

## **INFORMATION TO USERS**

**This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.**

**The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.**

**In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.**

**Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.**

**Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.**

**Bell & Howell Information and Learning  
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA  
800-521-0600**

**UMI<sup>®</sup>**



**Biochemical and molecular genetic studies of the metabolic role of methylcrotonyl-CoA  
carboxylase**

**by**

**Ping Che**

**A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY**

**Major: Biochemistry**

**Major professor: Basil J. Nikolau**

**Iowa State University**

**Ames, Iowa**

**2000**

**Copyright © Ping Che, 2000. All rights reserved.**

UMI Number: 9992449



---

UMI Microform 9992449

Copyright 2001 by Bell & Howell Information and Learning Company.

All rights reserved. This microform edition is protected against  
unauthorized copying under Title 17, United States Code.

---

Bell & Howell Information and Learning Company  
300 North Zeeb Road  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

**Graduate Collage**  
**Iowa State University**

**This is to certify that the Doctoral dissertation of**  
**Ping Che**  
**has met the dissertation requirements of Iowa State University**

Signature was redacted for privacy.

**Major Professor**

Signature was redacted for privacy.

**For the Major Program**

Signature was redacted for privacy.

**For the Graduate Collage**

## TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION .....	1
Biotin and Biotin Biochemistry .....	1
Biotin-Containing Enzymes.....	5
Plant Biotin-Containing Enzymes.....	7
Metabolic Control Gene Expression in Higher Plants .....	10
Thesis Organization.....	13
Literature Cited .....	14
CHAPTER 2. 3-METHYLCROTONYL-CoA CARBOXYLASE IS A COMPONENT OF THE MITOCHONDRIAL LEUCINE CATABOLIC PATHWAY IN PLANTS .....	28
Abstract.....	28
Introduction.....	29
Materials and Methods .....	31
Results .....	39
Discussion.....	45
Literature Cited .....	50
CHAPTER 3. DEVELOPMENTAL AND ENVIRONMENTAL REGULATION OF 3-METHYLCROTONYL-CoA CARBOXYLASE IN <i>ARABIDOPSIS</i> .....	71
Summary.....	71
Introduction.....	72
Results .....	74
Discussion.....	83
Material and Methods.....	87
References.....	93
CHAPTER 4. THE EFFECTS OF BIOTIN DEPLETION ON THE EXPRESSION OF BIOTIN-CONTAINING ENZYMES.....	118
Abstract.....	118
Introduction.....	119
Materials and Methods .....	122
Results and Discussion .....	125
Literature Cited .....	138
CHAPTER 5. CONCLUSIONS.....	160
APPENDIX I. MCCase BIOTINYLATED SUBUNIT GENOMIC DNA SEQUENCE FROM <i>ARABIDOPSIS THALIANA</i> (COLUMBIA).....	162
APPENDIX II. DATA FOR FURTHER STUDY .....	167
ACKNOWLEDGMENTS.....	172

## CHAPTER 1. INTRODUCTION

### Biotin and Biotin Biochemistry

Biotin (Figure 1), an essential water-soluble vitamin, is required by all living organisms for normal cellular functions and growth. It was first identified in 1901 as a growth factor in yeast and then isolated from egg yolk by Kögel and Tönms (1936). Kögel also reported the correct empirical formula  $C_{11}H_{18}O_3N_2S$  for biotin methylester in 1937. There are in total of eight biotin isomers, but only D-biotin has been found to be biologically active (Gaudy and Ploux, 1992). Biotin is synthesized in plants, some fungi, and most bacteria. The biosynthetic pathway for biotin was first elucidated in *E. coli* and *Bacillus* species through the growth and chemical supplementation of auxotrophic mutants (Eisenberg, 1973; Pai, 1975). Enzymes required for biotin biosynthesis have been purified and characterized from *Bacillus sphaericus* (Izumi et al., 1981; Ploux and Marquet, 1992; Ploux et al., 1992; Alexeev et al., 1994; Huang et al., 1995). Eukaryotic plants share a very similar biotin biosynthetic pathway. This common pathway for biotin synthesis is shown in Figure 2. Although most reactions in this pathway have been well defined, questions remain concerning the details of the initial and final reactions. Namely, what is the immediate precursor of pimeloyl-CoA, and what is the sulfur donor required for biotin synthase? Recently, Cys has been considered to be the sulfur donor (Birch et al., 1995).

The cDNA and gene of biotin synthase (BIO2), which catalyzes the final reaction in biotin biosynthesis, have been cloned from *Arabidopsis* (Weaver et al., 1996; Patton et al., 1996). So far, this is the first plant biotin biosynthetic gene to be cloned.

Two biotin auxotrophic mutants have been reported in *Arabidopsis*: *bio1* (Shellhammer and Meinke, 1990) and *bio2* (Patton et al., 1998). The *bio1* mutant seedlings can be rescued when grown in the presence of dethiobiotin, diaminopelargonic acid (DAPA) or biotin. The expression of *E. coli bioA* gene (encoding DAPA synthetase) in homozygous *bio1/bio1* plants eliminates the auxotrophic phenotype of *bio1* (Patton et al., 1996). These data indicate that the *bio1* gene of plants corresponds to the *bioA* gene of *E. coli* and that in the *bio1* mutant, biotin biosynthesis is blocked in the conversion of 7-keto-8-aminopelargonic acid to 7,8-diaminopelargonic acid (Fig. 2). The *bio2* mutant can only be rescued by biotin, but not by dethiobiotin. This indicates that the final step of biotin biosynthesis is blocked in the *bio2* mutants and the *bio2* gene corresponds to *bioB* gene (encoding biotin synthase) in *E. coli*.

Biotin is bound to specific proteins via an amide linkage between the biotin carboxyl group and a unique lysine amino group (Moss and Lane, 1971) (Figure 1). Usually the biotinylated lysine residue is near the carboxyl terminus of the protein. The amino acid sequence surrounding the biotinylated lysine residue is highly conserved among different biotin enzymes (i.e. Ala/Vala-Met-Lys-Met) in animals and bacteria.

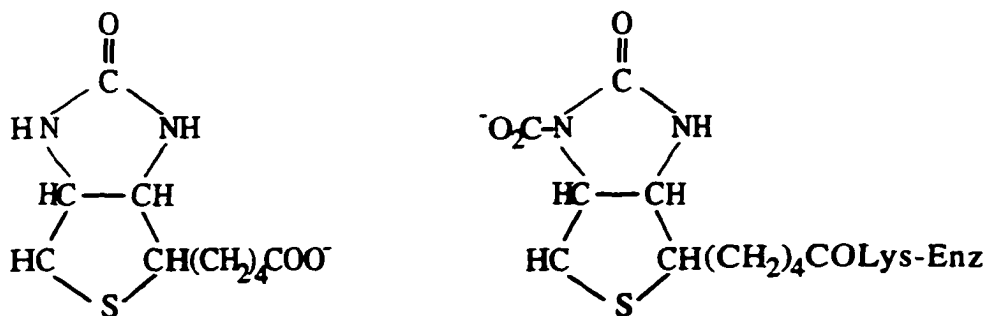


Figure 1. The structure of biotin and carboxyl-biotin-enzyme.



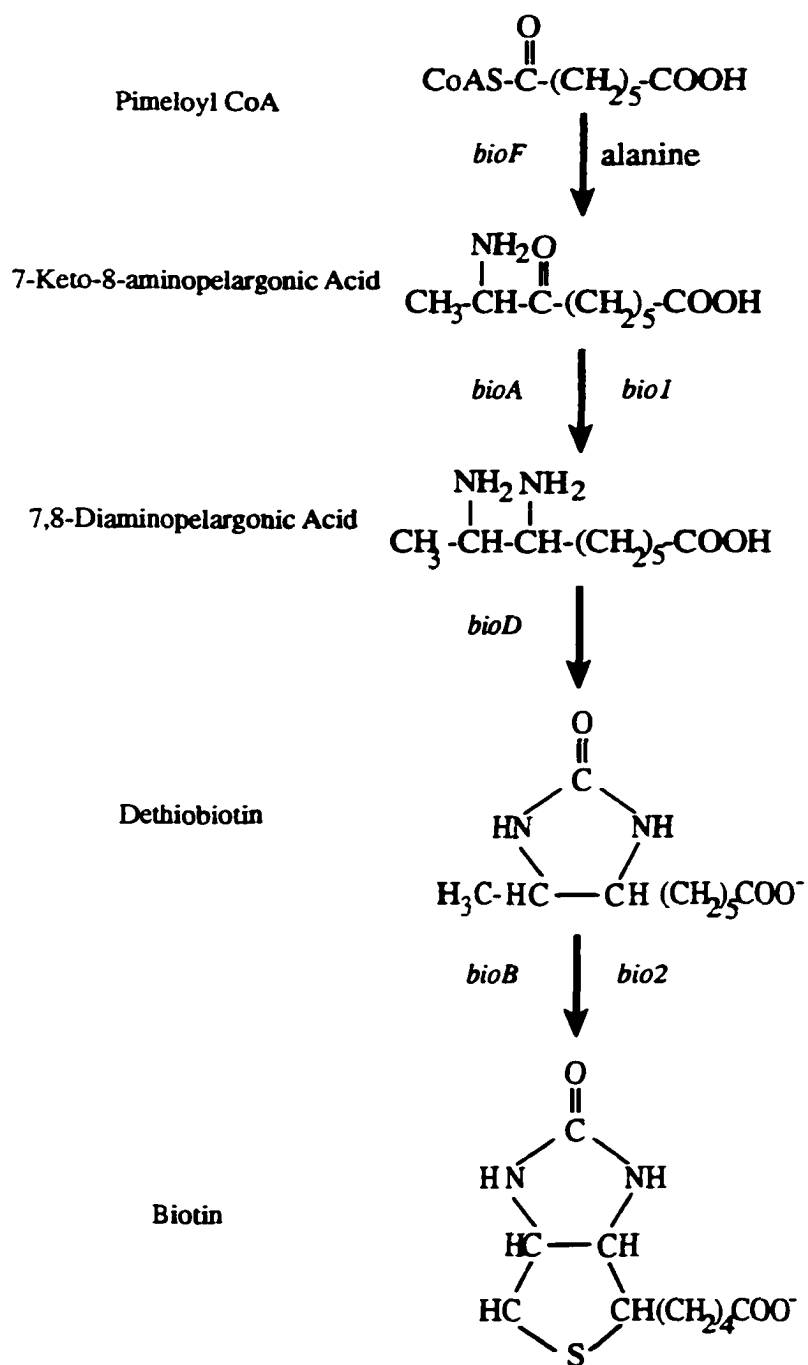
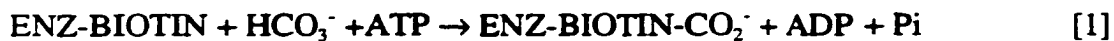


Figure 2. Biotin biosynthetic pathway in plants and microorganisms. Letters correspond to cloned *bio* genes of *E. coli*. Numbers correspond to mutant *bio* genes of *Arabidopsis*

Biotin acts as a small coenzyme and facilitates the transfer of CO<sub>2</sub> during carboxylation and decarboxylation reactions (Dakshinamurti and Bhagavan, 1985; Knowles, 1989).

The reactions catalyzed by biotin enzymes are involved in diverse metabolic processes including fatty acid biosynthesis, gluconeogenesis, and catabolism of branched-chain amino acids. Although each enzyme has distinct metabolic functions, the overall reactions catalyzed by biotin enzymes are in two similar steps. For carboxylases, the first step, an ATP and Mg<sup>2+</sup> dependent reaction, involves the carboxylation of biotin by the biotin carboxylase (BC) domain of the enzyme. Then, the carboxyl group is transferred from carboxybiotinyl enzyme intermediate to an appropriated acceptor substrate (such as acetyl-CoA, 3-methylcrotonyl-CoA, propionyl-CoA or pyruvate). This reaction is catalyzed by the carboxyltransferase domain.



Although the biological functions of biotin have been well recognized, little is known about its role in regulating gene expression and protein biosynthesis. Biotin operons (*bio* operons) from several prokaryotes have been sequenced and analyzed (Otsuka et al., 1988; Cronan, 1989; Gloeckler et al., 1990; Bower et al., 1996). Regulation of the *E. coli bio* operon is the best understood example of transcriptional regulation by biotin. *E. coli* contains only a single biotin-containing enzyme, the biotin carboxyl carrier protein (BCCP)

of acetyl-CoA carboxylase (ACCase). Biotin-protein ligase, BirA, which biotinylates BCCP in *E. coli*, is the repressor protein that regulates the transcription of the *bio* operon.

Repression of the transcription of the *bio* operon occurs when the cellular supply of biotin is in excess of that needed to biotinylate apoBCCP. Under these conditions, the BirA-biotinyl-AMP complex, which is an intermediate of the biotinylation reaction, accumulates. This complex binds to the *bio* operator, and represses transcription. Another example of regulation of gene expression by biotin is that of the enhanced the transcription of glucokinase by biotin in the starved rats (Chauhan and Dakshinamurti, 1991). However, the physiology significance of this regulation is still unknown.

### **Biotin-Containing Enzymes**

Different organisms contain a different set of biotin-containing proteins. *E. coli* contains only one such protein, the biotin carboxyl carrier subunit (BCCP) of ACCase. Other bacteria have one to three biotin-containing proteins, whereas eukaryotic cells contain four or five such proteins. So far, three biotin-containing enzymes have been reported in plants: ACCase (Sasaki et al, 1995; Ohlrogge et al., 1979; Stumpf, 1987; Harwood, 1988; Conn, 1981; Nikolau, et al., 1984), methylcrotonyl-CoA carboxylase (MCCase) (Alban et al., 1993; Chen *et al.*, 1993; Diez et al., 1994; Song et al., 1994; Wang et al., 1994, Wurtele and Nikolau, 2000; Weaver *et al.*, 1995; McKean *et al.*, 2000) and geranyl-CoA carboxylase (GCCase) (Guan et al., 1999). However, there are indications that additional biotin-containing enzymes occur in the extracts from plants (Wurtele and Nikolau, 1990).

Biotin-containing enzymes can be divided into three classes: carboxylases, decarboxylases, and transcarboxylases (Moss et al., 1971). All known eukaryotic biotin-

containing enzymes are carboxylases: ACCase, MCCase, pyruvate carboxylase (PCase), propionyl-CoA carboxylase (PCCase), geranyl-CoA carboxylase and urea carboxylase. Biotin-dependent decarboxylases are found in anaerobic bacteria: oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase and glutaconyl-CoA decarboxylase. The only known transcarboxylase is found in *Propionibacteria*.

All biotin enzymes consist of three domains: biotin carboxylase domain (BC), biotin-carboxyl-carrier domain (BCC) and the carboxyltransferase domain (CT). Each of the three catalytic domains can be found either on different polypeptides or in combination on larger polypeptides. This has led to the classification of biotin-containing enzymes into three types:

- 1) Heteromeric biotin-containing enzymes: Each of the three domains (BC, BCC and CT) is located on separate, dissociable subunits. ACCase from *E. coli* (Guchhait et al., 1974; Kondo et al., 1991; Li and Cronan, 1992a; 1992b) and plant chloroplasts (Sasaki et al., 1993; Choi et al., 1995; Shorrosh et al., 1995, 1996; Sun et al., 1997; Ke et al., 2000) are examples of this type biotin-containing enzyme.
- 2) “Intermediate” biotin-containing enzymes: This group of enzymes combine BC and BCCP domains in one peptide and the CT domain in another. This type of enzyme includes PCCase from animals and bacteria (Gravel et al., 1980; Henrikson and Allen, 1982), MCCase from animals (Lau et al., 1980), bacteria (Schiele et al., 1975; Fall and Hector, 1977) and plants (Chen et al., 1993; Alban et al., 1993; Diez et al., 1994; Wang et al., 1994 Song et al., 1994; Weaver et al., 1995; McKean et al., 2000), PCase and GCCase from *Pseudomonas citronellolis* (Utter et al., 1975; Hector and Fall, 1976; Fall and Hector, 1977) and acetyl-CoA carboxylase from *Myxococcus xanthus* (Kimura et al., 2000).

3) Homomeric biotin-containing enzymes: This type of enzyme consists of only one large polypeptide that has all three catalytic domains. ACCase from animals, yeast (Lopez-Casillas et al., 1988; Takai et al., 1988; Walid et al., 1992) and plant cytosol (Egin-Buhler et al., 1980; Gornicki et al., 1993; Roesler et al., 1994; Schulte et al., 1994; Shorrosh et al., 1994; Yanai et al., 1995) and urea carboxylase from yeast (Sumrada and Cooper, 1982; Genbauffe and Cooper, 1991) are examples.

### **Plant Biotin-Containing Enzymes**

Until recently, only three biotin-containing enzymes have been reported in plants, ACCase, MCCase and GCCase. However, there are indications that additional biotin-containing enzymes occur in the protein extracts from plants (Wurtele and Nikolau, 1990). The most thoroughly studied biotin-containing enzyme in higher plants is ACCase, which performs an essential function during the initial stages of fatty acid biosynthesis. There are two types of ACCase in plants (Sasaki et al, 1995): heteromeric type which is found in chloroplasts (Sasaki et al., 1993; Choi et al., 1995; Shorrosh et al., 1995, 1996; Sun et al., 1997; Ke et al., 2000), and the homomeric type which is found in the cytosol (Egin-Buhler et al., 1980; Gornicki et al., 1993; Roesler et al., 1994; Schulte et al., 1994; Shorrosh et al., 1994; Yanai et al., 1995). Malonyl-CoA generated in the plastids by heteromeric ACCase has a single known metabolic fate, the formation of fatty acids (Ohlrogge et al., 1979; Stumpf, 1987; Harwood, 1988). However, the cytosolic malonyl-CoA generated by the homomeric ACCase is not utilized for de novo fatty acid biosynthesis, but for the synthesis of a variety of phytochemical (Conn, 1981; Nikolau, et al., 1984), for example, epicuticular

waxes, suberin, flavonoids, stilbenoids, a variety of malonyl-derivatives chemicals, and free malonic acid.

Wurtele and Nikolau (1990) first reported the discovery of MCCase activity in plant protein extracts in 1990. Subsequently, this enzyme was purified from maize, carrot, pea, potato and soybean (Alban et al., 1993; Chen et al., 1993; Diez et al., 1994; Song et al., 1994; Wang et al., 1994, Wurtele and Nikolau, 2000). As in animals and bacteria, MCCase is composed of two subunits (Chen et al., 1993; Alban et al., 1993; Diez et al., 1994; Wang et al., 1994 Song et al., 1994; Weaver et al., 1995; McKean et al., 2000): the larger, biotinylated subunit (MCC-A) which is about 85 kDa and the smaller and non-biotinylated subunit (MCC-B) which is approximately 60 kDa. The cDNAs and genes coding for the MCC-A subunit have been cloned from tomato, soybean and *Arabidopsis* (Song et al., 1994; Wang et al., 1994; Weaver et al., 1995). At the amino-terminus, the initial 15-30 residues have the characteristics of a mitochondrial targeting transit peptide, consistent with the mitochondrial localization of the enzyme. Most recently, the cDNA and gene coding for MCC-B subunit have been cloned from *Arabidopsis* in our lab (McKean et al., 2000). Despite these molecular characterizations of MCCase, the physiological significance of this enzyme in plant metabolism has not been defined.

In animals and bacteria, this enzyme is known to be required for the catabolism of leucine (Leu) (Lau et al., 1980), which occurs in mitochondria. Studies on MCCase activity in tomato and soybean revealed that MCCase specific activity was always higher in the roots (non-photosynthetic organ) than in the leaves (photosynthetic organ) (Wang et al., 1995). It was also reported that darkness induced MCCase activity in barley seedlings (Maier et al., 1998; Clauss et al., 1993). These findings indicate that MCCase activity is inversely related

to plants' or plant organ's photosynthetic activity and implied MCCase's putative role in Leu catabolism. A study on MCCase activity during carbohydrate starvation in sycamore cells is consistent with MCCase's putative role in Leu catabolism (Aubert et al., 1996).

Furthermore, Leu catabolism may also occur in the peroxisomes, by a pathway that is not well defined, but it does not require MCCase (Gerbling and Gerhardt, 1988 and 1989; Gerbling, 1993). In this pathway, 3-methylcrotonyl-CoA, an intermediate from Leu degradation, is converted to isobutyryl-CoA, which in turn is converted via a modified  $\beta$ -oxidation pathway to acetyl-CoA.

In addition to Leu catabolism, MCCase also plays a role in the mevalonate shunt (Popják, 1971; Edmond and Popják, 1974). In the mevalonate shunt, mevalonate is converted to acetoacetate and acetyl-CoA. The net result of this shunt in animals is to reassimilate carbon that has been converted to mevalonate into primary metabolites rather than into isoprenoids. Isoprenoids are a diverse class of compounds that include scents, carotenoids, sterols, cardiac glycosides, taxol, gibberellins, abscisic acid, rubber, and the prenyl tail of chlorophyll and ubiquinones. These isoprenoids are synthesized from acetyl-CoA via the five-carbon intermediate isopentenyl pyrophosphate (IPP). In animals, this conversion (acetyl-CoA to IPP) occurs via the sequence acetoacetyl-CoA, HMG-CoA and mevalonate.

Most recently, GCCase (Guan et al., 1999), which plays an important role in isoprenoid catabolism, was purified and characterized from plants.

### **Metabolic Control of Gene Expression in Higher Plants**

Carbohydrates play multiple roles in biology by providing energy and carbon skeletons for the growth and development of an organism. They are also major regulatory molecules that control gene expression, metabolism, physiology, cell cycle and development in prokaryotes and eukaryotes.

The glucose-sensing pathway in *E. coli* is probably the best understood regulatory mechanism in which carbohydrates play a major role in regulation metabolism (Saier, 1991), and is known as catabolite repression. In *E. coli*, the expression of the *lac* operon proteins is repressed by the presence of glucose in the medium, and can be derepressed when glucose is removed. This phenomenon prevents the wasteful duplication of energy-producing enzyme systems. In this transduction pathway, cAMP and its receptor protein (CRP) are the major regulators of genes repressed by glucose. cAMP is synthesized by the enzyme adenylcyclase, and its concentration is regulated indirectly by glucose transport and metabolism. When bacteria are growing in a medium containing glucose, the cAMP concentration in the cells is quite low. However, when the bacteria are deprived of the energy source, the cAMP concentration increases. In the absence of glucose, the CRP-cAMP complex binds to the *lac* operon and stimulates transcription.

In yeast, genes required for growth on carbon sources other than glucose are repressed by the presence of glucose in the medium and can be derepressed when glucose is removed. Hexokinase (HK) PII, the enzyme that catalyzes the phosphorylation of hexose sugars as the first reaction of the glycolytic pathway, is considered to be the major sensing molecule for catabolite repression that is triggered by glucose (Carison, 1987; Entian and Frölich, 1984; Ma and Botstein, 1986; Ma et al., 1989; Rose et al., 1991). A functional



sucrose nonfermenting-1 (SNF1) complex, which is a serine/threonine kinase, is required for the down-stream signal transduction and essential for depression of all glucose-repressed genes (Celenza, 1986). The SNF1 forms a complex with other proteins, for example, the activating subunit SNF4 and other adapter proteins. The interaction between SNF1 and SNF4 is strongly regulated by glucose. The SNF4 protein binds to the SNF1 regulatory domain when glucose levels are low, whereas when glucose levels are high the regulatory domain of SNF1 binds to its own kinase domain, causing autoinhibition of the kinase activity (Jiang et al., 1996) and this regulation is also affected by HXK2, GLC7 (gene encodes a type 1 protein phosphatase) and REG1 (encodes a nuclear-localized protein), which means these components are upstream of SNF1 in glucose signaling pathway.

Compared to the understanding of sugar signal transduction pathway in *E. coli* and yeast, relatively little is known about the molecular and biochemical mechanisms underlying sugar response in the higher plants. A large and specific set of genes whose expressions are induced by sugar starvation has been reported. Some of them are photosynthetic genes, such as chlorophyll a/b binding protein (CAB) (Krapp et al., 1993; Harter et al., 1993). Some of them are non-photosynthetic genes, such as  $\alpha$ -amylase genes in rice suspension cells and germinating embryos (Huang, 1993; Yu et al., 1991 and 1996), sucrose synthase gene in maize root tips (Mass, 1990; Koch et al., 1992), and malate synthase and isocitrate lyase genes in cucumber (Grahma et al., 1994 and 1992). For all of these genes, sugar-repression appears to be regulated at the level of gene transcription. However, it is not entirely clear what the signal for sugar repression is and how that signal is transduced to alter gene transcription. Evidence that accumulation of sugars directly represses photosynthetic gene expression has been obtained in several cases. By analysis of promoter-reporter gene fusions, Sheen

(1990, 1994 and 1997) found that transcriptional activity of seven different promoters from photosynthetic genes of maize was repressed by glucose and sucrose. Furthermore, glucose transport alone is not sufficient to trigger the repression, glucose phosphorylation by hexokinase is required. The glucose analog 3-*O*-methylglucose, which is transported into cells but not phosphorylated via hexokinase, does not trigger repression. On the other hand, 2-deoxyglucose, which can be phosphorylated by hexokinase, but not further metabolized, triggers the repression. Furthermore, a hexokinase inhibitor (mannuheptulose) is able to reduce the glucose repression of a maize photosynthetic gene. These data support the hypothesis that plant hexokinase has dual-functions and plays a role in both sugar-sensing and phosphorylating hexoses. Two non-photosynthetic genes in the glyoxylate pathway, malate synthase and isocitrate lyase, have the similar characteristic of metabolic repression as these photosynthetic genes in maize (Grahma et al., 1994 and 1992).

However, it is still unknown how hexokinase stimulates the sugar-signaling pathway in plants and the notion that hexokinase is a primary sugar sensor was recently been challenged, and multiple sugar-sensing pathways are proposed to exist in plants (Halford et al., 1999).

Determination of the down stream components of sugar-sensing pathway in plants will help us fully and precisely understand the whole signal process. Recently, it is reported that plant homologues of SNF1, called SNF1-related protein kinase 1s (SnRK1), may play a role analogous to that of SNF1 in yeast and fungi (Purcell et al., 1998). Using the tobacco SNF1 homolog, it has been shown that both the glucose-regulated interaction with SNF4 and the specific interaction between the kinase and regulatory domains are conserved between yeast and plants (Jiang et al., 1996). However, the function of this protein in plants needs

further determination and it will be very interesting to determine whether the transcription of this gene is affected by hexokinase (Halford et al., 1999).

It is clear that the sugar-sensing system in plants is a much more complex system than glucose-sensing pathway in *E. coli* and yeast. In plants, sugars are not only involved in repressing gene expression, but they are also involved in stimulation gene expression and the induction is also controlled at the transcriptional level (see review Yu, 1999; Koch, 1996). Genes involved in starch biosynthesis, protein biosynthesis and storage, nitrogen reduction and assimilation, respiration, pigment formation and defense and metabolism of sucrose are enhanced by sucrose. For instance, exogenous sucrose induced the expression of genes for nitrate reductase in dark-adapted *Arabidopsis* (Cheng, 1992). Genes encoding patatin (Grierson et al., 1994; Liu et al., 1990), chalcone synthase (Tsukaya et al., 1991), and  $\beta$ -amylase and sporamine in sweet potato (Ishiguro et al., 1992) are all reported to be induced by sucrose. It is also reported that sugar-regulated gene expression pathway may communicate with other signal transduction pathway. For example, the expression of  $\alpha$ -amylase genes in barley embryos is under hormonal (GA and ABA) control as well as metabolic (sugar) control, suggesting the occurrence of an interaction between the GA and sugar signal transduction pathways (Perate, 1997).

### **Thesis Organization**

This dissertation is composed of five chapters which address three main questions: 1) What is the physiological role of MCCase in plants? 2) How is the MCCase expression regulated during plant development and in response to environmental stimuli? 3) Whether biotin plays a role in the regulation of gene expression of the biotin-containing enzyme,

MCCase, in plants? Chapter 1 provides an overview of biotin and biotin-containing enzymes. Chapter 2 is a paper published in the journal of *Plant Physiology*. This paper presents direct evidence that one of the roles of MCCase is catabolism of leucine in plant mitochondria and also demonstrated for the first time that plant mitochondria contain all the enzymes for this catabolic pathway. Ping Che, Jianping Song and Marc D. Anderson made equal contributions to this paper. My contribution to this paper includes the development of techniques for the analysis radioactive metabolites and determination of the Leu content of tissue by HPLC. In chapter 3, I demonstrated that the expression of the MCCase gene (*MCC-A* and *MCC-B*) is controlled by metabolic repression at the level of gene transcription. Chapter 4 describes, for the first time, that biotin regulation of MCCase accumulation occurs at the post-transcriptional and/or translational levels. Chapter 5 summarizes the results presented in chapter 2, 3 and 4 and discusses future research. Appendix I contains the MCCase biotinylated subunit genomic DNA sequence from *Arabidopsis thaliana*. Appendix II contains other data which are important for further studies on MCCase and ACCase.

### **Literature Cited**

- Aubert, S., Alban, C., Bligny, R. and Douce, R.** (1996) Induction of beta-methylcrotonyl-coenzyme A carboxylase in higher plant cells during carbohydrate starvation: Evidence for a role of MCCase in leucine catabolism. *FEBS Lett.* **383**, 175-180.
- Alban, C., Baldet, P., Axiotis, S. and Douce, R.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from higher plant mitochondria. *Plant Physiol.* **102**, 957-965.

- Alexeev, D., Bury, S.M., Boys, C.W.G., Turner, M.A., Sawyer, L., Ramsey, A.J., Baxter, H.C. and Baxter, R.L.** (1994) Sequence and crystallization of *Escherichia coli* dethiobiotin synthase, the penultimate enzyme of biotin biosynthesis. *J. Mol. Biol.* **235**, 774-776.
- Birch, O.M., Fuhrmann, M. and Shaw, N.M.** (1995) Biotin synthase from *Escherichia coli*, an investigation of the low molecular weight and protein components required for activity in vitro. *J. Biol. Chem.* **270**, 19158-19165.
- Bower, S., Perkins, J.B., Yocum, R.R., Howitt, C.L., Rahaim, P. and Pero, J.** (1996) Cloning, sequencing, and characterization of the *Bacillus subtilis* biotin biosynthetic operon. *J. Bacteriol.* **178**, 4122-4130.
- Celenza, J.L. and Carlson, M.** (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase, *Science* **233**, 1175-1180.
- Carlson, M.** (1987) Regulation sugar utilization in *Saccharomyces* species. *J. Bacteriol.* **169**, 4873-4877.
- Chen, Y., Wurtele, E.S., Wang, X. and Nikolau, B.J.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from somatic embryos of *Daucus carota*. *Arch. Biochem. Biophys.* **305**, 103-109.
- Chauhan, J. and Dakshinamurti, K.** (1991) Transcriptional regulation of the glucokinase gene by biotin in starved rats. *J. Biol. Chem.* **266**, 10035-10038.
- Cheng, C-L., Acdeo, D.N., Cristinsin, M. and Conkling, M.A.** (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. *Proc. Natl. Acad. Sci. USA* **89**, 1861-1864.

- Choi, J.-K., Yu, F., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning and characterization of cDNA coding for the biotin-containing subunit of the chloroplastic acetyl-CoA carboxylase. *Plant Physiol.* **109**, 619-625.
- Clauss, M., Motel, A. and Lichtenthaler, H.K.** (1993) Studies on the plant methylcrotonyl-CoA Carboxylase. *J. Plant Physiol.* **114**, 508-511.
- Conn, E.E.** (1981) The biochemistry of plants: A Comprehensive Treatise, Vol 7, Secondary plant metabolism. *Academic Press*, New York.
- Cronan, J.E. Jr.** (1989) The *E. coli* *bio* operon: transcriptional repression by an essential protein modification enzyme. *Cell* **58**, 427-429.
- Chauhan, J. and Dakshinamurti, K.** (1991) Transcriptional regulation of the glucokinase gene by biotin in starved rats. *J. Biol. Chem.* **266**, 10035-10038.
- Dakshinamurti, K. and Bhagavan, N.H., eds** (1985) Biotin. *Ann. NY Acad. Sci.* **447**, 1-441.
- Diez, T.A., Wurtele, E.S. and Nikolau, B.J.** (1994) Purification and characterization of 3-methylcrotonyl-coenzyme-A carboxylase from leaves of *Zea mays*. *Arch. Biochem. Biophys.* **310**, 64-75.
- Edmund, J. and Popják, G.** (1974) Transfer of carbon atoms from mevalonate to *n*-fatty acids. *J. Biol. Chem.* **249**, 66-71.
- Eisenberg, M. A.** (1973) Biotin: biogenesis, transport, and their regulation. *Adv. Enzymol.* **38**, 317-372.
- Egin-Buhler, B., Loyal, R. and Ebel, J.** (1980) Comparison of acetyl-CoA carboxylase from parsley cell cultures and wheat germ. *Arch. Biochem. Biophys.* **203**, 90-100.
- Entian, K.-D. and Barnett, J.A.** (1992) Regulation of sugar utilization by *Saccharomyces cerevisiae*. *Trends Biol. Sci.* **17**, 506-510.

- Fall, R.R. and Hector, M.L.** (1977) Acyl-coenzyme A carboxylase: Homologous 3-methylcrotonyl-CoA and geranyl-CoA carboxylases from *Pseudomonas citronellolis*. *Biochem.* **16**, 4000-4005.
- Guan, X., Diez, T., Prasad, T.K., Nikolau, B.J. and Wurtele, E.S.** (1999) Geranoyl-CoA Carboxylase: A novel biotin-containing enzyme in plants. *Arch. Biochem. Biophys.* **362**, 12-21.
- Gerbling, H.** (1993) Peroxisomal degradation of 2-oxoisocaproate. Evidence for free acid intermediates. *Bot. Acta.* **106**, 380-387.
- Gerbling, H. and Gerhardt, B.** (1988) Oxidative decarboxylation of branched-chain 2-oxo fatty acids by higher plant peroxisomes. *Plant Physiol.* **88**, 13-15.
- Gerbling, H. and Gerhardt, B.** (1989) Peroxisomal degradation of branched-chain 2-oxo acids. *Plant Physiol.* **91**, 1387-1392.
- Gloeckler, R., Ohsawa, I., Speck, D., Ledoux, C., Bernard, S., Zinsius, M., Villeval, D., Kisou, T., Kamogawa, K. and Lemoine, Y.** (1990) Cloning and characterization of the *Bacillus sphaericus* genes controlling the bioconversion of pimelate into dethiobiotin. *Gene* **87**, 63-70.
- Gornicki, P., Scappino, L.A. and Haselkorn, R.** (1993) Genes for two subunits of acetyl coenzyme A carboxylase of *Anabaena* sp. strain PCCC7120: biotin carboxylase and biotin carboxyl carrier protein. *J. Bacteriol.* **175**, 5268-5272.
- Gaudy, M. and Ploux, O.** (1992) In Modern Chromatographic Analysis of Vitamins, 2<sup>nd</sup> edition, (A. D. Leenheer, W. E. Lambert and H. J. Neilis, ed), Marcel Dekker Inc., pp441-467.

- Genbauffe, F.S. and Cooper, T.G.** (1991) The urea amidolyase (DUR1,2) gene of *Saccharomyces cerevisiae*. *DNA Sequence* **2**, 19-32.
- Graham, I.A., Denby, K.J. and Leave, C.J.** (1994) Carbon catabolite repression regulations glyoxylate cycle gene expression in cucumber. *Plant Cell* **6**, 761-772.
- Gray, J.C.** (1987) Control of isoprenoid biosynthesis in higher plants. *Adv. Bot. Res.* **14**, 23-91.
- Gravel, R.A., Lam, K.F., Mahuran, D. and Kronis, A.** (1980) Purification of human liver propionyl-CoA carboxylase by carbon tetrachloride extraction and monomeric avidin affinity chromatography. *Arch. Biochem. Biophys.* **201**, 699-673.
- Grierson C., Du J-S. Zabala M., Beggs K., Smith C., Holdsworth M. and Bevan M.** (1994) separate *cis* sequences and *trans* factors direct metabolic and developmental regulation of a potato tuber storage protein gene. *Plant J.* **4**, 507-516.
- Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D.** (1974) Acetyl coenzyme A carboxylase system of *Escherichia coli*. *J. Biol. Chem.* **249**, 6633-6645.
- Halford, N.G., Purcell, P.C. and Hardie, D.G.** (1999) Is hexokinase really a sugar sensor in plants? *Trends plants Sci.* **4**, 117—120.
- Harter, K., Talke-Messerer, C., Barz, W. and Schäfer, E.** (1993) Light- and sucrose-dependent gene expression in photomixotrophic cell suspension cultures and protoplasts of rap (*Brassica napus* L.) *Plant J.* **4**, 507-516.
- Harwood, J.L.** (1988) Fatty acid metabolism. *Ann. Rev. Plant Physiol. Plant & Mol. Biol.* **39**, 101-138.



- Haase, F.C., Henrikson, K.P., treble, D.H. and Allen, S.H.** (1982) The subunit structure and function of the propionyl coenzyme A carboxylase of *Mycobacterium smegmatis*. *J. Biol. Chem.* **257**, 11994-11999.
- Hector, M.L. and Fall, R.R.** (1976) Evidence for distinct 3-methylcrotonyl-CoA and geranyl-CoA carboxylase in *Pseudomonas citronellolis*. *Biochem. Biophys. Res. Comm.* **71**, 746-753.
- Huang, W., Jia, J., Gibson, K.J., Taylor, W.S., Rendina, A.R., Schneider, G. and Lindqvist, Y.** (1995) Mechanism of an ATP-dependent carboxylase, dethiobiotin synthetase, based on crystallographic studies of complexes with substrates and a reaction intermediate. *Biochem.* **34**, 10985-10995.
- Huang, N., Chandler, J., Thomas, B.R., Koizumi, N. and Rodriguez, R.L.** (1993) Metabolic regulation of  $\alpha$ -amylase gene expression in transgenic cell cultures of rice (*oryza sativa* L.). *plant Mol. Biol.* **23**, 737-747.
- Izumi, Y., Kano, Y., Inagaski, K., Kawase, N., Tani, Y. and Yamada, H.** (1981) Characterization of biotin biosynthetic enzymes of *Bacillus sphaericus*: a dethiobiotin producing bacterium. *Agric. Biol. Chem.* **45**, 1983-1989.
- Ishiguro, S. and Nakamura, K.** (1992) The nuclear factor SP8BF binds to 5'-upstream regions of three different genes coding for major proteins of sweet potato tuberous roots. *Plant Mol. Biol.* **18**, 97-108.
- Jiang, R. and Carlson, M.** (1996) Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. *Genes Dev.* **10**, 3105-3115.
- Jang, J., Leön, P., Zhoo, L. and Sheen, J.** (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* **9**, 5-19.

- Jang, J. and Sheen, J.** (1994) Sugar sensing in high plants. *Plant Cell* **6**, 1665-1679.
- Kleinig, H.** (1989) The role of plastids in isoprenoids biosynthesis. *Ann. Rev. Plant Physiol. & Plant Mol. Biol.* **40**, 39-59.
- Ke, J., Wen, T.-N., Nikolau, B.J. and Wurtele, E.S.** (2000) Coordinated regulation of the nuclear and plastidic genes coding for the subunits of the heteromeric acetyl-coenzyme A carboxylase. *J. Plant Physiol.* **122**, 1057-1071.
- Kimura, Y., Miyake, R., Tokumasu, Y. and Sato, M.** (2000) Molecular cloning and characterization of two genes from the biotin carboxylase and carboxyltransferase subunits of acetyl-coenzyme A carboxylase in *Myxococcus xanthus*. *J. Bacteriol.* **182**, 5462-5469.
- Knowles, J.R.** (1989) The mechanism of biotin-dependent enzymes. *Ann. Rev. Biochem.* **58**, 195-221.
- Kondo, H., shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M. and Tanabe, T.** (1991) Acetyl-CoA carboxylase from *Escherichia coli*: gene organization and nucleotide sequence of the biotin carboxylase subunit. *Proc. Natl. Acad. Sci. USA* **88**, 9730-9733.
- Koch K.E.** (1996) Carbohydrate-modulated gene expression in plants. *Ann. Rev. Plant Physiol. & Plant Mol. Biol.* **47**, 509-540
- Koch, K.E., Nolte, K.D., Duke, E.R., McCarty, D.R. and Avigne, W.T.** (1992) Sugar level modulate differential expression of maize sucrose synthase genes. *Plant cell* **4**:59-69.
- Kögel, F., and Tönnis, B.** (1936) *Z. Physiol. Chem.* **242**, 43-73.

