes during germination in the dark and in the light. A semiquantitative estimate of these changes was obtained by densitometric analysis of the autoradiograph shown. These results are shown in Figure 3b where they are compared with the MS activity measured at each stage. The data presented in Figure 3 are consistent with those obtained previously (27), in which enzyme activity was measured, but extend them by showing that the developmentally regulated changes in MS activity are the result of changes in the amount of MS polypeptide. These analyses were carried out with tissue homogenates equivalent to a fixed number of cotyledons at each stage and for each treatment, and show the changes in amount of MS per unit number of cells, since no cell division occurs during germination (3).

The observation that MS protein and enzyme activity reach a maximum at d 3 in light-grown tissue but at d 4 in dark-grown tissue might be due to the effect of illumination in raising the temperature of the tissue during its development. The striking difference between light-grown and dark-grown tissues is that MS is seen to persist in the cotyledons much longer in the dark than in the light, in agreement with previous results (27). The studies of \textit{Weir et al.} (27) showed that the amounts of MS mRNA translatable in vitro change with a developmental program similar to that of the enzyme activity, but slightly in advance of it. The possibility exists that the changes in the amount of translatable MS mRNA measured in that study are not simply a consequence of changes in the total amount of MS gene transcripts, but rather due to changes in the translatibility of the isolated RNA. To test this possibility, total RNA isolated from the cotyledons described above was subjected to Northern blot hybridization analysis with the cloned MS cDNA probe. Since the amount of RNA isolated by phenol extraction of cotyledons changes during germination and in response to light (Fig. 4), we did not load onto the agarose gel, equal amounts of RNA from each day of development. Instead, we analyzed RNA from a fixed number of cotyledons, thus providing an indication of the amount of MS-specific transcripts per unit number of cotyledon cells. The northern blot results show a single band of hybridization to the cDNA probe, corresponding to an RNA molecule of approximately 2000 nucleotides (Fig. 5). The intensity of this band of hybridization varies in parallel with the changes in the amount of MS enzyme (Fig. 3). To obtain a direct comparison of the results in Figures 3 and 5, the autoradiograph shown in the latter was quantitated by densitometry. The results are presented together with those for the Western blot analysis in Figure 6. These data are consistent with our conclusion that pMS730 encodes MS, and show that the changes in the amounts of MS polypeptides and transcripts are very closely coordinated, except that the changes in the amount of transcripts appear to precede those of the enzyme.
Fig. 3. Changes in amounts of malate synthase in cotyledons of seeds germinated in the light and in darkness. a, Total cotyledonary protein was solubilized with SDS and fractionated by PAGE. The polypeptides were transferred to nitrocellulose and incubated with malate synthase antiserum followed by 125I-labeled protein A, and subsequent autoradiography. The region of the autoradiograph encompassing polypeptides of M, between 50,000 and 65,000 is shown. Protein equivalent to 1/40 cotyledon was loaded in each lane. b, The amount of immunoreactive malate synthase was estimated by densitometry of the autoradiograph shown in (a). Malate synthase activity was assayed in extracts of soluble protein from cotyledons. DS corresponds to dry seed and 0 days of germination represents seeds imbied for 16 h at 4°C. Subsequent days of germination are timed from sowing at the end of the imbibition period.

Fig. 4. Amounts of total RNA extracted from cotyledons during germination. Total RNA was isolated by phenol extraction of cotyledons, followed by sodium chloride and ethanol precipitations. The RNA dissolved in water was quantitated by spectrophotometry. The amounts of RNA loaded onto agarose gels for Northern blot analyses (Fig. 5) are shown on the right. Days of germination are as described in the legend to Figure 3.

**DISCUSSION**

The data presented establish that pMS730 contains a cDNA sequence encoding MS. The evidence is as follows: (a) the cDNA uniquely selects mRNA which when translated *in vitro* yields a polypeptide of 57,000 mol wt, comigrating with MS during gel electrophoresis; (b) this polypeptide is immunoprecipitated by antisera raised against MS from *C. sativus* and *R. communis*, but not by antisera raised against ICL of *C. sativus*; (c) the cDNA hybridizes to a seemingly single species of RNA of approximately 2000 nucleotides, about 500 nucleotides longer than the required minimum to encode MS; and (d) the amount of this RNA changes during germination and in response to light in accordance with the changes in the amount of MS.

