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Molecular Characterization of the Non-biotin-containing Subunit of 3-Methylcrotonyl-CoA Carboxylase*

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The biotin enzyme, 3-methylcrotonyl-CoA carboxylase (MCCase) (3-methylcrotonyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.4), catalyzes a pivotal reaction required for both leucine catabolism and isoprenoid metabolism. MCCase is a heteromeric enzyme composed of biotin-containing (MCC-A) and non-biotin-containing (MCC-B) subunits. Although the sequence of the MCC-A subunit was previously determined, the primary structure of the MCC-B subunit is unknown. Based upon sequences of biotin enzymes that use substrates structurally related to 3-methylcrotonyl-CoA, we isolated the MCC-B cDNA and gene of Arabidopsis. Antibodies directed against the bacterially produced recombinant protein encoded by the MCC-B cDNA react solely with the MCC-B subunit of the purified MCCase and inhibit MCCase activity. The primary structure of the MCC-B subunit shows the highest similarity to carboxyltransferase domains of biotin enzymes that use methyl-branched thioesters as substrate or products. The single copy MCC-B gene of Arabidopsis is interrupted by nine introns. MCC-A and MCC-B mRNAs accumulate in all cell types and organs, with the highest accumulation occurring in rapidly growing and metabolically active tissues. In addition, these two mRNAs accumulate coordinately in an approximately equal molar ratio, and they each account for between 0.01 and 0.1 mol % of cellular mRNA. The sequence of the Arabidopsis MCC-B gene has enabled the identification of animal paralogous MCC-B cDNAs and genes, which may have an impact on the molecular understanding of the lethal inherited metabolic disorder methylcrotonylglycinuria.

The biotin enzyme, 3-methylcrotonyl-CoA carboxylase (MCCase) (3-methylcrotonyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.4), catalyzes the carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA. The reaction catalyzed by this enzyme appears to interconnect metabolic pathways of leucine catabolism and isoprenoid metabolism (1–5) (illustrated in Fig. 1). MCCase is not universally distributed, but it occurs in animals, plants, and some bacterial species (1, 5, 6, 46). In humans, deficiencies in MCCase result in the lethal condition methylcrotonylglycinuria (7).

Biotin enzymes have three structurally conserved functional domains: the biotin carboxylase domain, which catalyzes the carboxylation of biotin; the biotin carboxyl carrier domain, which carries the biotin prosthetic group; and the carboxyltransferase domain, which catalyzes the transfer of a carboxyl group from carboxybiotin to the organic substrate specific for each biotin enzyme (1). The carboxyltransferase domain of MCCase catalyzes the transfer of a carbonyl group from carboxybiotin to methylcrotonyl-CoA. Presumably because of differences in substrate specificities, carboxyltransferase domains are less conserved among biotin enzymes than are the biotin carboxylase or the biotin carboxyl carrier domains.

MCCase is composed of two nonidentical subunits: a larger, biotin-containing subunit of approximately 85 kDa (MCC-A), and a smaller, non-biotin-containing subunit of approximately 60 kDa (MCC-B) (1–4, 8–10). The amino acid sequence of MCC-A has been deduced from the corresponding cDNA clones, and this subunit contains the biotin carboxylase and biotin carboxyl carrier domains (11–13). Despite the metabolic importance of MCCase, the sequence and genetic regulation of the MCC-B subunit had not been characterized from any organism. We report here the primary structure of the MCC-B subunit from Arabidopsis, deduced from the isolated cDNA and gene, and that the MCC-A and MCC-B mRNAs accumulate in coordinate spatial and temporal patterns.

EXPERIMENTAL PROCEDURES

Reagents—The following materials were obtained from the Arabidopsis Biological Resource Center (Ohio State University); the Arabidopsis expressed sequence tag cDNA clone 1451L7; an Arabidopsis (ecotype Landsberg erecta) genomic library in the vector A10X (14); and a size-selected cDNA library (2–3-kb inserts) prepared from poly(A) RNA isolated from 3-day-old Arabidopsis (ecotype Columbia) seedling hypocotyls in the vector λZAPIII (15). An Arabidopsis (ecotype Columbia) cDNA library prepared from poly(A) RNA isolated from developing siliques in vector Actg10 was a gift from Dr. David W. Meinke (Oklahoma State University).

