Molecular, genetic, physiological and biochemical studies of 3-methylcrotonyl-CoA carboxylase and biotin carboxyl carrier protein-like proteins in Arabidopsis thaliana

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Molecular, genetic, physiological and biochemical studies of 3-methylcrotonyl-CoA carboxylase and biotin carboxyl carrier protein-like proteins in *Arabidopsis thaliana*

by

Geng Ding

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CHAPTER I GENERAL INTRODUCTION

RATIONALE AND OBJECTIVES

Biotin-containing proteins play important roles in metabolic pathways. 3-Methylcrotonyl-CoA carboxylase (MCCase) is one of the four biotin-containing enzymes that have been characterized in plants. MCCase contains two subunits, the biotinylated MCCA subunit and non-biotinylated MCCB subunit. The metabolic function of MCCase is identified as an enzyme for leucine (Leu) catabolism in mitochondria. However, the physiological functions of MCCase in plants are still unclear. Molecular, genetic, physiological and biochemical studies were conducted to investigate the physiological roles of MCCase. The carbon flux controlled by MCCase was analyzed and characterized in these studies. The relationships between mitochondrial and peroxisomal Leu catabolism pathways were analyzed.

There are indications that novel biotin-containing proteins might be present in plants. Three Arabidopsis genes encode proteins that show high sequence similarity with biotin carboxyl carrier protein (BCCP) of heteromeric acetyl-CoA carboxylase (htACCase). The specific identities and functions of these BCCP-like (BCCPL) proteins were studied by molecular, genetic and biochemical approaches.

Objective 1: Characterization of the physiological roles of MCCase in plants

Objective 2: Characterization of the potential relationships between two Leu catabolism pathways that are physically separated but functionally related

Objective 3: Identification and characterization of the biochemical and physiological functions of three BCCPL proteins
BACKGROUND AND SIGNIFICANCE

**Biotin and Biotin-Containing Enzymes**

Biotin, also known as Vitamin H, is an essential water-soluble enzyme cofactor required by all organisms for a series of metabolic processes. Biotin was first discovered in 1901 as a growth factor of yeast. In 1936, biotin was isolated for the first time from egg yolk by Kögl and Tönnsis, and later the structure of biotin was determined in early 1940s. Due to the three asymmetric carbon atoms in biotin, eight different stereoisomers could exist, but only D-biotin has been found to show biological activity (Gaudy and Ploux, 1992).

Biotin is biosynthesized in plants and a subset of microbes. Many archaea, bacteria, fungi and all animals, including humans, must uptake biotin from the environment or diets for normal growth and development. Biotin is covalently bound to a group of enzymes via an amide linkage between the biotin carboxyl group and the ε amino group of a specific lysine (Lys) residue located within a conserved -(A/I/V)MK(L/M/A/T)- biotinylolation motif (Chapman-Smith and Cronan, 1999). This covalent attachment is catalyzed by holocarboxylase synthetase, also known as biotin protein ligase (Chapman-Smith and Cronan, 1999).

Biotin-containing enzymes are involved in a variety of metabolic processes that are thought to be indispensable to normal cellular functions, including fatty acid biosynthesis, amino acid metabolism, gluconeogenesis, lipogenesis and secondary metabolism. Based on the chemical nature of the substrates, biotin-containing enzymes can be divided into three classes: carboxylases, decarboxylases, and transcarboxylases (Moss and Lane, 1971). Although each enzyme catalyzes distinct metabolic reactions, a common biochemical
mechanism is shared by all biotin-containing enzymes. Biotin prosthetic group plays a role as an intermediate carrier of the activated carboxyl group that is being transferred from donor substrate (D-CO$_2^-$) to acceptor substrate (A) (Moss and Lane, 1971). The overall reaction catalyzed by biotin-containing enzymes is carried out in two steps. The first step is the carboxylation of biotin on the enzyme (Enz) and the second step is the transfer of the carboxyl group from biotin-containing enzyme to the acceptor substrate (Knowles, 1989). In the case of carboxylases, which use bicarbonate as the donor substrate, the first step is an ATP and Mg$^{2+}$ dependent reaction.

\[
\text{Step 1: } \quad \text{D-CO}_2^- + \text{Enz-Biotin} \Rightarrow \text{Enz-Biotin-CO}_2^- + \text{D}
\]

\[
\text{Step 2: } \quad \text{A} + \text{Enz-Biotin-CO}_2^- \Rightarrow \text{Enz-Biotin} + \text{A-CO}_2^-
\]

\[
\text{Overall reaction: } \quad \text{D-CO}_2^- + \text{A} \Rightarrow \text{D} + \text{A-CO}_2^-
\]

All biotin-containing enzymes have three distinctive functional domains: biotin carboxylase domain (BC), biotin carboxyl carrier protein (BCCP) and carboxyl transferase domain (CT). BC domain is responsible for catalyzing the first step, whereas CT domain is required for the second step. BCCP, a structural domain, provides the conserved Lys residue on which the biotin prosthetic group is attached to and is thought to swing between the BC and the CT domains. The quaternary structural organization of these three domains varies among different biotin-containing enzymes and among the same enzymes from different organisms (Nikolau et al., 2003). Namely, each of these domains can be distributed among individually polypeptides, or two or all three domains can be fused into a single polypeptide.

To date, four biotin-containing enzymes have been characterized in plants: homomeric acetyl-CoA carboxylase (hmACCase) (Harwood, 1988; Konishi and Sasaki, 1994; Yanai et
al., 1995; Konishi et al., 1996), heteromeric acetyl-CoA carboxylase (htACCase) (Sasaki et al., 1993; Konishi and Sasaki, 1994; Choi et al., 1995; Konishi et al., 1996), 3-methylcrotonyl-CoA carboxylase (MCCase) (Alban et al., 1993; Chen et al., 1993; Diez et al., 1994; Song et al., 1994; Wang et al., 1994; Weaver et al., 1995; Anderson et al., 1998; McKean et al., 2000), and geranoyl-CoA carboxylase (GCCase) (Guan et al., 1999). Also, plants contain a specific seed storage biotin-protein (SBP) that is non-catalytic and thought to play a role in storing biotin (Duval et al., 1994). Interestingly, although biotin is still attached to a Lys residue, the biotinylation motif of SBP differs markedly from that of biotin-containing enzymes (Duval et al., 1994).

In addition, there are indications that additional biotin-containing enzymes, i.e., propionyl-CoA carboxylase and pyruvate carboxylase, may be present in the plant kingdom (Wurtele and Nikolau, 1990). Yet the actual proteins have not been identified, which raises the possibility that plant may have novel biotin-containing proteins.

**Metabolic Functions of MCCase**

MCCase (EC 6.4.1.4) catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA. MCCase was initially discovered in bacteria and animals over 40 years ago (Himes et al., 1963; Lane and Lynen, 1963; Moss and Lane, 1971). It is a heteromeric enzyme composed of a biotinylated MCCA subunit and a biotin-free MCCB subunit. MCCA subunit possesses the BC and BCCP domain, and is responsible for the carboxylation of the enzyme-bound biotin prosthetic group. The transfer of the carboxyl group is catalyzed by MCCB subunit that contains the CT domain. Indeed, the purification and characterization of this enzyme led to the discovery of the biochemical function of biotin as an enzyme cofactor (Himes et al., 1963; Moss and Lane, 1971).
In animals and microorganisms, the carboxylation of 3-methylcrotonyl-CoA is essential for the catabolism of Leu to acetoacetate and acetyl-CoA (Rodwell, 1969; Massey et al., 1976), which happens in mitochondria where MCCase is localized (Hector et al., 1980; Lau et al., 1980). In humans, the 3-methylcrotonylglycinuria (Eldjarn et al., 1970) is an autosomal recessive disorder of Leu catabolism resulted from the deficiency of MCCase (Stokke et al., 1972; Gompertz et al., 1973a; Gompertz et al., 1973b; Finnie et al., 1976). The deficiencies of both MCCA and MCCB subunits could result in this metabolic disease. Patients with MCCase deficiency excrete large quantities of abnormal metabolites, for instance, 3-methylcrotonylglycine and 3-hydroxyisovaleric acid, in the urine. However, patients show a wide range of clinical presentations, some that are lethal and others that are asymptomatic (Obata et al., 2001).

In addition to Leu catabolism, MCCase has been implicated as a component enzyme of the “mevalonate shunt” (Popjak, 1971; Edmond and Popjak, 1974; Fogelman et al., 1975; Edmond et al., 1976). The concept herein is that the amino acid, fatty acid and isoprenoid pathways are linked through a common route. The net result of the shunt pathway is to re-assimilate carbon that has been converted to mevalonate into primary metabolites (i.e., acetoacetate and acetyl-CoA), instead of into isoprenoids biosynthesis. Branching point of isoprenoids synthesis and the shunt is located at dimethylallyl pyrophosphate (DMAPP). In the shunt pathway, DMAPP is sequentially converted to 2-isopentenol, 2-isopentenaldehyde, 3-methylcrotonic acid, and 3-methylcrotonyl-CoA. The last metabolite could enter the Leu catabolism pathway. Recently, a study in fungus Aspergillus nidulans (Rodriguez et al., 2004) presented the first genetic evidence for the existence of a metabolic link involving MCCase between isoprenoid biosynthesis and leucine catabolism.
Moreover, MCCase could be involved in acyclic isoprenoids catabolism (Cantwell et al., 1978). A few Pseudomonas species use acyclic isoprenoids, such as citronellol and geraniol, as their sole carbon source for normal cellular functions. The catabolism of the acyclic isoprenoids requires GCCase, a biotin-containing enzyme, which catalyzes a carboxylation reaction from geranoyl-CoA to form γ-carboxygeranoyl-CoA. The carboxymethyl branch group of the product will then be eliminated and the resulting carbon skeleton is amenable for β-oxidation. Eventually, 3-methylcrotonyl-CoA would be generated by such a process and the further catabolism is common to the Leu catabolism, requiring MCCase.

**Characterization of MCCase in Plants**

Though the identification of MCCase from animals and bacteria could be traced back to more than 40 years ago, the first report of MCCase activity from plant is relatively recent (Wurtele and Nikolau, 1990). In the past decade, MCCase was purified from pea (Alban et al., 1993), potato (Alban et al., 1993), carrot (Chen et al., 1993), maize (Diez et al., 1994), tomato (Wang et al., 1994), and soybean (Song et al., 1994). The subcellular localization of MCCase in plant is consistent with that from animal, that is, in mitochondria (Baldet et al., 1992; Clauss et al., 1993). Notably, the genes coding for both subunits of MCCase were first isolated from plants (Song et al., 1994; Wang et al., 1994; Weaver et al., 1995; McKean et al., 2000) rather than from other kingdoms (Holzinger et al., 2001), and MCCase represents the first biotin-containing enzyme cloned from higher plants (Wang et al., 1994). As in animals and bacteria, plant MCCase is a heteromeric enzyme composed of two subunits: MCCA, the larger (about 75-80 kDa) biotinylated subunit, and MCCB, the smaller (about 60 kDa) non-biotinylated subunit (Wurtele and Nikolau, 2000; Nikolau et al., 2003). Southern blot analysis showed in Arabidopsis genome, each subunit is encoded by a single nuclear gene,
and these two genes are located on different chromosomes, i.e., \textit{MCCA} (At1g03090) is on chromosome 1 (Weaver et al., 1995), while \textit{MCCB} (At4g34030) is on chromosome 4 (McKean et al., 2000). In other plant species \textit{MCCA} and \textit{MCCB} are each encoded by a small gene family, e.g. tomato (Wang, 1993) and soybean (Song, 1993). The structural and biochemical properties of MCCase are conserved in plants. That is, in all plant cases, MCCA subunit contains two domains, the BC domain at the amino terminus and the BCCP domain at the carboxyl terminus; whereas MCCB contains the CT domain. Both subunits possess the characteristic mitochondrial targeting transit peptide on their amino terminus, consistent with the localization of the enzyme. Despite the subunit molecular weights are similar among species, two types of subunit stoichiometries were reported based on native molecular weights of the holoenzyme. MCCase from carrot (Chen et al., 1993), maize (Diez et al., 1994), tomato (Wang, 1993), and soybean (Song, 1993), appear to have an A_6B_6 quaternary structure, with a molecular weight of about 850 kDa, resembling the stoichiometry of animal MCCase (Lau et al., 1980). In contrast, MCCase from pea (Alban et al., 1993), potato (Alban et al., 1993) and Arabidopsis (Che et al., 2003) appear to have an A_4B_4 configuration, with a molecular weight of about 530 kDa, consistent with the quaternary structure of bacteria MCCase (Schiele et al., 1975). Using an Arabidopsis \textit{bio1} mutant which cannot biosynthesize biotin (Schneider et al., 1989; Shellhammer and Meinke, 1990), it has been shown that the subunit stoichiometry of MCCase is unaffected by the biotinylation status of the enzyme (Che et al., 2003). To date, how the two subunits are organized specially at the molecular level remains unknown.