This MS cDNA clone was the only one isolated from the 1500 clones made with RNA from cotyledons at a stage when glyoxysome biogenesis is taking place. If the cloning of different mRNA sequences occurs with equal efficiency, we could conclude that MS mRNA probably represents no more than about 0.1% of the polyadenylated RNAs in this tissue. Since parallel experiments failed to identify a cDNA clone encoding ICL, the mRNA for this enzyme may be no more abundant. These assumptions are consistent with *in vitro* translation experiments which yield numerous polypeptide products among which MS and ICL can only be recognized by immunoprecipitation (27).

The sequence data for pMS730 shows that the cDNA is derived from the 3' end of the mRNA. We do not know if the coding information for the carboxyl-terminal amino acids of MS is present in this clone, since we are unaware of any published nucleotide or amino acid sequences for MS from any organism. However, an open reading frame extending from the 3' end of the clone and comprising of 94 codons is a good candidate for this information. The deduced amino acid sequence is notable for the high frequency of charged residues (Glu + Asp = 16; Arg + Lys = 16). It might be of interest in the future to investigate how such a polar region of the MS polypeptide is disposed during MS import into the glyoxysome and subsequent anchoring to the inner surface of the glyoxysomal membrane (11). It might be that these charged amino acid residues participate in the specific aggregation phenomenon which accompanies MS import (12). Current research is aimed at isolating and determining the complete nucleotide sequence encoding MS which will enable us to begin to investigate MS structure, particularly as it relates to its import into microbodies.

These studies establish that the developmental regulation of MS synthesis in cotyledons of germinating cucumber seeds is brought about by changes in the steady-state amounts of the transcripts encoding this enzyme. These changes occur in the absence of cell division and are therefore the result of changes either in the rate of transcription of MS genes or in the rate of turnover of the resulting RNA. Similar results have been found for the regulation of ICL gene expression in germinating castor beans (17). Germination of cucumber seeds in the presence or absence of light dramatically affects the time for which MS gene transcripts are found in the cotyledons. In the dark, MS gene transcripts persist until the 7th d when the amount per cell is still 45% of the maximum on the 4th d (Fig. 6). In contrast, in the light, MS gene transcripts are below the level of detection 2 d after the maximum amount of the 3rd d. Thus, in the light either transcription stops sooner, or the turnover of transcripts is much more rapid than in the dark. Which ever alternative is correct, we can conclude that the half-life of MS gene transcripts
after the 3rd d in the light can be no more than 10 h (Fig. 6).

We believe that, with the isolation of a DNA probe encoding MS, we are now in a position to investigate, in detail, the molecular basis for the developmental program which leads to the activation of a specific plant gene, followed 48 h later by its inactivation. Together with the effect of light in regulating at least the second of these changes in gene expression, and with the effects of plant growth substances in affecting developmental processes in the cotyledons of some members of the Cucurbitaceae (8, 17, 25), we anticipate that studies of the early growth of cucumber seedlings will increase our understanding of the genetic and environmental controls of plant growth and development.

Fig. 5. Northern blot analyses of malate synthase gene transcripts in cotyledons of seeds germinated in the light and in darkness. Total RNA described in Figure 4 was fractionated by electrophoresis through agarose gels containing 6% (w/v) formaldehyde, and transferred to nitrocellulose filters by blotting. The amounts of RNA loaded into each gel lane are indicated in Figure 4. Filters were hybridized with pMS730 which had been 32P-labeled by nick translation. Autoradiographs of two complete gels are shown here. The mobilities of 25S, 18S, and 5.8S ribosomal RNAs were determined by ethidium bromide staining of parallel gel lanes. Days of germination are as described in the legend to Figure 3.

Acknowledgments—We thank J. M. Lord for a gift of antiserum against malate synthase of R. communis, H. Kindl for a gift of antiserum against malate synthase of C. sativus, W. Becker for gifts of antiserum against malate synthase and isocitrate lyase of C. sativus, D. Baulcombe and R. McGookin for constructing the cDNA clones, and S. Anderson for DNA sequencing.

LITERATURE CITED
15. MALPENSA, S, A NADOLSKA-ORCZYK 1983 In vitro culture of Cucumis sativus. 1. Regeneration of plantlets from callus formed by leaf explants. Z Pflanzen-
MALATE SYNTHASE GENE EXPRESSION IN CUCUMBER

physiol 111: 273–276
17. MARTIN, C, JR BESCHING, DH NORTHCOTE 1984 Changes in levels of transcripts in endosperm of castor beans treated with exogenous gibberellic acid. Planta (Berl) 162: 68–76
24. TOWBIN, H, T STAELIN, J GORDON 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. Proc Natl Acad Sci USA 76: 4350–4354