Plant Materials—Arabidopsis plants (ecotype Columbia) were grown under the conditions described previously (16). To obtain organs from 3-day old seedlings, sterile Arabidopsis seeds were imbibed in Petri plates on sterile, moist filter paper, and seedlings were harvested 3 days later. All other organs were harvested from plants grown in soil. The

* This work was supported in part by National Science Foundation Grant IBN-9507549 (to E. S. W. and B. J. N.), a Herman Frasch award (to E. S. W.), and an Iowa State University Graduate College research award (to E. S. W.). This is Journal Paper J-18155 of the Iowa Agricultural and Home Economics Experiment Station (Ames, IA), Project Nos. 2997 and 2913; supported by Hatch Act and State of Iowa funds. The biotin enzyme, 3-methylcrotonyl-CoA carboxylase (MCCase) (3-methylcrotonyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.4), catalyzes the carboxylation of 3-methylcrotonyl-CoA (3-methylcrotonyl-CoA carboxylase) (3-methylcrotonyl-CoA carboxylase) (3-methylcrotonyl-CoA carboxylase) to form 3-methylglutaconyl-CoA. The reaction catalyzed by this enzyme appears to interconnect metabolic pathways of leucine catabolism and isoprenoid metabolism (1–5) (illustrated in Fig. 1). MCCase is not universally distributed, but it occurs in animals, plants, and some bacterial species (1, 5, 6, 46). In humans, deficiencies in MCCase result in the lethal condition methylcrotonylglycinuria (7).

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Histological sections to hybridization with the respective 35S-labeled primers AM819 (5'-GGCAGGAATGTAGGCACCAC-3') and re-synthesis of 35S-labeled sense RNA probes. The radioactivity associated with each hybridization, and hybridization with radioactive 

The presence of this 66.5-kDa protein and the concomitant absence of the recombinant GST protein in p145GEX-containing cultures is consistent with the addition of 397 amino acids to the 27.5-kDa GST protein. This 66.5-kDa recombinant protein was recovered in inclusion bodies, and its purity was determined by SDS-PAGE. These analyses indicated that the 66.5-kDa recombinant protein accounted for over 80% of the protein associated with the isolated inclusion bodies. Hence, this protein was purified by preparative SDS-PAGE and used to generate antisera.

MCCase was purified from soybean seedlings by procedures previously used to purify this enzyme from carrot (8); these procedures included chromatography on Cibacron Blue, Q-Sepharose, and monomeric-avidin affinity matrices. Purification of the enzyme was monitored by determining the specific activity of MCCase in each fraction (8, 20). In addition, proteins were analyzed by SDS-PAGE (22), nondenaturing PAGE (47), and Western blotting (23). Purification of MCCase was repeated more than four times with similar results. MCCase and acetyl-CoA carboxylase activities were determined as the 3-methylcrotonyl-CoA- and acetyl-CoA-dependent rates of conversion of radioactivity from NaH14CO3 into an acid-stable product (5).

Antiserum was generated by immunizing rabbits with 1.25 mg of SDS-PAGE-purified protein antigen emulsified in Freund's complete adjuvant. One month later and at 2-week intervals thereafter, rabbits were challenged with intramuscular injection of protein antigen emulsified in Freund's incomplete adjuvant. Serum was collected 2.5 months after the primary injection.

**RESULTS AND DISCUSSION**

**Isolation of a cDNA Coding for a Subunit of a Biotin Enzyme**—To identify the MCC-B cDNA, we searched the Arabidopsis expressed sequence tag data base for sequences similar to carboxyltransferase domains of biotin enzymes, specifically those utilizing substrates chemically similar to methylcrotonyl-CoA.