- Krapp, A. and Stitt, M. (1995)** An evaluation of direct and indirect mechanisms for the 'sink-regulation' of photosynthesis in spinach: changes in gas exchange, carbohydrate, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source levels. *Planta* **195**, 313-323.
- Lau, E.P., Cochran, B.C. and Fall, R.R. (1980)** Subcellular localization of 3-methylcrotonyl-CoA carboxylase in bovine kidney. *Arch. Biochem. Biophys.* **205**, 352-359.
- Liu X.-J., Prat S., Willmitzer L. and Frommer W.B. (1990)** *Cis* regulatory elements directing tuber-specific and sucrose-inducible expression of a chimeric class I patatin promoter/GUS-gene fusion. *Mol. Gen. Genet.* **223**, 401-406.
- Lopez-Casillas, F., Bai, D.-H., Luo, X., Kong, I.-S., Hermodson, M.A. and Kim, K.-H. (1988)** Structure of coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. *Proc. Natl. Acad. Sci. USA* **85**, 5784-5788.
- Li, S.-J. and Cronan, J.E. Jr. (1992a)** The gene encoding the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. *J. Biol. Chem.* **267**, 855-863.
- Li, S.-J. and Cronan, J.E. Jr. (1992a)** The gene encoding the two carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. *J. Biol. Chem.* **267**, 16841-16847
- Ma, H. and Botstein, D. (1986)** Effects of null mutations in the hexokinase genes of *Saccharomyces cerevisiae* on catabolite repression. *Mol. Cell. Biol.* **6**, 4046-4052.
- Ma, H., Bloom, L.M., Walsh, C.T. and Botstein, D. (1989)** The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 5643-5649.

- Maier, A. and Lichtenthaler, H.K.** (1998) Methylcrotonyl-CoA carboxylase from barley (*Hordeum vulgare* L.): Purification, kinetic characterization and senescence-related induction of activity. *J. Plant Physiol.* **152**, 213-221.
- Mass, C., schaal, S. and Werr, W.** (1990) A feedback control element near the transcription start site of the maize *Shruken* gene determines promoter activity. *EMBO J.* **9**, 3447-3452.
- McKean, A.L., Ke, J., Song, J., Che, P., Achenbach, S., Nikolau, B.J. and Wurtele, E.S.** (2000) Molecular characterization of the non-biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase. *J. Biol. Chem.* **275**, 5582-5590.
- Moss, J. and Lane, M.D.** (1971) The biotin-dependent enzymes. *Adv. Enzymol.* **35**, 321-442.
- Otsuka, A.J., Buoncristiani, M.R., Howard, P.K., Flamm, J., Johsone, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J. and Matsuzaki, J.** (1988) The *Escherichia coli* biotin biosynthetic enzyme sequences predicated from the nucleotide sequence of the *bio* operon. *J. Biol. Chem.* **263**, 19577-19585.
- Nikolau, B.J. and Hawke, J.C.** (1984) Purification and characterization of maize leaf acetyl-CoA carboxylase. *Arch. Biochem. Biophys.* **228**, 86-96.
- Ohlrogge, J.B., Kuhn, D.N. and Stumpf, P.K.** (1979) Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. *Proc. Natl. Acad. Sci. USA* **76**, 1194-1198.
- Purcell, P.C., Smith, A.M. and Halford, N.G.** (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcription in leaves. *Plant J.* **14**, 195-202.

- Popják, G.** (1971) Specificity of enzymes in sterol biosynthesis. *Harvey Lect.* **65**, 127-156.
- Patton, D.A., Volrath, S. and Ward, E.R.** (1996) Complementation of the *bio 1* *Arabidopsis* biotin auxotroph with a bacterial biotin biosynthetic gene. *Mol. Gen. Genet.* **251**, 261-266.
- Patton, D.A., Johnson, M. and Ward, E.R.** (1996) Biotin synthase from *Arabidopsis thaliana*: cDNA isolation and characterization of gene expression. *Plant physiol.* **112**, 371-378.
- Pai, C.H.** (1975) Genetics of biotin biosynthesis in *Bacillus subtilis*. *J. Bacteriol.* **121**, 1-8.
- Perate, P., Matsukura, C., Vernieri, P. and Yamaguchi, J.** (1997) Sugar repression of a gibberellin-dependent signaling pathway in barley embryos. *Plant Cell* **9**, 2179-2208.
- Ploux, O. and Marquet, A.** (1992) The 8-amino-7-oxopelargonate synthase from *Bacillus sphaericus*: purification and preliminary characterization of the cloned enzyme overproduced in *Escherichia coli*. *Biochem. J.* **283**, 327-331.
- Ploux, O., Soularus, P., Marquet, A., Gloeckler R. and Lemoine, Y.** (1992) Investigation of the first step of biotin biosynthesis in *Bacillus sphaericus*: purification and characterization of the pimeloyl-CoA synthase, and uptake of pimelate. *Biochem. J.* **287**, 685-690.
- Roesler, K.R., Shorrosh, B.S. and Ohlrogge, J.B.,** (1994) Structure and expression of an *Arabidopsis* acetyl-coenzyme A carboxylase gene. *Plant Physiol.* **105**, 611-617.
- Rose, M., Albig, W. and Entian, K.-D.** (1991) Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinase PI and PII. *Eur. J. Biochem.* **199**, 511-518.

- Saier, M.H.** (1991) A multiplicity of potential carbon catabolite repression mechanism in prokaryotic and eukaryotic microorganisms. *New biol.* **3**, 1137-1147.
- Sasaki, Y., Hakamada, K., Suams, Y., Nagano, Y., Furusawa, I. and Matsuno, R.,** (1993) Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. *J. Biol. Chem.* **268**, 25118-25123.
- Schulte, W., Schell, J. and Topfer, R.** (1994) A gene encoding acetyl-coenzyme A carboxylase from *Brassica napus*. *Plant Physiol.* **106**, 793-794.
- Schiele, U., Niedermeier, R., Stürzer, M. and Lynen, F.** (1975) Investigations of the structure of 3-methylcrotonyl-CoA carboxylase from *Achromobacter*. *Eur. J. Biochem.* **60**, 259-266.
- Sasaki, Y., Konishi, T. and Nagano, Y.** (1995) The compartmentation of acetyl-coenzyme A carboxylase in plants. *Plant physiol.* **108**, 445-449.
- Shorrosh, B.S., Dixon, R.A. and Ohlrogge, J.B.** (1994) Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. *Proc. Natl Acad. Sci. USA* **91**, 4323-4327.
- Sorosh, B.S., Roesler, K.R., Shintani, D., van de Loo, F.J. and Ohlrogge, J.B.** (1995) Structure analysis, plastid localization, and expression of the biotin carboxylase subunit of acetyl-coenzyme A carboxylase from tobacco. *Plant physiol.* **108**, 805-812.
- Shellhammer, J. and Meinke, D.** (1990) Arrested embryos from the *bio 1* auxotroph of *Arabidopsis thaliana* contain reduced levels of biotin. *Plant Physiol.* **93**, 1162-1167.
- Sheen, J.** (1990) Metabolic repression of transcription in higher plants. *Plant Cell* **2**, 1027-1038.

- Song, J., Wurtele, E.S. and Nikolau, B.J.** (1994) Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase: Identification of the biotin carboxylase and biotin-carrier domains. *Proc. Natl Acad. Sci. USA* **91**, 5779-5783.
- Stumpf, P.K.** (1987) The biosynthesis of saturated fatty acids. In PK Stumpf, ed, The Biochemistry of Plant: A Comprehensive Treatise: Lipids: Structure and Function, Vol 9, Academic Press, New York, pp 121-136.
- Sun, J., Ke, J., Johnson, J.L., Nikolau, B.J. and Wurtele, E.S.** (1997) Biochemical and molecular biological characterization of CAC2, the *Arabidopsis thaliana* gene coding for the biotin carboxylase subunit of the plastidic acetyl-coenzyme A carboxylase. *Plant physiol.* **115**, 1371-1383.
- Sumrada, R.A. and Cooper, T.G.** (1982) Urea carboxylase and allophanate hydrolase are components of a multifunction protein in yeast. *J. Biol. Chem.* **257**, 9119-9127.
- Takai, T., Yokoyama, C., Wada, K. and Tanabe, T.** (1988) Primary structure of chicken liver acetyl-CoA carboxylase deduced from cDNA sequence. *J. Biol. Chem.* **263**, 2651-2657.
- Tsukaya H., Ohshima T., Naito S., Chino M. and Komeda Y.** (1991) Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis*. *Plant Physiol.* **97**, 1414-1421.
- Utter, M.F., Barden, R.D. and Taylor, B.L.** (1975) Pyruvate carboxylase: an evolution of the relationship between structure and mechanism and between structure and catalytic activity. *Adv. Enzymol.* **42**, 1-72.

- Wakil, A.-F., Chirala, S.S. and Wakil, S.J.** (1992) Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. *Proc. Natl. Acad. Sci. USA* **89**, 4534-4538.
- Wang, X., Wurtele, E.S. and Nikolau, B.J.** (1995) Regulation of  $\beta$ -methylcrotonyl-coenzyme A carboxylase activity by biotinylation of the apoenzyme. *Plant Physiol.* **108**, 1133-1139.
- Wang, X., Wurtele, E.S., Keller, G., McKean, A.L. and Nikolau, B.J.** (1994) Molecular cloning of cDNAs and genes coding for  $\beta$ -methylcrotonyl-CoA carboxylase of tomato. *J. Biol. Chem.* **269**, 11760-11769.
- Weaver, L., Yu, F., Wurtele, E.S. and Nikolau, B.J.** (1996) Characterization of the cDNA and gene coding for biotin synthase of *Arabidopsis thaliana*. *Plant physiol.* **110**, 1021-1028.
- Weaver, L.M., Lebrun, L., Franklin, A., Huang, L., Hoffman, N., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coenzyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1013-1014.
- Wurtele, E.S. and Nikolau, B.J.** (2000) Characterization of 3-methylcrotonyl-CoA carboxylase from plants. *Methods Enzymol.* **324**, 280-292.
- Wurtele, E.S. and Nikolau, B.J.** (1990) Plants contain multiple biotin enzymes: Discovery of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. *Arch. Biochem. Biophys.* **278**, 179-186.
- Yanai, Y, Kawasaki, T., Shimada, H., Wurtele, E.S. and Nikolau, B.J.** (1995) Genomic organization of 251 kDa acetyl-CoA carboxylase genes in *Arabidopsis*: tandem gene organization has made two differentially expressed isozymes. *Plant Physiol.* **36**, 779-787.



- Yu, S-M.** (1999) Cellular and genetic responses of plants to sugar starvation. *Plant Physiol.* **121**, 687-693.
- Yu, S-M., Kuo, Y-H., Sheu, Y-J. and Liu, L-F.** (1991) Metabolic depression of  $\alpha$ -amylase gene expression in suspension-cultured cells of rice. *J. Biol. Chem.* **266**, 21131-21137.
- Yu, S-M., Lee, Y-C., Fang, S-C., Chan, M-T., Hwa, S-F. and Liu, L-F** (1996) Sugars acts as signal molecules and osmotic to regulate the expression of  $\alpha$ -amylase genes and metabolic activities in germinating cereal grains. *Plant Mol. Biol.* **30**, 1277-1289.

## **CHAPTER 2. 3-METHYLCROTONYL-CoA CARBOXYLASE IS A COMPONENT OF THE MITOCHONDRIAL LEUCINE CATABOLIC PATHWAY IN PLANTS**

A paper published in the Journal of *Plant Physiology*

Marc D<sup>1</sup>. Anderson, Ping Che<sup>1</sup>, Jianping Song<sup>1</sup>, Basil J. Nikolau, and Eve Syrkin Wurtele

Department of Botany (M.D.A and E.S.W.) and Department of Biochemistry and Biophysics  
(P.C., J.S., and B.J.N.), Iowa State University, Ames, IA 50011

### **Abstract**

3-Methylcrotonyl-CoA carboxylase (MCCase) is a mitochondrial biotin-containing enzyme whose metabolic function is not well understood in plants. In soybean seedlings, the organ-specific and developmentally-induced changes in MCCase expression are regulated by mechanisms that control the accumulation of MCCase mRNA and that control the activity of the enzyme *per se*. During soybean cotyledon development, when seed storage proteins are degraded, leucine accumulation peaks transiently at 8 DAP. The coincidence between peak

---

<sup>1</sup> These authors contributed equally to this paper.

MCCase expression and the decline in leucine content provides correlative evidence that MCCase is involved in the mitochondrial catabolism of leucine. Direct evidence for this conclusion was obtained from radiotracer metabolic studies using extracts from isolated mitochondria. These experiments traced the metabolic fate of [U- $^{14}\text{C}$ ]leucine, and  $\text{NaH}^{14}\text{CO}_3$ , the latter of which was incorporated into methylglutaconyl-CoA via MCCase. These studies directly demonstrate that plant mitochondria can catabolize leucine via the following scheme: leucine  $\Rightarrow$   $\alpha$ -ketoisocaproate  $\Rightarrow$  isovaleryl-CoA  $\Rightarrow$  3-methylcrotonyl-CoA  $\Rightarrow$  3-methylglutaconyl-CoA  $\Rightarrow$  3-hydroxy-3-methylglutaryl-CoA  $\Rightarrow$  acetoacetate + acetyl-CoA. These findings demonstrate for the first time that the enzymes responsible for leucine catabolism are present in plant mitochondria. We conclude that a primary metabolic role of MCCase in plants is the catabolism of leucine.

### Introduction

3-Methylcrotonyl-CoA carboxylase (MCCase, EC 6.4.1.4) is a biotin-containing enzyme that catalyzes the Mg-ATP-dependent carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA. MCCase was discovered nearly 40 years ago in bacteria and mammals (see review by Moss and Lane, 1971) but its presence in plants was more recently reported (Wurtele and Nikolau, 1990). The reaction catalyzed by MCCase is required in several interconnected metabolic pathways (Fig. 1): leucine catabolism, isoprenoid catabolism, and the mevalonate shunt. The catabolism of leucine in animals and some bacteria requires the sequential action of six enzymes, which convert leucine to acetoacetate and acetyl-CoA. MCCase is the fourth enzyme in this catabolic pathway. In some species of bacteria, noncyclic isoprenoids, such as geranyl-CoA, are catabolized to acetyl-CoA

probably via a set of reactions analogous to  $\beta$ -oxidation; one of the reactions in this pathway is the carboxylation of MC-CoA (Seubert and Remberger, 1963). The mevalonate shunt converts mevalonate to acetoacetate and acetyl-CoA in animals (Popják, 1971; Edmond and Popják, 1974) and in plants (Nes and Bach, 1985). The net result of this shunt is to reassimilate carbon that has been converted to mevalonate into primary metabolites rather than into isoprenoids.

Despite the purification of MCCase from plants (Alban et al., 1993; Chen et al., 1993; Diez et al., 1994) and the isolation of cDNA and genomic clones for the biotin-containing subunit of MCCase (Song et al., 1994; Wang et al., 1994; Weaver et al., 1995), the physiological significance of this enzyme in plant metabolism has not been defined. Evidence for the catabolism of leucine has been provided by the observations that leucine-derived carbon is incorporated into sugars and organic acids (Stewart and Beevers, 1967; Sodek and Wilson, 1973) or isoprenoids (Overton, 1985; Koops et al., 1991) although there was no evidence implicating MCCase in the process. *In vitro* studies of leucine catabolism in plants indicate that this process occurs in peroxisomes by a mechanism that does not require MCCase (Gerbling and Gerhardt, 1988; 1989; Gerbling, 1993), a mitochondrial enzyme in plants (Baldet et al., 1992). To better understand the physiological role of MCCase in plants, we investigated the expression of MCCase during plant development, particularly focusing on cotyledon development when massive hydrolysis of seed storage proteins and the remobilization and catabolism of the amino acids occurs. We also examined the metabolism of leucine and MC-CoA in protein extracts from isolated mitochondria. Together, these studies demonstrate the involvement of MCCase in the mitochondrial catabolism of leucine.

## Materials and Methods

### Plant Material

Soybean seeds (*Glycine max* cv Corsoy 79) were germinated in a sterile mixture of 30% (v/v) black soil, 30% (v/v) peat moss, and 40% (v/v) perlite, in a greenhouse at 22 to 25 °C, under a cycle of 15 h illumination and 9 h darkness, with a maximum daily irradiance of no less than 1200  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Plants were watered daily and fertilized twice a week with a solution of 20/10/20 (N/P/K). Plants were grown to maturity in 25 cm pots. Plants that were to be sampled prior to 20 DAP were grown in 50 cm X 30 cm X 6 cm flats. All samples were harvested between 4 and 6 h after the beginning of the illumination period. Organs of plants at different stages of development were harvested and frozen in liquid N<sub>2</sub>. For long-term storage, plant materials for RNA isolation were stored at -70°C, and plant materials for protein isolation were stored in liquid N<sub>2</sub>. Mitochondria were isolated from shoots of pea (*Pisum sativum* cv Progress #9), 14 to 16 DAP, grown in flats under conditions similar to those described for soybeans.

### Isolation and Analysis of RNA

RNA was isolated as previously described (Logemann et al., 1987). The concentration of RNA was determined by absorbance at 260 nm. RNA samples were denatured with formaldehyde and formamide and were fractionated by electrophoresis using 1.0% agarose gels buffered with 0.02 M Mops, 8 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, pH 7.0 (Sambrook et al., 1989). After electrophoresis, the gel was washed twice with DEPC-treated water, soaked in 20 X SSC (1 X SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 10 min, and the RNA was transferred by capillary action with 20

X SSC to a nitrocellulose filter. The filter was subsequently baked for 2 h at 80°C in a vacuum oven, prehybridized for 2 h at 68°C in a solution containing 6 X SSC, 5 X Denhardt's [1 X Denhardt's is 0.02% (w/v) Ficoll, 0.02% (w/v) PVP-360, 0.025% (w/v) BSA], 50 mM Tris-HCl, pH 8.0, 0.2% (w/v) SDS, 10 mM EDTA, and 100 µg/mL sheared salmon sperm DNA, and hybridized overnight at 68°C in a similar solution containing 10% (w/v) dextran sulfate and <sup>32</sup>P-labeled DNA probes. The DNA probes were radioactively labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (10 mCi/mmol, Amersham) using the random primer labeling method (Feinberg and Vogelstein, 1983). After hybridization, the membrane was washed twice with 0.2 X SSC, 0.5% SDS for 10 min at room temperature followed by a third wash for 30 min at 68°C.

### **Preparation of Cell-Free Extracts**

All the procedures were performed at 4°C. Soybean organs (1 to 2 g fresh weight) were pulverized using a mortar and pestle in the presence of liquid N<sub>2</sub>. The frozen pulverized tissue was homogenized with 3 to 4 volumes of a buffer containing 0.1 M Hepes-KOH, pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 20 mM  $\beta$ -mercaptoethanol, 100 µg/mL PMSF, and 10 µM E-64. The extract was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 22,100 g<sub>max</sub> for 15 min. The extract was desalted in 200 µL portions using Sephadex G-25 columns (1 mL bed volume) and the pooled eluate was utilized for MCCase assays and western blot analyses.

## Enzyme Assays

MCCase activity was assayed by the incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into an acid-stable product (Wurtele and Nikolau, 1990). The reaction mixture contained 0.1 M Tricine-KOH, pH 8.0, 5 mM  $\text{MgCl}_2$ , 2.5 mM DTT, 5 mM  $\text{KHCO}_3$ , 5  $\mu\text{Ci}$   $\text{NaH}^{14}\text{CO}_3$  (58 mCi/mmol, Amersham), 1 mM ATP, and 0.2 mM MC-CoA. The assay was initiated by the addition of the protein extract and incubated in a final volume of 200  $\mu\text{L}$  at 37°C for 10 min. The reaction was terminated by the addition of 50  $\mu\text{L}$  6 N HCl. A sample (100  $\mu\text{L}$ ) was applied to a strip of Whatmann 3MM paper, dried, and the acid-stable radioactivity was determined by liquid scintillation. Assays were performed in duplicate. Control assays lacking the MC-CoA substrate were carried out for each protein extract. Cyt c oxidase activity was assayed in 1 mL reactions containing 0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.0, 0.1% (v/v) Nonidet P-40, mitochondria (10 to 25  $\mu\text{g}$  protein), and 0.4 mg reduced cyt c ( $\epsilon_{550} = 21 \text{ mM}^{-1} \text{cm}^{-1}$ ). The oxidation state of cyt c was adjusted to a standard level ( $A_{550} = 0.3$  for a 0.16 mg/mL cyt c solution) using sodium hydrosulfite as the reducing agent. Reactions were started with cyt c and oxidation was monitored by a change in absorbance at 550 nm. Hydroxypyruvate reductase was assayed according to Schwitzguebel and Siegenthaler (1984) and NADP-glyceraldehyde-3-phosphate dehydrogenase was assayed according to Heber et al. (1963) as modified by Kaethner and ap Rees (1985).

## Electrophoresis and Western Blot Analysis

SDS-PAGE was performed in 7.5% acrylamide gels as described previously (Laemmli, 1970). After electrophoresis, proteins were electrophoretically transferred to a

nitrocellulose membrane (Kyhse-Andersen, 1984), and the biotin-containing proteins were detected by using  $^{125}\text{I}$ -streptavidin (Nikolau et al., 1985), or the biotin-containing polypeptide of MCCase was immunologically detected with specific antiserum (Song et al., 1994), diluted 1:200, followed by an incubation with  $^{125}\text{I}$ -Protein A.

### **Quantitation of Chlorophyll, Leucine, and Protein**

The chlorophyll content of developing soybean cotyledons was measured by the method of Arnon (1949). Free leucine content of developing soybean cotyledons was determined following conversion of leucine to its dansyl derivative and separation by HPLC. Cotyledons (0.5 g) were homogenized with 5 mL 10% TCA and the insoluble material was removed by centrifugation at 10000  $g_{\text{max}}$  for 10 min. The supernatant was extracted three times with cold diethyl ether to remove the TCA. The dansylation procedure was modified from that of Tapuhi et al. (1981). Equal volumes (200  $\mu\text{L}$ ) of TCA-extracts, 0.2 M  $\text{NaHCO}_3$ , and 1.5 mg/mL dansyl chloride were mixed and incubated for 60 min at 21°C in darkness. The reaction was terminated by addition of 20  $\mu\text{L}$  2% (w/v) methylamine. The dansyl-leucine concentrations were determined from the absorbance at 254 nm following HPLC fractionation. The protein content of developing soybean cotyledons was determined from the protein concentration of the TCA-precipitate of the cotyledon extracts after an overnight incubation in 0.1 M NaOH to resolubilize the protein (Bradford, 1976, as modified by Jones et al., 1989).



### **Isolation of Mitochondria**

Mitochondria were isolated from pea shoots by a method modified from that of Sandalio et al. (1987). Reagents and equipment were maintained at 0-4°C. Chilled plant material (480 g) was homogenized with a mortar and pestle in 80 g batches with 240 mL of 0.35 M mannitol, 30 mM Mops-NaOH, 1 mM EDTA, 0.2% (w/v) BSA, 4 mM Cys, pH 7.5, and the homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged 1500  $g_{\max}$  for 10 min to yield a pellet containing predominantly intact chloroplasts and other dense material. The supernatant was centrifuged at 12000  $g_{\max}$  for 20 min to yield a pellet enriched in mitochondria and peroxisomes. Pellets were resuspended in wash medium (0.3 M mannitol, 20 mM Mops-NaOH, 1 mM EDTA, 0.2% (w/v) BSA, pH 7.2) and repelleted at 12000  $g_{\max}$  for 20 min. Washed pellets were resuspended in approximately 24 mL of wash medium and distributed among eight 40 mL polycarbonate tubes containing discontinuous Percoll gradients. Each gradient consisted of 6 mL 60% (v/v) Percoll, 16 mL 38% (v/v) Percoll, and 10 mL 26% (v/v) Percoll in a buffer of 0.25 M sucrose, 10 mM Mops-NaOH, 0.2% (w/v) BSA, pH 7.2. These step gradients separated mitochondria (which form a band at the 26%/38% interface) from peroxisomes (which form a band at the 38%/60% interface), and to some extent from thylakoid membranes (atop and within the 26% Percoll layer). After centrifugation at 15000  $g_{\max}$  for 45 min using a Beckman JA-20 rotor, mitochondria were removed, diluted at least 5-fold with wash medium, centrifuged at 12000  $g_{\max}$  for 20 min, and resuspended in a final volume of approximately 16 mL. This suspension was then distributed over four 40 mL polycarbonate tubes containing continuous Percoll gradients (self-generating gradients, initially 31% (v/v)

Percoll). Gradients were subjected to centrifugation at 41000  $g_{\max}$  for 30 min. The highly purified mitochondria (which form a band near the bottom of the continuous gradient) were collected and washed twice by dilution (at least 5-fold) with BSA-free wash medium and centrifugation at 12000  $g_{\max}$  for 20 min. Pooled mitochondrial pellets (generally 15 to 25 mg protein) were resuspended in a small volume of BSA-free wash medium (final volume of 1.5 to 2.5 mL). Mitochondrial purity was assessed by assaying cyt c oxidase activity (mitochondrial marker), hydroxypyruvate reductase activity (peroxisomal marker), and NADP-glyceraldehyde-3-phosphate dehydrogenase activity (chloroplastic marker) in samples taken from the crude filtrate and from the final mitochondrial preparation. In mitochondrial purifications, 12 to 21% of the cyt c oxidase activity was retained, resulting in a 47- to 68-fold increase in specific activity. The purification procedure resulted in the removal of over 99% of hydroxypyruvate reductase activity and over 99.9% of NADP-glyceraldehyde-3-phosphate dehydrogenase activity.

### ***In vitro* Mitochondrial Assays**

Suspensions of freshly isolated mitochondria were adjusted to 10 mM DTT and 0.01% (v/v) Triton X-100 and sonicated (15 sec/mL, 60% output power). Lysed mitochondria were immediately desalted on Sephadex G-25 columns (200  $\mu$ L extract/mL bed volume), using 0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.2, 1 mM DTT, as the elution buffer, and eluants were immediately frozen in liquid nitrogen and stored until later use.

The components of the leucine catabolic pathway were assessed as follows: Mitochondrial extracts were incubated with either 1  $\mu$ Ci L-[U- $^{14}\text{C}$ ]leucine (346 mCi/mmol, ICN), or with various intermediates of the leucine catabolic pathway (300  $\mu$ M MC-CoA, 1

mM IV-CoA, 1 mM  $\alpha$ KIC, or 1 mM L-leucine) in the presence of 20  $\mu$ Ci  $\text{NaH}^{14}\text{CO}_3$  (54 mCi/mmol, Amersham). The basal reaction components of these incubations were: 0.3 M mannitol, 5 mM  $\text{MgCl}_2$ , 10 mM KCl, 10 mM  $\text{KH}_2\text{PO}_4$ , 0.1% (w/v) BSA, 300  $\mu$ M thiamine pyrophosphate, 300  $\mu$ M  $\text{NAD}^+$ , 300  $\mu$ M  $\text{FAD}^+$ , 1 mM ATP, 500  $\mu$ M CoA, 300  $\mu$ g mitochondrial protein, pH 7.2 in a total volume of 600  $\mu$ L. In reactions containing either L-[U- $^{14}\text{C}$ ]leucine or nonradioactive leucine, 1 mM  $\alpha$ -ketoglutarate was added to support the transamination of leucine, and in reactions with L-[U- $^{14}\text{C}$ ]leucine, 300  $\mu$ M  $\text{NaHCO}_3$  was added to support the MCCase reaction. Mitochondrial extract was added immediately prior to the start of the reactions, which were initiated by the addition of the appropriate radioactively-labeled compound. Reactions were incubated at 21°C for the appropriate duration on a nutator, and terminated by adjusting the solution to 5% TCA. Precipitated protein was pelleted in a microcentrifuge and 10  $\mu$ L of the supernatant was transferred to a 0.75 cm x 0.75 cm section of Whatman 3MM paper, allowed to dry overnight to remove  $^{14}\text{CO}_2$ , and total acid-stable products were measured by liquid scintillation. The remainder of the supernatant was filtered through a 0.2 nm pore nylon syringe filter for HPLC analysis.

### HPLC Analysis

For the quantitation of leucine, dansylated amino acids were fractionated by HPLC, using an octadecylsilane column (Supelcosil LC-18, 25 cm x 4.6 mm i.d., 5  $\mu$ m particle size) with a 20  $\mu$ L injection volume and a flow rate of 1.2 mL/min. The solvent program was: isocratic at 7% B for 1 min, linear gradients of 7% B to 48% B over 15 min, 48% B to 65% B over 2 min, 65% B to 7% B over 7 min, and isocratic at 7% B for 3 min (buffer A was 10%

acetonitrile and buffer B was 30% acetonitrile, both in 25 mM trifluoroacetic acid, pH 7.6). Eluted peaks were identified by absorbance at 254 nm and quantification was performed with reference to standard dansyl-leucine.

Separation of radioactively-labeled metabolites was performed using octadecylsilane columns (Supelcosil LC-18, 25 cm x 4.6 mm i.d., 5  $\mu$ m particle size; Alltech Adsorbosphere, 25 cm x 4.6 mm i.d., 5  $\mu$ m particle size). The solvents and gradient profiles were the same as reported by King and Reiss (1985). Radioactively-labeled metabolites were detected using a Beckman 171 radioisotope detector with a scintillation cocktail (ScintiSafe Plus 50%, Fisher) flow rate of 2 mL/min. Radioactive peaks were identified by coelution with standards, measured at the maximal absorbance wavelength of the specific compounds (254 nm for acyl-CoA compounds and 210 nm for organic acids). The identity of acyl-CoA metabolites was confirmed by observing the disappearance of specific peaks after alkaline hydrolysis of the thioester bond (2.5 M NaOH, 3 h, 60°C). This treatment also affects  $\alpha$ KIC, which undergoes decarboxylation at alkaline pH.

### **Statistical Analyses**

MCCase activity and leucine, protein, and chlorophyll data from Figs. 2 and 3 are reported with standard errors of individual treatments. For all other data, standard errors were determined from the mean square of the error term from each respective analysis of variance. MCCase activity in Table I was analyzed as a factorial design with organ, developmental stage, and replication as the independent variables. The remaining data were analyzed as a factorial design with substrate, avidin, incubation duration, and replication (Fig. 5 and Table II) or reaction, incubation duration, and replication (Table III) as the

independent variables. Incubation duration was treated as a linear variable within the model. Interactions that did not significantly reduce the error or were not meaningful were removed from the model and only treatments giving a measurable response were included in the analysis. For the data in Tables II and III and Fig. 5, the variance was proportional to the mean; consequently, a log transformation was used.

## **Results**

### **MCCase Activity during Soybean Fruit Development**

MCCase activity was determined in extracts from the developing soybean fruit, its subtending leaf, and in vegetative leaves (Table I). In developing seeds, MCCase activity peaked at the onset of storage lipid and protein deposition at about 30 DAF, and subsequently declined to 1/3 of the peak activity as the seed matured. MCCase activity was also detectable in dry seeds. During seed set, MCCase activity was somewhat lower in the pod (pericarp) than in the developing seed, and mirrored the developmentally-induced changes that occurred in the seed until desiccation. In contrast to the seed, the pod did not retain MCCase activity upon desiccation. In the leaves subtending the fruit, MCCase activity changed little during the period when seeds were developing, until late in seed development, when the leaves began to senesce and MCCase activity declined. MCCase activity in vegetative leaves was higher than in the leaves subtending the fruit and was somewhat higher in the young leaves than in the mature leaves.

### **Organ-Specific Expression of MCCase**

The distribution of MCCase was examined among organs of soybean seedlings at 13 DAP. At this stage of maturity, the unifoliate leaves of the seedlings had fully unrolled (VC stage; Fehr and Caviness, 1977) and the cotyledons were beginning to senesce (Fig. 2A). MCCase activity was detectable in the shoot apex and in all organs of the seedling (Fig. 2B). The specific activity of MCCase was highest in roots and hypocotyls and lowest in unifoliate leaves and shoot apices. There was up to a 7-fold difference in the specific activities of MCCase among these organs.

To determine whether these differences in MCCase activity are due to the differences in the accumulation of the biotin-containing subunit of MCCase, samples of extracts from each organ containing equal MCCase activity were subjected to SDS-PAGE, followed by western analysis with antiserum against the biotin-containing subunit of MCCase (Fig. 2C). We would expect to observe equal intensities of the 85 kD MCCase subunit if the difference in activity among the organs was due to differences in the accumulation of the biotin-containing subunit. This was not the case; therefore, we conclude that the level of the biotin-containing subunit alone does not determine the activity of MCCase. One possible specific explanation that we tested is that MCCase activity is modified by the degree of biotinylation of the apoprotein, as was previously observed in tomato (Wang et al., 1995). However, in soybean, the differences in the intensity of the MCCase biotin-containing subunit band, revealed by western analysis with  $^{125}\text{I}$ -streptavidin, mirrored the changes detected by the antiserum to that subunit (cf. Figs. 2C and 2D), indicating that the relative state of biotinylation of the MCCase subunit was constant among the organs examined.

### **MCCase Expression in Developing Cotyledons**

One possible metabolic function of MCCase in plants is in the catabolism of leucine. Examination of MCCase in organs where free leucine is expected to be rapidly metabolized may provide insight into the role of MCCase in leucine catabolism. Developing soybean cotyledons were chosen as the model system. Using chlorophyll content as the criterion, under the growth conditions used in these experiments, development of the photosynthetic apparatus occurred between 6 and 10 DAP, thereafter the cotyledons maintained photosynthetic competence until 18 DAP when senescence became evident. Protein content declined dramatically from 6 to 14 DAP, reflecting the degradation of seed storage proteins (Fig. 3A). During this period of protein mobilization, free leucine accumulated, peaking at 8 DAP, and then sharply declined (Fig. 3B). During soybean cotyledon development, MCCase activity increased, reaching a peak at 10 DAP during the period of rapid protein degradation and coinciding with the decline in leucine concentration (Fig. 3B). The decrease in MCCase activity lags just behind the decline in leucine content, consistent with a role of MCCase in leucine catabolism.

To ascertain whether these developmentally associated changes in MCCase activity are ascribable to changes in MCCase enzyme accumulation or changes in enzyme efficiency, we determined the accumulation of the biotin-containing subunit of MCCase. Western blots were analyzed with either anti-MCCase serum to detect the subunit polypeptide *per se* (Fig. 3C) or  $^{125}\text{I}$ -streptavidin to detect the biotin prosthetic group of this polypeptide (Fig. 3D). In these analyses, gels were loaded with samples containing equal amounts of MCCase activity. Hence, if the changes in activity during cotyledon development are due to changes in the steady state levels of the MCCase biotin-containing subunit, we would expect to observe

equal intensity protein bands. However, the MCCase biotin-containing subunit exhibited a modulation in intensity. Therefore, as we concluded with the organs of soybean seedlings, the level of the biotin-containing subunit alone does not determine the activity of MCCase. Furthermore, the close correlation between the levels of the biotin-containing subunit (Fig. 3C) and the biotin prosthetic group on this subunit (Fig. 3D) indicates that the biotinylation status of the MCCase biotin-containing subunit is unaltered during cotyledon development.

### **Abundance of MCCase mRNA in Soybeans**

To ascertain whether the developmentally-associated changes in MCCase expression may also be regulated by the accumulation of the mRNA coding for the biotin-containing subunit of MCCase, we determined the abundance of this mRNA in organs of soybean plants at 13 DAP and in cotyledons from seedlings of various ages (Fig. 4). In plants at 13 DAP, the steady-state level of the 2.4 kb MCCase mRNA was highest in the cotyledon, followed by, in decreasing levels, the hypocotyl, shoot apex, root, and stem (Fig. 4C). Very little MCCase mRNA was detected in the leaf. In developing cotyledons, the steady state levels of the MCCase mRNA changed slightly from 4 to 8 DAP, increased by at least 10-fold between 8 and 10 DAP, and subsequently declined to about 1/5 of the level present at peak accumulation (Fig. 4D).

### **Mitochondrial Catabolism of MC-CoA**

Because MCCase is located in the mitochondria (Baldet et al., 1992), we investigated MCCase-related metabolism in this organelle. In the presence of MC-CoA and cofactors (see Materials and Methods), mitochondrial extracts catalyze the incorporation of



radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into acid-stable metabolites in a time-dependent manner (Fig. 5A). Incorporation of label was precluded by withholding MC-CoA or ATP (data not shown), or by preincubating mitochondrial extracts with avidin (Wurtele and Nikolau, 1990; Fig. 5A), consistent with a requirement for MCCase. Analysis of the products by HPLC revealed four radioactively-labeled peaks, eluting at ~3.3 min, ~5 min, ~19.2 min, and ~22.6 min (Figs. 6A and 6B). These were identified as acetoacetate, bicarbonate, HMG-CoA, and MG-CoA, respectively. Acetoacetate, HMG-CoA, and MG-CoA are predicted to be labeled if MCCase, MG-CoA hydratase, and HMG-CoA lyase are present and active in mitochondria (Fig. 1). The kinetics of metabolite accumulation (Figs. 5B, 5C, and 5D) support the sequential action of these enzymes. MG-CoA, the immediate product of MCCase, is labeled initially, followed sequentially by accumulation of [ $^{14}\text{C}$ ]HMG-CoA and [ $^{14}\text{C}$ ]acetoacetate. Interestingly, [ $^{14}\text{C}$ ]HMG-CoA accumulated to higher levels than [ $^{14}\text{C}$ ]MG-CoA, indicating that the hydratase reaction occurs at a faster rate than the lyase reaction, at least under the conditions we employed.

Additional evidence for the sequential conversion of MC-CoA to MG-CoA, HMG-CoA and acetoacetate was obtained in experiments in which avidin was used to inhibit MCCase activity after an initial incubation without avidin. In these experiments, we essentially carried out a pulse-chase experiment, where the incubation without avidin (for 2.5 min) represented the pulse phase, and the incubation following the addition of avidin was the chase phase. As avidin inhibited further incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$ , [ $^{14}\text{C}$ ]MG-CoA and [ $^{14}\text{C}$ ]HMG-CoA sequentially declined as [ $^{14}\text{C}$ ]acetoacetate accumulated. The accumulation of [ $^{14}\text{C}$ ]acetoacetate indicates that further metabolism of acetoacetate in

mitochondrial extracts is slower than the previous reaction catalyzed by HMG-CoA lyase, or that the catabolic mechanism (if it exists in mitochondria) has been disrupted in our mitochondrial extracts. These results clearly indicate the existence of MCCase, MG-CoA hydratase, and HMG-CoA lyase in plant mitochondria, enzymes that catalyze reactions common to leucine catabolism, isoprenoid catabolism, and the mevalonate shunt.

### **Mitochondrial Catabolism of Leucine**

If MCCase is involved in leucine catabolism, IV-CoA,  $\alpha$ -KIC, and leucine would be precursors of MC-CoA (Fig. 1). We addressed this hypothesis by testing whether IV-CoA,  $\alpha$ -KIC, and leucine could support the incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into MG-CoA, HMG-CoA, and acetoacetate. The results presented in Table II indicate that both IV-CoA and  $\alpha$ -KIC support the incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into acid-stable products in a time-dependent manner. As expected, this incorporation is avidin-sensitive. Both IV-CoA and  $\alpha$ -KIC support the incorporation of radioactivity into HMG-CoA, detectable after a 20 min incubation. In the case of the incubation with IV-CoA,  $[^{14}\text{C}]$ MG-CoA was detected after a 1 h incubation. These data indicate that both BCKDH and the branched-chain acyl-CoA dehydrogenase are present in mitochondria (Fig. 1). Leucine was unable to support detectable incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into MG-CoA or HMG-CoA (data not shown). A likely explanation is that leucine is too far upstream in the metabolic pathway from the MCCase reaction to be an effective source of MC-CoA.