Until recently the metabolic function of MCCase in plants was not well understood. Previously there are some correlative and indirect evidence suggests that plant MCCase plays
the same role in Leu catabolism as it does in animals and bacteria. First, though MCCase is expressed constitutively in pea, the activity of MCCase is remarkably increased during leaf senescence (Alban et al., 1993). In maize, primary leaves, where massive protein degradation occurs, MCCase activity is much higher in comparison to secondary, tertiary and quaternary leaves which are relatively young (Clauss et al., 1993). Moreover, MCCase is induced in isolated mitochondria from sycamore cells that undergo carbohydrate starvation (Aubert et al., 1996). Further, during soybean seed germination, Leu transiently peaks due to the degradation of seed storage proteins, and the decline of Leu content correlates in a timely manner with the accumulation of MCCase (Anderson et al., 1998). This correlation supports the hypothesis that MCCase is involved in Leu catabolism. The direct evidence of the metabolic role of MCCase came from radiotracer studies using cell free extracts from isolated mitochondria of pea. In this set of experiments, the metabolic fate of [U-14C] Leu and NaH14CO3 were determined and the incorporated NaH14CO3 into 3-methylglutaconyl-CoA via MCCase was demonstrated. Leu is catabolized via six consecutive reactions (Anderson et al., 1998): Leu $\rightarrow$ α-ketoisocaprate $\rightarrow$ isovaleryl-CoA $\rightarrow$ 3-methylcrotonyl-CoA $\rightarrow$ 3-methylglutaconyl-CoA $\rightarrow$ 3-hydroxy-3-methylglutaryl-CoA $\rightarrow$ acetoacetate + acetyl-CoA. MCCase is responsible for the forth reaction of this pathway. The results revealed for the first time that MCCase is involved in plant mitochondrial Leu catabolism. Therefore the primary metabolic role of MCCase in plants is proposed to be the catabolism of Leu.

Before the demonstration of Leu catabolism in mitochondria, Gerbling and Gerhardt proposed a Leu degradation pathway present in plant peroxisomes (Gerbling and Gerhardt, 1988, 1989; Gerbling, 1993). Leu is converted to acetyl-CoA in this pathway and the first
three reactions are common to mitochondria Leu catabolism. However, 3-methylcrotonyl-CoA is hydrolyzed to a free acid, 3-methylcrotonic acid, rather than carboxylated by MCCase to make 3-methylglutanoyl-CoA. Therefore, this pathway generates different intermediates, including isobutyryl-CoA and propionyl-CoA.

After asparagine, Leu is the second most abundant amino acid which transiently accumulates during carbon starvation (Genix et al., 1990; Aubert et al., 1996). Yet how Leu catabolism is regulated remains untouched. Some questions need to be addressed in the future: how these two physically separated metabolic pathways are related or coordinated with each other; how plants regulate these two pathways for normal growth and development; and what is the significance of plants possessing two Leu catabolic pathways.

As in animals and bacteria, MCCase in plants may be implicated in two other metabolic pathways: mevalonate shunt and isoprenoid catabolism. First, evidence from radiotracer studies in wheat suggests that the mevalonate shunt is present in plants (Nes and Bach, 1985; Bach, 1987). The acetate formed by the shunt pathway is mainly used for biosynthesis of long chain fatty alcohols having a chain length that is suitable for the membrane construction. Second, the purification and characterization of GCCase from both monocots and dicots suggest that plants may have the isoprenoid catabolism pathway that is proposed in Pseudomonas (Cantwell et al., 1978). Therefore, MCCase resides at the interconnected point of several metabolic pathways and may play important physiological roles in plants.

To understand the physiological functions of MCCase, the regulation of MCCase has been studied in plants. Lines of evidence to date have suggested that the regulation of MCCase may be complex and occur at multiple levels. MCCase is shown to be expressed at higher levels in non-photosynthetic organs (Wang et al., 1994; Anderson et al., 1998).
Transgenic plants containing the putative promoter regions of $MCCA$ and $MCCB$ fused with β-glucuronidase (GUS) gene, respectively, were used to study the expression of the two subunits (Che et al., 2002). GUS activities mediated by promoter regions of $MCCA$ and $MCCB$ are induced under light deprivation and CO$_2$-free conditions, and this induction is suppressed by carbohydrates which may be synthesized in photosynthetic organs. Also, upon sugar starvation, Leu and α-ketoisocaproate enhance the mRNA accumulation of MCCase and the branched-chain α-keto acid dehydrogenase complex (BCKDC), a multienzyme complex catalyzing the second reaction of Leu catabolism and catabolism of other branched-chain amino acids (BCAAs), i.e., isoleucine and valine (Fujiki et al., 2001). Therefore, maintaining the carbon status of the organism by Leu catabolism is considered as one of the physiological role of MCCase (Che et al., 2002).

Second, MCCase expression is regulated primarily at the level of gene transcription by sensing both environmental and metabolic signals. In all cases tested, including northern bolt, in situ RNA hybridization, western blot and transgenic plants expressing promoter-GUS fusion proteins, Arabidopsis $MCCA$ and $MCCB$ are coordinately expressed (McKean et al., 2000; Che et al., 2002), suggesting a mechanism that could harmonize both genes separated on different chromosomes.

Third, in tomato, the biotin containing MCCA subunit accumulates at approximately equal levels in roots and leaves, but the MCCase activity in leaves is only 10% of that in roots (Wang et al., 1994). The difference correlates with the organ-specific biotinylation status of the MCCA subunit. Further, a pool of non-biotinylated enzymes is present in leaves, although leaves have higher free biotin concentration (Wang et al., 1994). These findings
indicate a posttranslational regulation model for the expression of MCCase activity in different organs.

More recently, the non-catalytic functions of biotin in regulating MCCase expression have been demonstrated (Che, 2000; Che et al., 2003). Both subunits of MCCase hyper accumulate in response to the bio1 associated depletion of biotin (bio1 is a point mutation, specifically blocking biotin biosynthesis in Arabidopsis) (Schneider et al., 1989; Shellhammer and Meinke, 1990; Muralla et al., 2008), and this accumulation is the result of the enhanced translation of the mRNAs and/or the result of more stabilized protein. In addition, the transcription induction of both genes under light deprivation and CO₂-free conditions requires biotin. Finally, biotin status of an organism affects the accumulation of distinct charge isoforms of MCCase.

**Lipoic Acid**

Lipoic acid, another enzyme cofactor, plays a pivotal role in energy metabolism. It was discovered in the 1930s as a growth factor of certain bacteria and first isolated by Reed and coworkers in the 1950s (Reed et al., 1951). Lipoic acid is required for enzyme activities in multienzyme complexes which are known as α-keto acid dehydrogenase complexes (Reed et al., 1951; Reed, 1998) and glycine decarboxylase complex (Douce et al., 1994; Pares et al., 1994). Like biotin, the carboxyl group of lipoic acid is covalently linked to the ε amino group of a specific Lys residue through an amide bond. This Lys residue is located in the -DKA-motif which is conserved in all lipoylate-containing proteins (Reed and Hackert, 1966; Reche et al., 1998). Similar to biotin prosthetic group which serves as an intermediate carrier of carboxyl group, the lipoyl domain plays a role as an acyl group carrier in α-keto acid
dehydrogenase complexes, or as an aminomethyl group carrier in glycine decarboxylase complex (Reche and Perham, 1999).

**α-Keto Acid Dehydrogenase Complexes**

Defined by α-keto acid substrate specificity, three types complexes, pyruvate dehydrogenase complex (PDC), α-ketoglutarate dehydrogenase complex (KGDC) and branched-chain keto acid dehydrogenase complex (BCKDC), constitute the α-keto acid dehydrogenase complex family, whose molecular weight ranges from 4 to 9 MDa and is among the largest nonviral protein assemblies (Mooney et al., 2002). All complexes consist of three components: α-keto acid dehydrogenase/decarboxylase (E1), dihydrolipoyl acyltransferase (E2), and dihydrolipoyl dehydrogenase (E3). Lipoylated E2 is the core component of the complexes, with E1 and E3 non-covalently attached to it (Reed and Hackert, 1990; Berg and de Kok, 1997). The lipoyl-domain of E2 act as a swing-arm allowing the movement between active sites on E1 and E3 (Mooney et al., 2002). The overall reaction catalyzed by α-keto acid dehydrogenase can be represented as below (Reed and Hackert, 1990):

\[
RCO_{2}^{-} + \text{CoASH} + \text{NAD}^{+} \Rightarrow \text{RCO-S-CoA} + \text{CO}_{2} + \text{NADH}
\]

Plant cells uniquely contain two forms of PDCs, which are mitochondrial PDC (mtPDC) and plastidial PDC (ptPDC) (Reid et al., 1977; Mooney et al., 1999). mtPDC utilizes pyruvate to generate acetyl-CoA which links glycolysis to the Krebs cycle, whereas the ptPDC catalyzes the same reaction, but acetyl-CoA produced is used for *de novo* fatty acid biosynthesis in plastids (Mooney et al., 2002).
The KGDC catalyzes oxidative decarboxylation of α-ketoglutarate to produce succinyl-CoA, CO₂, and NADH. This reaction is located in mitochondria as part of the Krebs cycle (Millar et al., 1999).

The BCKDC is involved in catabolism of BCAAs. BCKDC catalyzes the oxidative decarboxylation of branched-chain α-keto acids which were generated by transamination reactions (Harper et al., 1984). In plants, it has been suggest that BCKDC activities present in both mitochondria and peroxisomes (Gerbling and Gerhardt, 1989; Anderson et al., 1998), providing evidence that BCAAs catabolism might occur in two subcellular compartments.

**Glycine Decarboxylase Complex**

In mitochondria, the glycine decarboxylase complex (GDC) and serine hydroxymethyltranserase catalyze two sequential reactions, which convert two glycine molecules into one each of serine, CO₂ and NH₃, with the reduction of NAD⁺ to NADH (Oliver, 1994). These reactions are considered to interconnect the metabolism of one-, two- and three-carbon compounds and are essential for photorespiration (Kikuchi, 1973; Oliver, 1994). The GDC consists of four different components, the P-, H-, T- and L-proteins. H-protein is the lipoylate-containing protein, and the linkage between the lipoyl moiety to H-protein provides a flexible arm which commutes successively to other three proteins (Douce et al., 1994; Oliver, 1994; Douce et al., 2001).
Dissertation Organization

This dissertation is composed of four chapters and four appendices.

The first chapter is a general introduction presenting the background knowledge of biotin, biotin-containing enzymes, previous studies on MCCase, as well as lipoic acid and lipoylate-containing enzymes. The purpose of this chapter is to set a context for the research that is described in the next two chapters.

The second chapter is a manuscript to be submitted to The Plant Cell. In this chapter, reverse genetic approaches were used to investigate the physiological functions of MCCase, and the relationships of two Leu catabolism pathways were analyzed. Four out of five T-DNA or transposon mutants were isolated and recovered by myself. The mccb-2 allele was isolated and recovered by Dr. Ping Che. The MCCase activity assay on mccb-2 allele and the histological analysis using MCCA::GUS and MCCB::GUS transgenic plants were also contributed by Dr. Che. The stereo microscopy (except the analysis on the intact mature seeds) and scanning electron microscopy analysis, and the experiment of removing seed coat and isolating embryos were undertaken by Dr. Hilal Ilarslan using the plant material generated by myself. All other experiments and data analyses were undertaken by myself.

The third chapter is a manuscript to be submitted to Plant Physiology. In this chapter, the identities of three BCCPL proteins were investigated using molecular, genetic, and biochemical approaches. All experiments and data analyses were designed, prepared and conducted by myself.

The last chapter contains general conclusions discussing and summarizing the studies presented in Chapter II and Chapter III.
The four appendices contain experimental results that are related to but not included in Chapter II and Chapter III.

This dissertation was under guidance, supervision and support of my major professor Dr. Basil J. Nikolau.

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CHAPTER II MOLECULAR, BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON 3-METHYLCROTONEYL-COA CARBOXYLASE

A manuscript to be submitted to *The Plant Cell*

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ABSTRACT

3-Methylcrotonyl-CoA carboxylase (MCCase) is a nuclear-encoded, mitochondrial-localized biotin-containing enzyme. The reaction catalyzed by this enzyme is required for leucine (Leu) catabolism in mitochondria, and it may also play a role in the catabolism of isoprenoids and in the mevalonate shunt. In Arabidopsis, two MCCase subunits (the biotinylated MCCA subunit and the non-biotinylated MCCB subunit) are each encoded by single genes (At1g03090 and At4g34030, respectively). A reverse genetic approach was undertaken to dissect the physiological role of MCCase in plants. We have recovered and characterized T-DNA and transposon knockout alleles of *MCCA* and *MCCB* genes.
Metabolite profiling studies indicate that mutations in either \textit{MCCA} or \textit{MCCB} block mitochondrial Leu catabolism, as inferred from the increased accumulation of Leu. Under light deprivation conditions, the hyper accumulation of Leu, 3-methylcrotonyl-CoA and isovaleryl-CoA suggests that two Leu catabolism pathway, i.e., the mitochondrial and the peroxisomal Leu catabolism pathways, are independently regulated. The biochemical block in mitochondrial Leu catabolism is associated with an impaired reproductive growth phenotype, which includes aberrant flower and silique development, and decreased seed germination. The decreased seed germination phenotype is only expressed when homozygous mutant seeds are collected from a parent plant that is itself homozygous, but not when collected from a parent plant that is heterozygous. These observations indicate that maternal mitochondrial Leu catabolism is required for normal seed development. These characterizations may shed lights on the role of catabolic processes in growth and development, an area of plant biology that is poorly understood.