The Arabidopsis partial expressed sequence tag cDNA clone 145L17T7 was identified by the BLAST algorithm because of its similarity to the carboxyltransferase domain of propionyl-CoA carboxylases. The 145L17T7 clone contains a 1330-nucleotide partial cDNA, which codes for 397-residue polypeptide. The corresponding full-length cDNA was isolated in two steps: first, by screening a Agt110 Arabidopsis cDNA library, which resulted in the isolation of p5CMB (a 1790-nucleotide partial cDNA clone), and second, by screening a size-selected AZAPII Arabidopsis cDNA library, which resulted in the isolation of the near-complete cDNA clone pMCC-B. The 1890-nucleotide MCC-B cDNA encodes a 587-amino acid polypeptide with a calculated molecular mass of 64 kDa. The 5' untranslated region is at least 78 nucleotides long, and the 3'-untranslated region contains the eukaryotic polyadenylation signal sequence AUAAA 29 nucleotides upstream of the poly(A) tail (24). The MCC-B cDNA hybridizes to a single mRNA of approximately 2.9 kb, indicating that it is nearly full-length.

**Identification of the Enzymatic Function of MCC-B**—To identify the biochemical function of the protein encoded by the MCC-B cDNA, we expressed a portion of this cDNA in E. coli as a GST fusion protein (termed p145GEX) (Fig. 2A). The expressed protein was used to generate anti-p145GEX serum.

MCCase was purified from 5-day-old soybean seedlings (Table I) (20) using a procedure similar to one previously used to purify this enzyme from carrot (8). Typically, this procedure gave a purified MCCase preparation with a specific activity about 400-fold higher than that found in the crude extract; in

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**Fig. 1.** Postulated interconnecting role of MCCase in metabolism. Leucine is catabolized to acetyl-CoA and acetoacetate in a mitochondrial pathway requiring MCCase in plants, animals, and some bacteria (1, 42, 46). The mevalonate shunt, identified in animals and plants (4, 44), metabolizes mevalonic acid (MVA) to acetyl-CoA and acetoacetate via MCCase and thus funnels carbon away from the biosynthesis of isoprenoids. Catabolism of isoprenoids to acetyl-CoA may proceed via geranyl-CoA and methylcrotonyl-CoA (MC-CoA) and requires geranyl-CoA carboxylase, a plastidic biotin-containing enzyme (45), and MCCase; this pathway is similar to that proposed for several pseudomonad species (2, 3). MC-CoA, methylglutaconyl-CoA, HMG-CoA, hydroxymethylglutaryl-CoA.
four repetitions of this purification, specific activity ranged between 300 and 650 milliunits/mg. These MCCase preparations were judged near homogeneous based upon two criteria. Analysis of these preparations by nondenaturing PAGE (47) revealed the presence of a single protein that migrated with a molecular weight of about 900,000 (Fig. 2D, lane 1). We concluded that this protein is MCCase because it contains biotin, which was detected with 125I-streptavidin-Western analyses (Fig. 2D, lane 2), and an Arabidopsis seedling extract (data not shown). Attempts to further purify MCCase by gel-filtration chromatography on Sephacryl S-400 did not increase the specific activity of the preparation. Furthermore, MCCase activity eluted from the Sephacryl S-400 column as a single peak corresponding to a molecular weight of about 900,000. This MCCase preparation contains biotin and reacts with anti-MCC-A serum (data not shown). Based on the structure of MCCase from other plant sources (8–10, 46) and from animals (21, 25) and bacteria (26, 27), the smaller, 60-kDa polypeptide was tentatively identified as the nonbiotinylated subunit of MCCase. Evidence in support of this identification was obtained by subjecting the purified MCCase preparations to nondenaturing PAGE followed by SDS-PAGE. In these analyses, the 90-kDa MCCase protein contained both the 85-kDa MCCase subunit and the 60-kDa polypeptide, and these were the only polypeptides detected in these preparations. These experiments were conducted with both the monomeric avidin affinity purified MCCase fraction and the MCCase-containing fraction obtained following a subsequent gel filtration chromatography purification step. These findings imply that the 60-kDa polypeptide is the MCC-B subunit.