To demonstrate that mitochondria possess the entire complement of leucine catabolic enzymes, the metabolic products of  $[\text{U}-^{14}\text{C}]$ leucine were examined by HPLC. Upon

incubation of mitochondrial extracts with [U- $^{14}\text{C}$ ]leucine, six primary peaks of radioactive metabolites were observed, eluting at ~3.6 min, ~5.1 min, ~13 min, ~20 min, ~36.5 min, and ~38.5 min. The peak at ~20 min coelutes with isovaleric acid, possibly arising from decarboxylation of  $\alpha\text{KIC}$ . Other peaks were identified as leucine (~5.1 min),  $\alpha\text{KIC}$  (~13 min), MC-CoA (~36.5 min), and IV-CoA (~38.5 min). The peak at ~3.6 min remains unidentified.  $\alpha\text{-}[^{14}\text{C}]\text{KIC}$  was first detected after 20 min and continued to accumulate to a maximum at 2 h (Table III). The conversion of [U- $^{14}\text{C}$ ]leucine to  $\alpha\text{-}[^{14}\text{C}]\text{KIC}$  as well as subsequent catabolism was dependent on the addition of  $\alpha$ -ketoglutarate, implicating the presence of the branched-chain amino acid aminotransferase in mitochondria. Accumulation of [ $^{14}\text{C}$ ]IV-CoA was detected between 1 and 2 h, demonstrating the sequential action of the aminotransferase and BCKDH. This appearance of [ $^{14}\text{C}$ ]IV-CoA was prevented by competition with nonradioactive  $\alpha\text{KIC}$  or by withholding free CoA, which is needed by BCKDH, from the incubation mix. [ $^{14}\text{C}$ ]MC-CoA was detectable after 4 h of incubation and its presence was enhanced by addition of avidin, which inhibits MCCase and prevents further catabolism of MC-CoA.

## Discussion

### Developmental Regulation of MCCase

As a first step in elucidating the physiological functions of MCCase in plants, we determined the accumulation of MCCase mRNA and protein in different organs of soybean and during cotyledon development. MCCase is expressed in all organs of the soybean plant, although there is considerable variation in the level of expression, presumably reflecting the relative importance of MCCase in these organs. These studies indicated that MCCase

expression can be regulated by at least two different mechanisms. Changes in the accumulation of the mRNA for the biotin-containing subunit of MCCase probably reflect developmentally-regulated changes in its gene transcription or mRNA stability. For example, during the development of the cotyledons, the accumulation of this mRNA increases with increasing age of the organ. In addition, MCCase activity does not always correlate with the level of accumulation of the MCCase biotin-containing subunit, indicating MCCase activity is controlled by a second mechanism. This lack of correlation could be explained by changes in the accumulation of the nonbiotinylated subunit (which we could not detect) or changes in the catalytic efficiency of the MCCase enzyme. The catalytic efficiency of the MCCase enzyme might be altered during development due to a post-translational mechanism (e.g. covalent modification of the enzyme) or due to the differential expression of isozymes of MCCase that differ in their catalytic efficiency. One post-translational modification that is essential for the activity of MCCase is biotinylation; MCCase activity is modulated by biotinylation in tomato (Wang et al., 1995). However, in seedling organs and developing cotyledons of soybean, differences in the abundance of the biotin prosthetic group closely paralleled differences in the abundance of the biotinylated subunit. Therefore, MCCase did not appear to be regulated by biotinylation in soybean. Clearly, isolation and characterization of the gene for the nonbiotinylated subunit of MCCase will facilitate studies of the regulation of the activity of this enzyme.

Cotyledons initially function as a source tissue supporting the growth of the entire seedling. At this stage of development, seed storage proteins are hydrolyzed and the released amino acids are incorporated into new proteins, catabolized during respiration, or utilized in the formation of new compounds. In soybean cotyledons, we observed that the maximal rate

of protein degradation occurred between 4 and 6 DAP, which correlated with the peak accumulation of free leucine content. Subsequently, between 8 and 10 DAP, the free leucine concentration declined and this correlated with the peak in MCCase activity. These data indicate that MCCase may have a role in leucine catabolism in cotyledons. This conclusion is consistent with the finding that in carbohydrate-starved sycamore cells, there is a correlation between a transient accumulation of leucine and the accumulation of the biotin-containing subunit of MCCase (Aubert et al., 1996). It is possible that leucine accumulation itself, or some consequence of leucine accumulation, is responsible for the induction of MCCase activity. Indeed, exogenously added leucine has been observed to induce MCCase activity in carrot cells (E.S Wurtele, B.J. Nikolau, unpublished). However, in sycamore cells, exogenous leucine did not induce accumulation of the biotin-containing subunit of MCCase (Aubert et al., 1996), suggesting that leucine itself was not the signaling molecule in this system.

### **Role of MCCase in Leucine Catabolism**

To provide more direct evidence for the metabolic function of MCCase in plants, we analyzed the fate of the MCCase product, MG-CoA, in extracts from isolated mitochondria. Incubation of mitochondrial extracts with MC-CoA and  $\text{NaH}^{14}\text{CO}_3$  resulted in the time-dependent incorporation of label into three compounds; MG-CoA, HMG-CoA, and acetoacetate. These three metabolites are those predicted to arise from MC-CoA catabolism via the pathway depicted in Fig. 1. Furthermore, the time-course of accumulation of radioactivity in these products relative to each other, as well as the depletion of [ $^{14}\text{C}$ ]MG-CoA and [ $^{14}\text{C}$ ]HMG-CoA and the simultaneous accumulation of [ $^{14}\text{C}$ ]acetoacetate following

addition of avidin to the mitochondrial extracts, are consistent with the interdependence of these three metabolites within a single pathway. Additional radiotracer experiments showed that mitochondrial extracts can metabolize  $\alpha$ KIC and IV-CoA to MC-CoA, which could then be carboxylated by MCCase. Finally, mitochondrial extracts are able to catabolize [U- $^{14}\text{C}$ ]leucine sequentially to  $\alpha$ -[ $^{14}\text{C}$ ]KIC, [ $^{14}\text{C}$ ]IV-CoA and [ $^{14}\text{C}$ ]MC-CoA. In combination, these radiotracer metabolic studies have shown that mitochondria have the capacity to catabolize leucine to acetoacetate and presumably, acetyl-CoA as illustrated in Fig. 1. These findings imply that the six enzymes involved in leucine catabolism are housed in the mitochondria.

In animals, the mitochondrial location of all of the enzymes of leucine catabolism is well established while in plants, these enzymes have received little attention. MG-CoA hydratase and HMG-CoA lyase have been studied in the context of their role in providing HMG-CoA for isoprenoid biosynthesis (Alam et al., 1991; Weber and Bach, 1993; van der Heijden et al., 1994; van der Heijden and Verpoorte, 1995). Studies of the branched-chain amino acid aminotransferase have concentrated on its role as the terminal enzyme in the biosynthesis of branched-chain amino acids (Aarnes, 1981; Pathre et al., 1987; 1989). Until our studies, the subcellular locations of these enzymes were unknown in plants.

Apart from MCCase, the only enzyme involved in mitochondrial leucine catabolism for which a cDNA or gene has been cloned is BCKDH (Fujiki et al., 1997; Luethy et al., 1997; Mooney et al., 1998). Interestingly, Fujiki et al. (1997) have observed enhanced accumulation of BCKDH mRNA in response to dark-induced starvation and senescence, conditions which induce MCCase accumulation (Aubert et al., 1996). A BCKDH has been shown to be present in peroxisomes and subsequent studies on the metabolism of  $\alpha$ KIC by

isolated peroxisomes have led to the prediction of a parallel, but MCCase-independent pathway for leucine catabolism (Gerbling and Gerhardt, 1988; 1989; Gerbling, 1993).

Our findings that mitochondria have the capacity to catabolize leucine by an MCCase-dependent mechanism, in combination with the studies of Gerbling and Gerhardt (1989) and Gerbling (1993), indicate that plants can catabolize leucine via two pathways that are separated into different subcellular compartments, mitochondria and peroxisomes. Studies are required to determine the fate of carbon catabolized via these two pathways and the mechanisms that regulate the commitment of leucine to each catabolic pathway. The conditions under which such partitioning is affected should reveal much about the mechanisms that control the utilization of carbon reserves during plant metabolism.

In addition to its role in the mitochondrial catabolism of leucine, MCCase has been suggested to participate in two important elements of isoprenoid metabolism in plants: the mevalonate shunt (Nes and Bach, 1985) and the catabolism of isoprenoids via geranoyl-CoA (X. Guan, T.A. Diez, B.J. Nikolau, E.S. Wurtele, unpublished). The ultimate product of these pathways, acetyl-CoA, has a wide array of potential fates including respiration, the glyoxylate cycle, lipogenesis, and the biogenesis of polyketides (Fig. 1). By virtue of its central position in leucine and isoprenoid metabolism, and the numerous potential fates of acetyl-CoA, MCCase might play a pivotal role in regulating the carbon flux among these pathways.

**Literature Cited**

- Aarnes H** (1981) Branched-chain amino acid aminotransferase in barley seedlings. *Z Pflanzenphysiol* **102**: 81-89
- Alam A, Britton G, Powls R, Goad J** (1991) Aspects related to 3-hydroxy-3-methylglutaryl-CoA synthesis in higher plants. *Biochem Soc Trans* **19**: 164S
- Alban C, Baldet P, Axiotis S, Douce R** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from higher plant mitochondria. *Plant Physiol* **102**: 957-965
- Arnon DI** (1949) Copper enzymes in chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* **24**: 1-15
- Aubert S, Alban C, Bligny R, Douce R** (1996) Induction of beta-methylcrotonyl-coenzyme A carboxylase in higher plant cells during carbohydrate starvation: Evidence for a role of MCCase in leucine catabolism. *FEBS Lett* **383**: 175-180
- Baldet P, Alban C, Axiotis S, Douce R** (1992) Characterization of biotin and 3-methylcrotonyl-coenzyme A carboxylase from higher plant mitochondria. *Plant Physiol* **99**: 450-455
- Bradford MM** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem* **72**: 248-254
- Chen Y, Wurtele ES, Wang X, Nikolau BJ** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from somatic embryos of *Daucus carota*. *Arch Biochem Biophys* **305**: 103-109



- Diez TA, Wurtele ES, Nikolau BJ** (1994) Purification and characterization of 3-methylcrotonyl-coenzyme-A carboxylase from leaves of *Zea mays*. Arch Biochem Biophys **310**: 64-75
- Edmund J, Popják G** (1974) Transfer of carbon atoms from mevalonate to *n*-fatty acids. J Biol Chem **249**: 66-71
- Fehr WR, Caviness CE** (1977) Stages of soybean development. Iowa State University Cooperative Extension Service **80**: 1-12
- Feinberg AP, Vogelstein B** (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132**: 6-13
- Fujiki Y, Sato T, Yoshikawa Y, Ito M, Watanabe A** (1997) Dark induced expression of genes for branched-chain alpha-keto acid dehydrogenase complex in *Arabidopsis* leaves (abstract No. 400). Plant Physiol **114**: S-95
- Gerbling H** (1993) Peroxisomal degradation of 2-oxoisocaproate. Evidence for free acid intermediates. Bot Acta **106**: 380-387
- Gerbling H, Gerhardt B** (1988) Oxidative decarboxylation of branched-chain 2-oxo fatty acids by higher plant peroxisomes. Plant Physiol **88**: 13-15
- Gerbling H, Gerhardt B** (1989) Peroxisomal degradation of branched-chain 2-oxo acids. Plant Physiol **91**: 1387-1392
- Heber U, Pon NG, Heber M** (1963) Localization of carboxydismutase and triosephosphate dehydrogenases in chloroplasts. Plant Physiol **38**: 355-360
- Jones CG, Hare JD, Compton SJ** (1989) Measuring plant protein with the Bradford assay. J Chem Ecol **15**: 979-993

- Kaethner TM, ap Rees T** (1985) Intracellular location of ATP citrate lyase in leaves of *Pisum sativum* L. *Planta* **163**: 290-294
- King MT, Reiss PD** (1985) Separation and measurement of short-chain coenzyme-A compounds in rat liver by reversed-phase high-performance liquid chromatography. *Anal Biochem* **146**: 173-179
- Koops AJ, Italiaander E, Groeneveld HW** (1991) Triterpenoid biosynthesis in the seedling of *Euphorbia lathyris* L. from sucrose and amino acids. *Plant Sci* **74**: 193-201
- Kyhse-Andersen J** (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* **10**: 203-209
- Laemmli UK** (1970) Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685
- Logemann J, Schell J, Willmitzer L** (1987) Improved method for the isolation of RNA from plant tissues. *Anal Biochem* **163**: 16-20
- Luethy MH, Miernyk JA, Randall DD** (1997) Molecular analysis of a branched-chain keto-acid dehydrogenase from *Arabidopsis thaliana* (abstract No. 696). *Plant Physiol* **114**: S-147
- Mooney BP, Miernyk JA, Randall DD** (1998) Nucleotide sequence of a cDNA encoding the dihydrolipoyltransferase (E2) subunit of the branched-chain alpha-keto acid dehydrogenase complex from *Arabidopsis thaliana* (accession no. AF038505) (PGR 98-071). *Plant Physiol* **117**: 331
- Moss J, Lane MD** (1971) The biotin-dependent enzymes. *Adv Enzymol* **35**: 321-442

**Nes WD, Bach TJ** (1985) Evidence for a mevalonate shunt in a tracheophyte. *Proc*

*Roy Soc Lond B Biol Sci* **225**: 425-444

**Nikolau BJ, Wurtele ES, Stumpf PK** (1985) Use of streptavidin to detect biotin-

containing proteins in plants. *Anal Biochem* **149**: 448-453

**Overton KH** (1985) The role of leucine in terpenoid metabolism: incorporation of

leucine into sesquiterpenoids and phytosterols by andrographic tissue culture. *In* KH

Neumann, W Barz, E Reinhard, eds, *Primary and Secondary Metabolism of Plant Cell*

*Cultures*, Springer-Verlag, Berlin, pp 225-234

**Pathre U, Singh AK, Sane PV** (1989) Inactivation of leucine aminotransferase with

diethylpyrocarbonate and rose bengal: Evidence for an active site histidine residue. *Ind J*

*Biochem Biophys* **26**: 136-139

**Pathre U, Singh AK, Viswanathan PN, Sane PV** (1987) Purification and properties of

leucine aminotransferase from soybean seedlings. *Phytochemistry* **26**: 2913-2917

**Popják G** (1971) Specificity of enzymes in sterol biosynthesis. *Harvey Lect* **65**: 127-156

**Sambrook J, Fritsch EF, Maniatis T** (1989) *Molecular Cloning: A Laboratory*

*Manual*. Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

**Sandalio LM, Palma JM, Del Rio LA** (1987) Localization of manganese superoxide

dismutase in plant peroxisomes isolated from *Pisum sativum* L. *Plant Sci* **51**: 1-8

**Schwitzguebel J-P, Siegenthaler P-A** (1984) Purification of peroxisomes and

mitochondria from spinach leaf by percoll gradient centrifugation. *Plant Physiol* **75**: 670-

674

**Seubert W, Remberger U** (1963) Untersuchungen über den bakteriellen Abbau von

isoprenoiden. II. Die Rolle der Kohlensäure. *Biochem Z* **338**: 245-264

- Sodek L, Wilson CM (1973)** Metabolism of lysine and leucine derived from storage protein during the germination of maize. *Biochim Biophys Acta* **304**: 353-362
- Song J, Wurtele ES, Nikolau BJ (1994)** Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase: Identification of the biotin carboxylase and biotin-carrier domains. *Proc Natl Acad Sci USA* **91**: 5779-5783
- Stewart CR, Beevers H (1967)** Gluconeogenesis from amino acids in germinating castor bean endosperm and its role in transport to the embryo. *Plant Physiol* **42**: 1587-1595
- Tapuhi YT, Schmidt DE, Lindner W, Karger BL (1981)** Dansylation of amino acids for high-performance liquid chromatography analysis. *Anal Chem* **48**: 1106-1109
- Van der Heijden R, de Boer-Hlupa V, Verpoorte R, Duine JA (1994)** Enzymes involved in the metabolism of 3-hydroxy-3-methylglutaryl-coenzyme A in *Catharanthus roseus*. *Plant Cell Tissue Organ Culture* **38**: 345-349
- Van der Heijden R, Verpoorte R (1995)** Metabolic enzymes of 3-hydroxy-3-methylglutaryl-coenzyme A in *Catharanthus roseus*. *Plant Cell Tissue Organ Culture* **43**: 85-88
- Wang X, Wurtele ES, Keller G, McKean AL, Nikolau BJ (1994)** Molecular cloning of cDNAs and genes coding for  $\beta$ -methylcrotonyl-CoA carboxylase of tomato. *J Biol Chem* **269**: 11760-11769
- Wang X, Wurtele ES, Nikolau BJ (1995)** Regulation of  $\beta$ -methylcrotonyl-coenzyme A carboxylase activity by biotinylation of the apoenzyme. *Plant Physiol* **108**: 1133-1139

**Weaver LM, Lebrun L, Franklin A, Huang L, Hoffman N, Wurtele ES, Nikolau BJ**

(1995) Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coenzyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol* **107**: 1013-1014

**Weber T, Bach TJ** (1993) Partial purification and characterization of membrane-

associated 3-hydroxy-3-methylglutaryl-coenzyme A lyase from radish seedlings. *Z*

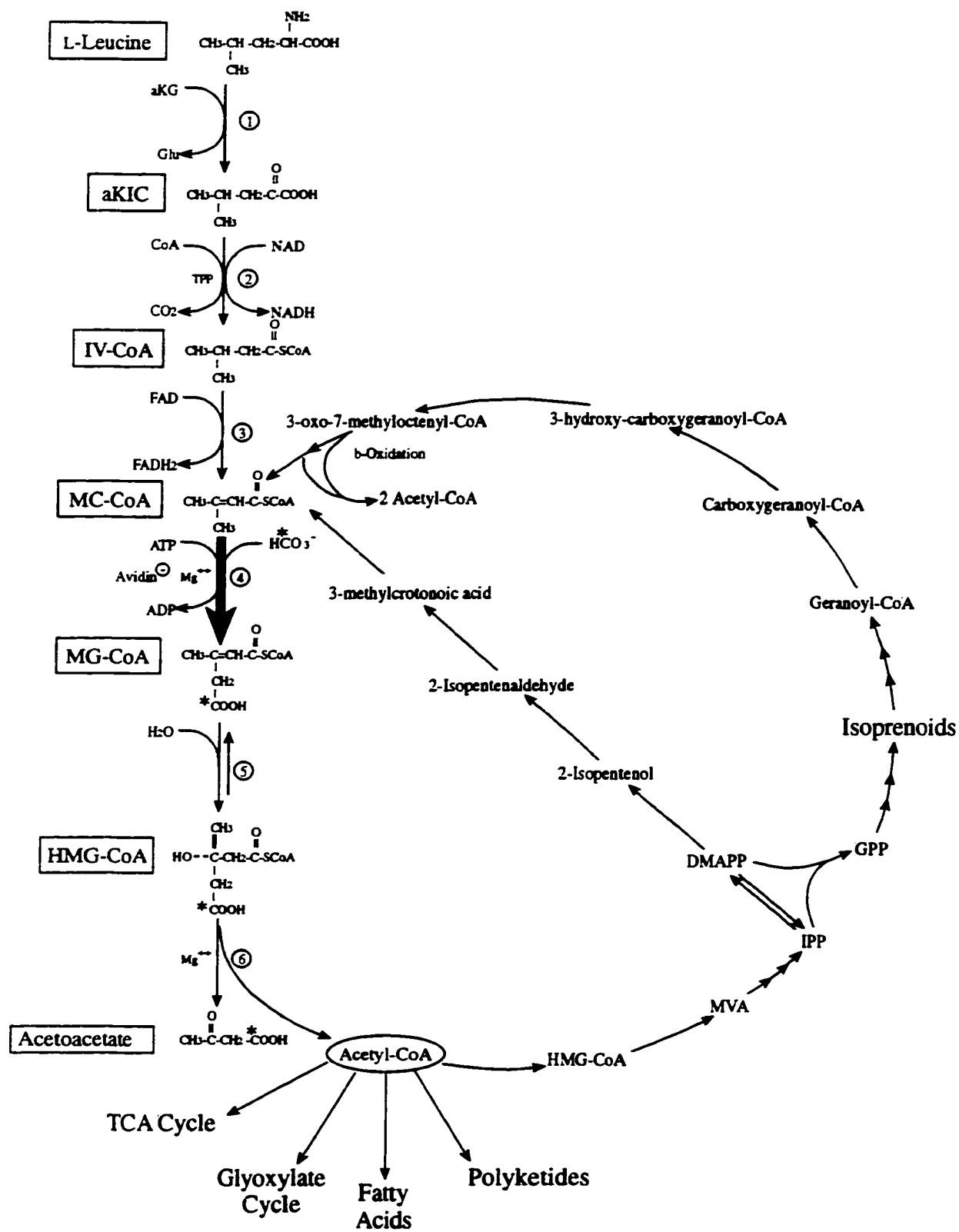
*Naturforsch* **C48**: 444-450

**Wurtele ES, Nikolau BJ** (1990) Plants contain multiple biotin enzymes: Discovery

of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate

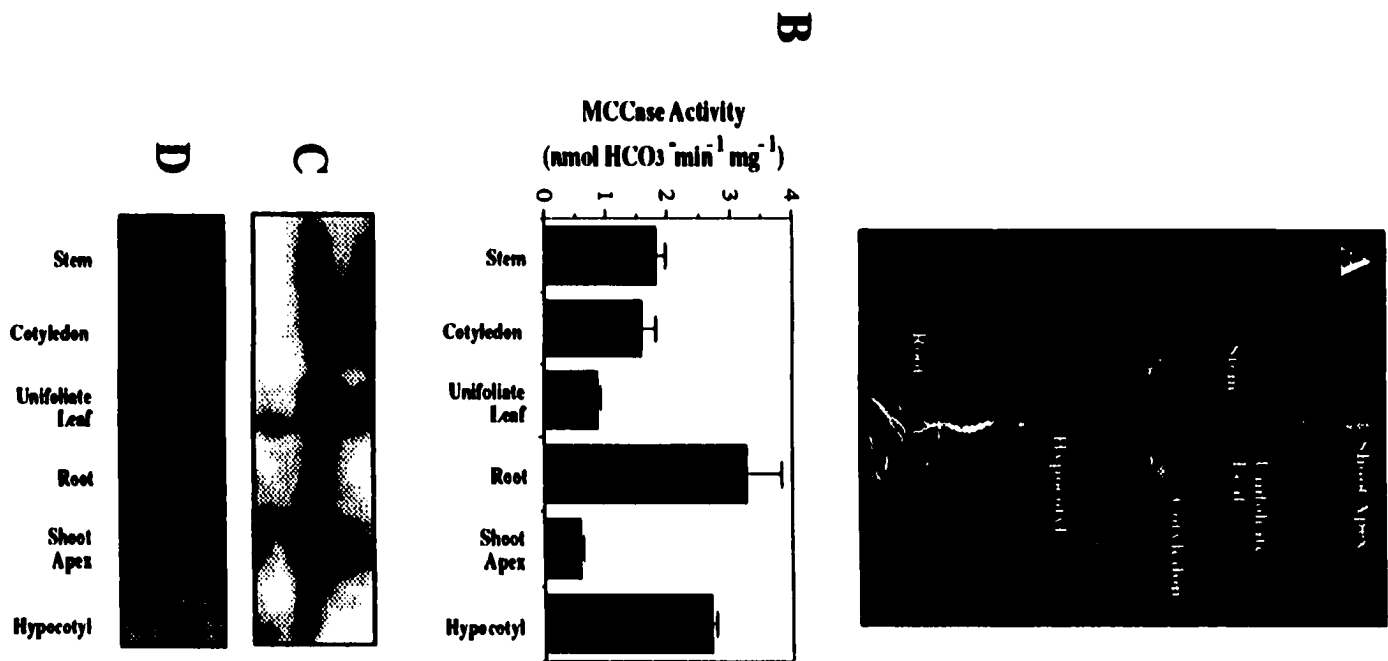
carboxylase in the plant kingdom. *Arch Biochem Biophys* **278**: 179-186.

**Figure 1.** Potential metabolic functions of MCCase. MCCase catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA (MC-CoA) to form 3-methylglutaconyl-CoA (MG-CoA). This reaction may be required in the catabolism of leucine to acetoacetate and acetyl-CoA (reactions 1 to 6). MCCase may also function to convert mevalonate (MVA) to acetoacetate and acetyl-CoA (via isopentenyl pyrophosphate [IPP] and 3-methylcrotonic acid) by the "mevalonate shunt." A third function of MCCase may be as a part of an isoprenoid catabolic pathway (via geranoyl-CoA). Reactions 4 to 6 are common to all three processes. The products of these processes, acetoacetate and acetyl-CoA, can be further metabolized to isoprenoids, polyketide derivatives (e.g. flavonoids, stilbenoids), fatty acids, glucose in tissues engaging the glyoxylate cycle, or respired to CO<sub>2</sub> in the tricarboxylic acid cycle. The enzymes of leucine catabolism are: 1) branched-chain amino acid aminotransferase, 2) branched-chain keto-acid dehydrogenase complex, 3) branched-chain acyl-CoA dehydrogenase, 4) 3-methylcrotonyl-CoA carboxylase, 5) 3-methylglutaconyl-CoA hydratase, 6) 3-hydroxy-3-methylglutaryl-CoA lyase. The asterisk denotes the carbon atom expected to be radioactively labeled when NaH<sup>14</sup>CO<sub>3</sub> is supplied for the MCCase reaction.

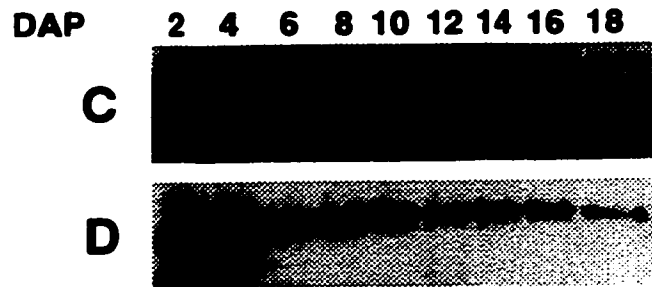
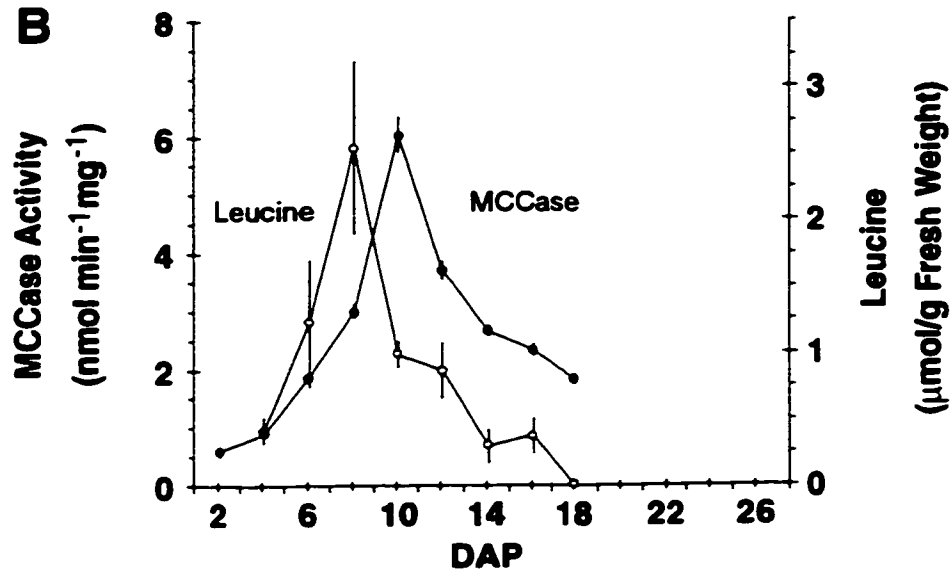
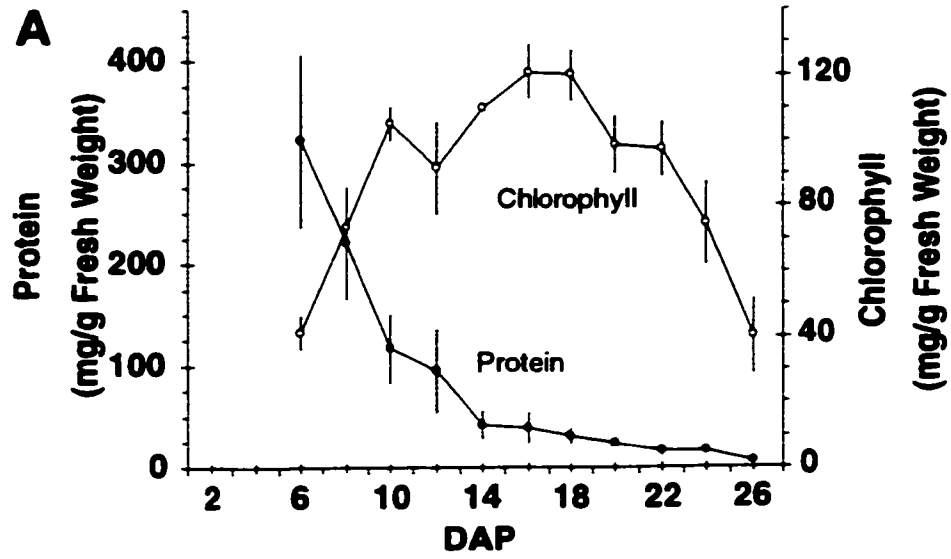


**Figure 2. MCCase activity and the accumulation of the biotin-containing subunit of MCCase in soybean seedlings at 13 DAP. Organs of a soybean seedling (A). MCCase activity (mean of five experiments  $\pm$  SE) (B). Western blot probed with antiserum to detect the biotin-containing subunit of MCCase (C). Western blot identical to that shown in panel C, but instead probed with  $^{125}$ I-streptavidin to detect the biotin prosthetic group on the biotin-containing subunit of MCCase (D). In panels C and D, protein samples were loaded on the basis of equal MCCase activity (0.1 nmol/min) to detect differences in the catalytic efficiency of the enzyme among organs. The data presented in panels C and D were gathered from a single experiment; five replicates of this experiment gave similar results.**

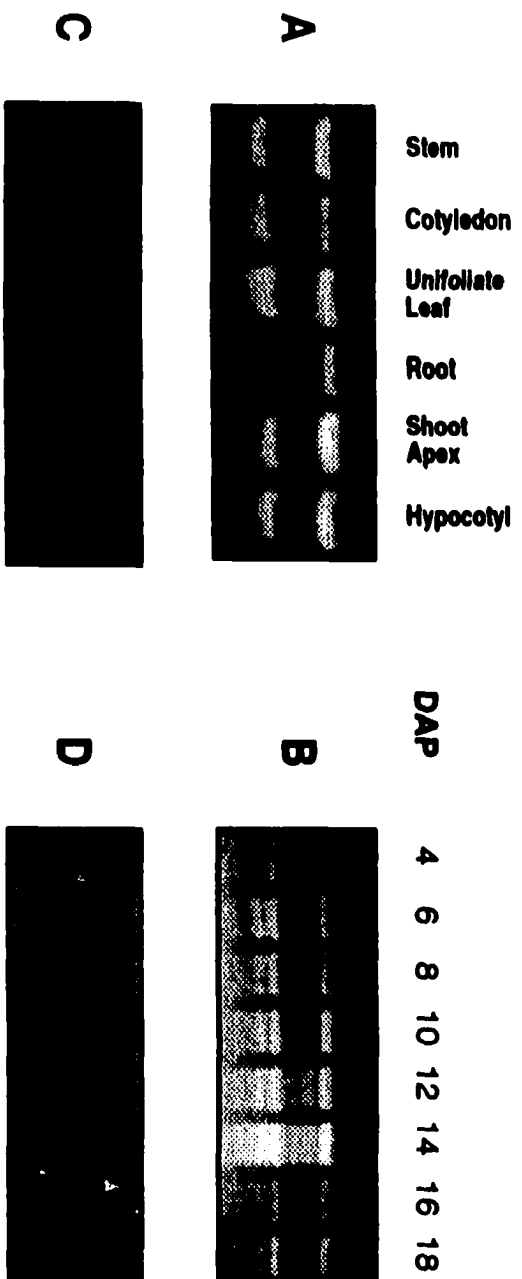




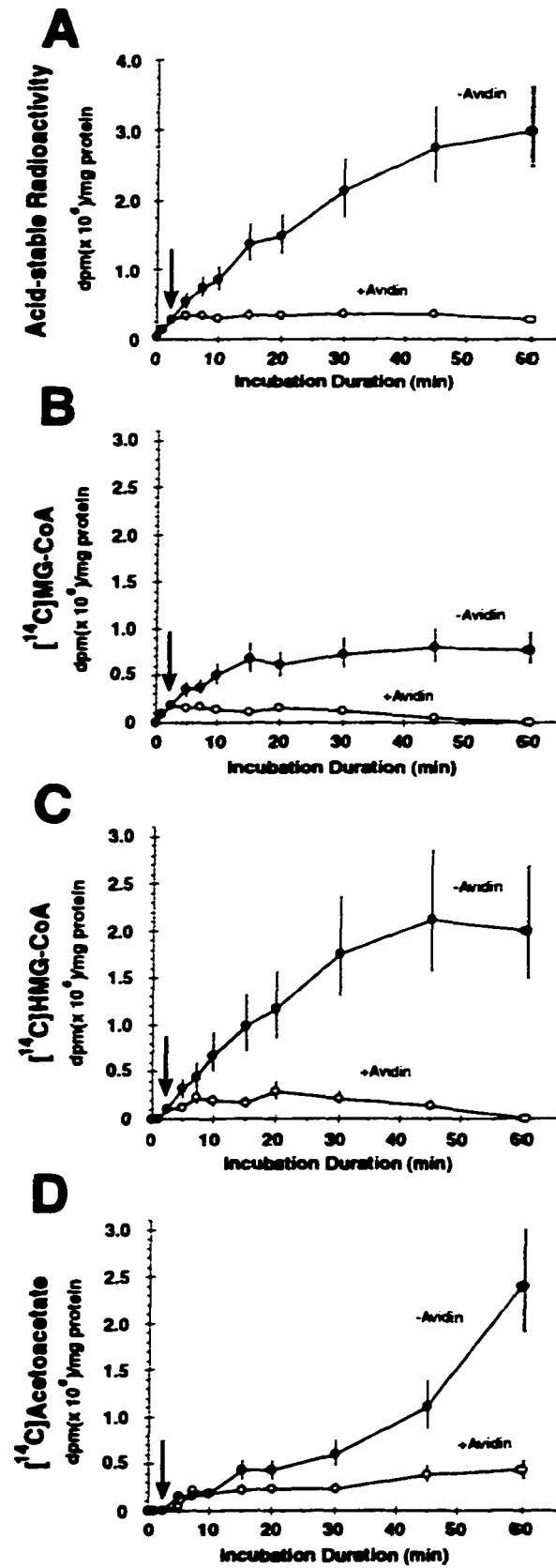
**Figure 3.** Effect of cotyledon development on MCCase activity, and leucine, protein, and chlorophyll accumulation. Chlorophyll and total protein contents (A) and MCCase activity and free leucine content (B). Western blot probed with antiserum to detect the biotin-containing subunit of MCCase (C). Western blot identical to that shown in panel C, but instead probed with  $^{125}\text{I}$ -streptavidin to detect the biotin prosthetic group on the biotin-containing subunit of MCCase (D). In panels C and D, protein samples were loaded on the basis of equal MCCase activity (0.1 nmol/min) to detect differences in the catalytic efficiency of the enzyme during cotyledon development. The data presented in panels A and B are means  $\pm$  SE from three replicates. The data presented in panels C and D were gathered from a single experiment; three replicates of this experiment gave similar results.



**Figure 4.** Steady state levels of the mRNA coding for the biotin-containing subunit of MCCase in organs of soybean seedlings at 13 DAP and during cotyledon development. Ethidium bromide-stained agarose gel containing approximately equal amounts (20  $\mu$ g) of RNA, isolated from various organs (A) or from cotyledons at different stages of development (B). Northern blot depicting the accumulation of the mRNA coding for the biotin-containing subunit of MCCase in various organs (C) or in cotyledons at different stages of development (D). The data presented in this figure were gathered from a single experiment. Five replicates (A and C) and three replicates (B and D) of these experiments gave similar results.



**Figure 5.** Kinetics of the incorporation of radioactively-labeled bicarbonate into acid-stable metabolites by mitochondrial extracts in the presence of MC-CoA and cofactors. Time-course of the incorporation of radioactivity into acid-stable products (A), MG-CoA (B), HMG-CoA (C), or acetoacetate (D). Incubations were carried out either without or with 10  $\mu\text{g}$  avidin (to inhibit MCCase activity), which was added 2.5 min after the start of the reaction (arrow). Each time point is an independent sample and represents two replicates. Due to the rapid incorporation of  $\text{NaH}^{14}\text{CO}_3$ , the background radioactivity was impossible to obtain. Therefore, the zero-minute time point was derived from samples incubated for 1 min in the presence of 10  $\mu\text{g}$  avidin.



**Figure 6.** Separation of radioactive metabolites by HPLC. Representative chromatograms depicting metabolites arising from the catabolism of MC-CoA by mitochondrial extracts in the presence of  $\text{NaH}^{14}\text{CO}_3$  and cofactors after 5 min (A) or 45 min (B) incubations. After 5 min,  $[^{14}\text{C}]\text{MG-CoA}$  and  $[^{14}\text{C}]\text{HMG-CoA}$  are at similar levels and  $[^{14}\text{C}]\text{acetoacetate}$  is barely detectable. After 45 min,  $[^{14}\text{C}]\text{HMG-CoA}$  has exceeded  $[^{14}\text{C}]\text{MG-CoA}$  and  $[^{14}\text{C}]\text{acetoacetate}$  is increased. Representative chromatograms depicting metabolites arising from the catabolism of  $[\text{U-}^{14}\text{C}]\text{leucine}$  by mitochondrial extracts in the presence of 10  $\mu\text{g}$  avidin after a 2 h (C) or 6 h (D) incubation. After 2 h,  $\alpha\text{-}[^{14}\text{C}]\text{KIC}$  has accumulated to a substantial level and  $[^{14}\text{C}]\text{IV-CoA}$  accumulation is in progress. After 6 h, the level of  $\alpha\text{-}[^{14}\text{C}]\text{KIC}$  has diminished, indicating that the rate of its disappearance has exceeded its formation.  $[^{14}\text{C}]\text{IV-CoA}$  has continued to accumulate and  $[^{14}\text{C}]\text{MC-CoA}$  has appeared.  $[^{14}\text{C}]\text{Isovaleric acid}$  is likely a decarboxylation product of  $\alpha\text{-}[^{14}\text{C}]\text{KIC}$ , possibly arising nonenzymatically.