**INTRODUCTION**

3-Methylcrotonyl-CoA carboxylase (MCCase; EC 6.4.1.4) is one of the four biotin-containing carboxylases characterized in plants (Nikolau et al., 2003). MCCase is composed of biotinylated MCCA subunit and non-biotinylated MCCB subunit. It catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA (MC-CoA) to form 3-methylglutaconyl-CoA (MG-CoA). The first step of this reaction is carboxylation of the enzyme-bound biotin (E-Biotin) prosthetic group by the biotin carboxylase (BC) domain on MCCA subunit. The second step is to transfer the carboxyl group to MC-CoA. It is catalyzed by the carboxyl
transferase (CT) domain on MCCB subunit. The biotin carboxyl carrier protein (BCCP) on MCCA subunit plays a structural role to provide a lysine residue within a conserved motif to which biotin is covalently attached.

\[
\text{Step1: } \text{HCO}_3^- + \text{ATP} + \text{E-Biotin} \rightarrow \text{E-Biotin-CO}_2^- + \text{ADP} + \text{Pi}
\]

\[
\text{Step2: } \text{E-Biotin-CO}_2^- + \text{MC-CoA} \rightarrow \text{E-Biotin} + \text{MG-CoA}
\]

\[
\text{Overall: } \text{HCO}_3^- + \text{ATP} + \text{MC-CoA} \rightarrow \text{MG-CoA} + \text{ADP} + \text{Pi}
\]

The identification of MCCase from animals and bacteria can be traced back to more than 40 years ago (Himes et al., 1963; Lane and Lynen, 1963; Moss and Lane, 1971). In these organisms, MCCase is involved in multiple metabolic processes. First, MCCase is required for the catabolism of leucine (Leu) to acetoacetate and acetyl-CoA (Rodwell, 1969; Massey et al., 1976) in mitochondria (Figure 1) (Hector et al., 1980; Lau et al., 1980). Second, MCCase is involved in acyclic isoprenoids catabolism in some Pseudomonas species (Cantwell et al., 1978; Diaz-Perez et al., 2004; Aguilar et al., 2006). The catabolism of the acyclic isoprenoids requires another biotin-containing carboxylase, geranoyl-CoA carboxylase. MCCase has also been implicated as a component enzyme of the “mevalonate shunt” proposed decades ago (Popjak, 1971; Edmond and Popjak, 1974; Fogelman et al., 1975; Edmond et al., 1976). The shunt pathway is thought to link amino acid, fatty acid and isoprenoid pathways together. A recent study in *Aspergillus nidulans* (Rodriguez et al., 2004) presented genetic evidence for the existence of a metabolic link involving MCCase between isoprenoid biosynthesis and Leu catabolism.

In plants, an initial clue of mitochondrial Leu catabolism pathway was the identification of MCCase activity (Wurtele and Nikolau, 1990). In the past decades, MCCase was purified from pea (Alban et al., 1993), potato (Alban et al., 1993), carrot (Chen et al., 1993), maize
(Diez et al., 1994), tomato (Wang et al., 1994), and soybean (Song et al., 1994). The subcellular localization of MCCase in plant is mitochondria (Baldet et al., 1992; Clauss et al., 1993). Both MCCA and MCCB subunits are encoded by single nuclear genes in Arabidopsis: 

\[ \text{MCCA (At1g03090)} \] is on Chromosome 1 (Weaver et al., 1995); \[ \text{MCCB (At4g34030)} \] is on Chromosome 4 (McKean et al., 2000).

In pea, radiotracer metabolic studies demonstrated that MCCase is required for Leu catabolism using cell-free extracts from isolated mitochondria (Anderson et al., 1998). MCCase is responsible for the forth reaction of this six-reaction pathway. Even earlier, a peroxisomal Leu catabolism pathway was proposed by Gerbling and Gerhardt in mung bean (Figure 1) (Gerbling and Gerhardt, 1988, 1989; Gerbling, 1993). The first three reactions are common in these two pathways. But in the peroxisomal pathway, instead of being carboxylated by MCCase, MC-CoA is hydrolyzed to a free acid, 3-methylcrotonic acid. Thus this pathway generates distinct intermediates, such as isobutyryl-CoA (IB-CoA) and propionyl-CoA. To date, some questions remain untouched, for instance: how these two physically separated metabolic pathways are related; how plants regulate these two pathways for normal growth and development; and what is the significance for plants to possess two Leu catabolism pathways.

The regulation of MCCase expression is very complex and occurs at multiple levels, including transcription, translation and post-translational modification. First, transgenic plants containing the putative promoter regions of \text{MCCA} and \text{MCCB} fused with \( \beta \)-glucuronidase (GUS) gene, respectively, were used to study the expression of the two subunits (Che et al., 2002). GUS activities are induced under light deprivation and CO\(_2\)-free conditions, and this induction is suppressed by carbohydrates which may be synthesized in
photosynthetic organs. Also, upon sugar starvation, Leu and α-ketoisocaproylate (intermediate of Leu catabolism) enhance the mRNA accumulation of MCCase and the branched-chain α-keto acid dehydrogenase (Fujiki et al., 2001). Second, MCCase hyper accumulates in response to the bio1-associated depletion of biotin (bio1 is a point mutation, specifically blocking biotin biosynthesis in Arabidopsis) (Schneider et al., 1989; Shellhammer et al., 1990; Muralla et al., 2008), and this accumulation is the result of the enhanced translation of the mRNAs and/or the result of more stabilized protein (Che, 2000; Che et al., 2003). Third, differential biotinylation status of MCCA subunit in different organs were found correlated with MCCase activity levels in tomato (Wang et al., 1994), implying the post-translational modification of MCCase. Finally in all cases reported, including northern blot, in situ RNA hybridization, western blot and transgenic plants expressing GUS reporter genes, Arabidopsis MCCA and MCCB are coordinately expressed (McKean et al., 2000; Che et al., 2002), suggesting a mechanism that could harmonize both genes separated on different chromosomes.

In this study, we used reverse genetic approaches to investigate the physiological functions of MCCase. Specifically, we recovered T-DNA and transposon tagged mutants of MCCA and MCCB gene, and characterized the molecular and biochemical features of these mutants. Furthermore, we analyzed the morphological, metabolic and physiological changes in the mutants. The mutants of MCCA and MCCB have a severe germination defect, and we conclude that this is due to a defect in the maternal tissues of the propagule. Finally we found that free amino acids and acyl-CoAs differentially accumulate in wild-type and mutant plants, especially when illumination is withheld. This study reveals the potential relationship between mitochondrial and peroxisomal pathways of Leu catabolism.
RESULTS

Characterization of T-DNA and Transposon Tagged Mutants in *MCCA* and *MCCB*

Genes

To study the physiological function of MCCase in plants, we used a reverse genetic approach to obtain the loss-of-function mutants. Five mutant lines for *MCCA* (At1g03090) and *MCCB* (At4g34030) were isolated and recovered from Arabidopsis T-DNA or transposon mutant collections. *mcca-1*, *mcca-2*, *mcca-3* and *mccb-1* alleles were found by searching the sequence-indexed Arabidopsis insertion database at The Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003). *mccb-2* was isolated from the T-DNA insertion collection at The Arabidopsis Knockout Facility at University of Wisconsin-Madison (Che, 2000; Sussman et al., 2000).

Seeds obtained from different collections were planted and genomic DNA was extracted from rosette leaves of individual progeny plants and subjected to PCR-based genotyping. Two sets PCR analyses were conducted to identify the genomic DNA sequences flanking T-DNA or transposon insertions. The first set of reaction was specific to the wild-type allele and used a pair of gene-specific primers that span the annotated position of the T-DNA or transposon insertions present in the mutant allele. Positive result of this PCR mean the plant being tested carries a wild-type allele in its genome. A second set of reactions was composed of two PCRs, which lead to the identification of the borders of the insertions by aligning the sequences of the PCR products with the Arabidopsis genomic sequence (Figure 2A, Tables 1 and 2). Each reaction of this set used one gene-specific primer in combination with a primer that is complementary to the T-DNA (left border, LB or right border, RB) or transposon (G-
edge, G, or H-edge, H) sequences. Positive results of this set of PCRs mean that the plant being tested carries a mutant allele. Moreover, if only the first set of reaction was positive, the plant was categorized as homozygous for the wild-type allele, and if only the second set of reactions was positive, the plant was categorized as homozygous for the mutant allele. If both sets of reactions were positive, the plant was categorized as heterozygote. PCR products were analyzed by gel electrophoresis and verified by sequencing (data not shown). For each mutant allele, we were able to recover all three categories of progeny, i.e., wild-type homozygous, mutant homozygous and heterozygous.

\textit{mcca-1} allele carries head-to-head chimeric copies of the T-DNA insertion which is associated with a 15-bp deletion in intron 11 (Figure 2A). In the \textit{mcca-2} allele, the genomic sequence flanking the left border of T-DNA insertion is located in the exon 1 of \textit{MCCA} gene (Figure 2A). However, the other border of the insertion could not be identified by PCR and thermal asymmetric interlaced (TAIL)-PCR (data not shown). The presence of \textit{MCCA} gene sequence downstream of the insertion was confirmed by PCR using the primers ALP1 and ARP1 (Figure 2A), indicating that the T-DNA insertion did not delete any sequences beyond the \textit{MCCA} locus. We postulated that there is a deletion in the other border of the insertion so that we can’t identify it using LB or RB primers. The transposon insertion in the \textit{mcca-3} allele was identified in the exon 1 of \textit{MCCA} gene and this insertion gave rise to a 10-bp tandem repeat flanking the transposon borders (Figure 2A). The \textit{mccb-1} allele is in agreement with the annotation in SALK database, and carries a single T-DNA insertion in the exon 10 of the \textit{MCCB} gene which caused a 12-bp deletion (Figure 2A). In \textit{mccb-2} allele, chimeric T-DNA insertions are oriented in a head-to-head manner and resulted in a 19-bp
deletion in exon 4 (Che, 2000). Because this allele was isolated in the ecotype Ws background, we outcrossed this allele to the ecotype Columbia plant for two generations.

For all analyses of mutant lines in this paper, wild-type, heterozygous mutant and homozygous mutant siblings in one comparison are always from a segregating family without further notation; special cases will be described.

**Are mcca and mccb Mutants Null Alleles?**

To test whether the mutant alleles fully disrupt the expression of *MCCA* and *MCCB* genes, investigations on the accumulation of mRNA, accumulation of protein, and enzyme activity were carried out. First, reverse transcriptase mediated (RT)-PCR was conducted to detect the expression of *MCCA* and *MCCB* mRNAs. At growth stage 6.50 (Boyes et al., 2001), about 40-50 days after planting (DAP) RNA was extracted from the aerial parts (including rosette leaves, cauline leaves, stems, buds, flowers and siliques) of plants that were homozygous either for the wild-type or for the mutant alleles; the exception being for the *mcca*-3 which was tested using heteroallelic mutant of *mcca-2/mcca-3*. To ensure that these assays were not confounded by genomic DNA that may contaminate the RNA preparations, the PCR primers were strategically placed to distinguish between products that contained introns (i.e., derived from genomic DNA) from those that lacked intronic sequences (i.e., derived from spliced mRNA templates). Primers were designed either to detect the transcripts that span the position of the insertion (*mcca-1, mccb-1* and *mccb-2*), or they were positioned downstream of the insertion (*mcca-2, mcca-2/mcca-3*) (Figures 2A, 2B and Table 2). PCR products were analyzed by agarose gel electrophoresis and sequenced; sequences were aligned against mRNA sequences of *MCCA* and *MCCB*. In all cases,
transcripts of *MCCA* or *MCCB* were not detected in the corresponding homozygous or heteroallelic mutants carrying the *mcca* or *mccb* mutation. We conclude therefore that T-DNA and transposon insertions have disrupted the accumulation of full length *MCCA* or *MCCB* mRNA in corresponding mutants.

Second, immunological analyses were carried out to evaluate the accumulation of MCCA and MCCB proteins in mutant lines using polyclonal antisera against MCCA (Weaver et al., 1995) and MCCB (McKean et al., 2000) subunits. Protein extractions from wild-type siblings, homozygous mutant siblings of *mcca-1, mcca-2, mccb-1* and *mccb-2*, and heteroallelic mutant siblings of *mcca-2/mcca-3*, were subjected to western blot analysis. Figure 2C shows that MCCA or MCCB proteins were undetectable in extracts from the mutants. In both *mccb-1* and *mccb-2* mutants, we detected immunologically reactive protein bands in these extracts but these were at lower molecular weight, which are not present in wild-type extracts. We propose that these are proteins maybe translated from truncated mRNAs of *MCCB* gene. These western blot analyses demonstrate that the T-DNA and transposon insertions have eliminated the accumulation of intact MCCA or MCCB protein, respectively.