Evidence in support of the conclusion that the pMCC-B clone codes for the nonbiotinylated subunit of MCCase was obtained from immunological analyses using the anti-145GEX serum. SDS-PAGE and Western analyses of soybean (Fig. 2C, lane 2) and Arabidopsis (Fig. 2C, lane 4) seedling extracts show that the anti-145GEX serum reacts with a single polypeptide in

### Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>milliunits</td>
<td>milliunits/mg</td>
<td>fold arbitrary units/mg</td>
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<tr>
<td>Crude extract</td>
<td>714.3</td>
<td>662.9</td>
<td>0.9</td>
<td>1.0</td>
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<tr>
<td>0–15% PEG</td>
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<td>594.5</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Quercetin Blue</td>
<td>103.7</td>
<td>724.3</td>
<td>6.8</td>
<td>7.6</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>80.1</td>
<td>548.7</td>
<td>6.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Monomeric avidin</td>
<td>0.9</td>
<td>302.9</td>
<td>336.6</td>
<td>374</td>
</tr>
</tbody>
</table>

* Determined by SDS-PAGE/Western analysis of each fraction, using 125I-streptavidin for the MCC-A subunit and anti-145GEX serum for the MCC-B subunit. The intensity of each subunit-band was quantified with the use of a PhosphorImager.

** ND, not determined.**
acetyl-CoA carboxylase activity. Specifically inhibited MCCase activity, without affecting MCCase. Twenty-nine hybridizing plaques (out of the 3.0 
late the gene encoding the non-biotin-containing subunit of 
MCCase—Arabidopsis—Southern blot analysis of 
ments establish that pMCC-A codes for the non-biotin-contain-
MCC-B was screened by hybridization with the 
3-Methylcrotonyl-CoA Carboxylase 5585 
FIG. 3. Immunoinhibition of MCCase activity. Aliquots of Arabidopsis extracts were incubated with the indicated amounts of either preimmune control serum (○) or anti-145GEX serum (●). Following a 1-h incubation on ice, each aliquot was assayed in triplicate for acetyl-CoA carboxylase (A) and MCCase (B) activity.

each extract that is about 60 kDa. In addition, this antiserum reacts with the 900-kDa purified MCCase protein (Fig. 2D, lane 3). To further demonstrate that pMCC-B codes for the nonbiotinylated subunit of MCCase, the various fractions obtained during the purification of the soybean MCCase were subjected to SDS-PAGE and Western blot analyses with either 125I-streptavidin or anti-145GEX serum. These characterizations demonstrate that there is a one-to-one correspondence between the specific activity of MCCase, the relative intensity of the 

Comparison of the sequences of the full-length MCC-B cDNA and gene demonstrate that the MCC-B subunit is encoded by a 2.78-kb stretch of the genomic sequence and that the transcribed region is interrupted by nine introns. There are only two sequence differences between the Landsberg gene and Columbia cDNA: a change of C to T at nucleotide 795 of the cDNA and a change of A to G at nucleotide 855, neither results in an amino acid change. The 10 exons of MCC-B range in length from 56 to 434 nucleotides. The nine intervening introns are from 77 to 164 nucleotides, larger than the minimum intron length of 70–73 nucleotides (28). The splice sites of each intron agree with plant consensus splice site sequences (29), and all nine introns contain the highly conserved dinucleotide sequence GT and AG at the 5’ and 3’ ends of the introns, respectively. In addition, the AU content of every intron is greater than 59%, which has been recognized as the minimum AU content for efficient splicing in dicots (28).

Primary Structure of the MCC-B Subunit and Comparison to Other Biotin Enzymes—The MCC-B subunit is a polypeptide of 587 amino acid residues. The N-terminal sequence of the MCC-B subunit of Arabidopsis (Fig. 4B) agrees with plant consensus splice site sequences (29), and all nine introns contain the highly conserved dinucleotide sequence GT and AG at the 5’ and 3’ ends of the introns, respectively. In addition, the AU content of every intron is greater than 59%, which has been recognized as the minimum AU content for efficient splicing in dicots (28).

An Arabidopsis (ecotype Landsberg) genomic DNA library was screened by hybridization with the MCC-B cDNA to isolate the gene encoding the non-biotin-containing subunit of MCCase. Twenty-nine hybridizing plaques (out of the $3.0 \times 10^4$ plaques that were screened) were identified. These represented overlapping clones of a single region of the Arabidopsis genome. Two clones, A2041 and A2102, were analyzed in detail. A 4.2-kb SalI fragment, which contained the 3’ end of the MCC-B gene (pMBG), and a 5.2-kb EcoRI fragment, which contained the 5’ end of the MCC-B gene (pMAGP), were subcloned and sequenced. Together, these two overlapping subclones contain the entire MCC-B gene (Fig. 4B).