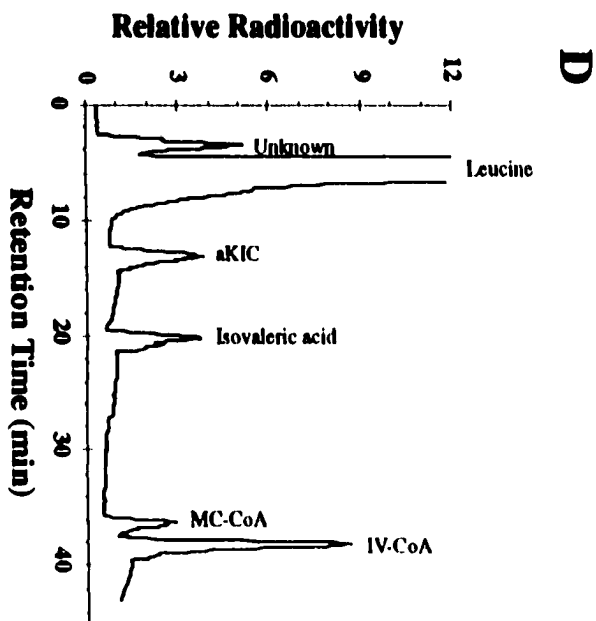
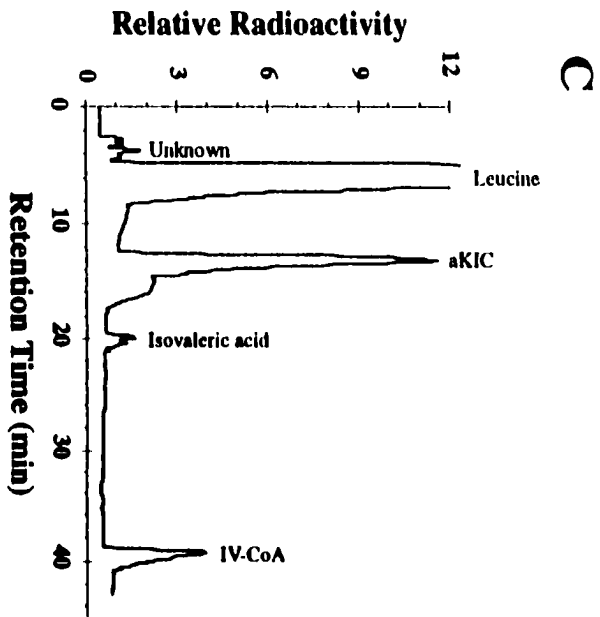
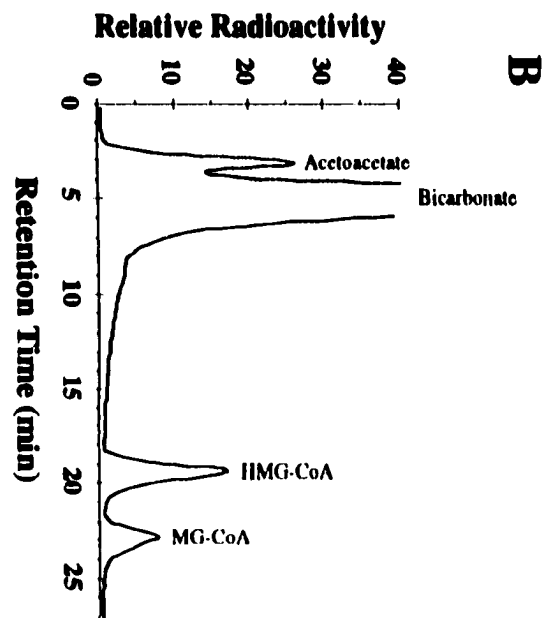
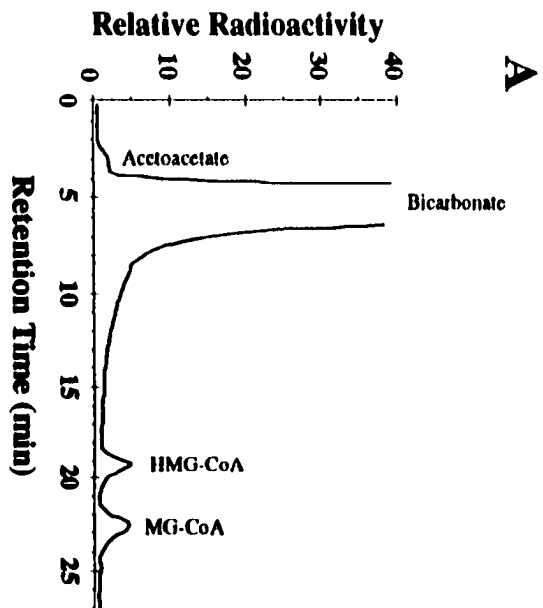


Table I. *MCCase activity in developing soybean organs*

Organ	Developmental	MCCase
	Stage	Activity <sup>a</sup>
	DAF <sup>b</sup>	nmol min <sup>-1</sup> mg
protein <sup>-1</sup>		
Developing Seeds	20	6.9
	30	9.9
	40	5.6
	50	3.3
Dry Seeds		4.3
Seed Pod Coat	10	2.5
	20	5.0
	30	6.0
	40	3.3
	50	2.3
	60	0.8
Reproductive Leaf <sup>c</sup>	10	1.2
	20	1.3
	30	1.3
	40	1.3
	50	0.02
Young Leaf	d	3.4
Mature Leaf	e	2.7

<sup>a</sup>Average of three replicates. SE = 0.7 nmol min<sup>-1</sup>mg protein<sup>-1</sup>

<sup>b</sup>Days after flowering.

<sup>c</sup>Reproductive leaf is the leaf next to a seed pod and is staged relative to flowering.

<sup>d</sup>Young leaf is the first true leaf of 14 day old plants.

<sup>e</sup>Mature leaf is the first true leaf of 30 day old plants, prior to flowering.

CoA	Acid-Stable		MG-CoA		HMG-		
	Metabolites						
	Incubation						
Substrate	Duration	-Avidin	+Avidin	-Avidin	+Avidin	-Avidin	
	+Avidin						
	min	dpm (x10 <sup>3</sup> )/mg protein			%		
IV-CoA	1	47.8(+10.3)	-	ND	-	ND	-
	5	80.3(+17.3)	64.6(+13.9)	ND	ND	ND	ND
	20	106.2(+22.8)	64.9(+13.9)	ND	ND	100	ND
	60	166.4(+35.8)	77.2(+16.6)	17	ND	83	ND
αKIC	1	49.9(+10.7)	-	ND	-	ND	-
	5	63.7(+13.7)	64.5(+13.9)	ND	ND	ND	ND
	20	88.5(+19.0)	67.1(+14.4)	ND	ND	100	ND
	60	146.3(+31.4)	100.8(+21.5)	ND	ND	100	ND

ND = Not detected

Table III. *Catabolism of leucine by mitochondrial extracts*

Mitochondrial extracts were incubated with [U-<sup>14</sup>C]leucine and cofactors for various lengths of time and the resulting radioactive products were analyzed by HPLC. Additional reactions were also conducted without added  $\alpha$ -ketoglutarate ( $\alpha$ KG) or CoA, or with the addition of 1 mM nonradioactive  $\alpha$ KIC or 10  $\mu$ g avidin.  $\alpha$ KG is required for the transamination of leucine; CoA is required for the decarboxylation and activation of  $\alpha$ KIC in the BCKDH reaction, nonradioactive  $\alpha$ KIC inhibits further metabolism of  $\alpha$ -[<sup>14</sup>C]KIC by competition, and avidin inhibits MCCase. Standard errors are reported as in Table II.

Additions/ Omissions	Incubation Duration	$\alpha$ KIC	IV-CoA	MC-CoA
	h	dpm ( $\times 10^3$ )/mg protein		
None	0.33	256.4 (+45.1)	ND	ND
	1	585.3 (+102.9)	19.7 (+2.3)	ND
	2	1303.2 (+229.1)	110.5 (+13.0)	ND
	4	697.6 (+122.7)	426.5 (+50.2)	8.6 (+4.2)
	6	362.1 (+63.7)	508.6 (+59.8)	29.9 (+14.4)
- $\alpha$ KG	2	89.4 (+15.7)	ND	ND
- CoA	2	1214.7 (+213.6)	ND	ND
+ 1 mM $\alpha$ KIC	2	1099.0 (+193.2)	ND	ND
	4	1835.0 (+322.6)	ND	ND
	6	2270.3 (+399.1)	ND	ND
+ avidin	2	936.8 (+164.7)	154.3 (+18.2)	ND
	4	800.7 (+139.9)	420.6 (+49.5)	51.4 (+24.7)
	6	602.4 (+105.9)	436.8 (+51.4)	71.7 (+34.5)

ND = Not Detected

# **CHAPTER 3. DEVELOPMENTAL AND ENVIRONMENTAL REGULATION OF 3-METHYLCROTONYL-CoA CARBOXYLASE IN *ARABIDOPSIS***

Ping Che, Eve Syrkin Wurtele and Basil J. Nikolau

*Department of Biochemistry, Biophysics and Molecular Biology (P.C. and B.J.N.) and*

*Department of Botany (E.S.W.), Iowa State University, Ames, IA 50011*

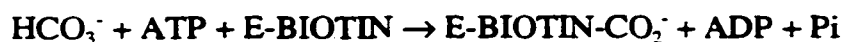
## **Summary**

3-Methylcrotonyl-coenzyme A carboxylase (MCCase) is a nuclear-encoded mitochondrial biotin-containing enzyme whose major metabolic role is the catabolism of leucine. In addition, MCCase may be required for isoprenoid catabolism and the mevalonate shunt. MCCase contains two subunits: the biotinylated MCC-A subunit and the non-biotinylated MCC-B subunit. In the work presented herein, the single-copy gene encoding the *Arabidopsis* MCC-A subunit was isolated and characterized. It contains 15 exons separated by 14 introns. We examined the regulation of expression of *MCC-A* and *MCC-B* genes in transgenic plants containing the 1.1-kb and 1.0-kb 5' upstream sequences of the two genes, respectively, fused to the  $\beta$ -glucuronidase gene. Transfer seedlings from light to dark induces MCCase expression, which is repressed by exogenous carbohydrates, especially sucrose. Several lines of evidence indicate that an inhibitor of MCCase expression is

synthesized in illuminated photosynthetic organs and can be translocated to non-photosynthetic organs. Furthermore, either artificially inhibiting carbohydrate biosynthesis by limiting atmospheric CO<sub>2</sub> or inhibiting carbohydrate translocation from leaves to roots by detaching the leaves, results in the induction of MCCase. These results are consistent with the hypothesis that the inhibitor is a carbohydrate, perhaps sucrose. The effect of hexoses and glucose analogues on MCCase expression indicate that phosphorylation of hexoses may be the signal for the repression. We conclude that MCCase expression is primarily controlled at the level of gene transcription and regulated by a complex interplay between the illumination status and its carbon balance.

### Introduction

MCCase (EC 6.4.1.4) was discovered 40 years ago in bacteria and mammals (Moss and Lane, 1971). This enzyme catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA (MC-CoA) to form 3-methylglutonyl-CoA (MG-CoA), in a two-step reaction mechanism:



MCCase is a heteromeric enzyme composed of two types of subunits: a biotinylated subunit (MCC-A) and a non-biotinylated subunit (MCC-B). The MCC-A subunit catalyzes the first half reaction, and MCC-B subunit catalyzes the second. The biotin prosthetic group acts as an intermediate carrier of the substrate carboxyl group that carboxylates MC-CoA.

Since the discovery of MCCase activity in plant extracts (Wurtele and Nikolau, 1990), this enzyme has been purified and characterized from several plant species (Alban *et al.*, 1993; Chen *et al.*, 1993; Diez *et al.*, 1994; Song *et al.*, 1994; Wang *et al.*, 1994; Weaver *et al.*, 1995; McKean *et al.*, 2000). It contains two subunits: MCC-A and MCC-B (Chen *et al.*, 1993; Alban *et al.*, 1993; Diez *et al.*, 1994; Weaver *et al.*, 1995; McKean *et al.*, 2000). Genic sequences for the two subunits have been isolated only for the plant enzyme.

In animals and in some bacteria and fungi, MCCase is required for the catabolism of leucine (Leu) (Lau *et al.*, 1980), for the operation of the mevalonate shunt (Popják, 1971; Edmond and Popják, 1974; Guan *et al.*, 1999) and in certain aspects of isoprenoid metabolism (Seubert and Remberger, 1963). Moreover, we recently demonstrated that plant mitochondria can convert Leu to acetoacetate and acetyl-CoA via a pathway analogous to that found in animals and bacteria (Anderson *et al.*, 1998). This earlier study not only provides direct evidence for the metabolic function of MCCase in plants, but also demonstrates that the entire Leu catabolic pathway occurs within plant mitochondria.

The molecular and biochemical regulation of Leu catabolism in plants is not fully understood. As a first step towards understanding MCCase regulation, and hence obtain more detailed insights into Leu catabolism, we isolated and characterized the genomic organization of the *MCC-A* gene and analyzed the regulation of *MCC-A* and *MCC-B* (McKean *et al.*, 2000) gene expression patterns. The data presented in this paper indicate that environmental and developmental signals affect MCCase expression and these are primarily regulated at the level of mRNA accumulation. Furthermore, these signals coordinately affect both the *MCC-A* and *MCC-B* genes. We also determined that artificially depleting carbohydrate synthesis induces the transcription of *MCC-A* and *MCC-B* genes in

*Arabidopsis* seedlings and roots. These results indicate that the expression of *MCC-A* and *MCC-B* genes is coordinately regulated by metabolic repression at the level of gene transcription and imply that the role of MCCase in plant metabolism is to respond to low carbohydrate availability during autophagic processes.

## Results

### Structure of *Arabidopsis thaliana* *MCC-A* gene

The *MCC-A* gene was isolated from an *Arabidopsis* genomic library in the vector  $\lambda$  Fix (see experimental procedures). Adjoining 2-kb and 4-kb *SacI* fragments were subcloned from this  $\lambda$  clone into plasmids (*pBP5* and *pBP4*, respectively) and sequenced. The complete sequence of *MCC-A* gene was obtained by combining the sequence of these two fragments. The structure of the *MCC-A* gene was identified by comparing the sequence with the corresponding cDNA (Weaver *et al.*, 1995). The transcribed region of the gene spans 6165 bp of genomic sequence and consists of 15 exons separated by 14 introns. All but one exon/intron junction displays the canonical 5'GT-intron-AG3' sequence (Fig. 1A). The only exception to this rule is the 3' end of intron 12 which has the sequence, 5'GT-intron-GG3'. The promoter region contains an apparent TATA box motif at position -243 to -236 (TTAATAAA).

### Light-mediated regulation of MCCase expression

Previously, we had found that MCCase activity was higher in roots (a non-photosynthetic organ) than in leaves (a photosynthetic organ) (Wang *et al.*, 1995; Anderson *et al.*, 1998). In addition, MCCase activity was found to be higher in barley seedlings (Maier



*et al.*, 1998; Clauss *et al.*, 1993) and plant cells (Aubert *et al.*, 1996) that were maintained in darkness. As a first step to investigate light-mediated regulation of MCCase expression, we examined the effect of illumination on MCCase activity. *Arabidopsis* seedlings were germinated on MS agar plates for 6 days, and then light was withheld by covering them with aluminum foil for a period of time. MCCase specific activity was determined in extracts prepared at various times over the next 4 days. As shown in Figure 2A, a significant increase in MCCase activity was induced by darkness in a time-dependent pattern. MCCase activity increased by three- and eight-fold after 0.5 and 2 days of dark treatment, respectively, and by 4 days, activity increased by more than 10-fold.

To further study the mechanism of MCCase induction, western-blot analysis were performed on extracts from the identical experiments. Aliquots of extracts containing equal amounts of protein were fractionated by SDS-PAGE, followed by western-blot analysis with antisera specific for the MCC-A and MCC-B subunits. These analyses (Fig. 2B) demonstrate that the accumulation of both MCC-A and MCC-B proteins increases in response to darkness, in parallel with the increase in MCCase activity. To ascertain whether the darkness-associated induction of MCC-A and MCC-B subunit accumulation is mediated by changes in the accumulation of the corresponding mRNAs, we determined the abundance of the respective mRNAs in the tissues collected from the same experiments. As shown in Figure 2C, the accumulation of *MCC-A* and *MCC-B* mRNAs was induced in tissues deprived of illumination, in parallel with the increases in MCC-A and MCC-B subunit accumulation. These data indicate that, in response to darkness, MCCase expression may be up-regulated in the level of gene transcription.

To further elucidate the mechanism by which MCCase expression is enhanced in response to darkness, we examined the response of promoter-reporter transgenes during light-dark transition. Specifically, we generated two sets of transgenic *Arabidopsis* lines, each of which carried either a *MCC-A:GUS* or *MCC-B:GUS* transgene as illustrated in Figures 1B and 1C. Time course experiments similar to those shown in Figure 2A were repeated with *MCC-A:GUS* and *MCC-B:GUS* transgenic *Arabidopsis* seedlings. GUS activities were measured using a fluorometric GUS assay (Jefferson, 1987). As shown in Figure 3A, transferring seedlings into darkness induced GUS activity for both transgenes; with about a 3-fold increase after 24 h of light deprivation, increasing to a 10-fold induction after 4 days of light deprivation. *In situ* staining of GUS activity in these seedlings indicated that the darkness-induced enhancement of *MCC-A*- and *MCC-B*-directed *GUS* expression occurred throughout the entire seedlings, including cotyledons, leaves, hypocotyls and roots (Fig. 3B and 5B). These results suggest that the sequences between -1150 to -12 of the *MCC-A* gene and -1110 to -64 of the *MCC-B* gene are sufficient to confer light-regulated expression on the *GUS* reporter gene. Furthermore, these results indicate that light-mediated changes in *MCC-A* and *MCC-B* gene expression are at least partially regulated by the changes in the transcription of the respective genes.

### **Regulation of MCCase expressions by sugars**

Sugars, particularly sucrose, the major translocated sugar, have been recognized as regulatory molecules that control gene expression (reviewed in Koch, 1996). To determine if sugars interact with the light-mediated changes in MCCase expression, GUS expression was studied in *MCC-A:GUS* and *MCC-B:GUS* transgenic plants grown on different

concentrations of sucrose either in continuous illumination or after transfer into darkness. As shown in Figures 4A and 5A, sucrose significantly repressed *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression, especially in seedlings grown in the dark. Histochemical staining of *MCC-A:GUS* expression in seedlings grown on different concentrations of sucrose showed that at low concentrations of sucrose, repression is confined to the roots and that as the exogenous sucrose concentration is raised, *MCC-A* expression is affected in the hypocotyls (Fig. 4B).

In these experiments, we noted that growth of the seedlings was inhibited in the presence of sucrose (Fig. 4B). To investigate whether repression of *MCC-A:GUS* and *MCC-B:GUS* expression is due directly to sucrose, rather than a general osmotic effect, we measured the effect of sorbitol on the expression of these transgenes. Although sorbitol, a non-metabolizable sugar, inhibits of *Arabidopsis* seedling growth as sucrose does (data not shown), it has the opposite effect of sucrose on *MCC-A*- and *MCC-B*-mediated *GUS* expression (Fig. 4C and 5A). These results indicate that the repression of *MCC-A*- and *MCC-B*-directed *GUS* expression by sucrose is not caused by the osmotic effect of the sugar, but is either the direct effect of sucrose or some metabolite derived from sucrose.

We also determined the metabolic regulation of *MCC-A* and *MCC-B* gene expression by glucose, fructose, galactose and xylose (Fig. 4D and 5A). Like sucrose, all these sugars repress *MCC-A*- and *MCC-B*-mediated *GUS* expression in darkness.

### **Regulation of *MCC-A:GUS* and *MCC-B:GUS* gene expression through information exchange between organs**

Sucrose is the major translocated sugar in plants and is thus the major form in which carbon is imported into non-photosynthetic cells. The data presented above indicate that there may be an interaction between the illumination status of the plant and sucrose levels in regulating *MCC-A* and *MCC-B* gene expression in photosynthetic organs. This was further investigated by studying MCCase expression in a non-photosynthetic organ, the root. Extracts were prepared separately from roots and leaves isolated from the identical plants that had been grown in soil for 21 days either under constant illumination or from plants that were deprived of illumination for the last 2 days prior to extraction. As previously reported for tomato (Wang *et al.*, 1995; Anderson *et al.*, 1998), MCCase activity is 3-4 fold higher in roots than in leaves. However, the interesting observation made here is that MCCase activity is induced in both leaves and roots of dark-adapted plants by a factor of 6-8 fold (Fig. 6A). This is presumably despite the fact that soil borne roots do not directly perceive the illumination status of the plant.

To further delineate how MCCase is induced in roots of dark-adapted plants, we determined *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression in transgenic plants treated as in the experiment shown in Figure 6A. As expected darkness induced *GUS* expressions in the leaves of these plants. Moreover, *GUS* expression was induced by a factor of 5-7 fold in roots of these dark-adapted transgenic plants (Fig. 6 B-C). These findings indicate that leaves communicate their illumination status to the roots and affect MCCase expression. One model to explain this result is that illuminated leaves produce a signal molecule (possibly sucrose), which acts to affect the suppression of *MCC-A* and *MCC-B*

gene expressions. Thus, when plants are placed in the dark, concentration of this suppressor signaling molecule declines, leading to the induced expression of MCCase in both leaves and roots.

To further develop this hypothesis, we conducted two analogous experiments in which illumination was withheld from a subset of the leaves on *Arabidopsis* rosettes. In one experiment, a single leaf of the second pair of emerged leaves, was selectively deprived of light, while the other leaves were continuously exposed to light. In the other experiment, light was withheld from all but one of the second pair of emerged leaves of 21-day old plants. In both experiments, *MCC-A*- and *MCC-B*-driven GUS activity were determined in the illuminated and non-illuminated leaves of the second pair of emerged leaves. As shown in Figure 7A and B, higher levels of GUS activities were detected in the selectively dark-treated leaves. However, these increased GUS activities in both cases were not as high as in those leaves of seedlings that were completely deprived of illumination. These data are consistent with the suppressor model outlined above. Namely, the shaded leaf ceases to produce *MCC-A* and *MCC-B* suppressor and thus GUS activities are induced. However, because the illuminated leaf or leaves synthesize the suppressor and it is translocated to the shaded leaf or leaves, and *MCC-A*- and *MCC-B*-mediated GUS activities were not fully induced (Fig.7A and B).

#### **Effect of limiting CO<sub>2</sub> on *MCC-A:GUS* and *MCC-B:GUS* gene expressions**

To begin the process of identifying the molecular signal that suppresses MCCase expression, we grew *Arabidopsis* seedlings in CO<sub>2</sub>-free atmosphere , but under constant illumination and examined the effect on the expression of MCCase.

As shown in Figures 8A and 8B, as compared to seedlings grown in a normal atmosphere, MCCase activity and accumulation of the MCC-A and MCC-B subunits were induced 8-9 fold when 6-day old seedlings were transferred to a CO<sub>2</sub>-free atmosphere for a 2-day period. The provision of exogenous sucrose in the growth medium repressed these induction of MCCase activity and accumulation of the MCC-A and MCC-B subunits.

In experiments identical to the above, *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression was determined in 8-day old transgenic seedlings which were transferred for 2 days to a CO<sub>2</sub>-free-air environment and kept under constant illumination. As shown in Figures 8C-D, in the absence of atmospheric CO<sub>2</sub>, *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression increased 10- and 11-fold, respectively. As with the induction of MCCase activity and subunit accumulation, the induction of *MCC-A*- and *MCC-B*-mediated *GUS* expressions in response to a CO<sub>2</sub>-free atmosphere was reversed by the inclusion of sucrose in the growth medium (Fig. 8C-D). Namely, in the presence of exogenous sucrose, *MCC-A-GUS* and *MCC-B-GUS* expression is not induced by the CO<sub>2</sub>-free-atmosphere.

We utilized this experimental system as a convenient means of identifying whether other biochemicals could substitute for sucrose and act as repressors of the induction process. We found that at equal molar concentrations, glucose, fructose, galactose and xylose were also able to repress the CO<sub>2</sub>-free-atmosphere induction of *MCC-A*- and *MCC-B*-mediated *GUS* expressions. However, these monosaccharides were not as strong repressors as sucrose (Fig. 8C-D). To ensure that the effects of these sugars on MCCase expression were not due to an osmotic stress of the tissue, sorbitol, a non-metabolizable sugar, was also tested. Sorbitol had no effect on the CO<sub>2</sub>-free-atmosphere induction of *MCC-A-GUS* and *MCC-B-GUS* expression.

Interestingly, if the 2-day CO<sub>2</sub>-free atmospheric treatment is conducted in darkness, *MCC-A*- and *MCC-B*-mediated GUS activities are still induced, and exogenous sucrose represses that induction (Fig. 8C-D), although these effects are not as strong as when conducted on illuminated plants.

### **Effects of sugars and glucose analogues on gene expression in the excised leaves and roots**

Roots are non-photosynthetic organs and obtain carbohydrates (mainly sucrose) from leaves via transport through the phloem. Detaching roots from leaves results in the depletion of the sucrose supply to the roots. Such detached roots can be sterilely maintained in culture media and MCCase expression can be monitored as the tissue is depleted of phloem-derived sucrose.

*Arabidopsis* seedlings were grown sterilely for two weeks in MS agar medium. The aerial portions of these seedlings were sterilely removed, and the detached roots were maintained for an additional one day in MS-based medium, supplemented with a variety of different carbohydrates. At the end of this incubation, tissue was extracted and MCCase activity and *MCC-A* and *MCC-B* subunit accumulations were determined in the roots (Fig. 9A and 9B). In the absence of any exogenous carbohydrate, MCCase activity and accumulation of the *MCC-A* and *MCC-B* subunits is about 2.5-fold higher in detached roots as compared to roots attached to seedlings. The addition of sucrose to the medium in which detached roots were maintained, represses this increase in MCCase activity (Fig. 9A) and accumulation of the two MCCase subunits (Fig. 9B). To gain further insights into the mechanism by which MCCase expression maybe regulated by sucrose metabolites, we also

tested the effect of glucose analogs on the detached roots. Of the two glucose analogs tested, 2-deoxyglucose repressed the induction in MCCase activity, and accumulation of MCC-A, and MCC-B subunits, whereas 3-*O*-methylglucose could not mimic the repressive ability of sucrose (Fig. 9A and 9B).

To further identify the mechanism by which MCCase expression is altered in detached roots, we studied *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expressions in these organs. As shown in Figures 9C and D, *MCC-A*- and *MCC-B*-mediated *GUS* expression is 4-fold higher in detached roots than in roots of intact plants. Consistent with the data presented in Figure 9A and 9B, incubating detached roots in presence of exogenous sucrose reversed this induction. Furthermore, the glucose analog 2-deoxyglucose also reversed this induction of *GUS* expressions, whereas 3-*O*-methylglucose did not. These findings indicate that the alterations in MCCase expression in detached roots are primarily the result of changes in the transcription of the *MCC-A* and *MCC-B* genes.

Lastly, monitoring *MCC-A*- and *MCC-B*-mediated *GUS* expressions in detached roots is a convenient experimental system for further identifying metabolites that affect MCCase expression. Using this experimental system, we found that as with the CO<sub>2</sub>-free atmosphere experiments, MCCase expression is repressed in detached roots by the exogenous application of glucose, fructose, galactose and xylose (Fig. 9C and 9D). The fact that sorbitol does not repress this expression indicates that these effects are due to metabolite repression of gene expression rather than the effect of osmotic stress.



## Discussion

Our previous work provided direct evidence that one of the metabolic roles of MCCase in plants is in the catabolism of Leu in mitochondria (Anderson *et al.*, 1998). This work also demonstrated for the first time that plant mitochondria contain all the enzymes for this catabolic pathway. Additional potential functions for MCCase include the interconnection between isoprenoid catabolism and general metabolism (Gray, 1987; Kleinig, 1989; Bach, 1995), and the mevalonate shunt (Nes and Bach, 1985; Bach, 1987). The key position occupied by MCCase in these metabolic networks implies that it may play an important role in plant development. In the work presented herein, we determined the full-length sequence and genomic organization of the *MCC-A* gene of *Arabidopsis*. Furthermore, we fused promoter regions from the *MCC-A* and *MCC-B* gene loci to the *GUS* reporter gene, and assayed for the inducibility of the fusion genes in transgenic *Arabidopsis* plants.

### Metabolic regulation of *MCC-A* and *MCC-B* gene expressions in *Arabidopsis*

The regulation of MCCase activity in plants has been reported in several cases. In tomato and soybean, the regulation of the expression of MCCase gene was investigated in different organs (Wang *et al.*, 1995; Anderson *et al.*, 1998). In both cases, the specific activity of MCCase was highest in the extracts from roots and lowest in the leaves. It was also reported that light has a significant effect on reducing MCCase activity (Maier *et al.*, 1998; Aubert *et al.*, 1996; Clauss *et al.*, 1993). Finally, MCCase activity is induced in senescing plants, and older plants or plant organs contain higher MCCase activity (Maier *et al.*, 1998; Clauss *et al.*, 1993; Alban *et al.*, 1993). However, the molecular mechanisms by

which MCCase activity is modulated remain unknown. The results presented in this paper demonstrate that in response to the withdrawal of illumination from plants, the expression of the *MCC-A* and *MCC-B* genes is up-regulated at the transcriptional level. Exogenous sugars repress these darkness-induced changes in gene transcription. A number of experiments indicate that the suppressor of MCCase expression (maybe sucrose) is synthesized during photosynthesis and is translocatable. For example, partial shading of seedlings moderates the effect of the suppressor on the shaded parts of the plant. Furthermore, depletion of the carbon source by artificially depriving plants of CO<sub>2</sub> or inhibiting carbohydrate translocation to roots by detaching leaves results in increased MCCase expression. In all instances, sucrose highly represses these inductions of MCCase expression. In all instances, changes in *MCC-A*- and *MCC-B*-promoter-mediated GUS activity were consistent with the changes in MCCase activity and accumulation of *MCC-A* and *MCC-B* subunit mRNAs and/or proteins. Furthermore, in all instances, changes in *MCC-A* and *MCC-B* gene expression were coordinated. Thus, the effect of light on *MCC-A* and *MCC-B* expression could be through light-mediated changes in sucrose (suppressor) accumulation. These results provide strong evidence that the transcription of the *MCC-A* and *MCC-B* genes are under metabolic control.

Sugars regulate the expression of a variety of genes which are essential for many different processes. Examples include genes that code for proteins localized to chloroplasts (photosynthetic genes) (Sheen, 1990; Jang and Sheen, 1994; Urwin and Jenkins, 1997), peroxisomes (malate synthase and isocitrate lyase need for glyoxylate metabolism) (Grahma *et al.*, 1994 and 1992), cytosol (nitrate reductase) (Cheng *et al.*, 1992) and mitochondria (MCCase, this paper). The sugar-sensing mechanism for the regulation of gene expression is not fully understood. Recently, it has been suggested that the initial signal for sugar sensing

is the phosphorylation of hexose sugars by hexokinase (Jang and sheen 1994; Jang *et al.*, 1997; Urwin and Jenkins 1997; Dai 1999; Grahma *et al.*, 1994; Grahma *et al.*, 1992). In agreement with this hypothesis, glucose, fructose and galactose all repressed *MCC-A* and *MCC-B* expression. In addition, the findings that the non-metabolizable glucose analogue 3-*O*-MG, which can not be phosphorylated by hexokinase, had no effect on *MCC-A* and *MCC-B* gene expression, but 2-dGlc, which can be phosphorylated by hexokinase, but not metabolized further, significantly repressed *MCC-A* and *MCC-B* gene expression, are consistent with the hexokinase hypothesis.

It has been reported that glucose is more efficient in repressing photosynthetic genes than sucrose (Jang and Sheen, 1994). However, in our system, sucrose was the most effective repressor, as compared to the hexoses tested. This difference maybe because sucrose is the main translocated sugar in plants and is translocated more efficiently from source to sink than other hexoses.

Interestingly, like MCCase, darkness also induced the expression of branch-chain keto-acid dehydrogenase (BCKDH) in *Arabidopsis* leaves, and this dark-induced expression is regulated by sugar levels in leaf cells (Fujiki *et al.*, 1997). BCKDH is a multienzyme complex that is also required for the Leu catabolic pathway (Fujiki *et al.*, 1997; Luethy *et al.*, 1997; Mooney *et al.*, 1998). Hence, the expression of two different enzymes in this pathway appear to be regulated by same mechanisms. This may imply that the entire pathway may be coordinately regulated by metabolic control.

#### **Potential functions of MCCase during carbohydrate starvation**

It has been reported that plants respond to carbohydrate starvation by increasing the enzymatic activities related to the fatty acid  $\beta$ -oxidation, the catabolism of proteins and

amino acids and the glyoxylate pathway that is needed for carbohydrate synthesis (for review, see Yu, 1999). Degradation of proteins and fatty acids are also observed during seed germination, senescence and limiting photosynthesis (Poirier *et al.*, 1999; Dieuaide *et al.*, 1992; Pistelli *et al.*, 1996; Ismail *et al.*, 1997; Lee *et al.*, 1998). The degradation of protein via autophagic processes results in increase accumulation of free amino acids (Genix *et al.*, 1990), which are capable of entering the tricarboxylic cycle and glyoxylate pathway to sustain respiration and metabolic processes during environmental stress.

Leu, isoleucine and valine are the major amino acids of membrane proteins in the plants. Acetyl-CoA, the final product of Leu catabolism, can be used in various processes including respiration, the glyoxylate cycle and fatty acid synthesis. Two enzymes in the glyoxylate pathway, malate synthase and isocitrate lyase, are also down-regulated by light and sugars, at the transcriptional level (Grahma *et al.*, 1994 and 1992). This correlation between Leu catabolism and the glyoxylate pathway indicates the potential fate of acetyl-CoA derived from Leu catabolism during carbohydrate limitation. In addition, *in situ* hybridization performed by McKean (2000) in different cell types and organs of *Arabidopsis* shows that *MCC-A* and *MCC-B* mRNAs accumulate at higher levels in rapidly growing and metabolically active tissues. These results indicate not only the tissues that are involved in MCCase biosynthesis, but may also indicate the tissues in which MCCase function is most important. The metabolic control of MCCase expression and the potential fate of acetyl-CoA imply that MCCase may play an important role in regulating the carbon and energy balance during plant development particularly in non-photosynthetic organs, and during carbon stress conditions. In support of this suggestion, it was reported that various amino acids, especially

Leu, strongly inhibited autophagic proteolysis in the isolated rat hepatocytes (Caro *et al.*, 1989).

## Experimental procedures

### Cloning and sequence analysis

An *Arabidopsis thaliana* genomic library, cloned in the bacteriophage vector  $\lambda$  FIX (Voytas *et al.*, 1990), was obtained from the Biological Resource Center (Ohio State University). The library was screened by hybridization with the *Arabidopsis* MCC-A cDNA (Weaver *et al.*, 1995). Hybridizing clones were isolated by plaque purification. Isolation and purification of bacteriophage DNA was conducted using the method described by Sambrook *et al.* (1989). DNA manipulation and subcloning of DNA fragments into *pBluescript KS* (*pBKS*, from Stratagene Cloning System, La Jolla, CA) was carried out according to standard procedures (Sambrook *et al.*, 1989). The MCC-A gene was isolated as a single  $\lambda$  clone. Two adjoining *SacI* fragments were subcloned into *pBKS* and named *pBP5* and *pBP4*. The junction of *pBP4* and *pBP5* was amplified by PCR using  $\lambda$  DNA containing MCC-A genomic DNA as the template. This amplified fragment was sequenced, which verified that *pBP4* and *pBP5* carried adjoining *Arabidopsis* genomic fragments. The nucleotide sequence of both strands of all plasmid clones was determined using an Applied Biosystem 373A Automated DNA Sequencer (DNA Sequencing Facility of Iowa State University). DNA sequences were analyzed using the GCG suite of sequence analysis software (University of Wisconsin Genetics Computer Group, Madison, WI).

### **Construction of the *MCC-A* and *MCC-B* promoter-*GUS* chimeric genes**

A *Sau3AI* fragment of *pBP4*, which contains the 5' end of the *MCC-A* gene, was subcloned into the *Bam*HI site of *pBKS*. The resultant plasmid, termed *pBP6*, was digested with *Hind*III and *Xba*I, and the DNA fragment from positions -1150 to -12 of the *MCC-A* gene was subcloned into the Ti-based plant transformation vector, *pBII101.2* (Clontech, Palo Alto, CA). Schematic of the resulting plasmid (*pBP8*) is shown in Figure 1B.

The promoter of the *MCC-B* gene was excised from the plasmid, *pMAGP* (McKean *et al.*, 2000) as a 1.2 kb *Xho*I-*Xmn*I fragment and subcloned into the *Xho*I and *Sma*I sites of *pBKS* (*pBP15*). This promoter fragment was PCR amplified with the vector associated T3 primer and the mutagenic primer of sequence 3' CTGTTAGTTCATCAGATCTAGT5' (A and T were added to create an *Xba*I site). This resulting PCR product was digested with *Xho*I and *Xba*I, and cloned into the *Sal*II and *Xba*I sites of *pBII101.2*. In the resulting plasmid (*pBP18*), the *MCC-B* promoter (from -1110 to -64 relative to the ATG translation start site) was fused with *GUS* reporter gene (Fig. 1C).

### **Plant transformation and regeneration**

The *pBP8* and *pBP18* plant transformation vectors were transformed into *Agrobacterium tumefaciens* C58C1 by electroporation. Transformation of *Arabidopsis* was done as described by Koncz and Schell (1996) but without applying a vacuum. Primary transgenic plants (T1 generation) were selected by germinating seeds on Murashige and Skoog medium (MS, BRL) containing 0.8% (w/v) Bacto-agar, 0.05% (w/v) MES (pH 5.7), 40 mg/l kanamycin, 250 mg/l vancomycin and 1% (w/v) sucrose. For each transgene construct, twenty independent transgenic lines were generated. T2 seeds obtained from these

twenty primary transformants were grown on MS medium supplemented with 40 mg/l kanamycin and scored for resistance to antibiotic. For each transgenic line six kanamycin-resistant plants were transferred to soil and T3 seeds were harvested. Ten independent transgenic plants were finally identified as being homozygous for the transgene on the basis of the segregation of the kanamycin-resistance trait. The transgenic nature of the kanamycin-resistant plants were further confirmed by Southern blot analysis (data not shown) .

### **Plant materials and growth conditions**

*Arabidopsis* seeds of the Columbia ecotype were germinated in sterile soil or MS media, and plants were grown in a growth room maintained at 22°C with 24-hour continuous illumination provided by 40 W Sylvania cold-white fluorescent light bulbs at white light irradiation of  $150 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ .

Seeds (T2 or T3 generation transgenic plants which are homozygous for the transgene) were surface-sterilized by treatment with 50% (v/v) ethanol for 1 min, followed by a 20 min treatment with 50% (v/v) bleach in 1% (v/v) Tween-20. Following extensive washing with sterile water, approximately 40 seeds were applied to each Petri dish, and plates were sealed with parafilm or gas-permeable surgical tape. In some experiments, the above media also contained different concentrations of sugars. Seedlings were grown under constant white light ( $150 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) or in completely darkness at 22°C. Day 0 was defined as the time when seeds were sown on the Petri dish.

Incubation of excised plant tissues was performed in Petri dishes containing sterile MS liquid medium with different concentration of sugars and glucose analogues for 24 hours in continuous light at 22°C.

**Isolation of RNA and blot hybridization analysis**

RNA was isolated from whole plant tissue by the method described by Logemann (1987). Twenty micrograms of each RNA sample was subjected to electrophoresis in a 1.4% (v/v) formaldehyde-agarose gel and blotted to nylon membrane by capillary transfer using 25 mM sodium phosphate buffer, pH 7.0. In order to ensure equal loading of RNA, ethidium bromide was included in the sample-loading buffer, and blots were viewed under UV illumination. After transfer, RNA was bonded to the membrane by baking at 90°C for 60 min. The filter was hybridized with <sup>32</sup>P-labeled *MCC-A* and *MCC-B* cDNA fragments. The blots were washed once with 1 x SSC, 0.1 (w/v) % SDS at 60°C for 15 min followed by 0.25 x SSC, 0.1% (w/v) SDS at 60°C for another 30 min.