Third, seeds of *Arabidopsis thaliana* Ws ecotype and homozygous mutant of *mccb-2* were germinated in the darkness for 7 days and then seedlings were collected and MCCase enzyme activity was determined. In extracts of Ws plants, the MCCase activity of was $1.2 \pm 0.1 \text{ nmol/(min\cdot mg)}$. In contrast, the MCCase activity was not detectable in *mccb-2* homozygous mutant, indicating the mutation completely blocked the expression of MCCase.

Based on analyses on three levels of expression of *MCCA* and *MCCB* genes in mutants, we conclude that these mutant alleles are indeed null alleles and the MCCase are fully disrupted in these mutants.
Maintenance/Turnover of *MCCA* and *MCCB* Is Independent of Each Other

Previous studies using northern bolt, *in situ* RNA hybridization, western blot and transgenic plants expressing promoter-GUS fusion proteins for both *MCCA* and *MCCB* genes have shown that these two genes were coordinately regulated (McKean et al., 2000; Che et al., 2002). The expression of these two genes probably is controlled by common environmental signals, such as sucrose and illumination status, which are coupled with transcription factors. The system herein allows us to evaluate the steady state level of mRNA and protein of one subunit while the other subunit is missing. To our surprise, RT-PCR and western blot analyses indicate that upon disruption of the *MCCA* gene, the accumulation of the *MCCB* mRNA and protein was unaffected as compared to wild-type siblings (Figure 3). Similarly, the accumulation of the *MCCA* mRNA and protein remains the same in *mccb* mutants as in wild-type plants (Figure 3). Hence, the elimination of one subunit does not affect the accumulation of the other subunit. Therefore, although there is machinery to coordinately regulates the expression of *MCCA* and *MCCB* genes, these results indicate that the maintenance/turnover of *MCCA* and *MCCB* is independent of each other at both mRNA and protein levels.

Two Types of *MCCA* mRNA Accumulate at Different Levels

It has previously been noticed that there are two types of full length *MCCA* cDNA sequences deposited at GenBank; accession numbers AY070723 and U12536 (Che et al., 2002). The difference between the two cDNAs is that AY070723 contains exon 6 (60-bp), which is absent from U12536 (Figure 2A). This observation indicates *MCCA* mRNA may
undergo alternative splicing and ultimately give rise to two types of MCCA proteins which vary in length by 20 amino acids.

RT-PCR has demonstrated that both transcripts exist in the mRNA extracted from wild-type Columbia plants (data not shown). We named these two transcripts *MCCA-L* (L, stands for long; 16-exon) and *MCCA-S* (S, stands for short; 15-exon). To compare the accumulation of the two transcripts, real-time RT-PCR was carried out with mRNAs extracted from 10 DAP, 14 DAP, 17 DAP and 22 DAP Arabidopsis Columbia seedlings. 18S rRNA was selected as the reference gene. Primers were strategically designed to distinguish the two transcripts (Figure 4 A and Table 2). A primer from each primer pair was using the expected cDNA sequence which spans two exons without the sequence of the intervening intron. PCR products were confirmed by gel electrophoresis after real-time RT-PCR. Our result indicates that *MCCA-L* is about two times more abundant than *MCCA-S*, and the relative abundance of the two transcripts were not affected during early stages of plant growth.

If both transcripts are translated to produce MCCA subunits, these two proteins will differ in molecular weight by 2 kDa, and pI by 0.1 units based on bioinformatic predictions (http://www.expasy.ch/tools/pi_tool.html). The 20 amino acids of *MCCA-L* are located at the BC domain of MCCA subunit. However, based on the results from conserved domain search at NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), the missing of the 20 amino acids in *MCCA-S* would not affect the characteristics for identification of the BC domain.
Transmission of *MCCA* and *MCCB* Genes

To test whether gamete transmission is affected by knocking out *MCCA* or *MCCB* gene, segregation analysis was conducted on heterozygous mutants. Two or three heterozygous mutant plants of each allele were planted and self-pollinated, and progeny seeds from each plant were collected individually and planted after breaking dormancy. Segregation ratio of each allele was determined by PCR-based genotyping the resulting seedlings (Table 3). Based on Chi-square test, segregation of *mcca-1*, *mcca-2* and *mccb-1* failed to reject the null hypothesis, which is that of normal Mendelian ratio for a recessive allele (1:2:1). However, tests on *mcca-3* and *mccb-2* reject the null hypothesis at 0.01 significance level.

Three possible explanations might be responsible for the differences in the Chi-square tests. First, there might be allelic differences among five alleles. Different alleles might have different effect on transmission. Second, different ecotype backgrounds could result in difference in transmission. Originally, *mcca-3* and *mccb-2* alleles were isolated in the ecotype Nössen and Ws background, respectively, whereas all other lines are in the ecotype Columbia background. Although *mccb-2* has been outcrossed twice with ecotype Columbia plants, the genetic background of this allele is scrambled as a mixture of the two ecotypes. Lastly, there is a mutation elsewhere in the genome of *mcca-3* and *mccb-2* alleles. Thus the altered transmission could be due to this background mutation.

To distinguish among these three explanations, allelic crosses between *mcca-1* with *mcca-2*, *mca-2* with *mcca-3*, and *mccb-1* with *mccb-2* were carried out. Heterozygous mutants of *mcca-1* were reciprocally crossed with heterozygous mutants of *mcca-2*. Seeds (F1) from crosses were collected from individual siliques on the parent plants, and after breaking dormancy these seeds were then grown and self-pollinated. These F1 plants of
individual siliques were genotyped by PCR. We had confirmed those siliques were the result of successful crosses, and we categorized F1 plants as wild-type, heterozygous mutant containing *mcca-1* allele, heterozygous mutant containing *mcca-2* allele, and heterozygous mutant containing both *mcca-1* and *mcca-2* allele (*mcca-1/mcca-2*, called as heteroallelic mutant). We used the same strategy and carried out the reciprocal crosses for *mcca-2* with *mcca-3*, and *mccb-1* with *mccb-2*. Heteroallelic mutant (F1) for *mcca-2* and *mcca-3* (*mcca-2/mcca-3*), and heteroallelic mutant (F1) for *mccb-1* and *mccb-2* (*mccb-1/mccb-2*) were successfully obtained.

Segregation ratio was analyzed using F1 plants from allelic crosses, i.e., *mcca-1* and *mcca-2*, *mcca-2* and *mcca-3*, and *mccb-1* and *mccb-2*. Based on PCR genotyping, the segregation ratios of all three allelic crosses showed that Chi-square tests failed to reject the null hypothesis (wild-types: heterozygous mutants: heterozygous mutants: heteroallelic mutants = 1:1:1:1) (Table 4). This result demonstrates that the gamete transmission is not affected by the mutations in *MCCA* or *MCCB* gene. Therefore, it indicates that non-Mendelian segregation of *mcca-3* and *mccb-2* may be due to background mutations in the genome of these two alleles.

**MCCase Mutants Express Complex Phenotypic Changes in Siliques and Seeds**

The expression of *MCCA* and *MCCB* was examined using transgenic plants carrying GUS driven by *MCCA* or *MCCB* promoter (Che et al., 2002). GUS is highly expressed in both transgenic lines in reproductive organs including flower buds, petals, sepals, stigmas, styles, pollen grains on the anthers, siliques, and developing seeds (Figures 5A to 5D). Noticeably, strong GUS activity was also detected on the base of the siliques, at the
abscission zone (Figure 5D). Based on these findings, we started our phenotype exploration by carefully examining the flowers and siliques in mutant plants.

For *mcca-1*, *mcca-2*, *mccb-1* and *mccb-2* alleles, homozygous mutant and wild-type populations generated from segregating heterozygous mutant parents were examined for morphological and developmental phenotypes. During vegetative growth, the overall physical appearance of the *mcca* and *mccb* mutant plants is similar to wild-type plants. For instance, the height of the mature bolts of mutants was similar to that of wild-type plants (Figures 6A to 6C, and 6F). However, features that distinguish the homozygous mutant plants from wild-type plants become more evident in the reproductive organs. Siliques of mutants were shorter compared to wild-type siblings and a large number of aborted siliques were found on the mutants (Figures 6A to 6C). To understand the silique elongation process, flower buds were tagged at 1 day after flowering (DAF), and length of growing siliques was measured on a daily basis. Elongation of the siliques on homozygous mutants exhibits large variation, whereas siliques on the wild-type siblings grow more uniformly (Figures 6D and 6E). Some of the mutant siliques can reach the same length as of wild-type siliques, but many mutant siliques failed to reach even half of the length of wild-type siliques. Some of these siliques were completely aborted at the beginning of silique development and did not elongate at all.

Because the elongation of siliques was completed at approximately 6-7 DAF in both wild-type and mutant plants (Figures 6D and 6E), we measured the silique length of *mcca-2*, *mccb-1* and *mccb-2* alleles at 8 DAF; as expected, mutants of these alleles also have shorter siliques as compared to wild-type siblings (Figure 6G).
There are two possible explanations for shorter siliques in mutant siblings. First, the developing seeds inside the siliques maybe healthy, but the defects in the structure of the siliques restrict the elongation (Gu et al., 1998; Roeder and Yanofsky, 2006). Second, which is more commonly encountered, the developing seeds inside the siliques are impaired, and hence the need for siliques to elongate is diminished (Choe et al., 2000; Kim et al., 2005; Liu and Makaroff, 2006). To distinguish between these two possibilities, siliques at 8 DAF from both wild-type and mutant siblings were dissected. In mcca-1, mcca-2, mccb-1 and mccb-2 alleles, the structure of the siliques appears the same in both wild-type and mutant siblings. However, we found that there are many aborted seeds besides the normal-looking developing seeds in the siliques of homozygous mutants but not in the wild-type siliques (Figures 7A to 7F). Aborted seeds are scattered at different positions within the siliques from tip to bottom, and are not located in any specific position (Figures 7B to 7D, and 7F; data of mccb-1 and mccb-2 not shown). In contrast, almost all developing seeds are normal in siliques of wild-type siblings, and aborted seeds are very rare (Figures 7A and 7E); and for those rarely seen aborted seeds found in wild-type siliques, they are often located at the bottom of a siliques (data not shown). Based on these observations, we conclude that the shorter siliques in the homozygous mutants are due to the aborted seeds, but not to a defect in the structure of the siliques.

To find out whether the developing seeds inside the mutant siliques have normal embryos, we removed the seed coat at 8 DAF and examined the embryos within by stereo microscopy. In the wild-type plants, the embryos were found to be uniformly at the walking-stick stage of development (Figure 7G). In contrast, in the mutants of all alleles analyzed, the
developmental stage of the embryos varied. Besides embryos at walking-stick stage, some embryos were at the torpedo stage (Figures 7H to 7L).

The morphology of the mature seeds was also analyzed under stereo microscopy and scanning electron microscopy (SEM). The overall physical appearance of the mutant seeds, including shape and color, is similar to wild-type seeds (Figures 7M to 7P). However, analyses on the mutant seeds demonstrated that mcca and mccb mutant generate seeds that are bigger in size, and heavier in weight than wild-type (Figures 8A and 8B).

We have found that many seeds in the mutant siliques are aborted (Figures 6 and 7), and these mutants generate heavier seeds (Figures 8B). Interestingly, these two effects are antagonistic to the seed yield per plant. The former decreases the seed yield and the latter increases it. Nonetheless, the actually seed yield per plant of mutants is significantly less than that of the wild-type siblings. For different mutant alleles, the seed yield varies between 50 to 70% of wild-type plants (Figure 8C). Therefore, the combination of two effects still resulted in reduced seed yield phenotype.

As mentioned before, GUS expression driven by MCCA or MCCB promoter was found to be concentrated in the abscission zone of siliques. Consistently, we found that the morphology of the nectary glands, including lateral nectary glands and medial nectary glands, was altered in mcca and mccb mutants (Figures 7Q to 7S; data of mccb-1 and mccb-2 not shown); the cells of the nectary gland from the mutants have a shrunken phenotype, resulting in smaller nectary gland. Yet the other tissues surrounding nectary glands appear normal. The Arabidopsis floral nectary is located at the base of stamen filaments, which is exactly the abscission zone for petals and sepals, and the place where MCCA and MCCB strongly is expressed (Figure 5D). This strong correlation between the expression of MCCA and MCCB
genes and the phenotype of \textit{mcca} and \textit{mccb} mutants indicated that MCCase may play an imported role in establishing the normal structure and function of nectary gland.