Isolation and Characterization of the MCC-B Gene of Arabidopsis—Southern blot analysis of Arabidopsis (ecotype Columbia) DNA digested with EcoRI, HindIII, BamHI, or KpnI and probed with the MCC-B cDNA reveals a single hybridizing band in each digest (Fig. 4A). Thus, the MCC-B subunit is probably encoded by a single gene.

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of the GCG Sequence Analysis package predicts that it will form an amphiphilic α-helix, a common feature of transit peptides (31). Proteolytic cleavage of the pre-MCC-B protein is predicted by the PSORT algorithm (32) to occur at residue 27, within the sequence IRP2GTD. This is consistent with the finding that an arginine residue is often present at residue relative to the cleavage site (33). Cleavage at residue 27 would result in a mature polypeptide with a calculated molecular weight of 60,900, similar to the apparent molecular weight of the polypeptide immunologically detected in Arabidopsis leaf extracts with anti-145GEX serum (Fig. 2C, lane 4). Furthermore, these findings agree with previous determinations of the molecular mass of the MCC-B subunit of MCCase purified from maize (58 kDa), carrot (65 kDa), pea (54 kDa), and potato (53 kDa) (8–10).

Fig. 5 depicts the sequences of the proteins most similar to the MCC-B subunit; all are carboxyltransferase subunits of biotin enzymes (30–39). The three known biotin enzymes most similar to MCC-B, methylmalonyl-CoA decarboxylase (35% identical), propionyl-CoA carboxylase (30% identical), and transcarboxylase (33% identical), catalyze the conversion of methylmalonyl-CoA to propionyl-CoA, or vice versa. This may reflect the importance of a methyl branch in the molecules that bind to the carboxyltransferase substrate-binding site of these enzymes. Comparison of the MCC-B subunit to the carboxyltransferase subunit of glutaroyl-CoA decarboxylase reinforces this hypothesis. The biotin enzyme glutaroyl-CoA decarboxylase catalyzes the decarboxylation of glutaroyl-CoA to form crotonyl-CoA. Glutaconyl-CoA and crotonyl-CoA differ from the MCCase substrate and product only by the absence of the methyl branch, yet the carboxyltransferase subunit of glutaroyl-CoA decarboxylase shows lower amino acid identity (23%) to MCC-B than do the carboxyltransferase subunits of methylmalonyl-CoA decarboxylase, propionyl-CoA carboxylase, and transcarboxylase, which all bind shorter, but branched, acyl-CoAs.

Acetyl-CoA carboxylases (40) show <15% identity to the MCC-B subunit of MCCase (not depicted); the identity is dispersed throughout the carboxyltransferase domain. The sequence of the MCC-B subunit of MCCase shows no significant similarity to pyruvate carboxylases, biotin enzymes that use a β-keto acid as substrate.

The MCC-B sequence of Arabidopsis has enabled us to identify several animal-derived sequences in the GenBank data base, the biochemical functions of which had not been defined; these probably represent clones of animal MCC-B. These include a protein (PID g6711) encoded by a hypothetical Caenorhabditis elegans gene (56% identity) and proteins encoded by expressed sequence tag cDNAs from Dictyostelium discoideum (GenBank accession numbers C90323 and C90323), mouse (GenBank accession numbers AA050443, AA463055, AA444444, and AA049241), and human (GenBank accession numbers R88931 and AA465612).

Spatial and Temporal Patterns of MCC-A and MCC-B mRNA Accumulation—The reaction catalyzed by MCCase is required for the catabolism of leucine and of isoprenoids and the mevalonate shunt (Fig. 1). To begin to comprehend the physiological roles of these metabolic processes in the growth and development of plants, we examined MCCase expression by determining the spatial and temporal patterns of MCC-A and MCC-B mRNA accumulation. This was conducted by RNA blot and in situ hybridization analyses (Figs. 6 and 7). Furthermore, because cDNA probes for both MCCase subunits were available (Ref. 13 and this study), these analyses enabled us to
the metabolic function of all plant cells. Tissues and cells with elevated levels of MCCase mRNAs probably have higher demands for metabolic processes that require this enzyme. Several peaks in MCCase expression are apparent (Figs. 6 and 7).