**Protein extracts**

*Arabidopsis* seedling tissue (0.1 g) was frozen with liquid N<sub>2</sub> and homogenized with an “Eppendorf pestle” in 1.5 ml microcentrifuge tubes. The resulting powder was homogenized at 4°C with 0.3 ml of extraction buffer (0.1 M Hepes-KOH pH 7.0, 20 mM 2-mercaptoethanol, 0.1 mg/ml PMSF, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v) glycerol) using the “Eppendorf pestle”. The mixture was immediately centrifuged at 12,200 g for 15 min at 4°C. The supernatant was recovered and frozen in liquid N<sub>2</sub> and the pellet was discarded. Protein concentrations were determined by the Bradford method (BioRad, Hercules, CA) using bovine serum albumin (BSA) as a standard (Bradford, 1976).



### **Histochemical staining of GUS activity**

Histochemical assay of GUS activity was conducted as described by Jefferson (1987) with minor modification. Whole seedlings and excised plant organs and tissues were incubated in 5-bromo-4-chloro-3-indole glucuronide (X-gluc) solution (0.5 mg/ml X-gluc in 50 mM Tris/NaCl buffer, pH 7.0, 0.5% (v/v) Triton X-100, 0.5 mM  $K_3[Fe(CN)_6]$ , 0.5 mM  $K_4[Fe(CN)_6]$ , 10 mM  $Na_2EDTA$ ). 100 mM X-gluc stock solution was prepared by dissolved 26.1 mg X-gluc in 0.5 ml DMSO just before use. Vacuum infiltration was carried out for 10 min. Tissue was then incubated at 37°C in the dark for 16 h or until color developed. To improve the contrast, Soluble pigments were removed by incubating the stained material in several changes of 70% (v/v) ethanol until the chlorophyll was cleared from the tissue. The stained tissue was examined under bright-field microscopy, using an Olympus BH2 microscope.

### **Fluorometric analysis of GUS activity**

GUS activity was determined in extracts with a fluorometric assay essentially as described by Jefferson (1987). Plant samples were collected and protein extracts prepared as described above. One hundred  $\mu$ l of protein extract was mixed with 500  $\mu$ l GUS assay buffer (2 mM 4-methylumbelliferyl  $\beta$ -glucuronide (MUG) in extraction buffer) and incubated in 37°C. Aliquots of 100  $\mu$ l were removed at timed intervals (generally 5, 15, 25, 35 and 45 min), and the reaction was terminated by adding 0.9 ml of 0.2 M  $Na_2CO_3$ . The fluorescent product was quantified using a fluorometer (Hitachi, Model F-2000). Excitation and emission wavelengths were 365 nm and 455 nm, respectively. Tissues from regenerated

non-transformed plants were used to quantify background GUS activity. All the experiments were repeated three times using three independently transformed plants.

### **MCCase assay**

MCCase activity was measured as the rate of incorporation of radioactivity from  $\text{NaH}^{14}\text{CO}_3$  into the acid-stable product (Wurtele and Nikolau, 1990). The reaction mixture contained 0.1 M Tricine-KOH, pH 8.0, 5 mM  $\text{MgCl}_2$ , 2.5 mM DTT, 5 mM  $\text{KHCO}_3$ , 5  $\mu\text{Ci}$   $\text{NaH}^{14}\text{CO}_3$  (58 mCi/mmol, Amersham), 1 mM ATP, and 0.2 mM methylcrotonyl-CoA. Aliquots of each extract were passed through individual 1 ml Sephadex G-25 columns preequilibrated with extraction buffer and centrifuged at 800 g for 1 min to remove lower molecular weight molecules. The MCCase assay was initiated by the addition of the protein extract and incubated in a final volume of 200  $\mu\text{l}$  at 37°C for 60 min. The reaction was terminated by the addition of 50  $\mu\text{L}$  6 N HCl. A sample (50  $\mu\text{l}$ ) was applied to a strip of Whatman 3MM paper, dried, and the acid-stable radioactivity was determined by liquid scintillation counting. Assays were performed in triplicate. For each protein extract, control assays lacking the methylcrotonyl-CoA substrate were carried out in parallel.

### **Electrophoresis and western blot analysis**

SDS-PAGE was performed in 10% (w/v) acrylamide gels as described previously (Laemmli, 1970). After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984). MCC-A and MCC-B subunits were immunologically detected with specific antisera (Weaver *et al.*, 1995; McKean *et al.*, 2000), diluted 1:2000,

followed by an incubation with  $^{125}\text{I}$ -Protein A. The biotin-containing MCC-A subunit was also detected by using  $^{125}\text{I}$ -streptavidin (Nikolau *et al.*, 1985).

### References

- Alban, C., Baldet, P., Axiotis, S. and Douce, R.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from higher plant mitochondria. *Plant Physiol.* **102**, 957-965.
- Anderson, M.D., Che, P., Song, J., Nikolau, B.J. and Wurtele, E.S.** (1998) 3-methylcrotonyl-coenzyme A carboxylase is a component of the mitochondrial leucine catabolic pathway in plant. *Plant Physiol.* **118**, 1127-1138.
- Aubert, S., Alban, C., Bligny, R. and Douce, R.** (1996) Induction of beta-methylcrotonyl-coenzyme A carboxylase in higher plant cells during carbohydrate starvation: Evidence for a role of MCCase in leucine catabolism. *FEBS Lett.* **383**, 175-180.
- Bach, T.** (1995) Some new aspects of isoprenoid biosynthesis in plants- A review. *Lipid* **30**, 191-202.
- Bach, T** (1987) Synthesis and metabolism of mevalonic in plants *Plant physiol. Biochem.* **25**, 163-178.
- Bradford, M.M.** (1976) A rapid and sensitive method for quantification for microgram quantities of protein utilization the principle of protein dye-binding. *Anal. Biochem.* **72**, 248-254.
- Caro, L.H.P., Plomp, P.J.A.M., Leverve, X.M. and Meijer, A.J.** (1989) A combination of intracellular leucine with either glutamate or aspartate inhibits autophagic proteolysis in isolated rat hepatocytes. *Eur. J. Biochem.* **181**, 717-720.
- Chen, Y., Wurtele, E.S., Wang, X. and Nikolau, B.J.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from somatic embryos of *Daucus carota*. *Arch. Biochem. Biophys.* **305**, 103-109.

- Clauss, M., Motel, A. and Lichtenthaler, H.K.** (1993) Studies on the plant methylcrotonyl-CoA Carboxylase. *J. Plant Physiol.* **114**, 508-511.
- Cheng, C., Acedo, G.N., Cristinsin, M. and Conkling M.A.** (1992) Sucrose mimic the light induction of *Arabidopsis* nitrate reductase gene transcription. *Proc. Natl Acad. Sci.* **89**, 1861-1864.
- Dieuaide, M., Brouquisse, R., Pradet, A. and Raymond, P.** (1992) Increased fatty acid  $\beta$ -oxidation after glucose starvation in maize roots. *Plant Physiol.* **99**, 595-600.
- Diez, T.A., Wurtele, E.S. and Nikolau, B.J.** (1994) Purification and characterization of 3-methylcrotonyl-coenzyme-A carboxylase from leaves of *Zea mays*. *Arch. Biochem. Biophys.* **310**, 64-75.
- Edmund, J. and Popják, G.** (1974) Transfer of carbon atoms from mevalonate to *n*-fatty acids. *J. Biol. Chem.* **249**, 66-71.
- Fujiki, Y., Sato, T., Yoshikawa, Y., Ito, M. and Watanabe, A.** (1997) Dark induced expression of genes for branched-chain alpha-keto acid dehydrogenase complex in *Arabidopsis* leaves (abstract No. 400). *Plant Physiol.* **114**, S-95.
- Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D.** (1999) Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. *Plant Cell* **11**, 1253-1266.
- Graham, I.A.** (1996) Carbohydrate control of gene expression in higher plants. *Res. in Microbio.* **147**, 572-580.
- Graham, I.A., Denby, K.J. and Leave, C.J.** (1994) Carbon catabolite repression regulations glyoxylate cycle gene expression in cucumber. *Plant Cell* **6**, 761-772.

- Genix, P., Bligny, R., Martin, J. and Douce, R.** (1990) Transient accumulation of asparagine in sycamore cells after a long period of sucrose starvation. *Plant Physiol.* **94**, 717-722.
- Gray, J.C.** (1987) Control of isoprenoid biosynthesis in higher plants. *Asv. Bot. Res.* **14**, 23-91.
- Guan, X., Diez, T., Prasad, T.K., Nikolau, B.J. and Wurtele, E.S.** (1999) Geranoyl-CoA Carboxylase: A novel biotin-containing enzyme in plants. *Arch. Biochem. Biophys.* **362**, 12-21.
- Halford, N.G., Purcell, P.C. and Hardie, G.** (1999) Is hexokinase really a sugar sensor in plants? *Trends in Plant Sci.* **4**, 117-120.
- Ismail, I., Debekis, L., Alpi, A. and Smith, S.M.** (1997) Expression of glyoxylate cycle genes in cucumber roots responds to sugar supply and can be activated by shading or defoliation of the shoot. *Plant Mol. Biol.* **35**: 633-640.
- Jang, J., León, P., Zhou, L. and Sheen, J.** (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* **9**, 5-19.
- Jang, J. and Sheen, J.** (1994) Sugar sensing in high plants. *Plant Cell* **6**, 1665-1679.
- Jefferson, R.A.** (1987) Assay chimeric genes in plants: The *GUS* gene fusion system. *Plant Mol. Biol. Reporter* **5**, 387-405.
- Koch K.E.** (1996) Carbohydrate-modulated gene expression in plants. *Ann. Rev. Plant Physiol. & Plant Mol. Biol.* **47**, 509-540.
- Kleinig, H.** (1989) The role of plastids in isoprenoid biosynthesis. *Ann. Rev. Plant Physiol. & Plant Mol. Biol.* **40**, 39-59.

- Koncz, C. and Schell, J.** (1986) Green lab protocol for vacuum infiltration transformation of *Arabidopsis*. *Mol. Gen. Genet.* **204**, 383-396.
- Kyhse-Anderson J.** (1984) Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203-209.
- Lau, E.P., Cocharan, B.C. and Fall, R.R.** (1980) Subcellular localization of 3-methylcrotonyl-CoA carboxylase in bovine kidney. *Arch. Biochem. Biophys.* **205**, 352-359.
- Lee, S.H., Chae, H.S., Lee, T.K., Kim, S.H., Cho, B.H., Cho, S.H., Kang, B.G. and Lee, W.S.** (1998) Ethylene-mediated phospholipid catabolic pathway in glucose-starved carrot suspension cells. *Plant Physiol.* **116**, 223-229.
- Luethy, M.H., Miernyk, J.A. and Randall, D.D.** (1997) Molecular analysis of a branched-chain keto-acid dehydrogenase from *Arabidopsis thaliana* (abstract No. 696). *Plant Physiol.* **114**, S-147.
- Laemmli, U.K.** (1970) Cleavage of structure protein during the assembly of the head of the bacteriophage T4. *Nature* (London) **227**, 680-685.
- Mooney, B.P., Miernyk, J.A. and Randall, D.D.** (1998) Nucleotide sequence of a cDNA encoding the dihydrolipoylacyl transferase (E2) subunit of the branched-chain alpha-keto acid dehydrogenase complex from *Arabidopsis thaliana* (accession no. AF038505) (PGR 98-071). *Plant Physiol.* **117**, 331.
- McKean, A.L., Ke, J., Song, J., Che, P., Achenbach, S., Nikolau, B.J. and Wurtele, E.S.** (2000) Molecular characterization of the non-biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase. *J. Biol. Chem.* **275**, 5582-5590.

- Maier, A. and Lichtenthaler, H.K.** (1998) Methylcrotonyl-CoA carboxylase from barley (*Hordeum vulgare* L.): Purification, kinetic characterization and senescence-related induction of activity. *J. Plant Physiol.* **152**, 213-221.
- Moss, J. and Lane, M.D.** (1971) The biotin-dependent enzymes. *Adv. Enzymol.* **35**, 321-442.
- Nes, W.D. and Bach, T.J.** (1985) Evidence for a mevalonate shunt in a tracheophyte. *Proc. Roy Soc. Lond B. Biol. Sci.* **225**, 425-444.
- Nikolau, B.J., Wurtele, E.S. and Stumpf P.K.** (1985) Use of streptavidin to detect biotin-containing proteins in plants. *Anal. Biochem.* **149**, 448-453.
- Poirier, Y., Ventre, G. and Caldelari, D.** (1999) Increased flow of fatty acids toward  $\beta$ -oxidation in developing seeds of *Arabidopsis* deficient in diacylglycerol acyltransferase activity or synthesizing medium-chain-length fatty acids. *Plant Physiol.* **121**, 1359-1366.
- Pistekki, L., Nieri, B., Smith, S.M., Alpi, A. and DeBekkis, L.** (1996) Glyoxylate cycle enzyme activities are induced in senescent pumpkin fruits. *Plant Sci.* **119**, 181-185.
- Popják, G.** (1971) Specificity of enzymes in sterol biosynthesis. *Harvey Lect.* **65**, 127-156.
- Sambrook, J., Fritsh, E.F. and Maniatis, T.** (1989) Molecular Cloning: A laboratory manual 2<sup>nd</sup>. eds. Cold Spring harbor Laboratory, Cold Spring Harbor, NY.
- Sheen, J.** (1994) Feed back control of gene expression. *Photosynthesis Res.* **39**, 427-438.
- Sheen, J.** (1990) Metabolic repression of transcription in higher plants. *Plant Cell* **2**, 1027-1038.
- Song, J., Wurtele, E.S. and Nikolau, B.J.** (1994) Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase: Identification of the biotin carboxylase and biotin-carrier domains. *Proc. Natl Acad. Sci. USA* **91**, 5779-5783.



- Taylor, C.B.** (1997) Sweet sensations. *Plant Cell* **9**, 5-19.
- Urwin, N.A.R. and Jenkins, G.** (1997) A sucrose repression element in the *Phaseolus vulgaris rbcS2* gene promoter resembles elements responsible for sugar stimulation of plant and mammalian gene. *Plant Mol. Biol.* **35**, 929-942.
- Voytas, D.F., Konieczny, A., Cummings, M.P. and Ausubel, F.M.** (1990) The structure, distribution and evolution of the *Ta1* retrotransposable element family of *Arabidopsis thaliana*. *Genetics* **126**, 713-721.
- Wang, X., Wurtele, E.S., Keller, G., McKean, A.L. and Nikolau, B.J.** (1994) Molecular cloning of cDNAs and genes coding for  $\beta$ -methylcrotonyl-CoA carboxylase of tomato. *J. Biol. Chem.* **269**, 11760-11769.
- Wang, X., Wurtele, E.S. and Nikolau, B.J.** (1995) Regulation of  $\beta$ -methylcrotonyl-coenzyme A carboxylase activity by biotinylation of the apoenzyme. *Plant Physiol.* **108**, 1133-1139.
- Weaver, L.M., Lebrun, L., Franklin, A., Huang, L., Hoffman, N., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coenzyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1013-1014.
- Wurtele, E.S. and Nikolau, B.J.** (1990) Plants contain multiple biotin enzymes: Discovery of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. *Arch. Biochem. Biophys.* **278**, 179-186.
- Yu, S.** (1999) Cellular and genetic responses of plants to sugar starvation. *Plant Physiol.* **121**, 687-693.

**Figure 1. A. Schematic representation of the structure of the *MCC-A* gene of *Arabidopsis*.**

**Black boxes represent the 15 exons and introns are indicated by the solid black lines.**

**Positions of the translation start codon (<sup>1</sup>ATG) and stop codon (<sup>4110</sup>TAA) and the unique *SacI***

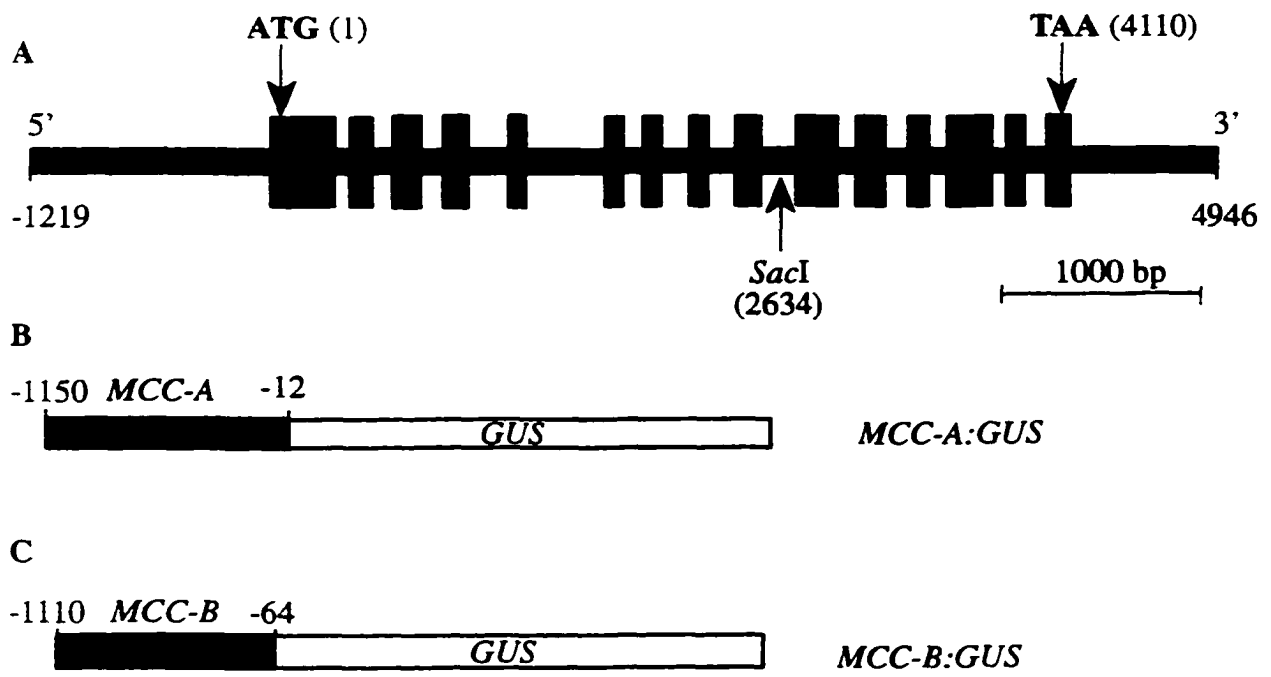
**site are indicated. B. Schematic representation of the *MCC-A:GUS* transgene, which is**

**composed of the 1.1 kb promoter region (-1150 to -12) of the *MCC-A* gene fused to the *GUS***

**reporter gene. C. Schematic representation of *MCC-B:GUS* transgene, which is composed of**

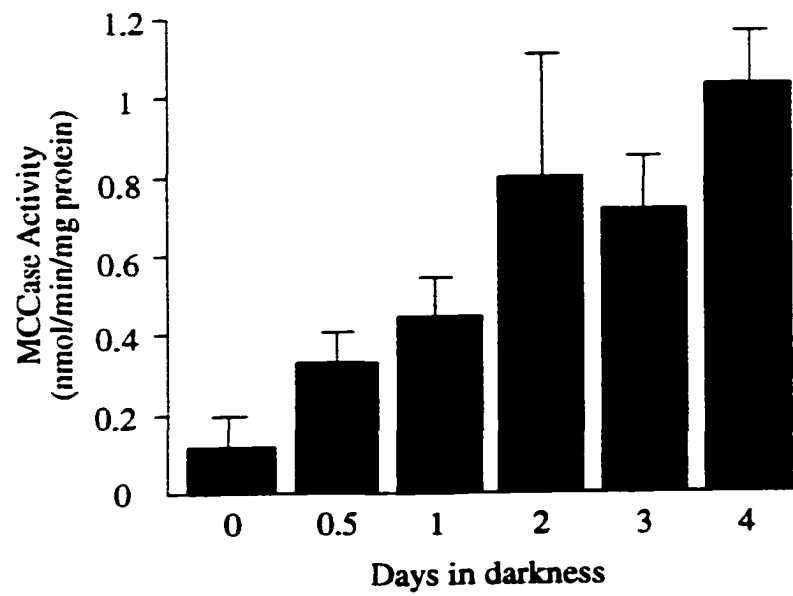
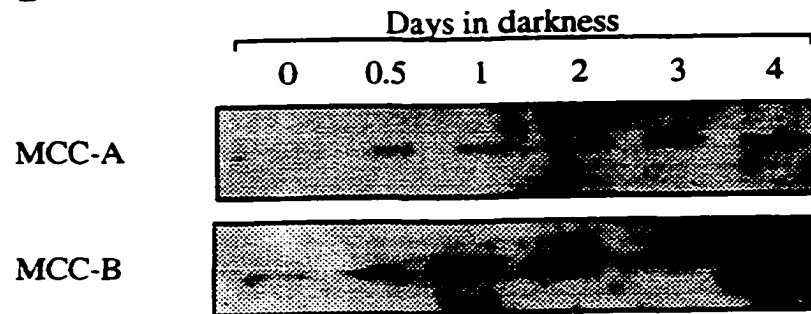
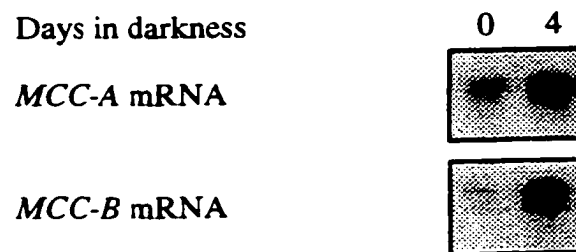
**the 1.0 kb promoter region (-1110 to -64) of the *MCC-B* gene fused to the *GUS* reporter**

**gene.**



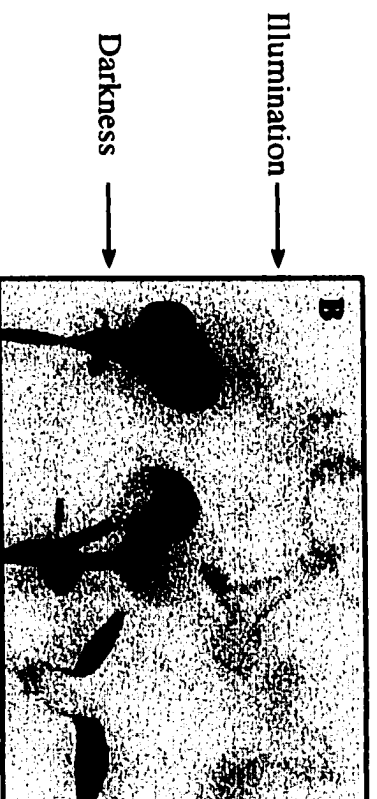
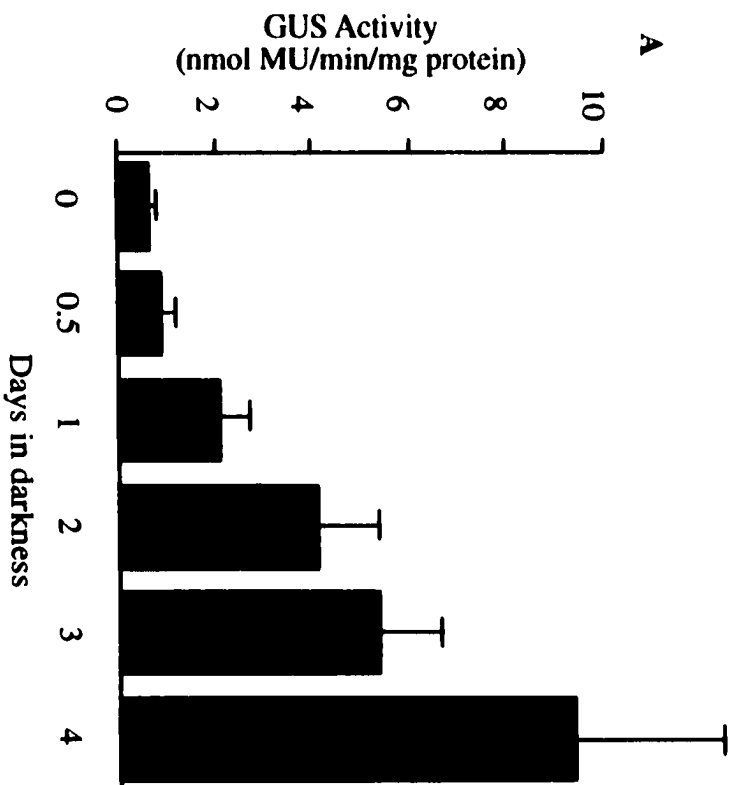
**Figure 2. Effect of dark treatment on MCCase expression.**

A. Changes in MCCase activity in 10-day-old *Arabidopsis* seedlings, which were initially grown under constant illumination and then transferred to darkness for the indicated time period. The data presented are means  $\pm$  SE from three replicates. B. Western-blot analysis of MCC-A and MCC-B accumulations in *Arabidopsis*, which were initially grown under constant illumination and then transferred to darkness for the indicated time period. Proteins were extracted from the samples and aliquots containing equal amounts of protein were fractionated by SDS-PAGE, followed by western-blot analysis with antiserum against MCC-A or MCC-B. C. Northern-blot analysis of *MCC-A* mRNA abundance in 10-day old *Arabidopsis* seedlings, which were initially grown under constant illumination and then transferred to darkness for the indicated time period. Equal amounts of RNA (30  $\mu$ g) isolated from each sample were fractionated by electrophoresis in a formaldehyde-containing agarose gel. The data presented in B and C were gathered from a single experiment; three replicates of this experiment gave similar results.

**A****B****C**

**Figure 3.** Effect of dark treatment on *MCC-A*-promoter-mediated GUS expression.

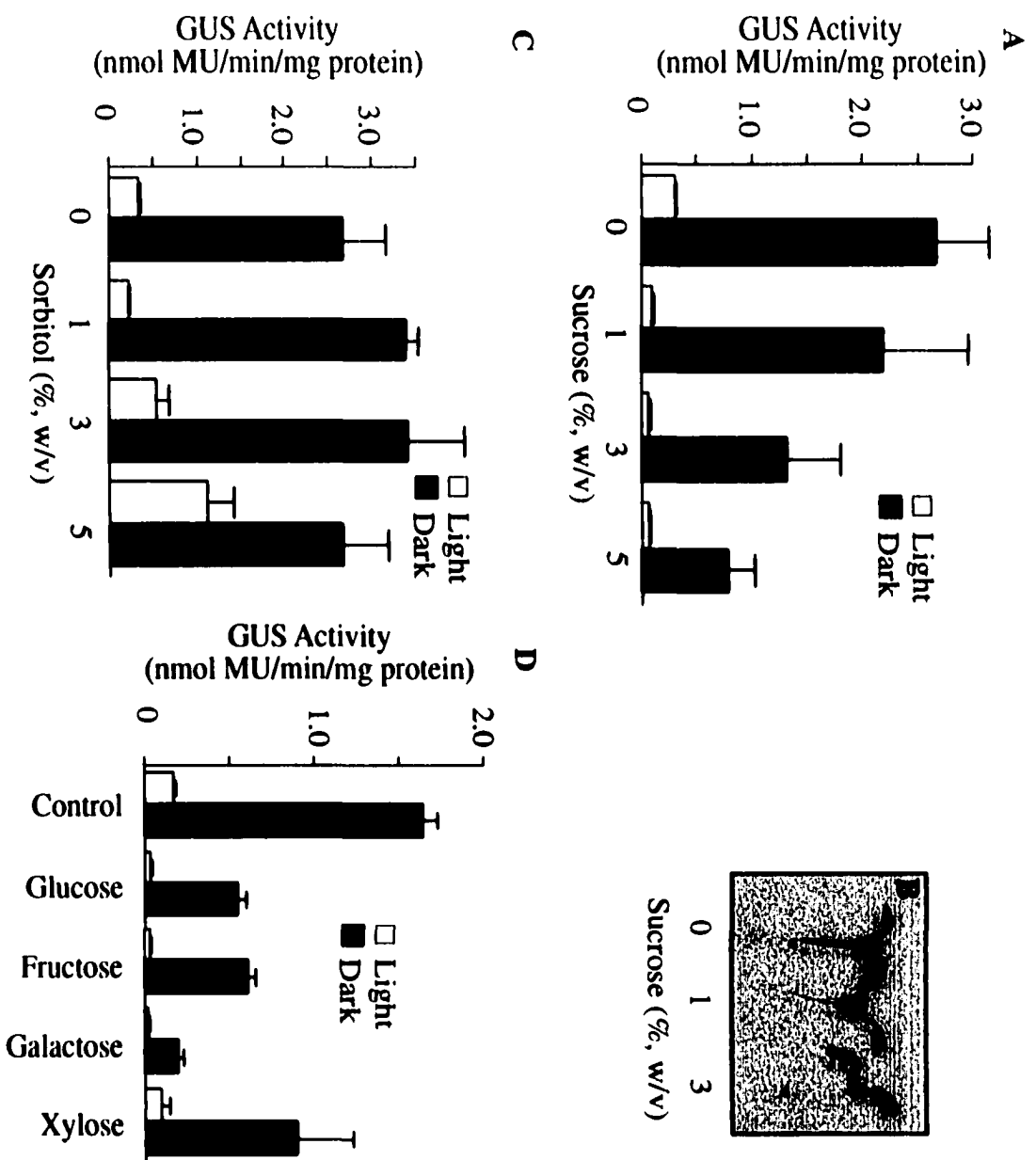
A. GUS activity in 10-day-old *MCC-A:GUS* transgenic *Arabidopsis* seedlings which were initially grown under constant illumination and then transferred to darkness for the indicated time period. The data are means  $\pm$  SE from three replicates using three independent transgenic lines. B. Histochemical localization of *MCC-A:GUS* expression in transgenic *Arabidopsis* plants. GUS activity is indicated by an indigo blue precipitate after staining with X-Gluc. Seedlings carrying *MCC-A:GUS* transgene were grown in continuous light for 5.5 days, half the seedlings were then moved into darkness. GUS expression was stained eight days after planting. The upper 3 seedlings (from three independent transgenic lines) were in continuous illumination and lower 3 seedlings (from same three independent transgenic lines) were in darkness for the last 60 hours prior to staining.



**Figure 4.** The effect of illumination and carbohydrates on *MCC-A*-promoter-mediated *GUS* expression.

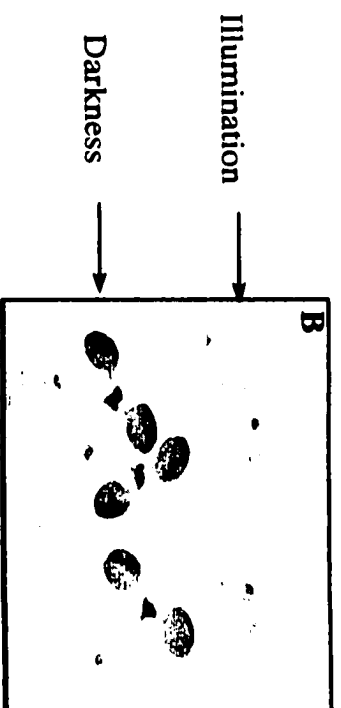
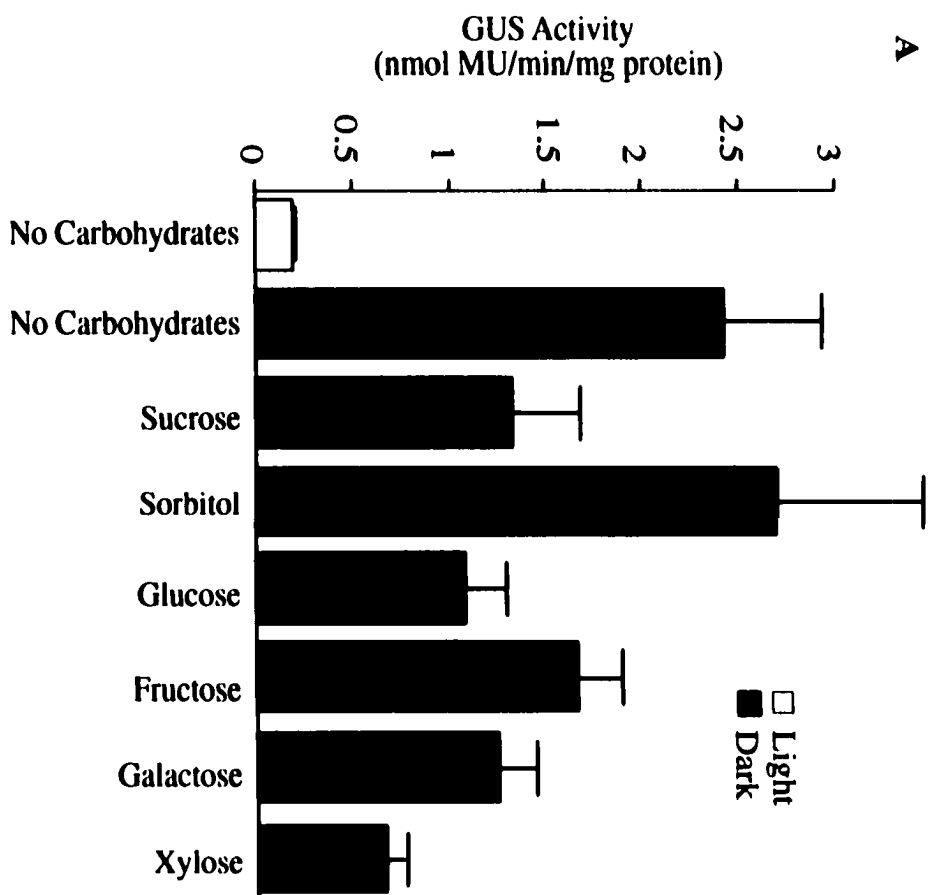
*MCC-A:GUS* transgenic *Arabidopsis* seedlings were grown for 8 days on MS medium containing the indicated concentration of sucrose (A) sorbitol (C) and 1.5% (w/v) of the indicated monosaccharides (D). Seedlings were grown either under continuous illumination (□) or transferred to darkness for the last 2 days of growth (■), and GUS activity was determined in protein extracts. The bars represent the mean  $\pm$  SE from three replicates using three independent transgenic lines. **B.** Histochemical staining of GUS activity in eight-day-old *MCC-A:GUS* transgenic *Arabidopsis* plants that were maintained in darkness for the last 2 days of growth. Seedlings were grown in MS agar medium containing the indicated concentration of sucrose.





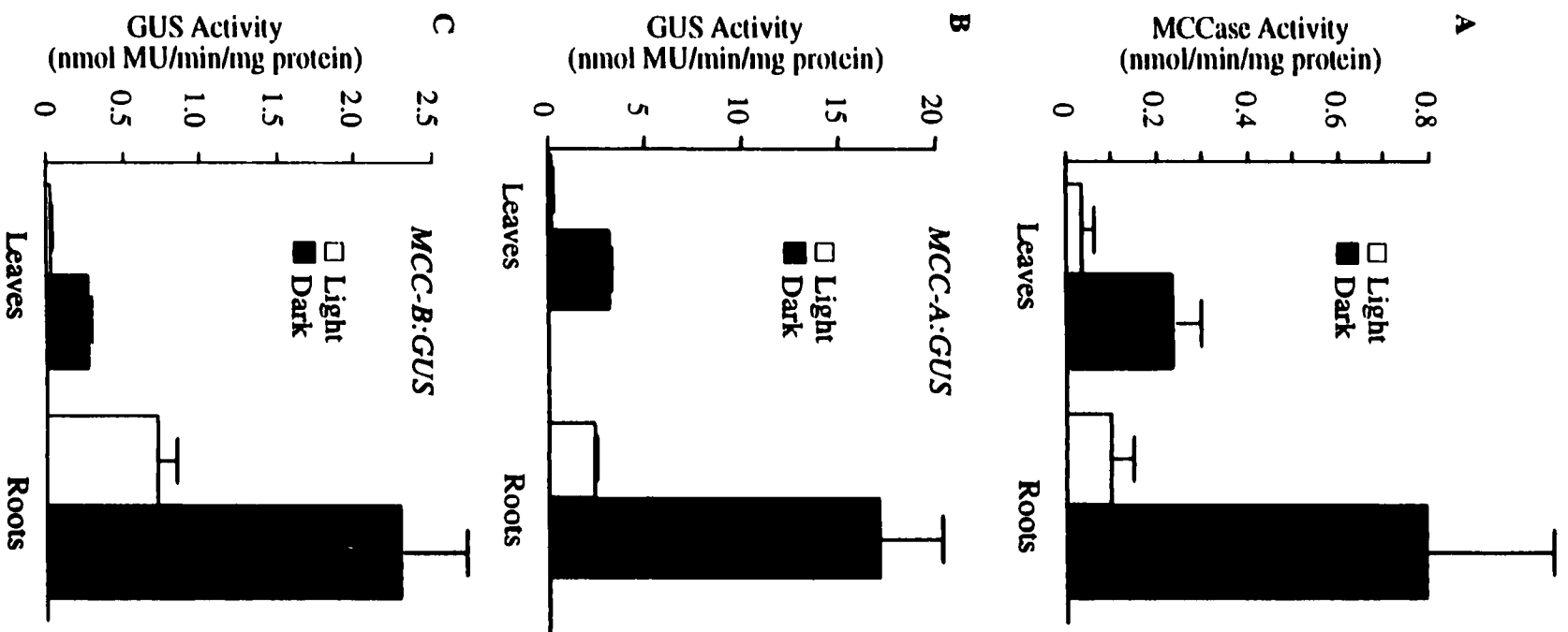
**Figure 5.** The effect of illumination and carbohydrates on *MCC-B*-promoter-mediated GUS activity.

A. *MCC-B:GUS* transgenic *Arabidopsis* seedlings were grown for 8 days on MS medium containing either 3% (w/v) of indicated disaccharide or 1.5% (w/v) of the indicated monosaccharide. Seedlings were grown under continuous illumination (□) or transferred to darkness for the last 2 days of growth (■). GUS activity was determined in protein extracts. The bars represent the mean  $\pm$  SE from three replicates using three independent transgenic lines. B. Histochemical staining of GUS activity in seedlings carrying the *MCC-B:GUS* transgene. Seedlings were grown in continuous light for 6 days, and then half the seedlings were moved into darkness for another 2 days. GUS activity was stained on the eighth day after planting. Upper seedlings (from three independent transgenic lines) were grown under continuous illumination, and lower seedlings (from same three independent transgenic lines) had been transferred to darkness for the last 2 days of growth.