\textbf{Germination Defect of \textit{mcca} and \textit{mccb} Mutants}

Seeds were collected from wild-type and homozygous mutant siblings that shared a common heterozygous parent. In order to minimize the impact of environment variance in these experiments, the growth conditions during seed development, the conditions used to store mature seeds and subsequent germination conditions were maintained identically for all genotypes at all generations (Bentsink and Koornneef, 2002). As we tried to propagate the seeds of homozygous \textit{mcca} and \textit{mccb} mutants, we noted that the germination rate of these mutants was very low (Figure 9A). Progeny seeds derived from a homozygous mutant of \textit{mcca-1}, \textit{mcca-2}, \textit{mccb-1} and \textit{mccb-2}, and F2 seeds from heteroallelic mutants of \textit{mcca-1/mcca-2}, \textit{mcca-2/mcca-3} and \textit{mccb-1/mccb-2} germinate at a rate that was lower than 50%, as compared to the wild-type siblings, whose germination rate was close to 100%. In contrast, we noted that the seeds derived from of heterozygous mutant parents carrying \textit{mcca-1}, \textit{mcca-2} and \textit{mccb-1} alleles germinate close to 100% (Table 5), meaning that mutant seeds from a heterozygous parent do not have the germination defect. Also, when we look at these mutants in the segregation analysis, their viability was comparable to wild-type siblings (with exception of \textit{mcca-3} and \textit{mccb-2}, there might be a background mutation in the genome of both alleles; discussed in segregation analysis). As we discussed, the segregation analysis on the progeny from allelic crosses display the normal Mendelian segregation ratio for a recessive allele. Based on these results, we conclude that only seeds derived from a parent that is homozygous mutant at \textit{mcca} or \textit{mccb} locus present the germination defect. The
homozygous mutant seeds that are progeny from a heterozygous parent, germinate at normal rates. Thus, in these alleles, seeds that develop on homozygous parents fail to germinate normally, but those homozygous mutant seeds that develop on a parent that is heterozygous for these alleles germinate normally. Therefore, the maternal tissue that is homozygous for the mutant alleles must fail to provide seeds some factor that is subsequently required for germination. In summary, the germination defect caused by insertions in \textit{mcca} or \textit{mccb} gene has a maternal effect.

We noticed that small percentage of the homozygous mutant seeds derived homozygous parents succeeded to germinate and grow to maturity (Figure 9A). These resulting plants share the same phenotypes (shorter siliques, more aborted seeds and less seed yield) as their parents (data not shown). To test whether the germination defect persisted in these plants, seeds were collected from these plants and sown on MS agar media. As illustrated in Table 6, the germination defect phenotype persisted in the progeny.

A possible explanation for the germination defect phenotype might be associated with breaking dormancy. Two types of physiological seed dormancy have been documented, embryo dormancy and coat dormancy (sometimes termed coat-enhanced dormancy) (Kucera et al., 2005). Because seed coat is a maternal tissue (originating from the integuments of ovules), mutations that affect the structure of seed coat can result in seed germination defects (Bentsink and Koornneef, 2002). To test whether seed coat is the constraint responsible for the germination defect, the seed coats of mature wild-type and mutant seeds of \textit{mccb-1} allele were removed and the embryos were placed directly on MS agar media. However, the germination defect of \textit{mccb} mutants could not be alleviated by the removal of the seed coat (Figures 9B to 9E). Therefore, we concluded that the structure of the seed coat in the mutant
is not the cause of the germination defect. We have noticed that the morphology of embryos from mutants appears normal as compared to that of wild-types (Figures 7O and 7P), in terms of the overall embryo structures and the epidermis cells of the embryos. But these mutant embryos are larger in size and heavier in weight, indicating that defects might be developed during embryogenesis. Therefore, the growth potential of the embryo itself might inherit a physiological defect from the maternal parent due to loss of MCCase function.

**The Phenotypes Are Due To Loss of MCCA or MCCB Gene Functions**

The allelism tests were carried out by examining the phenotypes of heteroallelic MCCA and MCCB F1 hybrids. Those heteroallelic hybrids (mcca-1/mcca-2, mcca-2/mcca-3, and mccb-1/mccb-2) express the phenotypes, including shorter siliques, more aborted seeds, less seed yield and germination defect, which were shown in the homozygous mutants (Figures 6A, 6B and 9A), indicating that the phenotypes we observed are indeed due to the loss of functional MCCA or MCCB gene in respective mutants.

**Effect of Light Deprivation on Free Amino Acids and Acyl-CoAs Quantities in mcca and mccb Mutants**

Previous studies indicated that branched-chain amino acids (BCAAs), i.e., Leu, isoleucine (Ile) and valine (Val), undergo catabolic metabolism in peroxisome (Gerbling and Gerhardt, 1988, 1989; Gerbling, 1993). This peroxisomal catabolic pathway for Leu is different from the pathway demonstrated in mitochondria (Anderson et al., 1998), but these two pathways, share some common acyl-CoA intermediates (i.e., isovaleryl-CoA [IV-CoA], MC-CoA and acetyl-CoA; Figure 1). However, MG-CoA and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) are unique intermediates of the mitochondrial pathway, while isobutyryl-
CoA (IB-CoA) and propionyl-CoA are unique intermediates of the peroxisomal pathway. Due to the loss of MCCase function in the mutants, we would expect that the mitochondrial Leu catabolism pathway is blocked. By monitoring the concentration of Leu and other intermediates of both pathways, we hoped to get novel insights into possible connections between these two pathways.

Moreover, it has been shown that the lack of illumination induces the expression of MCCA and MCCB genes in young Arabidopsis seedlings, and the hyper accumulation of MCCA and MCCB proteins is paralleled with the increases of MCCase activity (Che et al., 2002).

Therefore, in this study, we combined these two effects: one is light deprivation, which induces the MCCase activity; and the mutation in MCCA and/or MCCB, which knocked out MCCase activity. The rationale was to expose plants to an environment which requires high MCCase activity, but at the same time remove MCCase, and measure the metabolic response of these plants.

mcca-1 and mccb-1 homozygous mutants and their wild-type siblings as controls were grown under constant light for 23 days (for acyl-CoA analysis) or 26 days (for amino acid analysis). These plants were either kept under same conditions or treated with 2-day darkness before tissue collection. The rosette leaves were harvested immediately at the end of 25 DAP (for acyl-CoA analysis) or 28 DAP (for amino acid analysis). At this age, normal Arabidopsis plants are at rosette growth stage, parallel with inflorescence emergence (Boyes et al., 2001). Free amino acids (distinguished from protein-bound amino acids) and a list of acyl-CoAs (Table 7) were targeted for analysis to determine their steady-state levels of accumulation. All amino acids, except arginine (Arg) and cysteine (Cys), were measured and quantified
individually. Henceforth, without further notation “total amino acids” represent all amino acids with the exception of Arg and Cys.

We measured the concentration of 18 amino acids and calculated the sum of the concentration of total amino acids. We found that under constant illumination total free amino acids accumulated at rather low levels, however upon dark treatment, total amino acids in both wild-type plants and mutant plants (*mcca-1* and *mccb-1* alleles) increased, with a higher increase in the mutants (Figure 10A and Table 8). The increase in free amino acids due to darkness is in agreement with previous studies (Koch, 1996; Brouquisse et al., 1998; Buchanan et al., 2000). During prolonged dark treatment, sugar starvation dramatically changes the pattern of gene expression in plants, which also initiates changes in substantial physiological, biochemical and metabolic processes to recycle cellular components. Protein remobilization is one of the processes that occur during sugar starvation (Yu, 1999).

When we look specifically at Leu concentration, we found it showed similar accumulation patterns as total amino acids (Figure 10B). Under constant illumination, free Leu in both wild-type and mutant siblings accumulated at about 0.05 µmol/g FW, accounting for less than 1% of the total amino acids. In contrast, after two days dark treatment, Leu concentrations in wild-type and mutant plants increased significantly, with the mutants having a much higher increase. More specifically, Leu of wild-type plants increased about 10-fold, constituting 3% of total amino acids, whereas in these homozygous mutants, Leu concentration increased more than 40-fold, constituting more than 10% of total amino acids (Figure 10B, Table 8). This result confirms that dark treatment induces the demand for MCCase activity to a higher level, but due to the MCCase mutations, Leu catabolism could not keep up with this demand.
Based on the behavior of every amino acid in this experiment, we analyzed the potential relationships between Leu and each of all the other amino acids (Table 9) using Pearson’s correlation coefficients (SAS). We found that Ile and Val are highly positively correlated (>0.85) with Leu. Therefore, loss of MCCase function, not only disrupts Leu catabolism, but also affects the accumulation of all the other BCAAs. This finding suggests that the catabolisms of BCAAs are tightly interconnected.

Next we measured the concentration of acyl-CoA intermediates. In plants grown under constant illumination, IV-CoA, the product of the second reaction of both mitochondrial and peroxisomal Leu catabolism pathways, accumulates at the same level in the mutants and wild-type siblings (Figure 11E and Table 10). Leu, under the same conditions, displays the same behavior (Figure 10B and Table 8). In contrast, MC-CoA, the product of the third reaction of both pathways, and the substrate of MCCase in mitochondrial Leu catabolism pathway, accumulated significantly more in mcca and mccb mutants than wild-type siblings under constant illumination conditions (Figure 11F and Table 10). The increased MC-CoA accumulation confirmed that the mutations knocked out MCCase activity and blocked the Leu catabolism pathway at the MCCase step. In the mean time, we didn’t observe any significant change in the concentrations of IB-CoA and propionyl-CoA (two intermediates from the peroxisomal pathway) in the comparisons of mutants with wild-type plants under constant illumination (Figures 11D, 11G and Table 10), indicating that peroxisomal pathway is not induced upon shutting down the mitochondrial pathway.

Due to dark treatment, both wild-type and mutant siblings accumulate 3-5 times more IV-CoA as compared with plants under constant illumination (Figure 11E). As for MC-CoA, wild-type plants accumulate 6-7 times more than wild-type plants under constant
illumination. Mutants accumulate comparable amount of MC-CoA as wild-type plants in these conditions, with a 2-fold increase as compared to mutants under constant illumination (Figure 11F). These observations demonstrate that the metabolite flux of Leu catabolism, at least from the first three reactions, dramatically increases under dark treatment in both wild-type and mutant plants. In the mean time, IB-CoA and propionyl-CoA do not display such hyper accumulation under dark treatment. Although we found changes that are statistically significant (p <0.05) in the comparison between constant illumination and darkness treatments in the wild-type siblings of mcca-1 allele for IB-CoA and propionyl-CoA (Table 10), the changes here are much less than the changes found in IV-CoA and MC-CoA. Therefore, we conclude that the peroxisomal Leu catabolism pathway is not contributing to the overall increase of the carbon flux of Leu catabolism and it is not induced under dark treatment.

Combining the amino acid and acyl-CoA analyses, we demonstrated that Leu catabolism is been blocked due to the loss of MCCase function. More importantly, under sugar starvation conditions, hyper accumulation of Leu and other Leu catabolism intermediates that are upstream of MC-CoA (including MC-CoA) in the MCCase mutants, while no hyper accumulation of peroxisomal Leu catabolism intermediates that are downstream of MC-CoA, indicating that the demand for mitochondrial Leu catabolism is increased dramatically, the supply of the Leu catabolism could not keep up with this demand. The demand-supply conflict suggests that the peroxisomal pathway does not compensate for the loss of mitochondrial Leu catabolism pathway.

In this experiment, we also determined the concentration of acetyl-CoA and HMG-CoA (Figures 11B and 11C, Table 10). We did not find any significant change of HMG-CoA in
this experiment, yet we found some changes in acetyl-CoA upon dark treatment and knocking out MCCase (Figure 11B and Table 10). It is well established that acetyl-CoA is involved in multiple primary and secondary metabolic pathways, for instance, Krebs cycle and fatty acid biosynthesis. Due to these complex metabolic fates of acetyl-CoA, we did not try to conclude the concentration changes of acetyl-CoA are based on dark treatment and the loss of MCCase function.

**DISCUSSION**

The initial biochemical characterization of MCCase as an enzyme was correlated with the identification of biotin as an enzyme cofactor (Lynen et al., 1959). Since then the metabolic pathways requiring MCCase have progressively unfolded, and these include mitochondrial Leu catabolism, mevalonate shunt and isoprenoid metabolism (Popjak, 1971; Lau et al., 1980; Fall, 1981; Anastasis et al., 1985; Bach, 1987; Kleinig, 1989; Bach et al., 1991; Bach, 1995). More recently, the identification of plant MCCase (Wurtele and Nikolau, 1990) facilitated the demonstration of mitochondrial Leu catabolism pathway in plants (Anderson et al., 1998). Later studies have focused on the developmental and environmental regulation of the genes encoding MCCase, i.e., *MCCA* and *MCCB* (Wang et al., 1995; Che et al., 2002; Che et al., 2003). These studies indicated the regulation of MCCase is at different levels, including transcription, translation and post-translational modification. Interestingly, it has been shown that MCCase is highly induced by sugar starvation (Aubert et al., 1996a; Che et al., 2002). In the work presented here, we used reverse genetic approaches to investigate the physiological roles of MCCase. Phenotypic changes were characterized, and the possible metabolic
relationship between mitochondrial and peroxisomal Leu catabolism pathways were analyzed by using targeted metabolic profiling.

**Physiological Function of MCCase**

We used T-DNA or transposon insertional mutagens to knockout *MCCA* and *MCCB* genes. We have shown that at the mRNA, protein and enzyme activity levels, MCCase has been totally eliminated in the mutants. The overall vegetative growth was quite normal in these mutants as compared to their wild-type siblings, yet the reproductive growth of these mutants had a major defect. Silique development, seed yield, seed size, morphology of nectary gland, and most important, germination rate are affected. Consistent with these findings, the expression analysis of *MCCA* and *MCCB* genes (McKean et al., 2000; Che et al., 2002) demonstrate that MCCase are highly expressed in rapidly growing, metabolically active, yet non-photosynthetic organs, including buds, flowers, siliques, and developing seeds. Combined these two lines of evidence, we conclude that one of the physiological roles of MCCase is to maintain normal reproductive growth, and mitochondrial Leu catabolic pathway is required for balancing carbon and energy when photosynthesis is limited.