During reproductive development, accumulation of MCC-A and MCC-B mRNAs is elevated in flower buds (Fig. 6, A–C, lane B; Fig. 7, A and B) and flowers just before opening (Fig. 6, A–C, lane F; Fig. 7, C and D). Within the flower, these mRNAs are concentrated in the ovary and enclosed ovules (Fig. 7, C–F). Following pollination, which in Arabidopsis occurs just after flower opening, the ovary develops into the siliques, and the ovules within develop into seeds. The accumulation of MCC-A and MCC-B mRNAs remains high in the siliques at 1 DAF, when siliques are most rapidly expanding (Fig. 6, A–C). Subsequently, as the siliques develop, accumulation of these mRNAs initially declines and then rises to peak levels in siliques at 6–7 DAF. This second peak in the accumulation of these mRNAs occurs at a period when seed storage products are being rapidly deposited in the embryos (Fig. 6, A–C). Indeed, in situ hybridization analyses of the siliques indicate that the two MCCase subunit mRNAs are most highly concentrated within the developing embryos (Fig. 7, G–N). Within the developing embryos, peak accumulation of the MCC-A and MCC-B mRNAs occurs in torpedo stage embryos at 5–7 DAF (Fig. 7, I–L). Subsequently, accumulation of these two mRNAs within the embryo declines, so that they are barely detectable in mature embryos (Fig. 7, O and P).

Seed germination is initiated upon the imbibition of water by the mature seed and the embryo within and is completed by the emergence of the radicle from the seed. Under the growth conditions used in our experiments, Arabidopsis seeds germinated within about 2–2.5 days after imbibition. During this process, the accumulation of MCC-A and MCC-B mRNAs initially increases in the seedlings relative to the levels found in mature seed embryos (cf. Fig. 7, R and S, which depicts seedling 1 day after imbibition, to Fig. 7, O and P, which shows mature seed embryos). Within the germinating seedling, these mRNAs are evenly distributed throughout the root and cotyledons (Fig. 7, R and S). Once germination is completed, the accumulation of MCC-A and MCC-B mRNAs is concentrated to the provascular region of the seedling root (Fig. 7, T–W). Upon further development of the cotyledons, the accumulation of these mRNAs initially declines until 5 days after imbibition (Fig. 6, D–F). Subsequently, their level of accumulation steadily increases to reach a peak during senescence (22–24 days after imbibition) and then declines in late senescence (Fig. 6, D–F).

The accumulation of the MCC-A and MCC-B mRNAs in leaves (Fig. 6, A–C) is somewhat lower than peak accumulation levels. Within young, expanding leaves, these two mRNAs accumulate evenly among all the cell-types of the leaf (Fig. 7, X and Y).

We interpret elevated MCCase mRNA levels to reflect higher rates of leucine (1, 5, 41–43) and/or isoprenoid (2, 42, 44) catabolism. We hypothesize that such increased catabolism is needed to satiate demands for ATP generation, particularly in organs and tissues that are net photosynthetic. For example, flowers, flower buds, and germinating cotyledons would require catabolically derived ATP for growth. Developing embryos would have increased demand for ATP to support biogenesis of seed storage products. The elevated accumulation of MCCase mRNAs in senescing cotyledons may reflect an enhanced need for ATP to support export of metabolites that are being translocated to sink tissues. Furthermore, the occurrence of high levels of MCCase mRNAs in germinating seedlings and in senescing cotyledons is coincident with a massive hydrolysis

FIG. 6. Temporal changes in the accumulation of MCC-A and MCC-B mRNAs. RNA blots were hybridized with MCC-A-specific (A and D) and MCC-B-specific (B and E) 32P-labeled antisense RNA probes. RNA was isolated from young expanding leaves (L), flower buds (B), flowers (F) and developing siliques at the indicated days after flowering (A–C) and developing cotyledons at the indicated days after planting (D–F). The concentrations of the MCC-A (white columns) and MCC-B (black columns) mRNAs were determined with the use of in vitro generated RNA standards, as described under “Experimental Procedures” (C and F). The data presented in A, B, D, and E were gathered from a single experiment. Error bars in C and F represent the S.D. obtained from two replicates of this experiment.

address whether MCC-A and MCC-B mRNAs accumulate coordinately during development.