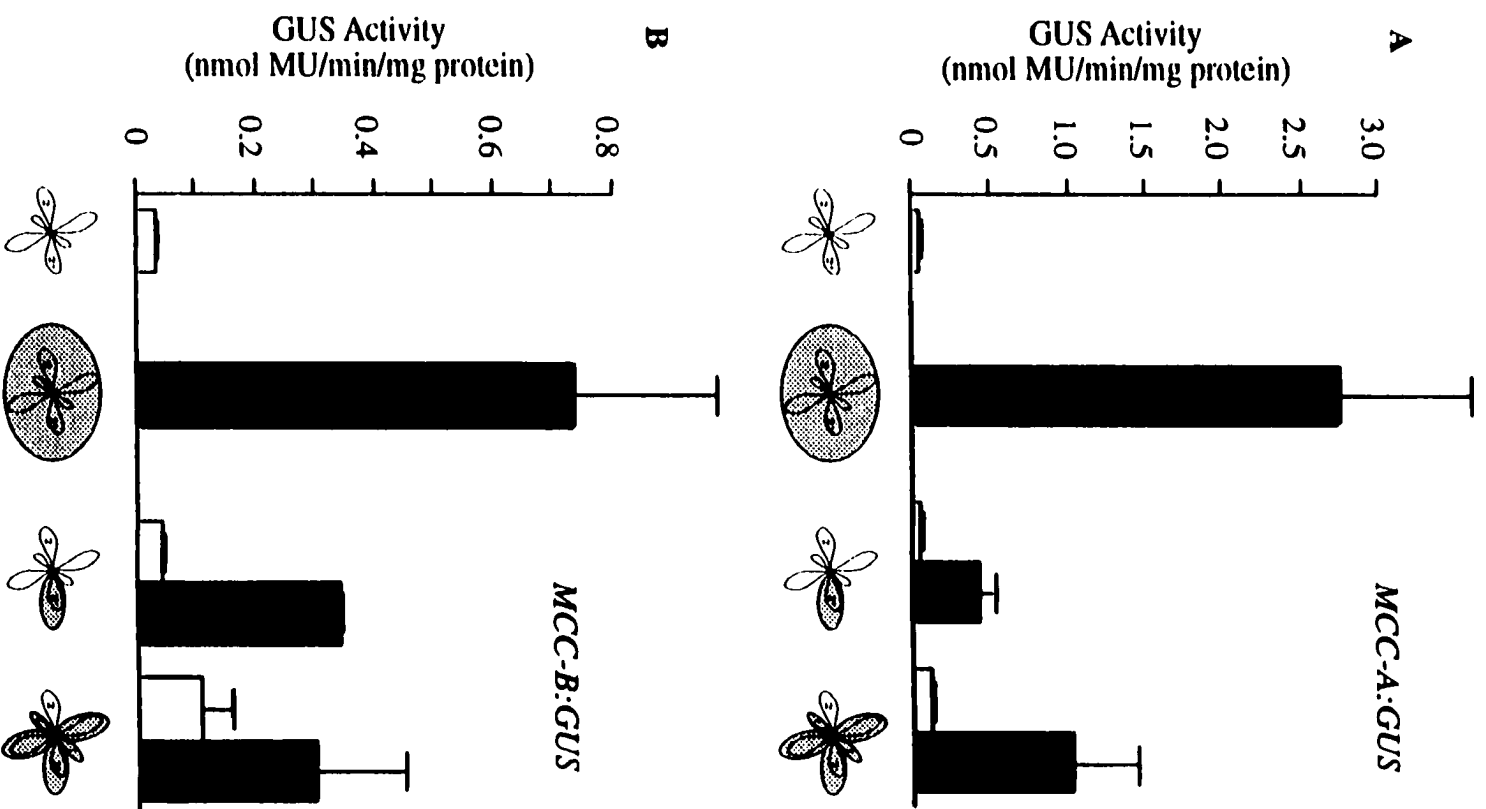
**Figure 6.** Effect of the illumination status of plants on MCCase expression in roots and leaves.

Three-week-old wild-type (A), *MCC-A:GUS* (B) or *MCC-B:GUS* (C) transgenic *Arabidopsis* plants were grown in soil under constant illumination (□) or under constant illumination until the last 2 day of growth, when they were placed in darkness (■). MCCase activity (A) and GUS activities (B and C) were determined in protein extracts from leaves and roots. The data are means  $\pm$  SE from three replicates. In panel B and C, the data are means  $\pm$  SE from three replicates using three independent transgenic lines.



**Figure 7. Effect of partial shading on MCCase expression.**

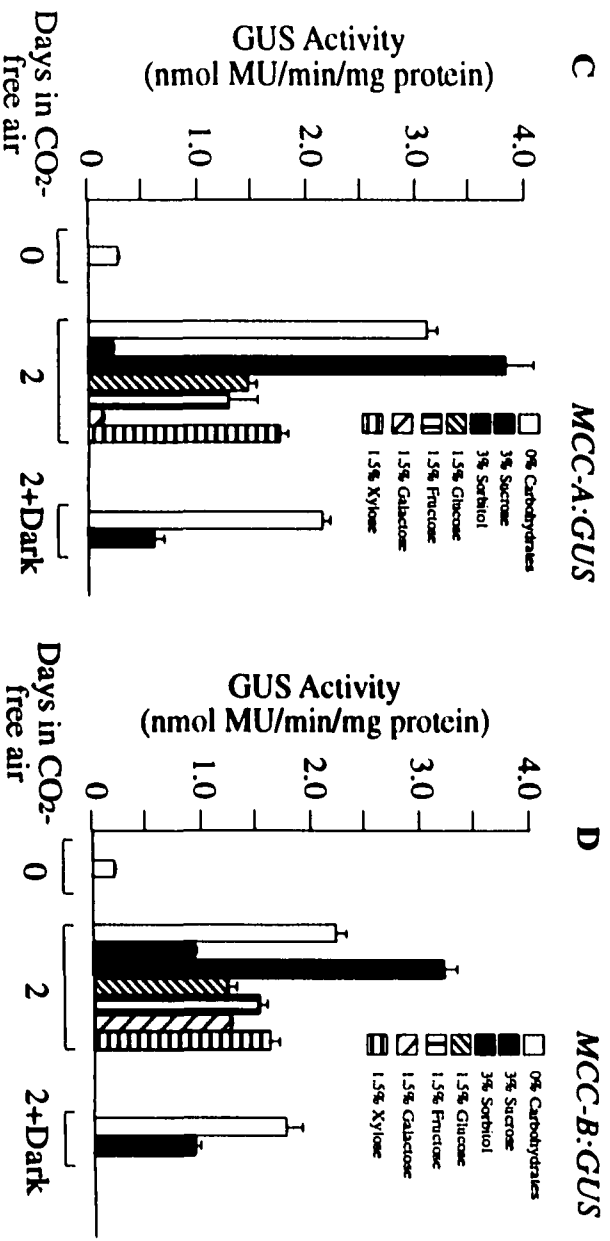
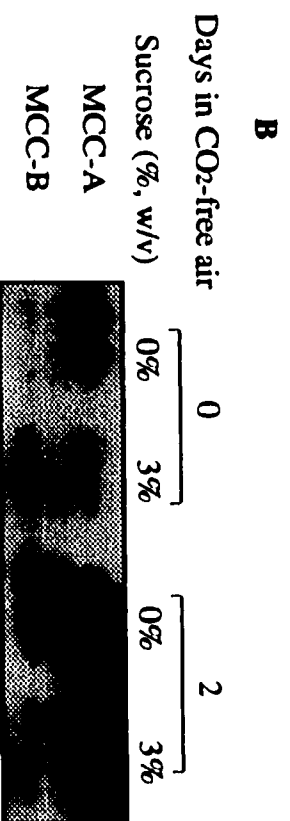
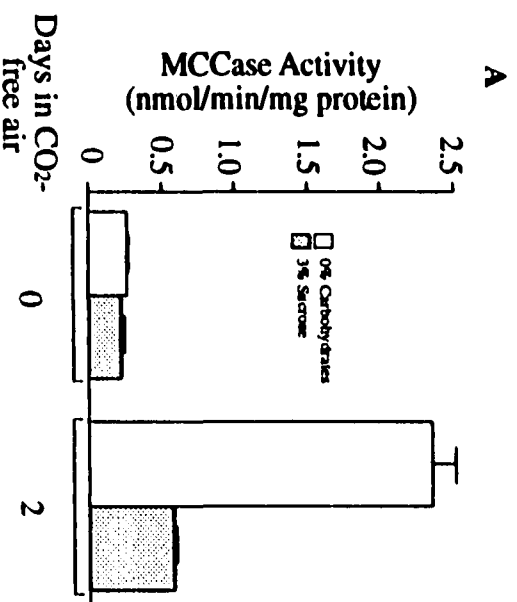
Three-week-old *MCC-A:GUS* (A) and *MCC-B:GUS* (B) transgenic *Arabidopsis* plants at the four-leaf stage were maintained either in constant illumination or in constant illumination until the last 2 days of growth when the indicated subset of leaves were shaded by wrapping in foil. GUS activity was determined in protein extracts prepared from individual shaded (■) or illuminated (□) second pair of leaves. The data are means  $\pm$  SE from three replicates using three independent transgenic lines.



**Figure 8.** The effect of limiting atmospheric CO<sub>2</sub> on MCCase expression .

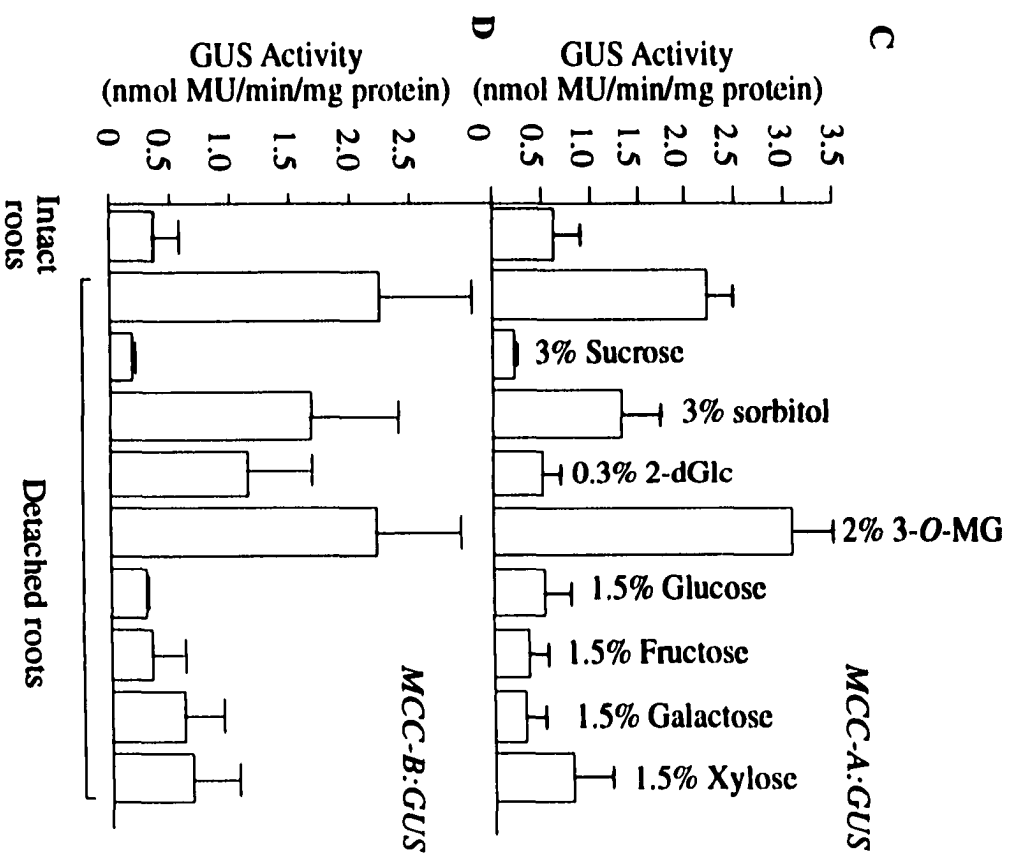
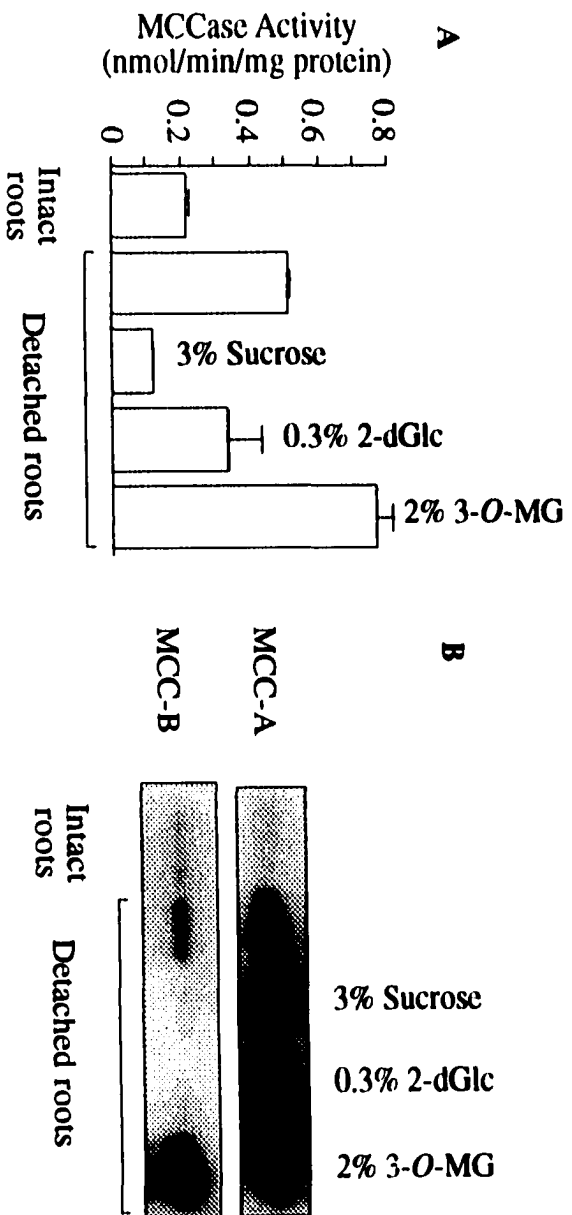
Eight-day old wild-type (A, B), *MCC-A:GUS* (C) and *MCC-B:GUS* (D) transgenic plants were grown on MS-agar medium supplemented with the indicated carbohydrates (% w/v). Seedlings were grown in either a normal atmosphere or in a normal atmosphere for the first 6 days of growth followed by 2 days of growth in a CO<sub>2</sub>-free atmosphere. MCCase (A) and GUS (C, D) activities were determined in protein extracts prepared from these seedlings. The accumulation of the MCC-A and MCC-B subunits were detected by western analysis of protein extracts (B). The data in panel A are means  $\pm$  SE from three replicates. The data in panel B were gathered from a single experiment; three replicates of this experiment gave similar results. The data in panel C and D are means  $\pm$  SE from three replicates using three independent transgenic lines.





**Figure 9. Regulation of MCCase gene expression in detached roots.**

Roots were detached from the aerial portions of two-week-old wild-type (A, B) or *MCC-A:GUS* (C) and *MCC-B:GUS* (D) transgenic *Arabidopsis* seedlings and incubated in MS media containing the indicated carbohydrates (% w/v) for 1 day. MCCase (A) and GUS (C, D) activities were determined in protein extracts. The accumulation of the MCC-A and MCC-B subunit was determined by western analysis (B). The data in panel A are means  $\pm$  SE from three replicates. The data in panel B were gathered from a signal experiment; three replicates of this experiment gave similar results. The data in panel C and D are means  $\pm$  SE from three replicates using three independent transgenic lines.



## **CHAPTER 4. The Effects of Biotin Depletion on the Expression of Biotin-Containing Enzymes**

Ping Che, Lisa M. Weaver, Eve Syrkin Wurtele and Basil J. Nikolau

*Department of Biochemistry, Biophysics and Molecular Biology (P.C., L.M.W. and BJN) and*

*Department of Botany (E.S.W.), Iowa State University, Ames, IA 50011*

### **Abstract**

Regulation of biotin-containing enzyme expression by biotin was investigated in wild-type and biotin-depleted *bio1* mutant *Arabidopsis* plants. Our results showed for the first time that the accumulation of 3-methylcrotonyl-CoA carboxylase (MCCase) subunits (the biotinylated MCC-A subunit and the non-biotinylated MCC-B subunit) was inversely related to the biotin content of the tissue. In contrast, abundance of *MCC-A* and *MCC-B* mRNAs and *GUS* expression mediated by the *MCC-A* and *MCC-B* promoters were not affected by the depletion of biotin. We conclude that *MCC-A* and *MCC-B* accumulation is down-regulated by biotin and this down-regulation is mediated at the translational and/or post-translational level of gene expression. Although biotin had no effect on *MCC-A* and *MCC-B* gene transcription, as biotin was depleted in *bio1* plants, these genes gradually lost their ability to be induced by darkness or in a CO<sub>2</sub>-free-atmosphere. These results indicate that biotin is required for the metabolic control of *MCC-A* and *MCC-B* gene transcription.

The molecular weight of MCCase reveals that different species have different subunit stoichiometries. The subunit stoichiometry of *Arabidopsis* MCCase is  $A_4B_4$ . Biotinylation has no effect on MCCase subunit stoichiometry. We also determined that charge isoforms of MCCase exist in both wild-type and *bio1* mutant plants.

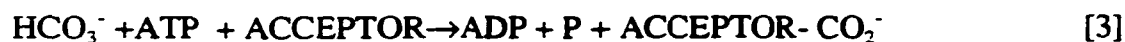
### Introduction

Biotin is an essential water-soluble vitamin required by all living organisms for normal cellular functions and growth. It is biosynthesized by plants, some fungi, and most bacteria. The biosynthetic pathway for biotin was first elucidated in bacteria by studying the growth behavior of auxotrophic mutants (Eisenberg, 1973; Pai, 1975). Enzymes and genes required for biotin biosynthesis have been characterized initially in bacteria (Izumi et al., 1981; Ploux and Marquet, 1992; Ploux et al., 1992; Alexeev et al., 1994; Huang et al., 1995). Analogous, but more limited studies of eukaryotes indicate that these organisms share a very similar biotin biosynthetic pathway. The cDNA and gene for biotin synthase (*BIO2*), which catalyzes the final reaction in biotin biosynthesis, has been cloned from *Arabidopsis* (Weaver et al., 1995; Patton et al., 1996). To date, this is the only plant biotin biosynthetic gene to be isolated from plants.

Two biotin auxotrophic mutants have been reported in *Arabidopsis*: *bio1* (Shellhammer and Meinke, 1990) and *bio2* (Patton et al., 1998). The *bio1* mutant seedlings can be rescued when grown in the presence of dethiobiotin, diaminopelargonic acid (DAPA) or biotin. The expression of *E. coli bioA* gene (encoding DAPA synthetase) in homozygous *bio1/bio1* plants eliminates the auxotrophic phenotype (Patton et al., 1996). This evidence indicates that the plant *bio1* gene corresponds to the *bioA* gene of *E. coli*. Hence, in the *bio1*

mutant of *Arabidopsis*, biotin biosynthesis is blocked in the conversion of 7-keto-8-aminopelargonic acid to 7, 8-diaminopelargonic acid. The *bio2* mutant can only be rescued by biotin, but not by dethiobiotin. This indicates that the final step of biotin biosynthesis is blocked in the *bio2* mutants. Molecular characterization of this mutant indicates that the *bio2* gene corresponds to *bioB* gene of *E. coli*, which encodes biotin synthase.

Biotin acts as a small coenzyme that binds covalently to a lysine amino group of carboxylases to facilitate the transfer of  $\text{CO}_2$  during carboxylation and decarboxylation reactions (Moss and Lane, 1971). The reactions catalyzed by these enzymes are involved in diverse metabolic processes including fatty acid biosynthesis (ACCase), gluconeogenesis (pyruvate carboxylase), and the catabolism of amino acids (MCCase and propionyl-CoA carboxylase). Although each enzyme has distinct metabolic functions, the overall reactions catalyzed by biotin-dependent carboxylases are carried out in two similar steps. The first step, the ATP and  $\text{Mg}^{2+}$  dependent reaction, involves the carboxylation of biotin on the enzyme. Then the carboxyl group is transferred from the carboxybiotinyl enzyme intermediate to an appropriated substrate, which serves as an acceptor.



Different organisms contain a different complement of biotin-containing proteins. Bacteria have one to three biotin-containing protein depending on the species. For instance, *E. coli* contains only one such protein, the biotin carboxyl carrier subunit (BCC) of ACCase. Eukaryotic organisms contain four or five such proteins. Three biotin-containing enzymes

have been demonstrated in plants, ACCase, MCCase and geranyl-CoA carboxylase (GCCase) (Guan et al., 1999). However, additional biotin-containing polypeptides are present (Wurtele and Nikolau, 1990).

The most thoroughly studied biotin-containing enzyme in higher plants is ACCase, which catalyzes an essential reaction during the initial stages of fatty acid biosynthesis. There are two types of ACCase found in plants (Sasaki et al., 1995). The heteromeric type occurs in chloroplasts and is composed of four subunits. These are the nuclear-coded biotin carboxyl carrier subunit (*CAC1*), biotin carboxylase subunit (*CAC2*),  $\alpha$ -carboxyltransferase subunit (*CAC3*) and plastid-coded  $\beta$ -carboxyltransferase subunit (*accD*) (Sasaki et al., 1993; Choi et al., 1995; Shorrosh et al., 1995, 1996; Sun et al., 1997; Ke et al., 2000). The homomeric ACCase is found in the cytosol of plant cells (Egin-Buhler et al., 1980; Gornicki et al., 1993; Roesler et al., 1994; Schulte et al., 1994; Shorrosh et al., 1994; Yanai et al., 1995).

MCCase is a mitochondrial enzyme whose major metabolic role in plants is the catabolism of leucine (Anderson et al., 1998). It contains two subunits, the biotinylated, MCC-A subunit and non-biotinylated, MCC-B subunit (Chen et al., 1993; Alban et al., 1993; Diez et al., 1994; Weaver et al., 1995; McKean et al., 2000). The cDNA and genomic clones for subunits of ACCase and MCCase have been isolated and the proteins have been purified and characterized from different plants. Most recently, GCCase (Guan et al., 1999), which plays an important role in isoprenoid catabolism, was purified and characterized from plants.

Although the biological functions of biotin have been well recognized, little is known about the role of this cofactor in regulating gene expression, especially in plants. Biotin operons (*bio* operons) from several prokaryotes have been sequenced and analyzed (Otsuka

et al., 1988; Cronan, 1989; Gloeckler et al., 1990; Bower et al., 1996). Regulation of the *E. coli bio* operon is the best understood example of transcriptional regulation by biotin. In eukaryotes, it has been reported that biotin enhances the transcription of glucokinase in starved rats (Chauhan and Dakshinamurti, 1991). However, the physiological significance of this regulation is still unknown.

As an initial investigation to ascertain whether and how the expression of biotin-containing enzymes are regulated by their prosthetic group, biotin, we performed western blot analysis and compared MCCase expression patterns between wild-type and *bio1* mutant *Arabidopsis* plants. We also measured the GUS activity mediated by *MCC-A* and *MCC-B* promoters in both wild-type and *bio1* genetic backgrounds. These studies led to the discovery that biotin does indeed play a role in the regulation MCCase expression.

## Materials and Methods

### Plant materials and growth conditions

Wild-type Columbia ecotype of *Arabidopsis thaliana*, homozygous *bio1* mutant (Shellhammer and Meinke, 1990), *MCC-A:GUS* and *MCC-B:GUS* transgenic *Arabidopsis* (Che et al., Chapter 3 this thesis), soybean (*Glycine max* cv Corsoy 79) and pea (*Pisum sativum* cv Progress #9) seeds were germinated in sterile soil or MS agar media in Petri dishes. Plants were grown in a controlled growth-room maintained at 22°C under continuous illumination with 40 W Sylvania cold-white fluorescent bulbs. The white light irradiation was at 150  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ .



### Genetic crosses

Homozygous *MCC-A:GUS* and *MCC-B:GUS* transgenic lines (Che et al., Chapter 3 this thesis) (used as male) were crossed with homozygous *biol* mutants (as female). The resulting F1 progeny were selected on the basis of kanamycin resistance, a trait tightly linked to each of the transgenes. F2 progeny, obtained after self-pollination of F1 plants, were grown on MS agar medium without biotin. Those progeny that showed the *biol* phenotype were selected and rescued by transferring the seedlings to soil and watered daily with a 1 mM biotin solution. Finally, the F3 progeny obtained by self-pollination of F2 plants were tested for kanamycin resistance in the presence of biotin in the media. Those F3 families that did not show segregation of the kanamycin resistance trait were judged to be homozygous for both the promoter-mediated *GUS* transgene and the *biol* gene. These plants were used for further experimentation.

### Protein extracts

*Arabidopsis* seedling tissue (0.1 g) was frozen with liquid N<sub>2</sub> and homogenized with an “Eppendorf pestle” in 1.5 ml microcentrifuge tubes. The resulting powder was homogenized at 4°C with 0.3 ml of extraction buffer (0.1 M Hepes-KOH pH 7.0, 20 mM 2-mercaptoethanol, 0.1 mg/ml PMSF, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v) glycerol) using the “Eppendorf pestle”. The mixture was immediately centrifuged at 12,200 *g* for 15 min at 4°C. The supernatant was recovered and frozen in liquid N<sub>2</sub> and the pellet discarded. Protein concentrations were determined by the Bradford method (BioRad, Hercules, CA) using bovine serum albumin (BSA) as a standard (Bradford 1976).

### **Electrophoresis and western blot analysis**

SDS-PAGE was performed in 10% (w/v) polyacrylamide gels as described previously (Laemmli, 1970). Non-denaturing polyacrylamide gel electrophoretic procedures were applied by two methods. One is the method described by Hedrick and Smith (1968) in which the mobility of proteins are size limited by the polyarylamide gel pore size. In this method, gels were composed of a linear gradient of 3%-30% (w/v) polyacrylamide and protein were separated on the basis of the molecular weight. The other method was developed by Lambin and Fine (1979), and it fractionates proteins according to both charge and size. In this method, electrophoresis is conducted in a gel of 2-16% (w/v) polyacrylamide linear gradient. After electrophoresis, gels were either stained with Coomassie Brilliant Blue or subjected to western blot analysis

For western blot analysis, proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979). In this study western blots were probed with antisera directed against the MCCase subunits (MCC-A and MCC-B) (Wang et al., 1994; Song et al., 1994; McKean et al., 2000). Biotin-containing proteins were also detected by using <sup>125</sup>I-streptavidin (Nikolau et al., 1985).

### **Fluorometric analysis of GUS activity**

GUS activity was determined in extracts with a flourometric assay essentially as described by Jefferson (1987). Plant samples were collected and protein extracts prepared as described above. One hundred  $\mu$ l of protein extract was mixed with 500  $\mu$ l GUS assay buffer (2 mM 4-methylumbelliferyl  $\beta$ -glucuronide (MUG) in extraction buffer) and incubated in 37°C. Aliquots of 100  $\mu$ l were removed at timed intervals (generally 5, 15, 25, 35 and 45

min), and the reaction was terminated by adding 0.9 ml of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. The fluorescent product was quantified using a fluorometer (Hitachi, Model F-2000). Excitation and emission wavelengths were 365 nm and 455 nm, respectively. Tissues from regenerated non-transformed plants were used to quantify background GUS activity. All the experiments were repeated three times using three independently transformed plants.

### **Isolation of RNA and blot hybridization analysis**

RNA was isolated from whole plant tissue by the method described by Logemann (1987). Twenty micrograms of each RNA sample was subjected to electrophoresis in a 1.4% (v/v) formaldehyde-agarose gel and blotted to nylon membrane by capillary transfer using 25 mM sodium phosphate buffer, pH 7.0. In order to ensure equal loading of RNA, ethidium bromide was included in the sample-loading buffer and blots were viewed under UV illumination. After transfer, RNA was bonded to the membrane by baking at 90°C for 60 min. The filter was hybridized with <sup>32</sup>P-labeled *MCC-A* and *MCC-B* cDNA fragments. The blots were washed once with 1 x SSC, 0.1% (w/v) SDS at 60°C for 15 min followed by 0.25 x SSC, 0.1% (w/v) SDS at 60°C for another 30 min.

## **Results and Discussions**

### **The effects of biotin depletion on MCCase expression**

Studies to ascertain the effect of biotin on gene expression need to be conducted in a tissue that contains low levels of endogenous biotin, optimally the tissue should contain no biotin. This condition however is difficult to find, particular in organisms that produce and/or store this cofactor. Because the *bio1* mutant of *Arabidopsis* (Shellhammer and

Meinke, 1990) cannot biosynthesize biotin, it is ideally suited for investigations into the effect of biotin on plant gene expression. The homozygous *bio1* mutant is recessive embryonic lethal, but can form normal plants in the presence of exogenous biotin (Shellhammer and Meinke, 1990). Therefore, in absence of exogenous biotin, homozygous *bio1* mutant seedlings fail to develop beyond the cotyledon stage as the seedling depletes residual biotin obtained from the parental tissue.

As an initial step to ascertain the rate of biotin depletion in the *bio1* mutant, we measured and compared MCCase activity in *bio1* and wild-type *Arabidopsis* seedlings. To better control the biotin content in these experiments and prevent biotin contamination from microorganisms, in all these experiments seedlings were grown on sterile media in Petri dishes. *Arabidopsis* seedlings grown in Petri dishes on Whatman 3MM paper saturated with 0.25 x Hoagland's solution were harvested at different stages after planting. MCCase activity was determined in extracts prepared from these samples. As shown in Figure 1A, during seedling development, MCCase activity increased initially, peaking at almost the same maximum MCCase activity at 8 and 10 day after planting (DAP) for both *bio1* and wild-type plants. Two days after this peak, MCCase activity in *bio1* and wild-type plants sharply declines to lower levels where it is maintained for next 20 days. However, during these 20 days, MCCase activity in the *bio1* mutant is 3-fold lower than that in the wild-type plants. These findings indicate that residual biotin obtained from the parental plant is depleted within the first 10 days of growth. Addition of exogenous biotin to the *bio1* plants did not change the pattern of MCCase expression, but higher MCCase activity was expressed.

Because our previous studies have shown that MCCase expression is influenced by the illumination status of the plant (Che et al., Chapter 3 this thesis), studies of the effect of biotin depletion on MCCase expression are better conducted with seedlings grown in continuous illumination.

The effect of biotin depletion on MCC-A subunit accumulation can be determined in the *bio1* seedlings either by measuring MCCase activity or by western blot analysis using <sup>125</sup>I-streptavidin or anti-MCC-A serum. As shown in Figure 2A, in comparison to wild-type plants, covalent bound by biotin on the MCC-A subunit gradually declined in *bio1* seedlings. Biotin content on the MCC-A subunit declined significantly in 11-day old *bio1* mutant seedlings, reaching the lowest level by 20 DAP. This profile is consistent with the pattern of decreasing MCCase activity seen in the seedlings (Fig.1). In contrast to the declining biotin-content of the MCC-A subunit, the accumulation of the MCC-A subunit itself, revealed by western blot analysis using MCC-A-specific antibodies, increased in *bio1* plants to reach a maximum in 20-day old seedlings (Fig. 2B). Analogous analysis indicate that the accumulation of the MCC-B subunit also increased in response to biotin depletion (Fig. 2C). Addition of exogenous biotin to *bio1* mutant seedlings decreased the accumulation of both the MCC-A and MCC-B subunits to the levels found in wild-type seedlings (Fig. 2B-C). These results indicate that limiting biotin content in the tissue results in increasing accumulation of apo-MCCase.

The interpretation of the above results are complicated by the factor that *bio1* mutant seedlings show altered morphological traits in response to biotin deprivation as compared to wild-type seedlings. Namely, whereas 20-day old wild-type seedlings have fully developed cotyledons, leaves and roots, 20-day old *bio1* mutant seedlings have only expanded

cotyledons, and do not fully develop roots and leaves. Hence, to ensure that the experiment described in Figure 2A-C fairly reflect an increase in apo-MCCase accumulation in response to biotin deprivation, the identical experiment was conducted and MCC-B accumulation was determined in the cotyledon. In this experiment, MCC-B protein expression patterns were compared in the same organ. As shown in Figure 2D, identical results were obtained using only cotyledons. These results indicate that there is no significant difference in the conclusion to be drawn relative to MCCase expression in response to biotin-deprivation between experiments that use whole seedlings or cotyledons. Therefore, to simplify the experiments, whole seedlings were used as the experimental material for the following studies.

The above findings demonstrated for the first time that biotin plays a role in the regulation of MCCase gene expression. The accumulation of the MCC-A and MCC-B proteins is inversely related to the biotin content of the tissue.

A number of observations have been made to date which may indicate that MCCase expression in a particular tissue is inversely related to the biotin-content of that tissue. However, the mechanism by which this relationship is established appears to vary. For instance, in tomato, soybean and *Arabidopsis*, the specific activity of MCCase is higher in the roots than that in the leaves (10-, 4- and 3-fold higher in tomato, soybean and *Arabidopsis*, respectively) (Wang et al., 1995; Anderson et al., 1998, Che et al. Chapter 3 this thesis). However, the total biotin content of leaves is 4- or 5-fold higher in roots of tomato (Wang et al., 1995), which is inversely related to MCCase activity. Furthermore, in *Arabidopsis*, the biotin content in cauline leaves (younger leaves) is 11-fold higher than that in rosette leaves (older leaves) (Shellhammer and Meinke, 1990).

Biotin synthase (BIO2), which catalyzes the addition of a sulfur atom to dethiobiotin and converts it to biotin, is down-regulated by light and BIO2 expression is also inversely related to the age of the tissue (Patton et al., 1996). These findings imply that dark-treated plants and older plants may contain less biotin. However, MCCase activity and accumulation of MCCase subunits are higher in such dark-treated plants or older plant organs (Maier et al., 1998; Aubert et al., 1996; Clauss et al., 1993; Che et al. Chapter 3 this thesis). Regardless of the mechanisms that are involved in the regulation MCCase expression, all of these previous studies support our hypothesis that the accumulation of the MCC-A and MCC-B proteins and MCCase activity are inversely related to the biotin content of tissue.

#### **Biotin regulation of MCCase expression is at the post-translational level**

To further elucidate the mechanism by which MCCase expression is enhanced in response to biotin depletion, we compared the accumulation of *MCC-A* and *MCC-B* mRNA in 20-day old *bio1* and wild-type plants. As shown in Figure 3A, the abundance of the *MCC-A* and *MCC-B* mRNAs was unaltered between wild-type and *bio1* mutant seedlings.

Furthermore, we analyzed *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression in a biotin-deficient genetic background and compared the expression obtained when these transgenes were in a wild-type background. This was achieved by intercrossing homozygous *MCC-A:GUS* or *MCC-B:GUS* transgenic lines (Che et al., Chapter 3 this thesis) with homozygous *bio1* mutant plants (see method for detail). The developmental and environmental regulation *GUS* expression was analyzed by using homozygous *MCC-A:GUS/bio1* and *MCC-B:GUS/bio1* transgenic plants grown in the absence and presence of biotin and comparing these expression patterns to those obtained from the same transgene

maintained in a wild-type background. As shown in Figure 3B-C, there was no significant change in both the *MCC-A*- and *MCC-B*-promoter-mediated GUS expression in both wild-type and *bio1* genetic background plants, consistent with the northern blot analysis (Fig. 3A).

Based on these results, we conclude that the depletion of biotin has no effects on the transcription of *MCC-A* and *MCC-B* genes and on the accumulation of the respective mRNAs. Hence, the enhanced accumulation of MCC-A and MCC-B proteins in response to biotin deprivation is not due to an alteration in the expression of these genes at the transcriptional and post-transcriptional level of control. Most likely, the enhanced accumulation of these proteins are due to either changes in the translation of the respective mRNAs or changes in the stability of the MCC-A and MCC-B proteins.

Our previous studies demonstrate that *MCC-A* and *MCC-B* genes are down-regulated by carbohydrates at the level of gene transcription (Che et al., Chapter 3 this thesis), namely the transcription rates of these genes can be repressed by carbohydrates, such as sucrose. To test if the increased accumulation of at least the MCC-B subunit in the *bio1* mutant seedlings (Fig. 2B-D) is affected by exogenous sucrose, *bio1* mutant and wild-type plants were grown in the presence or absence of sucrose and MCC-B accumulation determined. As shown in Figure 3D, exogenous sucrose had no effect on the enhanced accumulation MCC-B subunit the *bio1* mutant seedlings. This result indicates that the increased accumulation of the MCC-B (and probably MCC-A) protein in response to biotin depletion probably is not due to enhanced *MCC-B* gene transcription, otherwise, sucrose would have altered the observed expression pattern. These results further support our conclusion that biotin regulation of MCCase expression is not regulated the level of gene transcription.



### **Biotin is required for metabolic control MCCase gene expression**

Our previous studies showed that *MCC-A* and *MCC-B* genes are down-regulated by carbohydrates at the level of gene transcription via a metabolic repression mechanism (Che et al., Chapter 3 this thesis). Both darkness and a CO<sub>2</sub>-free atmosphere induce MCCase expression by enhancing the transcription of the respective genes. To test whether biotin has any role in this regulation, homozygous *MCC-A:GUS/bio1* and *MCC-B:GUS/bio1* transgenic plants were grown in MS medium under either continuous illumination or transferred to darkness for the last 2 days of growth. As shown in Figure 4A-B, as *MCC-A:GUS/bio1* and *MCC-B:GUS/bio1* transgenic plants aged, they gradually lost their ability to respond to the darkness treatment, as compared to the respective transgenes maintained in a wild-type genetic background. This was especially apparent with 15- and 20-day old seedlings, which did not respond to darkness at all and had the same GUS activity as those plants grown under constant illumination (Fig. 3B-C). Interestingly, *MCC-A*- and *MCC-B*- mediated GUS activity declined significantly in the *bio1* mutant background on eleventh day, which was the same day biotin content on the *MCC-A* subunit declined in these plants (Fig. 2A). These results indicate that biotin is required in the metabolic regulation MCCase gene expression.

To further study the effect of biotin on the regulation of MCCase expression, exogenous biotin was applied to *MCC-A:GUS* and *MCC-B:GUS* transgenic seedlings of the wild-type and *bio1* genetic background. These seedlings were grown for 13 days under constant illumination in a normal atmosphere and then they were either maintained in the same growth condition or transferred to either darkness or a CO<sub>2</sub>-free atmosphere for an additional 2 days of growth. Consistent with our findings that biotin has no effect on the transcription of MCCase genes, exogenous biotin had no effect on *MCC-A* -and *MCC-B*-

promoter-mediated GUS activity in both the wild-type and *biol* genetic background plants grown under constant illumination (Fig. 5A-B). Both dark-treatment or a CO<sub>2</sub>-free atmosphere (Fig. 5A-B) induced the *MCC-A* and *MCC-B* promoter-mediated GUS activity in wild-type genetic background, but not in the *biol* genetic background. Addition of exogenous biotin did not alter the darkness-induced or CO<sub>2</sub>-free atmosphere induced *MCC-A* and *MCC-B* promoter-mediated GUS expression when the transgenes were in a wild-type genetic background. However, exogenous biotin enable the transgenes to be at least partially induced by darkness and the CO<sub>2</sub>-free atmosphere, while they were in a *biol* genetic background (Fig. 5A-B). These results indicate that biotin is required for the metabolic regulation of *MCC-A* and *MCC-B* genes at the transcriptional level.

These initial studies on biotin regulation of MCCase expression suggest that the regulatory pathway that controls the expression of this enzyme may be very complex. Biotin appears to play two roles in the regulation MCCase expression. First, the accumulation of MCCase subunits is up-regulated by biotin deprivation, and this occurs by a translational and/or post-translational mechanism. Second, although biotin has no effect on the regulation of the transcription of the two MCCase genes when plants are in normal growth conditions, biotin is required in order for the MCCase genes to respond to environmental and metabolite signals.

In contrast to MCCase, the regulation of another biotin enzyme, the chloroplastic heteromeric ACCase, is complete different (Fig. A4 and Fig. A5 in appendix II). Although, the biotin content on the CAC1 subunit declines during biotin deprivation as *biol* mutant plants develop (same as the biotin depletion on the MCC-A subunit), the accumulation pattern of ACCase subunits, CAC1, CAC2 (data not shown) and CAC3, do not change in

response to this deprivation (Fig. A5 in appendix II). Furthermore, depletion of biotin in the *bioI* mutant results in decreased *CACI*-promoter-mediated GUS expression and addition of exogenous biotin enhanced the GUS activity in both a wild-type and the *bioI* genetic background (Fig. A4 in appendix II). These results indicate that different genes may be regulated by different mechanisms in response to biotin.

As an essential vitamin, biotin plays a variety of roles in cell growth and development. Biotin is a dual-function compound. It may act as a regulator, for example, directly regulating the *bio* operon of *E. coli* (Cronan, 1989) or act as a prosthetic group to ensure the activity of biotin-containing enzyme. In plants, depletion of biotin will inhibit biotin-containing enzyme activities and result in the blocking of sequential reactions in pathways requiring biotin-containing enzymes (for instance, fatty acids biosynthesis and branched-chain amino acids catabolism). Although, it is still not clear what the signaling mechanisms are for the increased accumulation MCCase during biotin depletion and whether these processes are dependent of biotin's enzymatic function, using biotin analog, such as dehydrobiotin which binds to the active site of the biotin-containing enzyme and inhibits the enzymatic function of the protein, may help us solve this puzzle.