**The Maternal Effect of MCCase**

Based on the fact that seed germination defect of homozygous *mcca* and *mccb* mutants only occur when the parent plant of the seeds is also homozygous mutant, we demonstrate it is a maternal effect of mutations in MCCase which affects the germination rate. For seeds to break dormancy and germinate, viable embryos have to overcome the surrounding physical constraints provided by the diploid maternal structure, namely the seed coat. Two types of seed dormancy are likely involved in germination defect, coat dormancy and embryo
dormancy (Kucera et al., 2005). Because the seed coat (originated from integuments of ovules) is maternal tissue, phenotypes of mutants that change the structure of the seed coat can result in failure of the germination (Bewley, 1997; Bentsink and Koornneef, 2002). Yet in our case, we have shown in \textit{mccb} mutants that removing the seed coat does not allow for the germination of the isolated embryos, indicating that the defect of the mutants is not the incapability to break seed coat. Furthermore, maternal effect can contribute to embryo dormancy via physiology (Bentsink and Koornneef, 2002). The developing embryos have tight linkages to the female organs which provide micro-environment and are physically surrounding them. The nutrients for embryogenesis depend upon the metabolism of the maternal parent. Also, cytoplasmic substances of the maternal parent, such as plastids and mitochondria, can be directly transferred to the progeny during zygote formation (Roach and Wulff, 1987), which imprints the progeny. The data we have gathered to date does not allow us to distinguish whether the physiological defect or the cytoplasmic substances of the maternal parent is responsible for low germination rate in these mutants.

We have shown that the MCCase mutant seeds are bigger and heavier than that of wild-type, yet we don’t know whether the seed size changes are due to maternal effect or not. Two possible experiments could be done to address this question. First, by measuring individual seeds from a heterozygous parent and identify the genotype of each seeds. In this experiment, we could determine if the seed size and weight variations are already established in heterozygous progenies. Second, reciprocal crosses between homozygous mutant (\textit{mcca} or \textit{mccb}) to wild-type could provide direct evidence (Roach and Wulff, 1987; Ohto et al., 2005; Sundaresan, 2005). The strategy is to use homozygous mutant as either maternal parent or paternal parent to carry out the cross, and then measure the seed size and weight of the
progenies. If the seed size is bigger, and weight is heavier in the progenies which are from the mutant maternal parent than those progenies from wild-type maternal parent, then the seed size and weight phenotype can be credited to maternal effect.

**Coordinate Regulation vs. Independent Expression of MCCA and MCCB**

Several lines of evidences have demonstrated that the expression patterns of *MCCA* and *MCCB* genes are very similar both temporally and spatially during development and under certain metabolic stresses (McKean et al., 2000; Che et al., 2002). Since the MCCase holoenzyme requires both subunits in order to function, one would assume it would not be economical for a plant to accumulate one subunit of the enzyme when the other subunit is missing. Nonetheless, in this study we have seen that when the MCCA subunit is missing, it does not affect the accumulation of the MCCB subunit at mRNA and protein levels, and *vice versa* (Figure 3). Consistent with these findings, abundance of *MCCB* transcript accumulation was not affected in antisense *MCCA* plants (Qian, 2002). Clearly, when one subunit of MCCase is totally eliminated, plants don’t recycle the ineffective counterpart. Thus, the mechanism for degradation and/or turnover of the one subunit is independent of the abundance of the other subunit. Also, the mechanism for coordinate regulation (such as common transcription factors) of these two genes might be unrelated to the mechanism for maintenance of these two gene products.

**Two MCCA Transcripts vs. Two Forms of MCCase**

Two mRNAs of *MCCA* gene that differ by 60 bp are present in Arabidopsis. In this study, we confirmed that *MCCA* mRNA undergoes alternative splicing and generates transcripts containing or omitting exon 6 (60 bp). The transcript that contains exon 6 is expressed at
higher levels than the transcript lacks this exon. If two types of MCCA subunits are produced from the alternative slicing variants, and the molecular weight of two forms MCCA subunits only differs in about 2 kDa. In western blot analyses using anti-MCCA serum, we could only identify one distinct band (Figures 2C and 3B). One explanation is that one of the transcripts is not translated to produce MCCA subunit. An alternative explanation is that the resolution of SDS-PAGE is not high enough to distinguish two bands differing by only 2 kDa.

Interestingly, two forms of MCCase were purified from soybean leaf extract using anionic exchange chromatography (Song, 1993). Enzyme kinetic analysis were carried out and revealed that except Km for ATP, two forms of MCCase have nearly identical kinetic property. More recently, the Arabidopsis holo-MCCase was revealed as an A4B4 heterooctomer with molecular weight at 530 kDa, and two charge isoforms of MCCase were detected in Arabidopsis (Che et al., 2003). It was believed that these two forms of MCCase were sharing same molecular weight but only different on charge status. If both forms of MCCA transcripts are expressed and contribute to formation of holoenzyme, the maximum difference on molecular weight between the two forms of MCCase would be 8 kDa. In fact, the gradient polyacrylamide gels used in Che et al (2003) could not resolve two bands with only 8 kDa differences. This opens a possibility that two forms of MCCA subunit could be responsible for isoforms of MCCase.

The Regulation of BCAAs Catabolism

In higher plants, the catabolism of BCAAs is thought to occur in both peroxisomes (Gerbling and Gerhardt, 1988, 1989; Gerbling, 1993) and mitochondria (Anderson et al., 1998; Taylor et al., 2004). The first three reactions of these two pathways are the same. The
mitochondria localized enzymes responsible for these reactions have been studies in recent years (Daschner et al., 1999; Fujiki et al., 2000; Daschner et al., 2001; Faivre-Nitschke et al., 2001; Diebold et al., 2002; Graham and Eastmond, 2002; Taylor et al., 2004; Schuster and Binder, 2005).

BCAAs are first transaminated to produce the respective branched-chain α-keto acid by the branched-chain amino acid transaminase (BCAT). This is a reversible reaction and it is also responsible for the last step of BCAAs biosynthesis. In Arabidopsis, although BCATs comprise a small gene family, only AtBCAT-1 is localized in mitochondria. AtBCAT-1 is capable to initiate degradation of all BCAAs in almost all tissues of Arabidopsis (Diebold et al., 2002; Schuster and Binder, 2005).

Second reaction involves the branched-chain α-keto acid dehydrogenase complex (BCKDC), which is composed of E1, E2 and E3 components. Only one gene for each BCKDC component is found in the Arabidopsis mitochondria and the enzyme complex is able to process all α-keto acids generated from the transamination of all three BCAAs. The products of this reaction are branched-chain acyl-CoAs (Fujiki et al., 2000; Taylor et al., 2004).

The third enzyme is called branched-chain acyl-CoA dehydrogenase. It catalyzes the oxidation of branched-chain acyl-CoAs. In case for Leu catabolism, isovaleryl-CoA dehydrogenase (IVD) catalyses the conversion of IV-CoA to MC-CoA. One IVD gene was found in Arabidopsis with mitochondrial localization peptide (Daschner et al., 1999). Heterologous expression the AtIVD protein in Escherichia coli demonstrated that it can process branched-chain acyl-CoAs generated from both Leu and Val (Daschner et al., 2001). Similar findings were observed from IVD purified from potato tuber mitochondria (Faivre-
Thus, it is suggested branched-chain acyl-CoA intermediates are oxidized via a single enzyme located in the mitochondria (Daschner et al., 2001; Graham and Eastmond, 2002; Taylor et al., 2004).

Therefore, plants appear to use single enzymes for the first three reactions of BCAAs catabolism in mitochondria. Subsequent reactions of BCAAs catabolism diverge, with MCCase involved in Leu catabolism, β-oxidations involved in Ile and Val catabolism.

Based on our findings, BCAAs concentrations exhibit very similar behavior in response to loss of MCCase function and light deprivation, indicating that the catabolism of all BCAAs maybe very tightly linked. One possible explanation is that the failure to degrade Leu might generate metabolic signals to control the upstream reactions of Leu catabolism. Since those upstream reactions are apparently using common enzymes for all BCAAs degradation, one regulatory machinery could have an effect on all three BCAAs catabolism pathways. Consistent with this hypothesis, expression of BCKDC, IVD and MCCase are coordinately induced due to sugar starvation (Fujiki et al., 2000; Daschner et al., 2001; Fujiki et al., 2001; Che et al., 2002).

The Relationship between Mitochondrial and Peroxisomal Leu Catabolism Pathways

When grown under constant illumination, although the concentration of MC-CoA is increased in meca or mecbb mutants, concentrations of Leu, IV-CoA, IB-CoA and propionyl-CoA are similar in wild-type and mutant plants. This observation demonstrates that MCCase mutants disrupt the mitochondrial Leu catabolic pathway at the step of MC-CoA to MG-CoA. Moreover, the fact that the overall Leu accumulation is not affected indicates that under
normal condition plants only require basal level of Leu catabolism, which can be conducted either in mitochondria or peroxisomes.

When illumination is withdrawn for a prolonged time, photosynthesis is ceased and the plant is under sugar starvation. Reserves, such as fatty acids and proteins are being remobilized to provide energy for survival. MCCase is highly induced under these conditions, and considered to be important in this process, called autophagy (Aubert et al., 1996a; Aubert et al., 1996b). Under these conditions, we found that IV-CoA and MC-CoA are increased more than 2 folds in mcca and mccb mutants, as well as in wild-type plants, indicating that the Leu catabolism (at least the first three reactions that are upstream of MCCase) is induced. This is consistent with the coordinate expression of BCKDC, IVD and MCCase which are induced during sugar starvation (Fujiki et al., 2000; Daschner et al., 2001; Fujiki et al., 2001; Che et al., 2002). At the same time, the concentrations of IB-CoA and propionyl-CoA, the intermediates that are specific to peroxisomal Leu catabolism pathway, are not increased correspondingly, indicating the peroxisomal pathway is not induced due to the sugar starvation. Therefore, the accumulation of IV-CoA and MC-CoA under dark treatment might be only contributed by the induction of mitochondrial Leu catabolism pathway.

Furthermore, Leu is dramatically increased in wild-type plants under dark treatment, and the accumulation of Leu in MCCase mutants is much higher than that of the wild-type plants. Thus the dark treatment and the elimination of MCCase have synergetic effect to Leu accumulation. However, we did not see any significant increase of IB-CoA and propionyl-CoA in MCCase mutants, indicating the peroxisomal pathway does not respond to the block of mitochondrial Leu catabolism pathway. In consequence, the higher demand for Leu catabolism (sugar starvation), the block of the mitochondrial Leu catabolism pathway
(MCCase mutants), and no compensation from peroxisomal pathway, result the plant in hyper accumulation of Leu.

In summary, based on these analyses, we propose that physiological roles of the mitochondrial and peroxisomal Leu catabolism pathways are different. Under normal conditions, both pathways are functioning and the block in the mitochondrial pathway could be balanced by peroxisomal pathway. However, when illumination is withdrawn, the mitochondrial pathway actively responds to the sugar starvation condition, indicating that it has important function for carbon and energy balance when plants are “hungry”. However, peroxisomal pathway is not induced under this condition, and does not compensate the elimination of mitochondrial pathway, indicating the regulation of this pathway is independent from the mitochondrial pathway under this condition.

MATERIALS AND METHODS

Plant Materials

Seed stocks of *Arabidopsis thaliana* wild-type (ecotype Columbia) and T3 T-DNA tagged mutant lines carrying *mcca-1* (SALK_039622), *mcca-2* (SALK_137966) and *mccb-1* (SALK_011463) alleles were obtained from ABRC (Arabidopsis Biological Resource Center, Columbus, OH). The transposon line carrying the *mcca-3* allele (RATM12-2608-1_G) (ecotype Nössen) was obtained from Ds insertion collection at RIKEN BioResource Center (Tokyo, Japan). T2 T-DNA tagged seed stocks carrying the *mccb-2* allele (ecotype Ws) were isolated by screening the Arabidopsis knockout collections at the University of Wisconsin (Krysan et al., 1999; Sussman et al., 2000).
**Growth Conditions**

Seeds were surface-sterilized by treating with 75% (v/v) ethanol for 1 min, and followed by a 10 min treatment with 50% (v/v) bleach containing 0.1% (v/v) Tween 20. After 3 washes with sterile water, seeds were placed in Petri dish (BD Labware, Franklin Lakes, NJ) on Murashige and Skoog (MS) agar solid media (Sigma-Aldrich, St. Louis, MO) containing 1× multivitamin (Sigma-Aldrich) and 1% sucrose. These dishes were first placed at 4 ºC for 2 days in order to break dormancy, and were then transferred to a growth room which was maintained at 22ºC under constant illumination provided by 40 W Sylvania cold-white fluorescent light bulbs at white light irradiation of 150 µmol m⁻² s⁻¹. If needed, seedlings were gently transferred in LC1 Sunshine Mix soil (Sun Gro Horticulture, Canada) 10-12 DAP (including the first 2 days in 4 ºC).