MCC-A and MCC-B mRNAs are detectable in all cell types of cotyledons, leaves, flower buds, seedling roots, and embryos, but development affects the level of their accumulation (Figs. 6 and 7). The ubiquitous accumulation of the MCC-A and MCC-B mRNAs reinforces the concept that MCCase is important for
of proteins in these organs, which would provide free leucine as a substrate for catabolism. These data expand previous studies in soybean and pea, which indicate that MCCase activity is higher in metabolically active organs (42) and increases in response to carbohydrate starvation (41). The data presented herein, in combination with these previous studies (41, 42), indicate that changes in MCCase activity are at least partially attributable to changes in MCCase mRNA accumulation.

Finally, the changing patterns of MCC-A and MCC-B mRNA accumulation are similar both temporally and spatially during development (Figs. 6 and 7). Indeed, quantitative analyses indicate that these two mRNAs accumulate at approximately equal molar ratios (Fig. 6, C and F). These findings imply that the expression of the MCC-A and MCC-B genes is coordinately regulated. Accumulation of MCC-A (and MCC-B) mRNAs ranges from about 2 to 15 fmol/mg RNA. Assuming that 1% of

FIG. 7. Spatial distribution of MCC-A and MCC-B mRNAs in Arabidopsis. Histological tissue sections were hybridized with 35S-labeled antisense RNA probes (A–P and R–Y) or, for controls, with 35S-labeled sense RNA probes (Q and Z), and stained with Toluidine Blue. Black spots visualized by autoradiography are silver grains reflecting location of the MCC-A or MCC-B mRNAs. All hybridizations conducted three times with similar results. Each type of section was probed with all four probes, and representative results are shown. The distribution of the MCC-A and MCC-B mRNAs (as labeled) is shown in flower buds (A and B), in a flower viewed at lower magnification (C and D) and at higher magnification to show the ovary (E and F), in siliques at 3 DAF (G and H), in siliques at 5 DAF (I and J), in siliques at 7 DAF (K and L), in siliques at 9 DAF (M and N), in siliques at 12 DAF (O and P), in seedlings at 1 day after imbibition (R and S), in seedlings at 2 days after imbibition (T and U), in seedlings at 3 days after imbibition (V and W), and in young expanding leaves (X and Y). Control hybridizations are shown for seedlings at 2 days after imbibition with sense MCC-A probe (Q) and for young leaves with sense MCC-B probe (Z). All control hybridizations show negligible signal. The MCC-A and MCC-B mRNAs accumulate in very similar spatial patterns. r, receptacle; ov, ovary; o, ovule; a, anther; s, sepal; p, petal; oI, outer integument; ii, inner integument; w, wall of ovary; ge, globular embryo; te, torpedo embryo; cot, cotyledon; rt, root; sc, seed coat (derived from inner and outer integument); pc, provascular cambium. Bars, 885 μm in A–D and G–Z and 41 μm in E and F.
total RNA is mRNA and that the average mRNA is 2 kb, 15 fmol/mg of MCC-A (or MCC-B) mRNA would be equivalent to 0.1 mol % of the cellular mRNA.

**Biological Implications**—MCCase catalyzes a reaction at a branchpoint between leucine and isoprenoid catabolism (5, 12, 38, 40, 46) (Fig. 1). To understand the metabolic significance of these catabolic pathways in plants, which are net anabolic organisms, we isolated and characterized, for the first time from any organism, the MCC-B gene. The observed ubiquitous accumulation of MCCase mRNAs indicates that pathways requiring MCCase may be required throughout the life cycle of the plant. In addition, the elevated accumulation of MCCase mRNAs in metabolically active, nonphotosynthetic organs, may indicate an amplified demand for these catabolic processes to augment ATP generation.

The characterization of the MCCase genes may prove important in elucidating the molecular basis of methylcrotonylglycuria, a fatal genetically inherited human metabolic disorder characterized by the absence of MCCase (7). In addition, MCCase is of significance in comprehending how the mevalonate shunt can divert carbon away from the biosynthesis of isoprenoids, such as cholesterol, which has major implications in the prevention of vascular degenerate diseases.

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3-Methylcrotonyl-CoA Carboxylase
Molecular Characterization of the Non-biotin-containing Subunit of 3-Methylcrotonyl-CoA Carboxylase

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