### **The effect of biotin on MCCase subunit stoichiometry**

The subunit stoichiometry of MCCase has been investigated in several plant species (Chen et al., 1993; Alban et al., 1993; Diez et al., 1994; Weaver et al., 1995; McKean et al., 2000). In all cases, MCCase is composed of two subunits: a larger, biotinylated MCC-A subunit and a smaller, non-biotinylated MCC-B subunit. Different species have very similar subunit molecular weights. The molecular weights of MCC-A and MCC-B subunits are

about 80 kDa and 60 kDa, respectively. However, the native molecular weight of MCCase is reported to be quite different in different species. For example, the native molecular weight of soybean MCCase is about 850 kDa (Song et al., 1993), but only 530 kDa for the pea enzymes (Alban et al., 1993). Based on these types of observations, the enzyme from pea (Alban et al., 1993) and potato (Alban et al., 1993) has been proposed to have an  $A_4B_4$  quaternary structure, whereas the enzyme from carrot (Yang et al., 1993), maize (Diez et al., 1994), soybean (Song et al., 1993), and tomato (Wang et al., 1993) appear to have an  $A_6B_6$  quaternary structure. In the present work, we determined the molecular weight of the native MCCase from *Arabidopsis* and deduced the subunit's stoichiometry of the enzyme. Proteins from 20-day-old *Arabidopsis* seedlings, 7-day old soybean cotyledons and 16-day old pea leaves were analyzed by non-denaturing electrophoresis in a 5-30% linear gradient polyacrylamide gels. By conducting electrophoresis as described by Hedrick and Smith (1968), proteins migrated to position in the gel at which the pore radius of the gel limited the movement of each protein. In this way, proteins were fractionated in relation to their molecular weight. The molecular weight of the native MCCase was estimated by comparing its migration to proteins of known molecular weight. We determined that the molecular weight of the native MCCase in both pea and *Arabidopsis* to be 500 kDa, whereas soybean MCCase is about 900 kDa (Fig. 6A). These data confirmed that different species have different MCCase molecular weights, probably due to different subunit stoichiometries ( $A_6B_6$  for soybean and  $A_4B_4$  for pea and *Arabidopsis*). However, it is still not clear what causes these differences and what physiological significance is indicated by these differences. That MCCase in different species have different quaternary structure ( $A_4B_4$  and  $A_6B_6$ ) may provide new and interesting possibilities about how MCCase activity could be regulated.

We also investigated whether biotinylation effects MCCase subunit stoichiometry. Protein extracts from 20-day old wild-type (containing holo-MCCase) and *biol* mutant seedlings (containing apo-MCCase) were analyzed by non-denaturing electrophoresis in 5-30% linear gradient polyacrylamide gels. As shown in Figure 6B, both apo-MCCase and holo-MCCase have the same molecular weight and hence the same subunit stoichiometry, A<sub>4</sub>B<sub>4</sub>. Thus, biotinylation of MCCase has no effect on the molecular weight of MCCase and does not affect the subunit stoichiometry of the enzyme.

It is still not entirely clear whether the biotinylation occurs prior to or after the importation of biotin-containing proteins into organelles, such as mitochondria. The distribution of total biotin and its partition between free and protein-bound biotin in plant cells varies widely between different plant species (Shellhammer and Meinke, 1990; Baldet, 1993a; Mozafi, 1993). In pea leaves most of the biotin is unbound and found mostly in the cytoplasm (Baldet, 1993a), which is consistent with the findings in tomato (Wang et al., 1995). However, in *Arabidopsis*, most of the biotin resides in the chloroplast and mitochondria and is bound to proteins (Shellhammer, 1991). There is evidence which indicates that biotin holocarboxylase synthetase, which catalyzes the biotinylation of apo-proteins to form the holo-proteins, exists in the cytosol, chloroplast and mitochondria of pea leaves (Tissot et al., 1996; 1997). Therefore, biotinylation of biotin-containing proteins may take place in all three of these subcellular locations. Furthermore, since the biotin-containing subunit of propionyl-CoA carboxylase can be imported into mitochondria independent of its biotinylation status (Taroni and Rosenberg, 1991), it was proposed that the apo-MCCase subunit should accumulate in mitochondria (Wang et al., 1995).

It is most likely that the import of the MCC-B peptide into mitochondria is independent of the biotinylation status of the MCC-A subunit. If this is the case, because both the apo-MCCase and holo-MCCase have same subunit stoichiometry in *Arabidopsis*,  $A_4B_4$ , it would indicate that apo-MCC-A and MCC-B subunits were indeed in the same location, the mitochondria. This would imply that apo-MCC-A subunit can be imported into mitochondria without being biotinylated. The remaining question is whether the biotinylation occurs prior to or after the combination of apo-MCC-A with MCC-B subunits to form the  $A_4B_4$  complex. Whenever the biotinylation occurs, the co-existence of apo- and holo-MCCase in the mitochondria may indicate that the post-translation biotinylation modification MCCase is a mechanism for regulating MCCase activity (Wang et al., 1995).

#### **The effect of biotin on the formation of MCCase charge isoforms**

Two MCCase isoforms have previously been purified from soybean (Song, 1993). These two MCCases, which were separated on the basis of charge differences, show different kinetic properties, but they have the same molecular weights. Because in soybean, MCCase subunits are encoded by a small gene family, these charge isoforms may be products of different genes. We examined whether charge isoforms of MCCase occur in *Arabidopsis*. This was conducted by subjecting protein extracts to non-denaturing polyacrylamide electrophoresis. By using the method of Lambin and Fine (1979), proteins were separated according to charge and molecular weight. Knowing that all MCCase molecule in these extracts have the identical molecular weight (see Fig. 6), any isoforms detected in this latter analysis must be charge isoform.

After electrophoresis, MCCase was detected by western blot analysis of the resulting gels using anti-MCC-B serum (same results were obtained by using anti-MCC-A serum, data not shown). As shown in Figure 7, there are two immunoreactive bands in wild type protein extracts. This was a surprise finding, as the two MCCase subunits are each coded by a single gene in *Arabidopsis* (Weaver et al., 1995; McKean et al., 2000). Therefore, these charge isoforms must be the result of a post-translational process. One such process that can potentially generate charge isoforms of MCCase is the post-translational biotinylation of the MCC-A subunit. Biotin is covalently bound to this subunit by an amide linkage to a specific Lys side chain to form the biocytin residue. Therefore, biotinylation make the protein less positively charged. To investigate if differential biotinylation may be the cause of the different charge isoforms of MCCase, we subjected protein extracts from *biol* seedlings to non-denaturing electrophoresis. Three MCC-B immunoreactive bands were detected in *biol* extracts. Two of these were the same as those detected in extracts from wild-type seedlings (Fig. 7). These results are partially consistent with biotinylation being the cause of the charge isoforms. If this hypothesis is correct, five bands should be observable in total, each corresponding to the degrees of biotinylation of MCCase ( $A_4B_4$ ,  $A_4(\text{biotin})B_4$ ,  $A_4(\text{biotin})_2B_4$ ,  $A_4(\text{biotin})_3B_4$ ,  $A_4(\text{biotin})_4B_4$ ). However, only three bands were observed in the *biol* mutant. It is possible that the two bands seen in wild-type seedlings and the three bands in the *biol* mutant are the most dominate forms of MCCase, To resolve this dilemma, the specific biotinylation status corresponding to each charge isoform needs to be determined.

### Literature Cited

- Alban, C., Baldet, P., Axiotis, S. and Douce, R.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from higher plant mitochondria. *Plant Physiol.* **102**, 957-965.
- Anderson, M.D., Che, P., Song, J., Nikolau, B.J. and Wurtele, E.S.** (1998) 3-methylcrotonyl-coenzyme A carboxylase is a component of the mitochondrial leucine catabolic pathway in plant. *Plant Physiol.* **118**, 1127-1138.
- Aubert, S., Alban, C., Bligny, R. and Douce, R.** (1996) Induction of beta-methylcrotonyl-coenzyme A carboxylase in higher plant cells during carbohydrate starvation: Evidence for a role of MCCase in leucine catabolism. *FEBS Lett.* **383**, 175-180.
- Alexeev, D., Bury, S.M., Boys, C.W.G., Turner, M.A., Sawyer, L., Ramsey, A.J., Baxter, H.C. and Baxter, R.L.** (1994) Sequence and crystallization of *Escherichia coli* dethiobiotin synthase, the penultimate enzyme of biotin biosynthesis. *J. Mol. Biol.* **235**, 774-776.
- Dakshinamurti, K and Bhagavan, N.H. eds** (1985) Biotin. *Ann. NY Acad. Sci.* **447**, 1-441.
- Baldet, P., Alban, C., Axiotis, S., Douce, R.** (1993a) Localization of free and bound biotin in cells from green pea leaves. *Arch. Biochem. Biophys.* **303**, 67-63.
- Baldet, P., Gerbling, H., Axiotis, S., Douce, R.** (1993b) Biotin biosynthesis in plants: identification of intermediates. *Eur. J. biochem.* **217**, 479-485.
- Bower, S., Perkins, J.B., Yocum, R.R., Howitt, C.L., Rahaim, P. and Pero, J.** (1996) Cloning, sequencing, and characterization of the *Bacillus subtilis* biotin biosynthetic operon. *J. Bacteriol.* **178**, 4122-4130.



- Bradford, M.M.** (1976) A rapid and sensitive method for quantification for microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **72**, 248-254.
- Che, P., Wurtele, E.S., and Nikolau, B.J.** (2000) Developmental and Environmental Regulation of 3-Methylcrotonyl-CoA Carboxylase in *Arabidopsis*. In preparation.
- Chen, Y., Wurtele, E.S., Wang, X. and Nikolau, B.J.** (1993) Purification and characterization of 3-methylcrotonyl-CoA carboxylase from somatic embryos of *Daucus carota*. *Arch. Biochem. Biophys.* **305**, 103-109.
- Choi., J.-K., Yu, F., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning and characterization of cDNA coding for the biotin-containing subunit of the chloroplastic acetyl-CoA carboxylase. *Plant Physiol.* **109**, 619-625.
- Clauss, M., Motel, A. and Lichtenthaler, H.K.** (1993) Studies on the plant methylcrotonyl-CoA Carboxylase. *J. Plant Physiol.* **114**, 508-511.
- Cronan, J.E. Jr.** (1989) The *E. coli* *bio* operon: transcriptional repression by an essential protein modification enzyme. *Cell* **58**, 427-429.
- Chauhan, J., Dakshinamurti, K.** (1991) Transcriptional regulation of the glucokinase gene by biotin in starved rats. *J. Biol. Chem.* **266**, 10035-10038.
- Diez, T.A., Wurtele, E.S. and Nikolau, B.J.** (1994) Purification and characterization of 3-methylcrotonyl-coenzyme-A carboxylase from leaves of *Zea mays*. *Arch. Biochem. Biophys.* **310**, 64-75.
- Egin-Buhler, B., Loyal, R., Ebel, J.,** (1980) Comparison of acetyl-CoA carboxylase from parsley cell cultures and wheat germ. *Arch. Biochem. Biophys.* **203**, 90-100.

- Eisenberg, M. A.** (1973) Biotin: biogenesis, transport, and their regulation. *Adv. Enzymol.* **38**, 317-372.
- Gornicki, P., Scappino, L.A., Haselkorn, R.** (1993) Genes for two subunits of acetyl coenzyme A carboxylase of *Anabaena* sp. strain PCCC7120: biotin carboxylase and biotin carboxyl carrier protein. *J. Bacteriol.* **175**, 5268-5272.
- Gloeckler, R., Ohsawa, I., Speck, D., Ledoux, C., Bernard, S., Zinsius, M., Villeval, D., Kisou, T., Kamogawa, K. and Lemoine, Y.** (1990) Cloning and characterization of the *Bacillus sphaericus* genes controlling the bioconversion of pimelate into dethiobiotin. *Gene* **87**, 63-70.
- Guan, X., Diez, T., Prasad, T.K., Nikolau, B.J. and Wurtele, E.S.** (1999) Geranoyl-CoA Carboxylase: A novel biotin-containing enzyme in plants. *Arch. Biochem. Biophys.* **362**, 12-21.
- Huang, W., Jia, J., Gibson, K.J., Taylor, W.S., Rendina, A.R., Schneider, G. and Lindqvist, Y.** (1995) Mechanism of an ATP-dependent carboxylase, dethiobiotin synthetase, based on crystallographic studies of complexes with substrates and a reaction intermediate. *Biochem.* **34**, 10985-10995.
- Hedrick, J.L. and Smith, A.J.** (1968) Size and charge isomer separation and estimation of molecular weight of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* **126**, 155-164.
- Izumi, Y., Kano, Y., Inagaski, K., Kawase, N., Tani, Y., Yamada, H.** (1981) Characterization of biotin biosynthetic enzymes of *Bacillus sphaericus*: a dethiobiotin producing bacterium. *Agric. Biol. Chem.* **45**, 1983-1989.

- Jefferson, R.A.** (1987) Assay chimeric genes in plants: The *GUS* gene fusion system. *Plant Mol. Biol. Reporter* **5**, 387-405.
- Ke, J., Wen, T.-N., Nikolau, B.J. and Wurtele, E.S.** (2000) Coordinated regulation of the nuclear and plastidic genes coding for the subunits of the heteromeric acetyl-coenzyme A carboxylase. *J. Plant Physiol.* **122**, 1057-1071.
- Kyhse-Anderson J.** (1984) Electrophoretic transfer of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203-209.
- Laemmli, U.K.** (1970) Cleavage of structure protein during the assembly of the head of the bacteriophage T4. *Nature* (London) **227**, 680-685.
- Logemann, J., Schell, J., Willmitzer** (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16-20.
- Lambin, P. and Fine, J.M.** (1979) Molecular weight estimation of proteins by electrophoresis in linear polyacrylamide gradient gels in the absence of denaturing agents. *Anal. Biochem.* **98**, 160-168.
- McKean, A.L., Ke, J., Song, J., Che, P., Achenbach, S., Nikolau, B.J. and Wurtele, E.S.** (2000) Molecular characterization of the non-biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase. *J. Biol. Chem.* **275**, 5582-5590.
- Maier, A. and Lichtenthaler, H.K.** (1998) Methylcrotonyl-CoA carboxylase from barley (*Hordeum vulgare* L.): Purification, kinetic characterization and senescence-related induction of activity. *J. Plant Physiol.* **152**, 213-221.
- Mozafar, A.** (1993) Plant vitamins: agronomic, physiological, and nutritional aspects. *CRC Press*, London.

- Moss, J. and Lane, M.D. (1971) The biotin-dependent enzymes. *Adv. Enzymol.* **35**, 321-442.
- Nikolau, B.J., Wurtele, E.S. and Stumpf P.K. (1985) Use of streptavidin to detect biotin-containing proteins in plants. *Anal. Biochem.* **149**, 448-453.
- Otsuka, A.J., Buoncristiani, M.R., Howard, P.K., Flamm, J., Johnsone, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J. and Matsuzaki, J. (1988) The *Escherichia coli* biotin biosynthetic enzyme sequences predicated from the nucleotide sequence of the *bio* operon. *J. Biol. Chem.* **263**, 19577-19585.
- Patton, D.A., Johnson, M. and Ward, E.R. (1996) Biotin synthase from *Arabidopsis thaliana*: cDNA isolation and characterization of gene expression. *Plant physiol.* **112**, 371-378.
- Patton, D.A., Volrath, S., Ward, E.R. (1996) Complementation of the *bio 1* *Arabidopsis* biotin auxotroph with a bacterial biotin biosynthetic gene. *Mol. Gen. Genet.* **251**, 261-266.
- Patton, D.A., Schetter, A.L., franzmann, L.H., Nelson, K., Ward, E.R. and Meinke, D.W. (1998) An embryo-defective mutant of *Arabidopsis* disrupted in the final step of biotin synthesis. *Plant Physiol.* **116**, 935-946.
- Pai, C.H. (1975) Genetics of biotin biosynthesis in *Bacillus subtilis*. *J. Bacteriol.* **121**, 1-8.
- Ploux, O. and Marquet, A. (1992) The 8-amino-7-oxopelargonate synthase from *Bacillus sphaericus*: purification and preliminary characterization of the cloned enzyme overproduced in *Escherichia coli*. *Biochem. J.* **283**, 327-331.

**Ploux, O., Soullard, P., Marquet, A., Gloeckler R. and Lemoine, Y. (1992)**

Investigation of the first step of biotin biosynthesis in *Bacillus sphaericus*: purification and characterization of the pimeloyl-CoA synthase, and uptake of pimelate. *Biochem. J.* **287**, 685-690.

**Roesler, K.R., Shorrosh, B.S., Ohlrogge, J.B., (1994)** Structure and expression of an *Arabidopsis* acetyl-coenzyme A carboxylase gene. *Plant Physiol.* **105**, 611-617.

**Schulte, W., Schell, J., Topfer, R. (1994)** A gene encoding acetyl-coenzyme A carboxylase from *Brassica napus*. *Plant Physiol.* **106**, 793-794.

**Song, J., Wurtele, E.S. and Nikolau, B.J. (1994)** Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase: Identification of the biotin carboxylase and biotin-carrier domains. *Proc. Natl Acad. Sci. USA* **91**, 5779-5783.

**Song, J. (1993)** Molecular cloning and characterization of 3-methylcrotonyl-CoA carboxylase from soy bean. Ph.D. Thesis, Iowa State University.

**Shellhammer, J. (1991)** Analysis of biotin auxotroph of *Arabidopsis thaliana*. PhD thesis, Oklahoma State University, Stillwater.

**Shellhammer, J. and Meinke, D. (1990)** Arrested embryos from the *bio 1* auxotroph of *Arabidopsis thaliana* contain reduced levels of biotin. *Plant Physiol.* **93**, 1162-1167.

**Sasaki, Y., Hakamada, K., Suams, Y., Nagano, Y., Furusawa, I., Matsuno, R., (1993)** Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. *J. Biol. Chem.* **268**, 25118-25123.

**Sasaki, Y., Konishi, T. and Nagano, Y. (1995)** The compartmentation of acetyl-coenzyme A carboxylase in plants. *Plant physiol.* **108**, 445-449.

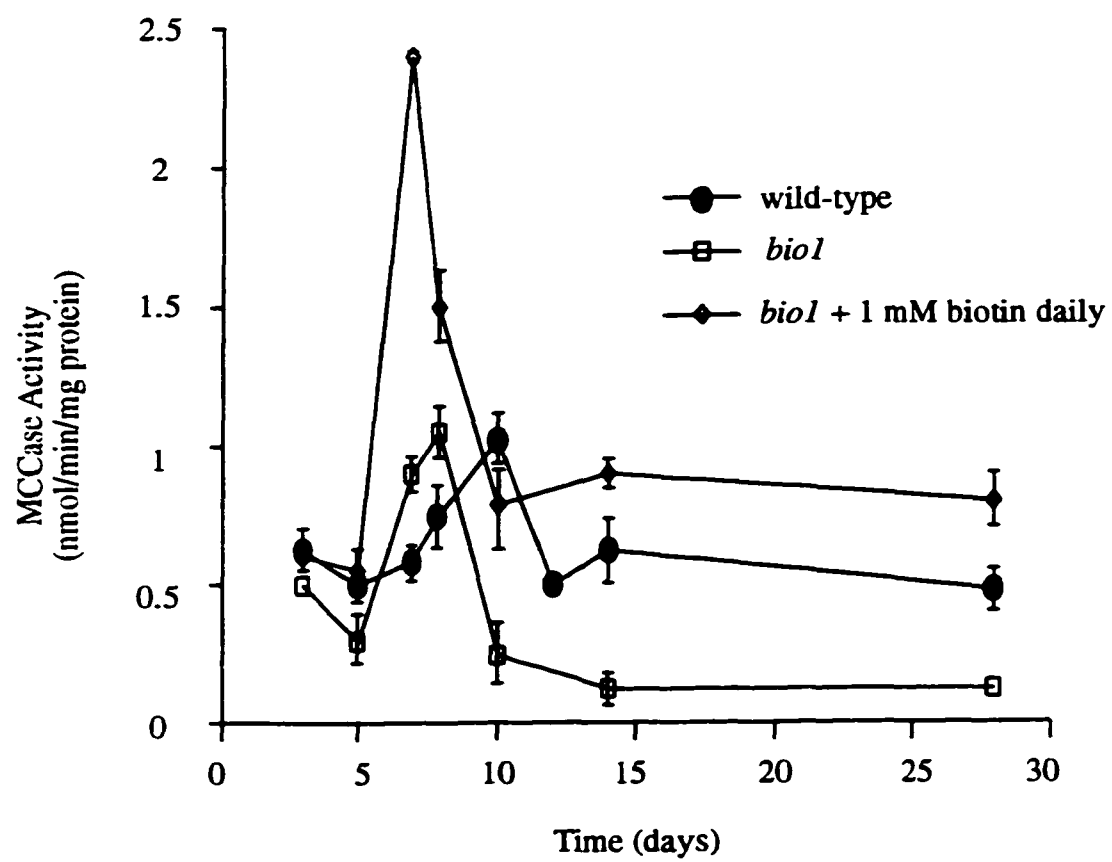
- Shorrosh, B.S., Dixon, R.A., Ohlrogge, J.B.** (1994) Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. *Proc. Natl Acad. Sci. USA* **91**, 4323-4327.
- Sorosh, B.S., Roesler, K.R., Shintani, D., van de Loo, F.J., Ohlrogge, J.B.** (1995) Structure analysis, plastid localization, and expression of the biotin carboxylase subunit of acetyl-coenzyme A carboxylase from tobacco. *Plant physiol.* **108**, 805-812.
- Sun, J., Ke, J., Johnson, J.L., Nikolau, B.J. and Wurtele, E.S.** (1997) Biochemical and molecular biological characterization of CAC2, the *Arabidopsis thaliana* gene coding for the biotin carboxylase subunit of the plastidic acetyl-coenzyme A carboxylase. *Plant physiol.* **115**, 1371-1383.
- Taroni, F., Rosenberg, L.E.** (1991) The precursor of the biotin-binding subunit of mammalian propionyl-CoA carboxylase can be translocated into mitochondria as apo- and holo-protein. *J. Biol. Chem.* **266**, 13267-13271.
- Tissot, G., Douce, R., Alban, C.** (1997) Evidence for multiple forms of biotin holocarboxylase synthetase in pea (*pisum sativum*) and in *Arabidopsis thaliana*: subcellular fractionation studies and isolation of a cDNA clone. *Biochem. J.* **323**, 179-188.
- Tissot, G., Job, D., Douce, R., Alban, C.** (1996) Protein biotinylation in higher plants: characterization of biotin holocarboxyllase synthetase activity from pea (*pisum sativum*) leaves. *Biochem. J.* **314**, 391-395.
- Towbin, H., Staehelin, T. and Gordon, J.** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet; Procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350-4354.

- Wang, X., Wurtele, E.S., Keller, G., McKean, A.L. and Nikolau, B.J.** (1994) Molecular cloning of cDNAs and genes coding for  $\beta$ -methylcrotonyl-CoA carboxylase of tomato. *J. Biol. Chem.* **269**, 11760-11769.
- Wang, X., Wurtele, E.S. and Nikolau, B.J.** (1995) Regulation of  $\beta$ -methylcrotonyl-coenzyme A carboxylase activity by biotinylation of the apoenzyme. *Plant Physiol.* **108**, 1133-1139.
- Wang, X.** (1993) Characterization of  $\beta$ -methylcrotonyl-CoA carboxylase of Tomato, a Newly identified biotin enzyme in plants. Ph.D. Thesis, Iowa State University.
- Weaver, L.M., Lebrun, L., Franklin, A., Huang, L., Hoffman, N., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coenzyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1013-1014.
- Wurtele, E.S. and Nikolau, B.J.** (1990) Plants contain multiple biotin enzymes: Discovery of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. *Arch. Biochem. Biophys.* **278**, 179-186.
- Weaver, L.M., Lebrun, L., Franklin, A., Huang, L., Hoffman, N., Wurtele, E.S. and Nikolau, B.J.** (1995) Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coenzyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1013-1014.
- Yanai, Y, Kawasaki, T., Shimada, H., Wurtele, E.S. and Nikolau, B.J.** (1995) Genomic organization of 251 kDa acetyl-CoA carboxylase genes in *Arabidopsis*: tandem gene organization has made two differentially expressed isozymes. *Plant Physiol.* **36**, 779-787.

**Figure 1.** The effect of plant age on MCCase activity.

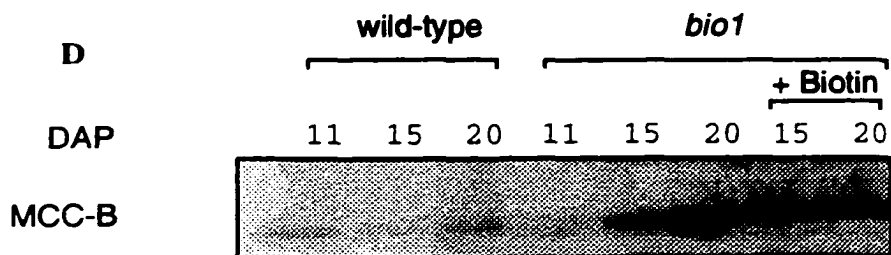
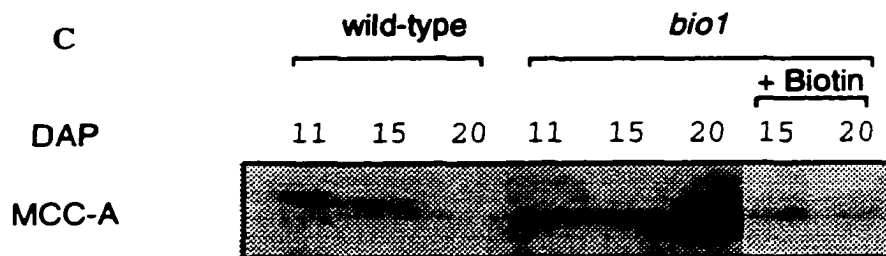
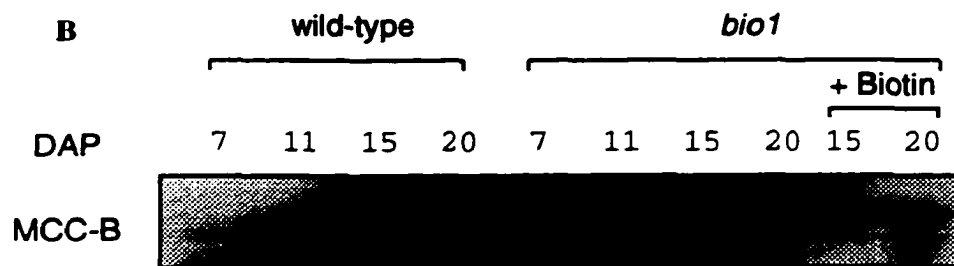
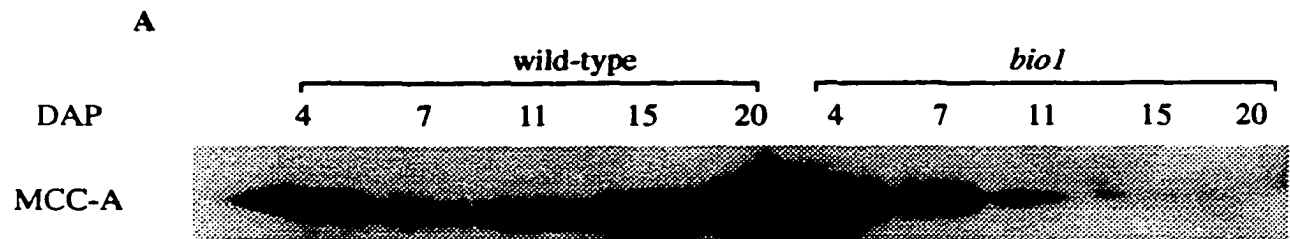
MCCase specific activity was determined in protein extracts of seedlings between 3 and 28 days after sowing. seedlings were grown in a cycle of 15 h illumination and 9 h of darkness in wild-type, *bio1* and *bio1* plant s supplemented daily with 1 mM biotin. Measurements are the average of four determinations.





**Figure 2.** The effect of biotin on the biotinylation status and the expression of MCCase.

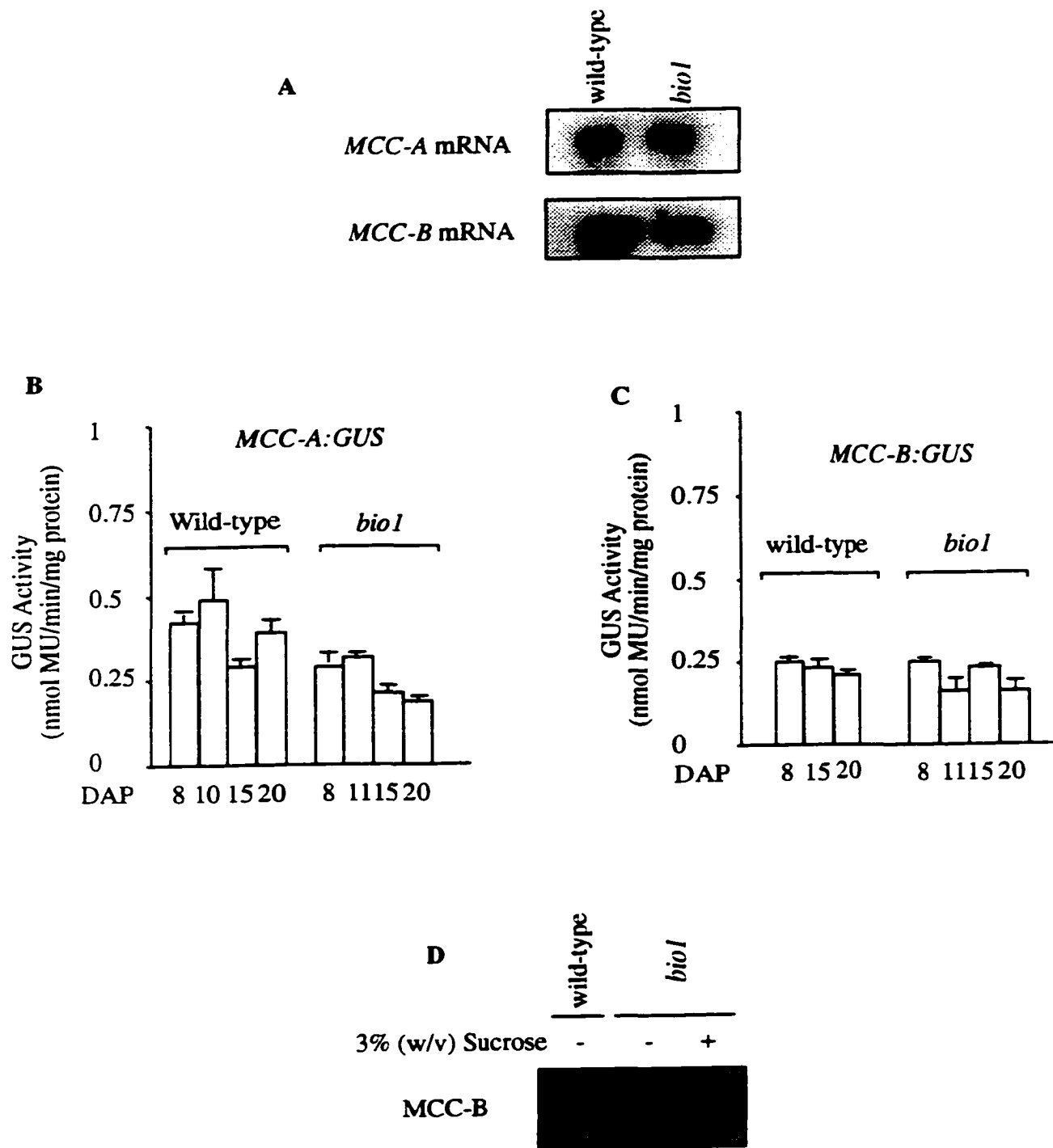
Protein extracts were prepared from whole seedlings (A, B and C) or cotyledons (D) of wild-type and *bio1 Arabidopsis* plants at the indicated days after planting (DAP). Aliquots of extracts containing equal amounts of protein (150 µg) were subjected to SDS-PAGE, followed by western blot analysis with <sup>125</sup>I-streptavidin (A) or immunological detection with antibodies to MCCase subunits (B, C and D). Biotin (0.25 mM) was supplemented to *bio1* mutants 2 days before harvest. The data presented were gathered from a single experiment; three replicates of this experiment gave similar results.



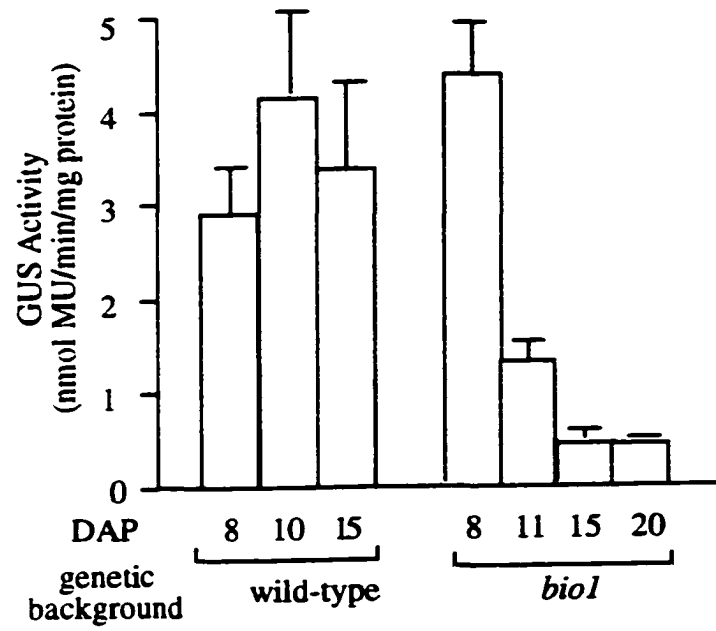
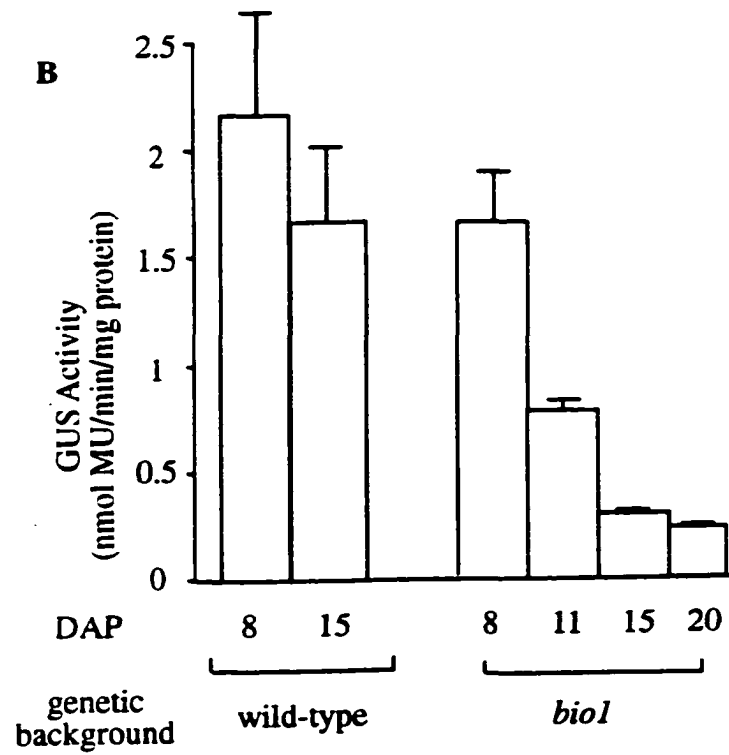
Cotyledons

**Figure 3.** The effect of biotin depletion on MCCase gene transcription.

(A) Northern analysis of *MCC-A* and *MCC-B* mRNA accumulations in wild-type and *bio1* *Arabidopsis* plants. Equal amounts (50 µg) of RNA isolated from 20-day old wild-type and *bio1* plants were subjected to electrophoresis in formaldehyde-containing agarose gels. MCCase mRNAs were detected by hybridization with radioactively labeled cDNAs coding for *MCC-A* and *MCC-B*. Different ages of *MCC-A:GUS* (B) and *MCC-B:GUS* (C) transgenic *Arabidopsis* seedlings with either wild-type or *bio1* genetic background grown on MS agar medium for indicated time period under constant illumination. GUS activity was determined in protein extracts. Data are the mean  $\pm$  SE from three replicates. (D) Protein extracts were prepared from 20-day old wild-type, *bio1* and *bio1* plants supplemented with 1.5% (w/v) sucrose. Aliquots of extracts containing equal amounts of protein (150 µg) were subjected to SDS-PAGE, followed by immunological detection with antibodies to MCC-B subunit. The data presented were gathered from a single experiment; three replicates of this experiment gave similar results.

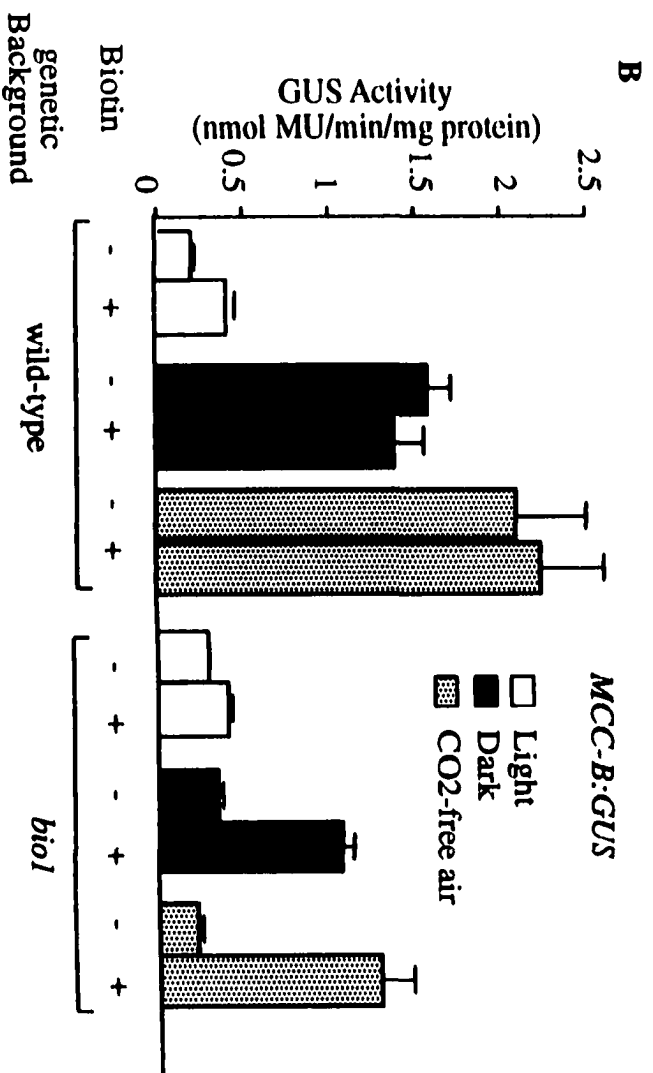
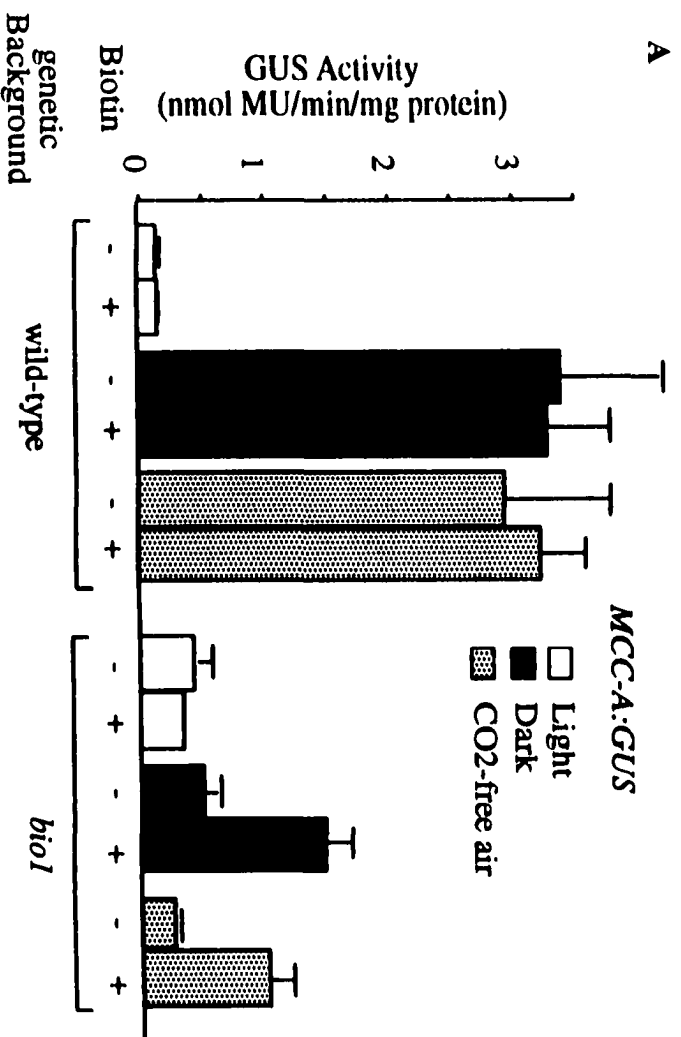


**Figure 4.** The effect of depletion biotin on *MCC-A* and *MCC-B* promoter-mediated *GUS* expression in darkness. *MCC-A:GUS* (A) and *MCC-B:GUS* (B) transgenic *Arabidopsis* seedlings with either wild-type or *biol* genetic background grown on MS agar medium for indicated time period. These plants were transferred to darkness for the last 2 days of growth. GUS activity was determined in protein extracts. Data are the mean  $\pm$  SE from three replicates.