**Plant DNA Extraction, PCR and Segregation Analysis**

Plant DNA extractions and PCR amplifications were performed using the protocols provided by The Arabidopsis Knockout Facility at the University of Wisconsin (Sussman et al., 2000).

The mutant allele that carries a T-DNA or a transposon insertion was detected by a PCR reaction using a gene-specific primer and a T-DNA or transposon border primer. The wild-type allele was detected by a PCR reaction using a pair of gene-specific primers that flank the T-DNA or transposon insertion location. The PCR products were analyzed by agarose gel electrophoresis. All primers sequences were listed in Table 2. The segregation analysis is based on scoring the numbers of the plants from each genotype category. Chi-square test was then performed.
RNA Extraction and RT-PCR Analysis

About 100 mg plant tissues were flash-frozen in liquid N$_2$ and stored at -70°C. The frozen tissue was ground into fine powder with a Kontes pellet pestle (Fisher Scientific, Pittsburgh, PA) in eppendorf tube. Total RNA was extracted with RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The RNA quality was evaluated by agarose gel electrophoresis and the quantity was measured by absorbance at 260 nm and 280 nm. One $\mu$g of RNA was used to synthesize first-strand cDNA using the SuperScript$^\text{TM}$ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer primers provided in the kit. With 18S rRNA as the reference gene, cDNAs were relatively quantified using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) by comparing the intensity of the PCR products after electrophoresis and stained with ethidium bromide. Equal amount of cDNAs were used for further PCRs with gene-specific primers.

Real-Time RT PCR

MS agar media for this experiment contains 1mM D-biotin (USB, Cleveland, Ohio). Whole seedlings of Columbia were collected at 10 DAP, 14 DAP, 17 DAP and 22 DAP in aliquots of 0.1 g. Tissues were flash-frozen in liquid N$_2$ and stored at -70°C. Another set of tissue samples was collected from an independent experiment and used as a biological replicate. The RNA extraction and the synthesis of cDNA were as described. Real-time RT-PCR experiments were performed using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) with GeneAmp 5700 Sequence Detection System (Applied Biosystems). The primers for real-time RT-PCR were designed using Primer Express (Applied Biosystems). 18S rRNA was used as the reference gene. Primer efficiencies were analyzed
on all primer pairs. Quantification method was modified from literatures (Pfaffl, 2001; Muller et al., 2002). The $\Delta C_T$ ($C_T$, threshold cycle) was used to calculate the relative expression level of each transcript.

**Protein Extraction**

The protein extraction procedures were modified from previous paper (Che et al., 2002). The extraction buffer contained 0.3% (v/v) Protease Inhibitor Cocktail for plant cell extracts (Sigma-Aldrich). Protein concentration was determined by Bradford method (Bradford, 1976) with BioRad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

**Protein Electrophoresis and Western Blot Analysis**

Proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984). The membrane was first blocked in 5% (w/v) Instant Nonfat Dry Milk (Nestle, Vevey, Switzerland) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h, and then was incubated with MCCA (Weaver et al., 1995) or MCCB (McKean et al., 2000) antiserum which has been diluted 1:10,000 in 5% milk in TBST for 1 h. Following three times rinses with TBST, the membrane was incubated for 1 h with horseradish peroxidase (HRP) linked anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, United Kingdom), diluted 1:7,500 in 5% milk in TBST. After washing four times with TBST, MCCA and MCCB subunits were detected by ECL Chemiluminescent Detection System (Amersham Biosciences) on X-ray films (Kodak, Rochester, New York).
MCCase Activity Assay

MCCase activity assay was described in Che et al. (2002).

Histochemical Localization of \textit{MCCA::GUS} Transgene

The construction of the \textit{MCCA::GUS} transgene, plant transformation and regeneration, and the histochemical staining were described in Che et al. (2002).

Stereo Microscopy

Elongated fresh siliques at 8 DAF were dissected by removing one ovary wall to reveal the developing seed. Fresh siliques were slit open by using hypodermic fine-pointed needles. Developing seeds were dissected from siliques to collect embryos for examination at 8 DAF. Embryos were dissected by removing seed coats from the seeds and photographed using stereo microscope. Digital images of siliques, mature seeds and embryos were acquired by using an Olympus SZH-10 stereo microscope (Leeds Precision Instruments, Minneapolis, MN) with Zeiss AxioCam HRC digital camera (Carl Zeiss Inc., Thornwood, NY, USA).

Scanning Electron Microscopy (SEM)

Nectary glands and mature embryos were analyzed using SEM. For nectary glands, flowers which just opened were dissected by removing the sepals, petals. For mature embryos, seeds were dissected by removing the seed coats. Nectary and mature embryos were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, under vacuum (pressure: 18 psi Hg) for 7 h at room temperature, and then overnight at 4°C. Fixed samples were washed three times with the same buffer, post-fixed in buffer 1% OsO₄ for 2 h, washed two times in the same buffer followed by deionized water,
and dehydrated in an ethanol series to absolute ethanol and placed in 100% ethanol. Samples were subsequently critical point-dried in a DCP-1 Denton critical point-drying apparatus (Denton Vacuum Inc., Cherry Hill, NJ), and mounted on aluminum stubs with double-sided sticky tape and silver cement. Nectary and embryos were sputter coated with gold and palladium in a Denton Vacuum LLC Desk II Cold Sputter Unit (Denton Vacuum Inc., Moorestown, NJ) for 120 seconds, and viewed with JEOL 5800LV SEM (JEOL, Japan Electron Optics Laboratory, USA Inc., Peabody, MA) at 10 kV.

Amino Acid Analysis

Arabidopsis plants were first grown on MS agar media in Petri dish and later transferred to soil at 10 DAP. At 26 DAP, half of the plants were covered with carton boxes. After two days, rosette leaves of the darkness-treated plants and the plants maintained under constant illumination were immediately harvested under light in aliquot of about 0.05 g. Specifically, for those plants treated with darkness, the tissue collecting was conducted in a timely manner. The maximum time for the darkness-treated samples exposed to light before flash-frozen in liquid N\textsubscript{2} was less than 20 min. After tissue collection, each sample was weighted, flash-frozen in liquid N\textsubscript{2} and stored at -70°C until use. The whole experiment was repeated once as biological replicate.

Plant tissue samples spiked with 4 nmol internal standard (norvaline) were homogenized with 250 µL 10% (w/v) trichloroacetic acid (TCA) (Sigma-Aldrich) using a Kontes pellet pestle. The pestle was washed with an additional 100 µL 10% TCA and the extract (total volume of 350 µL) was incubated at room temperature for 5 min. Following centrifugation at 25,200g for 3 min, the supernatant was transferred to a 2 mL glass vial, the amino acid
extracts were derivatized and analyzed as described in EZ: Faast™ Free (Physiological) Amino Acid Analysis Kit (Phenomenex, Torrance, CA). Amine-containing analytes were analyzed using a GC series 6890 from Agilent (Agilent Technologies, Palo Alto, CA) equipped with an HP-1 silica capillary column (Agilent 19091-60312). The GC was coupled to an Agilent 7863 G2613A auto-sampler and flame ionization detector controlled by MSD ChemStation (D.00.01) software. Hydrogen and synthetic air were used as auxiliary gasses for the FID detector. Analytes were separated at a flow rate of 1.2 mL/min using helium as carrier gas. The injector was held at 250°C. The oven was initially held at 110°C for 1 min, and then ramped to 290 °C at 30 °C/min and held for 7.5 min. Resulting chromatograms were acquired by Agilent’s ChemStation software (D.00.01). Peaks were identified by comparing their retention time to those of the standards. Calibration was conducted using the internal standard and standards mixtures provided by EZ: Faast™. Three replicates at each data point were used to make the standard curves for each amino acid. Concentration (µmol/g FW) of each amino acid was normalized to internal standard and calculated based on standard curve and tissue mass. Statistical analysis was carried out using SAS (SAS Institute Inc. Cary, NC). Student’s t tests were applied on certain comparison.

Acyl-CoA Analysis

Chemicals: Water, methanol (HPLC grade) and all acyl-CoA standards as either sodium or lithium salts were purchased from Sigma-Aldrich. Standards were dissolved in 10 mM formate pH 4.6 ammonium formate (Sigma-Aldrich) and absorbance was measured by spectrometer at 260 nm. Concentration was calculated based on $\varepsilon_{260} = 16400 \text{ M}^{-1} \cdot \text{cm}^{-1}$.
(Dawson et al., 1969). Dilution series were made from acyl-CoA standard mixtures for calibration and quantification.

**Plant tissues:** Tissues for acyl-CoA extraction were same as those for amino acids, except that the dark treatment was applied at 23 DAP and rosette leaves were collected at 25 DAP. About 0.1 g tissue was weighed accurately and flash-frozen in liquid N\(_2\) until use. Three samples of each genotype per mutant allele were collected. The tissue preparation was independently repeated once as biological replication. These two batches of samples were processed at the same time.

**Extraction:** Upon extraction, 20 µL of 7.5 µM 2-butenoyl-CoA as internal standard was added into each sample. Tissues were grinded with a glass homogenizer in 1 mL 10% ice-cold TCA (Fisher Scientific, certified ACS). The homogenizer was washed with another 0.3 mL 10% TCA. The total volume of 1.3 mL extract was transferred into eppendorf tube and centrifuged at 10000 g for 10 min at 4°C. The supernatant was saved and transferred into a 15 ml glass tube. To remove TCA, the supernatant was extracted with ice-cold ethyl ether (Fisher Scientific, certified ACS) for three times. Trace amount of ether was evaporated by blowing a stream of N\(_2\) gas to the sample for 5 min. Extract was filtered with ethanol pre-wet PTFE filter (0.45 µm) (Sigma-Aldrich) into 1.5 mL eppendorf tube. The liquid was flash-frozen in liquid N\(_2\), and then lyophilized overnight without heat using a SpeedVac system (Savant instruments, Farmingdale, NY). The resulting pellet with a color of dark brown was dissolved by vigorous vortex in 100 µL 10 mM ammonium formate (pH 4.6). The 100 µL extract was stored at -70°C until injection. For each sample, 20 µL was injected and each sample was injected and analyzed three times.
LC-MS/MS: The LC-MS/MS analysis was modified from (Perera et al., 2008). The analysis was performed on an Agilent 1100 Series HPLC system coupled with a diode array detector and an Agilent MSD Ion trap mass spectrometer (model SL) (Agilent Technologies). HPLC was carried out with an Ascentis® C18 (10 cm x 2.1 mm, 3 µm) column, equipped with an Ascentis® Supelguard™ C18 (2 cm x 4.0 mm, 5 µm) pre-column (Sigma-Aldrich). The columns were maintained at 25°C. The auto sampler was maintained at 4°C. The LC mobile phases were 90% 10 mM ammonium formate, 10% methanol (solvent A), and 10% 10 mM ammonium formate, 90% methanol (solvent B); both were filtered with bottle-top vacuum filter equipped with Nylon membrane (Corning Incorporated, Corning, NY). The original 10 mM ammonium formate was adjusted to pH 4.6, before making solvent A and B. The eluting gradient was as follows: 0-4 min 100% solvent A: 0% solvent B; 4-12 min linear gradient to 0% solvent A: 100% solvent B; 12-25 min maintained at 0% solvent A: 100% solvent B; 25-30 min linear gradient to 100% solvent A: 0% solvent B. After each sample, a 12 min post run in 100% solvent A: 0% solvent B was applied. The flow rate was 0.2 mL/min. Positive mass spectrometry was performed using the following parameters: source voltage at 3.5 kV, skimmer at 40.0 V, capillary exit at 153.9 V, nebulizer pressure at 20 psig, drying gas (nitrogen) temperature at 350°C and flow rate at 10 L/min. Helium was used as the collision gas. In MS/MS mode, parent ions were isolated at a bandwidth of 4 AMU and after 2 ms, fragmented at amplitude of 1.8 V for HMG-CoA and 1.5 V for all other acyl-CoAs, and scanned between 200 and 1200 m/z. The collision energy was optimized for neutral loss and multiple-reaction monitoring (MRM). All the MS/MS experiment were using 30,000 ions or 300 ms in ion charge control. Acyl-CoAs were identified by 3 ions. They are 2
characteristic ions for acyl-CoAs, i.e., 428 and 410, and one characteristic ion for individual acyl-CoA listed in Table 7. The detection limit for acyl-CoAs is also listed in Table 7.

Quantification: Calibration curves of each acyl-CoA standard were generated (Figure 11A). The concentration of acyl-CoAs from plant samples were normalized against internal standard, and divided by tissue fresh weight.

REFERENCES


**Figure 1.** The mitochondrial and peroxisomal Leu catabolism pathways. The two pathways are summarized from Anderson et al. (1998) and Gerbling and Gerhardt (1989). The common acyl-CoA intermediates shared by both pathways are shaded with yellow boxes. The acyl-CoA intermediates that are unique in mitochondria pathway are shaded with orange boxes. The acyl-CoA intermediates that are unique in peroxisomal pathway are shaded with green boxes. The arrows with solid lines represent a single reaction. The arrows with dashed lines in peroxisomal pathway represent modified β-oxidation sequences. HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA. IB-CoA, isobutyryl-CoA. IV-CoA, isovaleryl-CoA. MC-CoA, 3-methylcrotonyl-CoA. MG-CoA, 3-methylglutaconyl-CoA.
Figure 2. Molecular characterization of *mcca* and *mccb* mutant alleles.
Figure 2. (Continued).