**A****B**

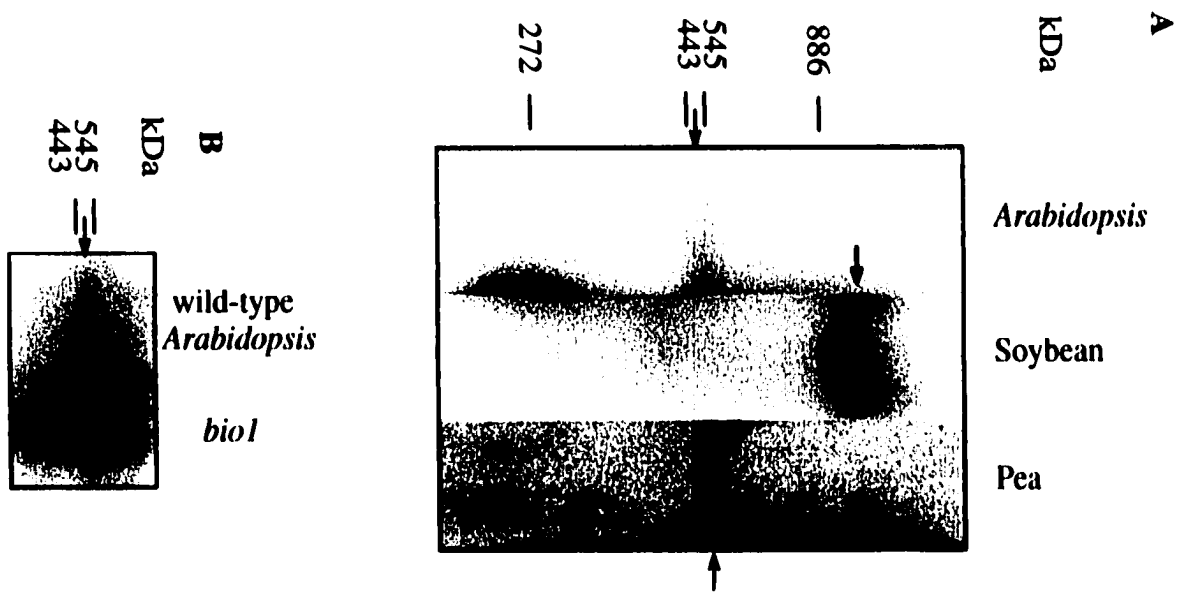
**Figure 5.** Fifteen-day old *MCC-A:GUS* (A) and *MCC-B:GUS* (B) transgenic *Arabidopsis* seedlings with either wild-type or *bio1* genetic background grown on MS agar. These plants were kept in the constant illumination (□), transferred to darkness (■) or CO<sub>2</sub>-free air (▤) for the last 2 days of growth. In all of these growth conditions, 0.25 mM biotin was supplied to the MS medium for the last 2 days of growth as indicated in the figure. GUS activity was determined in protein extracts. Data are the mean ± SE from three replicates.





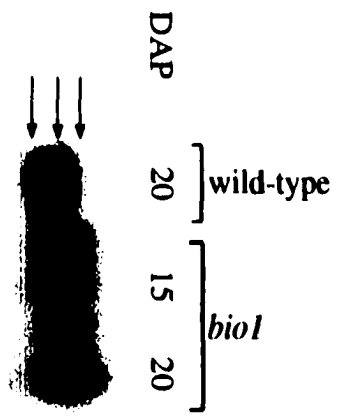
**Figure 6.** Determination of the molecular weight of the native MCCase by nondenaturing polyacrylamide gel electrophoresis.

An aliquot of protein extracts from wild-type, *bio1* mutant *Arabidopsis*, soybean and pea were subjected to electrophoresis in a gel composed of a linear gradient of 5-30% (w/v) polyacrylamide. After electrophoresis at 200 volts for 3 days, MCCase was detected by western blot analysis of the resulting gels. The blots were incubated with the anti-MCC-B serum (A, B). Same results were obtained using either anti-MCC-A serum or <sup>125</sup>I-streptavidin( data not shown). The molecular weight of MCCase was determined by comparing its migration to the standard proteins (apoferritin dimer, 886 kDa; apoferritin monomer, 443 kDa; urease dimer, 545 kDa; urease monomer, 272 kDa). Position of MCCase is indicated by arrows.



**Figure 7. Analysis MCCase charge isoforms.**

Aliquots of protein extracts from 20-day old wild-type and *bio1 Arabidopsis* plants were subjected to electrophoresis in a linear 5-20% gradient polyacrylamide gel. After electrophoresis at 70 volts for 17 hours, MCCase was detected by western blot analysis with anti-MCC-B serum.



## CHAPTER 5. CONCLUSIONS

Comprehensive studies of MCCase have attempted to address three questions: 1) what is the physiological role of MCCase in plants? 2) how is MCCase expression regulated during plant developmental and in response to environmental stimuli? 3) whether biotin plays a role in the regulation of MCCase gene expression?

The radiotracer metabolic studies using extracts from isolated mitochondria and incubating with [U- $^{14}\text{C}$ ]leucine and  $\text{NaH}^{14}\text{CO}_3$ , respectively, demonstrate that plant mitochondria can catabolize leucine via the following scheme: leucine  $\Rightarrow$   $\alpha$ -ketoisocaproate  $\Rightarrow$  isovaleryl-CoA  $\Rightarrow$  3-methylcrotonyl-CoA  $\Rightarrow$  3-methylglutaconyl-CoA  $\Rightarrow$  3-hydroxy-3-methylglutaryl-CoA  $\Rightarrow$  acetoacetate + acetyl-CoA. These findings demonstrate for the first time that the enzymes responsible for leucine catabolism are present in plant mitochondria. We conclude that one of the primary metabolic role of MCCase in plants is the catabolism of leucine. In addition to Leu catabolism as demonstrated here, MCCase may also operate in the mevalonate shunt and isoprenoid metabolism. Further studies on the role of MCCase in these pathways may illustrate the pivotal role of MCCase in manipulating these pathways during plant development.

A single-copy gene encoding the *Arabidopsis* MCC-A subunit was isolated and characterized. It contained 15 exons separated by 14 introns. The light mediated repression of MCCase expression reveals that the expression of the *MCC-A* and *MCC-B* genes are under metabolic control at transcriptional level. The role of MCCase in the Leu catabolism and metabolic control MCCase activity imply that MCCase plays an important role in regulating

the carbon and energy balance during plant development and particularly in non-photosynthetic organs and during stress conditions. I also isolated *Arabidopsis* line containing T-DNA insertional mutation in the *MCC-B* gene (Fig. A1, Appendix II). Further biochemical and genetic characterizations of this mutation will be helpful for determining the biological function of MCCase in plants.

Biotin, an essential water-soluble vitamin, is required by all living organisms for normal cellular functions and growth. It may also play very important roles in the regulation of protein expression. Our studies showed for the first time that MCCase is down-regulated by biotin at translational and/or post-translational level. Although biotin had no effects on MCCase gene transcription, it is required for the MCCase response to plant metabolic changes at the transcriptional level. On the other hand, ACCase is up-regulated by biotin at transcriptional level, which is opposite to the mode of regulation of MCCase.

The comparison of the molecular weight of MCCase reveals that different plants species have different MCCase subunit stoichiometry. We determined herein that the subunit of *Arabidopsis* MCCase is  $A_4B_4$ . The co-existence of apo- and holo-MCCase and different charge isoform of MCCases in the *Arabidopsis* indicate that biotinylation may play a role in the regulation of MCCase expression during plant developmental and environmental changes. The determining whether the quaternary structure of apo- and holo-MCCase are different will provide new and interesting information about the regulation and structure of biotin enzyme.

**APPENDIX 1. MCCase BIOTINYLATED SUBUNIT**  
**GENOMIC DNA SEQUENCE FROM *ARABIDOPSIS***  
***THALIANA* (COLUMBIA)**

```

1  gagctctcgg tccactttcg tcaccctttg tttttttctc cacgttcgct 50
                                Δ Sau3A I pBP 8      CAAT-box
51  ttttagattcg acttattgcg atcttgcggtg tcaaactttt tccaatcatc 100
101  taaaagttga gtcggagtta gagttacgtt gttcataact tttagttaaa 150
                                GATA-motif
151  ctttgcaaga aaaaagtata tacgatatat ggtggccttat cgttatgtat 200
201  catttcattc agcttttctaa aattttataat ttaaaaaata atattataat 250
251  taagattaaa aacgaaaaat tcaaatttaa ttgactgaga atgttttttg 300
                                Δ Nsi I pBP12
301  tgtttggtat gcattacttg tttgacacaa cttatatatc atcctttatt 350
351  tgttactcaa acgaagattc acgaaacgga caaataaaact atccacctat 400
401  tgggaacaaa taggttgtct gacttttaag catcacaaac gggttatatac 450
                                T-box
451  tattttacat aaacgtttcg agtgtgttaa gattcattta attgtaaaact 500
501  aaataatcaa cagcgtgact tcaaatacaa ttgtcttctt ataagtttat 550
                                CAAT-box
551  tactaacata tattcttggt cagtttggtg ccaatattga attaattaaa 600
601  aggatttgtt atattttcct taattgtatt gttttttttg tcggttatag 650
                                Δ Hinc II pBP 13
651  aaatgcaaaa caaaaagaaa tgaatagagg agtcaacact caacagccac 700
                                E-box/ABRE motif
701  tacctaacaa acacttttaa tagttccagc tcgtatcggt ttcatgcctc 750
751  caaagatttt actactattt attatgattt tcaaaatcat ttataactat 800
                                E-box      GATA-motif
801  ccctccacat gtcttttaata gtcagattga tattatatta aaactctata 850
                                E-box      CAAT-box
851  tagtaagacc aagtgaattt taaaattaca tcaatgcgat ttatatggat 900
                                Δ Stu I pBP14
901  gattcaaaag tatttatatt tcaaaactga aacaaggcct tgcagccttt 950
                                GATA-motif      TATA-box CAAT-box
951  cttaatgctt cgtgcagata ggcccattaa taaaacaata aagcccatat 1000
1001  ttgtaaaatc aggtaaagac gcaacgcaag cggcacatca gacacatata 1050
1051  cattccgacc aaaccatgat aaagtatatg ccacgtcact attcatcgct 1100

```



1101 gattagtttt tacgaaattg accaacatac cctcgttggt tggttttatc 1150  
           E-box                                  E-box  
 1151 tacaacgcag ttgctgagtc acacttggtt tcttcactcg gaaaattcga 1200  
           Δ Sau3A I  
 1201 aaatcgagAT CAAATGTCGA TGATGACGGC GTGGGCACTT CGACGGAATG 1250  
 1251 TTCGTCGGAA AAATCACTCG ATGCTCGTGA GATATATTTC CGGCTCCGCA 1300  
 1301 TCGATGAAGC CTAAGGAGCA ATGTATTGAG AAGATTTTGG TGGCGAACAG 1350  
 1351 AGGCGAGATT GCTTGCCGGA TCATGAGAAC GGCGAAGCGT TTAGGGATTC 1400  
 1401 AGACCGTTGC GGTTACAGT GACGCTGATA GAGATTCGCT CCATGTGAAA 1450  
 1451 TCTGCAGACG AAGCGGTACG GATTGGCCCG CCCTCGGCTA GACTTAGTTA 1500  
 1501 CCTCAGCGGT GTGACTATCA TGGAGGCAGC GGCTCGGACT GGTGCACAGg 1550  
 1551 tatgagtttt gatccccgaa aaagaataat cgtgttcttg tagctgatgt 1600  
 1601 cttctttgaa ttatataact agGCAATACA CCCTGGCTAT GGGTTCTTGT 1650  
 1651 CAGAAAGTTC TGACTTTGCC CAACTCTGTG AGGATTCGGG TCTAACATTC 1700  
 1701 ATTGGACCTC CAGCATCTGC TATTAGAGAC ATGGGTGATA AAAGgtacca 1750  
 1751 ttacaggcct cctattacga cttgcttctt ctttgcacaa gttgttattt 1800  
 1801 tattctctct tgaatagttt ctgaggacat gtccctcatg tgtatttatg 1850  
 1851 aagTGCATCC AAGAGAATTA TGGGAGCTGC TGGAGTTCCT CTCGTGCCTG 1900  
 1901 GATATCATGG CCATGAGCAA GATATTGATC ATATGAAGTC AGAAGCTGAA 1950  
 1951 AAGATTGGAT ACCCTATAAT AATCAAGCCA ACACATGGAG GTGGGGGGAA 2000  
 2001 Ggtatgttat gatatacggc attgattttt tccaatagat ggctttttgt 2050  
 2051 aatagtcaaa gtttgatacc cttttaaaat ctattgggtt cgaagcaaag 2100  
 2101 taagctcttg ttttttagGG AATGAGGATA GTGCAAAGTG GGAAGGATTT 2150  
 2151 TGCTGACTCG TTTTGGGGG CCCAACGTGA AGCTGCTGCT TCTTTTGGCG 2200  
 2201 TTAACACTAT TCTTCTCGAG AAATACATTA CTCGACCGAG GCATATAGAA 2250  
 2251 GTCCAGgtag tgagtcgtga aatttcattg tgtttttttt tttcatgaaa 2300  
 2301 ggctgggttat ggcgcattga aaccagttta ttgtatatag aaagaggaca 2350  
 2351 aatgttaacc ttaacttgta tttcattttt ggcgtactca ttgagtaacc 2400  
 2401 atttctggct tttctcctt ttttctagca ttttaactaa ttttgcttcg 2450  
 2451 gtgtttccac tgcttttagA TATTGGTGA CAAACATGGA AATGTTCTTC 2500  
 2501 ATCTATACGA AAGGGATTGC AGTGTGCAAA GAAGACACCA AAAGATTATT 2550

2551 GAAGAAGCTC CTGCGgtaat gaccatattt ctttctgtta ttgattcata 2600  
 2601 tattttcttag cataaatgga tgtgggtctg attgacacta tactgggtgca 2650  
 2651 gcctaataata tctgaaaagt tccgcgcaaa cttaggccaa gctgctgtct 2700  
 2701 ctgcggcacg ggtatccctt taaatcctgc tttcatcttt tacagatttc 2750  
 2751 atgggtcttca gtgagaaaagc ttagccgaaa atgtttcatt acaatgctct 2800  
 2801 gtatatgca ttcattcata gtatgctccg ggtgtctcta ttttatatag 2850  
 2851 tgcattacta atattgatca tgagttattt gatcaaagtt tactattggt 2900  
 2901 aatgttttagt tcaattgata tgtagtcttt tgaaacctct tcttgctagg 2950  
 2951 ctatctttca cttcaacttc cacaagaagc gcttatattc tgttaccttc 3000  
 3001 tgatatttaa agGCAGTTGG ATATTATAAT GCTGGGACGG TGGAATTTAT 3050  
 3051 AGTTGACACC GAGTCTGATC AATTTTATTT TATGGAAATG AACACCCGTC 3100  
 3101 TACAGgtgct tctttgatct atatgtttat taaatttaac tcgataaaca 3150  
 3151 tttggctttc caaaggaaca ctgatgatcc ttcttagGTC GAACATCCTG 3200  
 3201 TAACTGAGAT GATTGTTGGT CAAGATCTTG TGGAATGGCA AATTCGGGTT 3250  
 3251 GCCAATGGGG AACCTCTCCC CTTAAGCCAA TCCGAGGTGC CAATGTCAGg 3300  
 3301 tattgctctt cctcctaaaa ataaatggaa aatagtgggt tggtttactc 3350  
 3351 tcattcgcta ttccgtactg aatccatgat ttttcaagat gttacttgtg 3400  
 3401 gcagaaaaaa atctgaacaa catgaccac atatgtatct tcttggcagG 3450  
 3451 TCATGCCTTT GAGGCCAGGA TATATGCTGA AAACGTTCCA AAAGGATTTC 3500  
 3501 TTCCTGCAAC TGGGGTCCTC AATCATTATC GCCCTGTTGC AGTCTCACCA 3550  
 3551 TCAGgtgaag tacaaaattg tgtatacaac tgttttttcc tatcttatag 3600  
 3601 aaagtttttt tttctctcgt atatatctga ccctgttttt gtctgttgt 3650  
 3651 ttacctctct catgttgga tttgtctgaa acagTTCGGG TTGAAACTGG 3700  
 3701 AGTTGAGCAA GGAGACACTG TTAGCATGCA CTATGATCCT ATGATTGCAA 3750  
 3751 AGCTTGTTGT CTGGGGAGGT AATCGTGGCG AAGCTTTAGT GAAACTGAAG 3800  
 3801 GATTGCTTGT CTAAC TTCA Ggtagattcc ctgagaacct gtattttgga 3850  
           ↓*Sac* I  
 3851 gctctctctg caattttaat tatacctcag cataactata aatcttcaag 3900  
 3901 gccttatggt tccattgtga atttcttaag aaaccttttt gagtttctcg 3950

3951 cacatcgctt cttagttacc acaatagttt agtaattaca tggatatttg 4000  
 4001 ttgacagGTA GCAGGTGTAC CTACGAACAT AAATTTTCCTT CAAAAACTTG 4050  
 4051 CTAGTCATAA GGAGTTTGCA GTGGGCAATG TAGAAACTCA TTTTATTGAG 4100  
 4101 CACCATAAAA GTGATCTATT TGCTGACGAA AGCAATCCAG CTGCAACAGA 4150  
 4151 AGTGGCATAA AAGGCAGTCA AGCATAGTGC AGCATTGGTG GCTGCTTGTA 4200  
 4201 TCTCCACAAT CGAGCATTCT ACTTGGAAATG AAAGTAATCA TGgtcagttc 4250  
 4251 gcattgggtc tagtctcttc ttcacaactt acatgattcc cgagcatgtc 4300  
 4301 ttaatttatt atgtgtaaac tcatccagGG AAAGTTCCAT CGATATGGTA 4350  
 4351 TTCGAATCCT CCTTTTAGGG TCCATCATGA AGCCAAACAA ACCATTGAGC 4400  
 4401 TAGAATGGAA TAATGAATGC GAGGGAAGTG GCTCTAACCT CATATCACTC 4450  
 4451 GGTGTAAGAT ATCAACCAGA TGAAGCTAT CTCATTGAGg tttaaaactt 4500  
 4501 cacatcctcc ttttagattc acttttagac tcatatactt catttatgag 4550  
 4551 tcttataatt ggaattctta tggtttgtca acattccacg agacagGAAG 4600  
 4601 GCAATGATTC TCCAAGTTTA GAACTCAGAG TAACACGAGC AGGAAAGTGC 4650  
 4651 GATTTTAGAG TTGAAGCGGC CGGATTGAGC ATGAGTGTTA GTCTAGCTGC 4700  
 4701 GTAATTGAAG gtaaatcatt agcattatgt tcaagcaact cttaacagct 4750  
 4751 ctcatactct ttctgtctct ctcgtaattg ctgcttatga tgtatgtttg 4800  
 4801 gttacaggGA TGGTTATAAG CATATCCATA TATGGCATGG TTCAGAACAC 4850  
 4851 CATCAGTTCA AGCAGAAGGT AGGAATCGAA TTCTCTGAAG ATGAAGAAGG 4900  
 4901 TGTCCAACAC AGAACCAGCT CCGAAACATC ATCACACCCT CCAGGAACCA 4950  
 4951 TTGTGGCCCC CATGGCTGGT TTGGTAGTCA AGGTCCTTGT CGAAAACGAA 5000  
 5001 GCCAAAGTAG ATCAAGGTCA ACCTATATTA GTCCTAGAGG CAATGAAGAT 5050  
 5051 GGAGgttagt atataaacat cttcatatac cataatataa gaaacattta 5100  
 5101 agtaattatc gttttattct cgttcagCAC GTTGTGAAAG CACCATCCTC 5150  
 5151 TGAAGCATA CAGGACCTCA AAGTTAAAGC AGGCCAACAG GTTTCAGATG 5200  
 5201 GCAGTGCTCT ATTCAGGATC AAAGgttagt ggtaatttag taccagcatt 5250  
 5251 ttttccttta tacatttcat atcatccttt tgaaaatttc atccaactcc 5300  
 5301 accaatttca atatctttgc agGGTAAATA ACACGATTG CGATTGATCA 5350  
 5351 ACCATTCGCT GGCTTACGCG AAACCTGAAC TAATAAGCAT TTGGGTGTGT 5400

```

5401  ATATAAACCG AAATCTTACA AAAGATAAAT AAATCCAAAG CTTTATAaat 5450
5451  gtttttactg cctctatata gatctgtgcg gctagagatt gaaggtaact 5500
5501  tcttagtata caaaatggtg attacattaa tattcaagat agtccgagcc 5550
5551  atttcttgaa tactaaacca gcttctctct tcttgagctg agactcgctc 5600
5601  tgaacttttg gagctttgcc ttataagca gtcttgatgat acttcaagaa 5650
5651  gagcatccga tacttgact tatcaacaaa catatttttt tctccatca 5700
5701  tctccacaac ttcattagcc cgggaaaaaa acccgctct aacaaatgta 5750
5751  taaagcaccg catcaagcaa ctctgatca aacttcatcg atgaagtagc 5800
5801  tgcagctatg gatttcattt ctcccataa ctcagtgacc tctgtgtatt 5850
5851  tactcccaat ggctgcatat cctgtgacca tagaatgaaa agtttgagca 5900
5901  tttggcgaat gcccaagact tctcatctc tttaatgcct tctctgcac 5950
5951  ctgcatcaaa cctttcttgc taaaaaatg gatcacattg ttccaatcat 6000
6001  gaacaccagc atccaaactc tgcacttctc tgatttctct caagagtttt 6050
6051  gacatgagtc ccgcctctgc gtttcttca catcccttta agagcttctc 6100
6101  aaatttctga ttccgcctc gtagtatctt cgctctttc atttccttaa 6150
6151  acacattgag agctc 6165

```

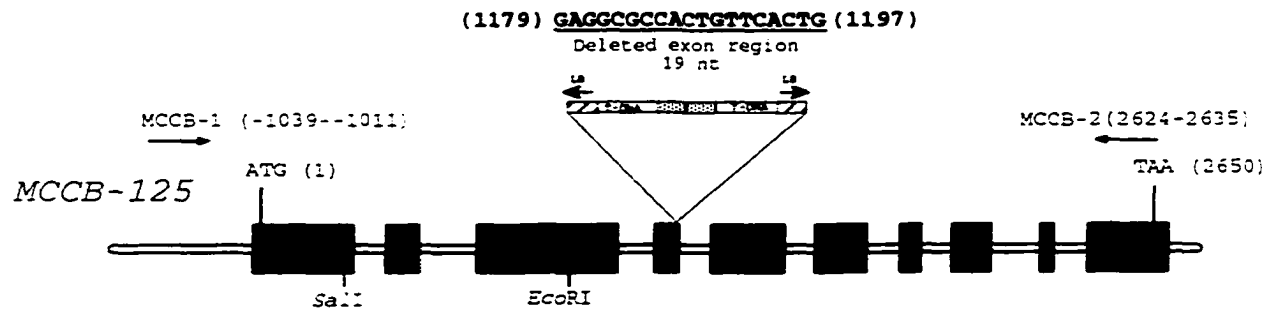
UPPERCASE: MCCase biotinylated subunit exons

**bold lowercase underline**: TATA box

**BOLD UPERCASE**: Initial site

5' promoter deletions to -12 (relative to the translation start site) were indicated as *pBp8*, *pBP12*, *pBp13*, *pBP14*, *pBP20*, *pBP22*.

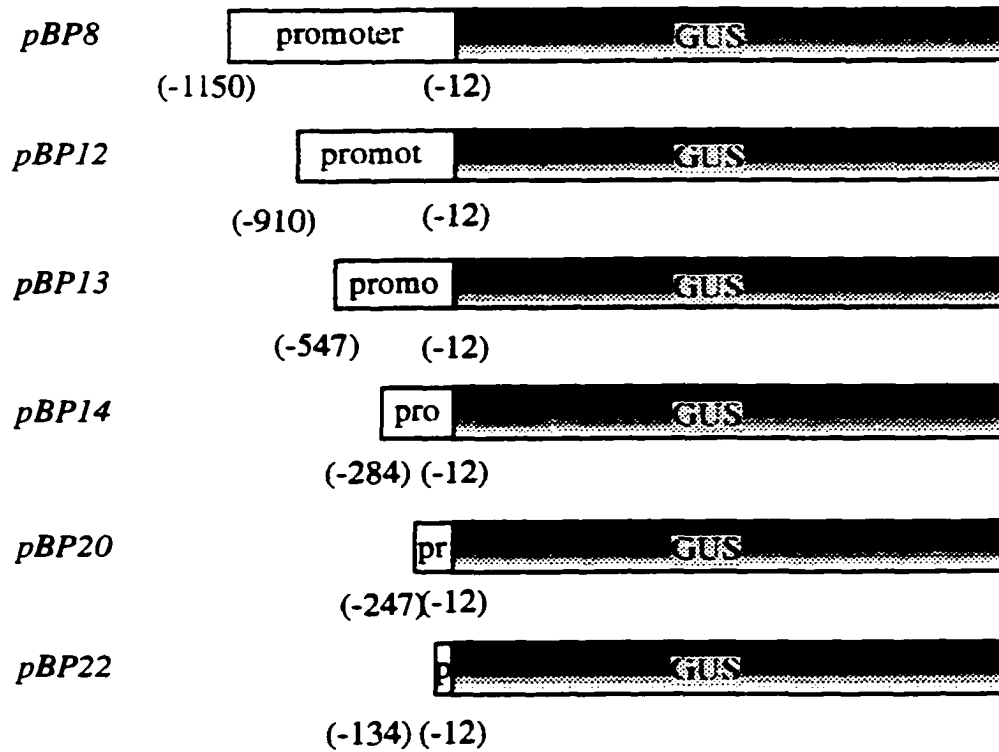
## APPENDIX II DATA FOR FURTHER STUDY



**Figure A1.** Schematic representation of the structure of T-DNA insertion in the *MCC-B* gene.

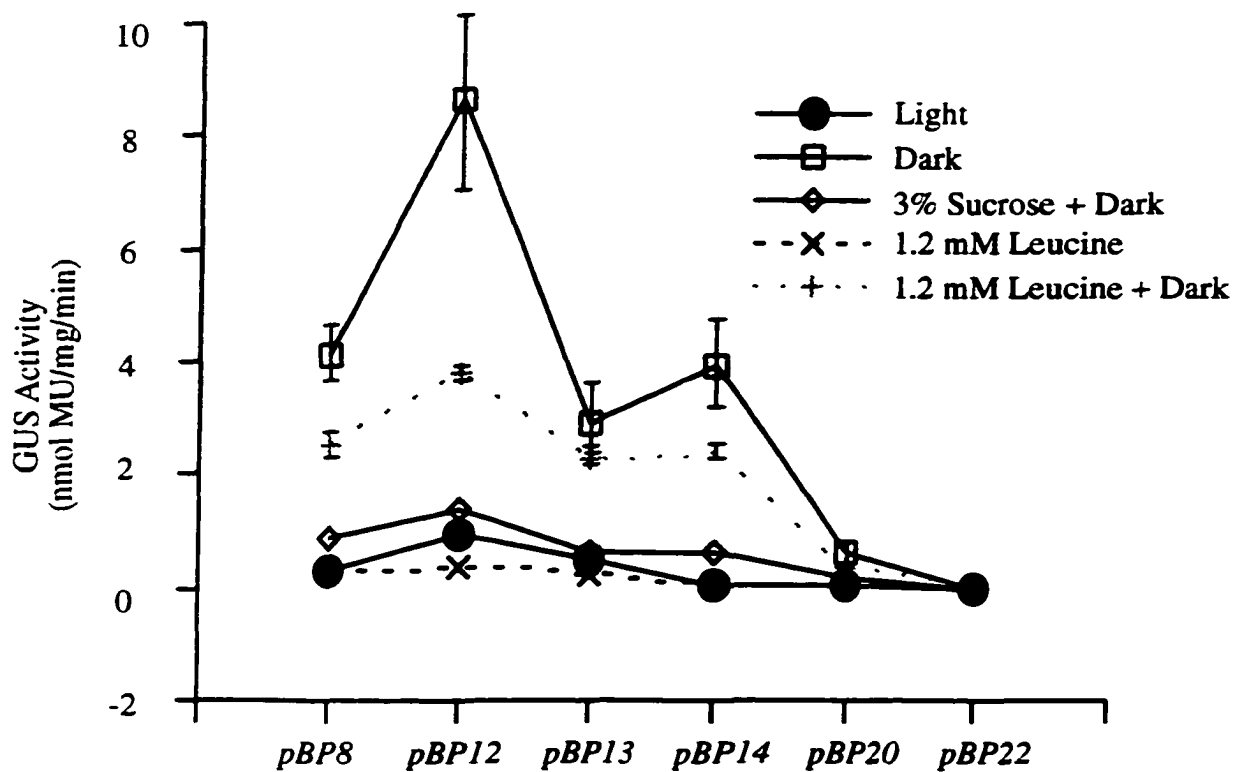
The physical map of the *MCC-B* gene is present. Black boxes represent the 10 exons. Introns are indicated by the solid black lines. Positions of the translational start codon (<sup>1</sup>ATG) and stop codon (<sup>2650</sup>TAA) are indicated. The mutant alleles contains a concatameric T-DNA insertion in the fourth exon with LB at each junction. The 19 nt of *MCC-B* sequence deleted from the mutant allele is shown above the site of insertion (underlined) and positions are indicated. Primers specific for the *MCC-B* gene or T-DNA border sequence are indicated by arrows.

## Plasmid name



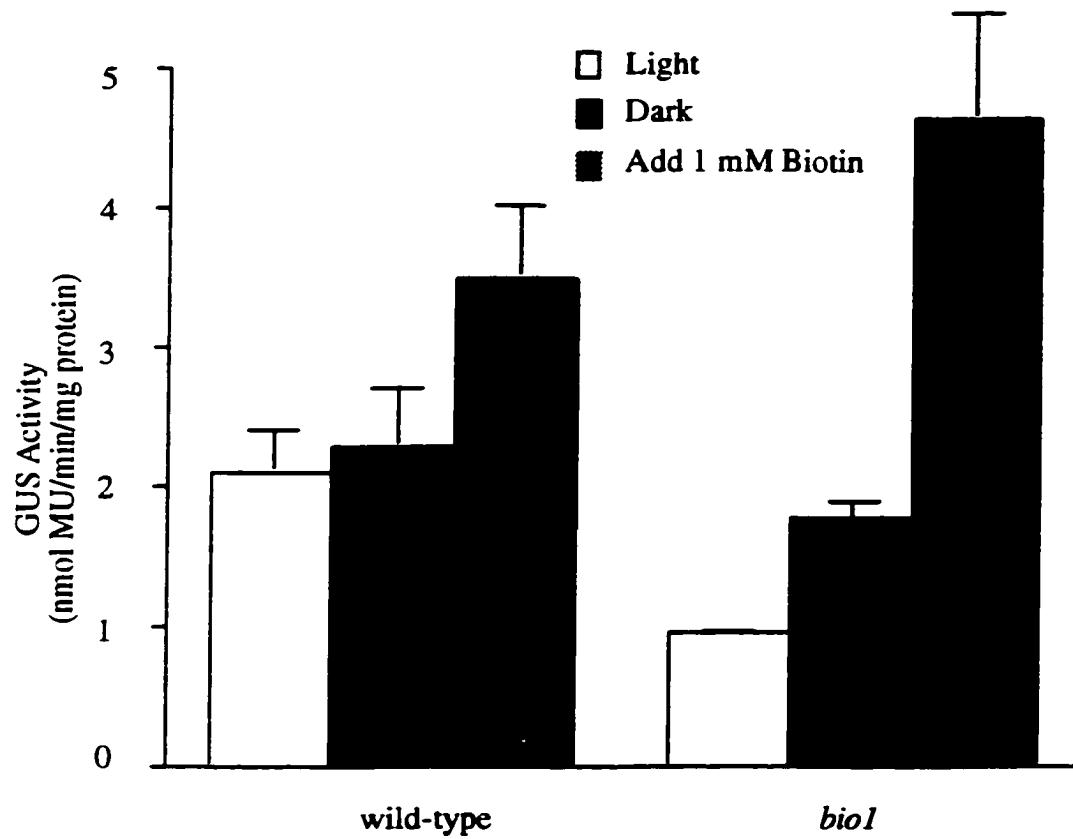
**Figure A2.** Schematic illustration of the 5' deletion mutants of *MCC-A:GUS* transgenes.

Six promoter-GUS fusion plasmids were constructed. The number under the constructs refer to 5' deletion points in the promoter and the translation start site (ATG) of *MCC-A* is denoted as position +1.



**Figure A3.** Deletion analysis *MCC-A:GUS* promoter constructs.

GUS activities of the transgenic T2 seedlings grown for six days on MS medium containing the indicated. Seedlings were then transferred to darkness or maintained in constant illumination for another two days. The data are the mean  $\pm$  SE from three replicates using three independent transgenic lines.

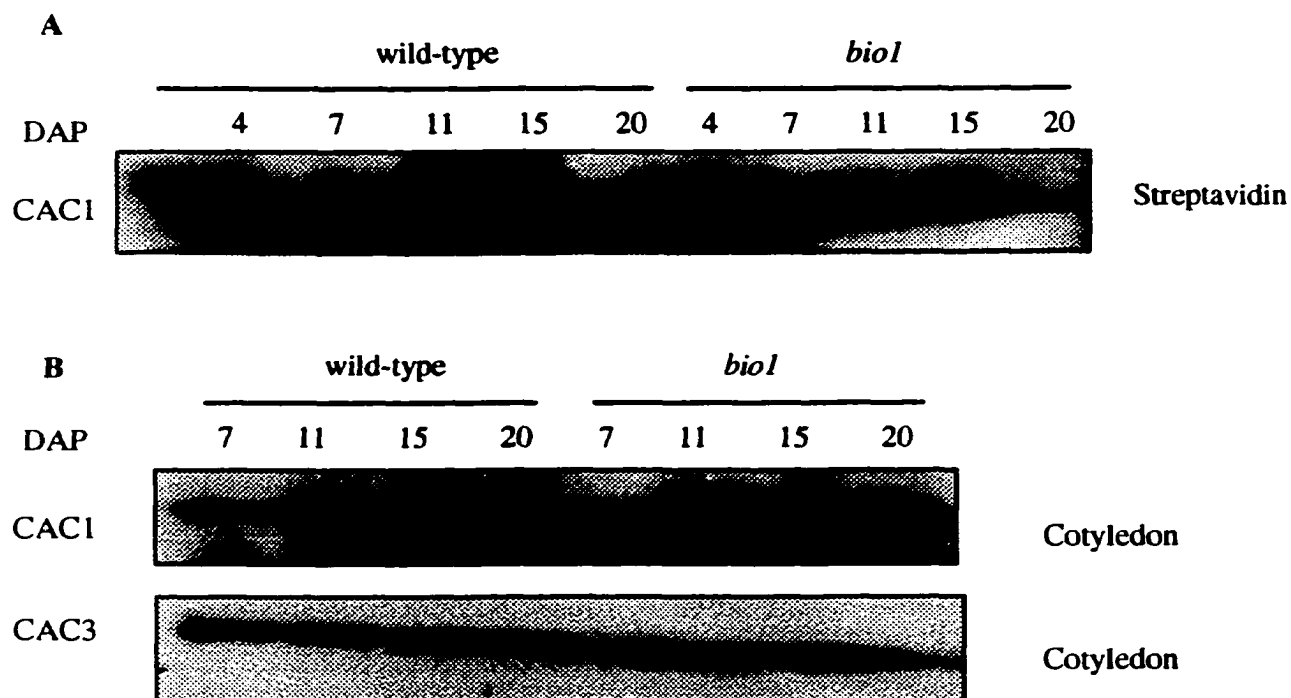


**Figure A4.** Biotin induces *CAC1*-promoter-mediated GUS expression.

*CAC1:GUS* transgenic *Arabidopsis* seedlings either in a wild-type or homozygous *biol* genetic background were grown on MS agar medium for 13 days and then transferred for an additional 2 days to darkness or maintained under constant illumination with or without 5 ml 1mM biotin. Proteins were extracted from these seedlings and GUS activity determined.

The bars represent the mean  $\pm$  SE from three replicates.





**Figure A5.** The effect of biotin on the biotinylation status and the expression of heteromeric ACCase. Protein extracts were prepared from whole seedlings (**A**) or cotyledons (**B** and **C**) of wild-type and homozygous *bioI* *Arabidopsis* plants at the indicated days after planting (DAP). Aliquots of extracts containing equal amounts of protein (150  $\mu$ g) were subjected to SDS-PAGE, followed by western blot analysis with  $^{125}$ I-streptavidin (**A**) or immunological detection with antibodies to ACCase subunits (**B** and **C**). Biotin (0.25 mM) was supplemented to *bioI* mutants 2 days before harvest. The data presented were gathered from a single experiment; three replicates of this experiment gave similar results.

## **ACKNOWLEDGMENTS**

I would like to express my sincere appreciation of Dr. Basil J. Nikolau, my major professor, for his teaching, guidance and help in all aspects of my graduate works at Iowa State University. His encouragement, support and friendship will last forever.

I am also very grateful to Dr. Eve Syrkin Wurtele, Dr. Martin H. Spalding, Dr. Alan Myers, Dr. Andy Norris for serving on my committee and providing their opinions and helpful discussions in my work. In addition, I acknowledge their efforts of reviewing my papers and making suggestions in the preparation of their manuscript.

Besides, this dissertation is to the memory of Dr. James A. Olson who enthusiastically help me in my work. This memory will always live.

I would also like to thank all my colleagues in Dr. Nikolau's laboratory for their cooperation, help and friendships

Finally, I would like to dedicate this thesis to all my families for their love, understanding and support.