(A) Schematic representation of the molecular structure of the *MCCA* and *MCCB* loci in the mutant alleles. Solid boxes represent the exons and the open boxes represent the 5’ and 3’ UTRs. Thick lines represent introns. The stripped box in *MCCA* gene represents exon 6 which can undergo alternative splicing. The triangles represent the T-DNA or transposon insertions. The T-DNA and transposon insertions are not to scale. The vertical lines linking the triangles to the genes show the position of insertions. Deletions associated with the insertion of the T-DNA or transposon in the *meca*-1, *mccb*-1 and *mccb*-2 alleles are 15-bp, 12-bp and 19-bp, respectively. The dashed vertical line for the *meca*-2 allele indicates the 3’ border of the insertion is unknown. The arrowheads on *meca*-3 allele shows that the transposon insertion caused a 10-bp tandem repeat. Arrows under each gene structure show the positions and direction of 5’ and 3’ primers used for genotyping PCR and RT-PCR. The dashed line in some of these primers indicates that the primer sequence is composed of sequences from two adjacent exons without the sequence of the intervening intron.

(B) RT-PCR analysis on *mcca* and *mccb* mRNAs in *meca*-1, *meca*-2, *mccb*-1, *mccb*-2 mutant alleles and *mcca*-2/*meca*-3 heteroallelic mutants. RNA was isolated from aerial parts of 40-50 DAP sibling plants that carry either *MCCA* and *MCCB* wild-type alleles (WT), or *mcca* or *mccb* mutant alleles (Mu). Primers used in PCR for *MCCA* and *MCCB* transcripts are indicated. Primers used for 18s rRNA are 18SFW and 18SRV. Sequences of primers are shown in Table 2.

(C) Western blot analysis of MCCA and MCCB subunits in *meca*-1, *meca*-2, *mccb*-1, *mccb*-2 mutant alleles and *mcca*-2/*meca*-3 heteroallelic mutants. All proteins were extracted from sibling plants. Aliquots of protein extract from 12 DAP seedlings containing 20 µg proteins (lanes 1-8 and 13-20) or from aerial parts of 40-50 DAP plants containing 10 µg proteins (lanes 9-12) were subject to SDS-PAGE. After electrophoresis, gels were either stained with Coomassie Blue (lanes 1-4, 9, 10, 13-16) to show equal loading, or transferred to nitrocellulose membranes. Western blot analysis was conducted using with anti-MCCA serum (lanes 5-8, 11 and 12) or anti-MCCB serum (lanes 17-20).
Figure 3. The effect of *mcca* alleles on *MCCB* gene expression and the effect of *mccb* alleles on *MCCA* gene expression.

(A) RT-PCR analysis of *MCCB* and *MCCA* mRNA accumulation in stocks carrying *mcca-1*, *mcca-2*, *mccb-1*, *mccb-2* mutant alleles and the *mcca-2/mcca-3* heteroalleles. RNA was isolated from aerial parts of 40-50 DAP sibling plants that carry either *MCCA* and *MCCB* wild-type alleles (WT), or *mcca* or *mccb* mutant alleles (Mu). 18S rRNA as positive control is shown in Figure 2B. Primer pairs used in PCR for *MCCA* and *MCCB* transcripts are indicated. Sequences of primers are shown in Table 2.

(B) Western blot analysis of MCCA and MCCB subunits accumulation in stocks carrying *mcca-1*, *mcca-2*, *mccb-1*, *mccb-2* mutant alleles and the *mcca-2/mcca-3* heteroalleles. All proteins were extracted from sibling plants. Aliquots of protein extract from 12 DAP seedlings containing 20 µg proteins (lanes 1-4 and 7-10) or from aerial parts of 40-50 DAP plants containing 10 µg proteins (lanes 5 and 6) were subject to SDS-PAGE. After electrophoresis, gels transferred to nitrocellulose membranes. Western blot analysis was conducted using anti-MCCA serum (lanes 7-10) or anti-MCCB serum (lanes 1-6). Equal loading among samples is shown in Figure 2C by Coomassie Blue staining.
Figure 4. Relative amount of the two *MCCA* mRNAs.

(A) Schematic representation of the alternative splicing of exon 6 of *MCCA*. Solid boxes represent exons and thick lines represent introns (only shows part of the full length mRNA from exon 5 to exon 7). Thin lines represent the splicing events. Positions of the primers used in RT-PCR assays for the two transcripts are indicated by arrows. Sequences of primers are shown in Table 2.

(B) mRNAs from seedlings of wild-type Columbia were collected at 10 DAP, 14 DAP, 17 DAP and 22 DAP. The accumulation of *MCCA-L* and *MCCA-S* was measured by real-time RT-PCR and normalized to 18S rRNA. Each value represents the mean of two biological replicates. And each biological sample was measured three times. The error bars represent ± SE. The number on each comparison represents the ratio of the amount of *MCCA-L* to the amount of *MCCA-S*.
Figure 5. Histochemical localization of $MCC-A::GUS$ transgene in Arabidopsis.

(A) Flower buds.

(B) Mature flowers.

(C) Siliques of different age.

(D) The base of a silique.
Figure 6. Phenotypic changes of *mcca* and *mccb* mutants.
Figure 6. (Continued).

Representative wild-type (WT, ■) or homozygous mutant (Mu, □) siblings from plant carrying mcca-2/mcca-3 (A), mccb-1/mccb-2 (B), and mcca-1 mutant alleles (C).

(D) and (E) Siliques of the mcca-1 mutant are shorter and elongate at heterogeneous rates, as compared to wild-type siblings. Flower buds of wild-type and homozygous mcca-1 mutant sibling lines were tagged at 1 DAF using a loop of thread without disturbing the growth. Starting at 3 DAF the lengths of developing siliques were carefully measured using a mechanical caliper. The measurement was carried out till the siliques started to dry. Number of the siliques measured is represented, and number of the plants from which the siliques were selected is represented in parentheses. (D) Data from individual siliques. (E) Average of silique development. Bars represent ± SE.

(F) Bolt height. The height of individual plants was measured at maturity when the plants were completely dry. Student’s t test indicates that there is no significant difference between the wild-type and mutant siblings. Number of plants analyzed is represented. Bars represent ± SE.

(G) Length of siliques at 8 DAF. Flower buds were tagged individually using a loop of thread at 1 DAF. Length of growing siliques was measured at 8 DAF using a mechanical caliper. Student t test indicates that the difference between wild-type and mutant siblings is significant. *, p <0.05; **, p <0.01. Number of siliques measured is represented, and number of plants from which the siliques were collected is represented in parentheses. Bars represent ± SE.
Figure 7. *meca* and *mceb* mutants have complex phenotypes associated with siliques, embryos, seeds and nectary glands.
Figure 7. (Continued).

(A) to (D) 8 DAF siliques of wild-type (WT) (A) and mutant siblings (Mu) ([B] to [D]) of *mcca-1* allele. White bars represent 0.5 mm in (A) to (N).

(E) and (F) 8 DAF siliques of wild-type (E) and mutant siblings (F) of *mcca-2* allele.

(G) to (L) 8 DAF embryos of wild-type (G) and mutant siblings ([H] to [L]) of *mcca-2* allele. Embryos were collected from freshly dissected siliques.

(M) and (N) Stereo micrographs of mature seeds of wild-type (M) and homozygous mutant siblings (N) of *mcca-2* allele.

(O) and (P) Scanning electron micrographs of embryos of mature seeds from wild-type siblings (O) and homozygous mutant (P) of *mcca-2* allele. Seed coat has been removed. Yellow bars represent 0.1 mm.

(Q) Scanning electron micrograph of the base of a wild-type flower shows the locations of lateral stamen (lst), medial stamen (mst), lateral nectary gland (ln), and medial nectary gland (mn). Flowers were collected when they just opened. Petals were removed.

(R) and (S) Higher magnification of the medial nectary glands of mature flowers from wild-type (R) and mutant sibling (S) of *mcca-2*. s, stomata. Black bars represent 0.1 mm or 0.02 mm as indicated.
Figure 8. Comparisons of wild-type and mutant seeds in length, weight of individual seeds, and seed yield per plant.

(A) The length of the seeds of wild-type (■) or homozygous mutant (□) of mcca-1, mcca-2, mccb-1 and mccb-2 alleles. The length was measured at seeds’ longest diameter under stereo microscope using AxioVision. Number of the seeds measured is indicated, and the number of the plants from which the seeds were collected is represented in the parentheses. Bars represent ± SE.
Figure 8. (Continued).

(B) The weight of the seeds of wild-type and homozygous mutant of mcca-1, mcca-2, mccb-1 and mccb-2 alleles. Single seed weight was calculated based on the total weight of the entire seed batch (normally 500 seeds) and the actual number of the seeds. The average values of single seed weight ± SE are presented. Number of the seeds measured is indicated, and the number of the plants from which the seeds were collected is represented in the parentheses. Bars represent ± SE.

(C) Seed yield of wild-type and mutant of mcca-1, mcca-2, mccb-1 and mccb-2 alleles. Seeds of individual plants were collected when plants were completely dry. Number of plants analyzed is presented. Bars represent ± SE.

Student’s t tests were performed between wild-type and mutant siblings within each allele for (A) to (C). *, p<0.05; **, p <0.01.
Figure 9. Germination defect of *mcca* and *mccb* mutants.
Figure 9. (Continued).

(A) Germination rate of wild-type (■) or homozygous mutant (□) plants. Seed batches from sibling plants of
mcca-1, mcca-2, mccb-1 and mccb-2 alleles were obtained from several heterozygous mutants. Seed batches
from sibling plants of hetero-allelic mutants (mcca-1/mcca-2, mcca-2/mcca-3 and mccb-1/mccb-2) are F2 plants
from the allelic crosses. About 100 seeds from each seed batch were randomly selected and planted on MS agar
media plates after cold treatment to break dormancy. Germination rates were recorded at 8 DAP. The numbers
of seed batches for each line were indicated. Student’s t tests were performed to test if the difference between
wild-type and mutant is significant. **, p <0.01. Bars represent ± SE.

(B) to (E) Removing seed coat does not rescue the germination defect of mccb-1 mutants. Seeds of wild-type
and mutant siblings were sterilized and soaked in water for 4 hours. Half of the seeds were placed directly on
MS agar media ([B] and [C]). Another half of the seeds were dissected under microscope and seed coats were
carefully removed without disturbing the embryos ([D] and [E]). Embryos were then transferred immediately
onto the MS agar media. All plates were treated in 4°C for 2 days and then transferred into normal growth
condition. Germinated seedlings were scored at 8 DAP as shown in the table. Experiment has been repeated and
has similar result.
**Figure 10.** Effect of light deprivation on amino acid accumulation in *mcca* and *mccb* mutants.

Sibling plants that are wild-type (■) and *mcca-1* and *mccb-1* mutants (□) were grown on MS agar media for 10 days before transferring to soil. At 26 DAP, plants were either covered with carton box to withhold illumination for 2 days (shaded area), or kept under illumination (open area). Free amino acids (not from proteins) were extracted at 28 DAP and subjected to GC-FID analysis. Number of the plants analyzed is indicated. Bars represent ± SE. See Table 8 for *p*-value of comparisons.

(A) Total amino acid concentration. Calculation based on sum of all amino acids (Arg and Cys are not included). Sample sizes were indicated. FW, fresh weight.

(B) Free Leu concentration. Sample sizes are same as (A).
Figure 11. Effect of light deprivation on acyl-CoA accumulation in meca and mec b mutants.
Figure 11. (Continued).
Sibling plants that are wild-type (■) and mcca-1 and mccb-1 mutant (□) were grown on MS agar media for 10 days before transferring to soil. At 23 DAP, plants were either covered with carton box to withhold illumination for 2 days (shaded area), or kept under normal illumination (open area). Acyl-CoA esters were extracted at 25 DAP and subjected to LC-MS/MS analysis. Student’s t tests were formed. See Table 10 for p-value of comparisons. ISTD, internal standard. HMG, 3-hydroxy-3-methyl-glutaryl-CoA. IB-CoA, isobutyryl-CoA. IV-CoA, isovaleryl-CoA. MC, 3-methylcrotonyl-CoA.

(A) The calibration curves of acyl-CoA standards. Curves were fitted by the equations shown on the table.
(B) to (G) Concentration of acyl-CoAs in wild-type (■) or mutant (□) siblings of mcca-1 and mccb-1 lines with or without dark treatment. FW, fresh weight. Bars represent ± SE. a, average of 5 samples. b, average of 4 samples. All other cases, average of 6 samples.
Table 1. Characterization of T-DNA and transposon tagged lines of MCCA and MCCB genes.

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<td>mccb-2</td>
<td>3</td>
<td>mccb-2/MCCB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-2/mccb-2</td>
</tr>
</tbody>
</table>

*The H₀ for all Chi-square tests is proposed as: wild-types: heterozygous mutants: homozygous mutants = 1:2:1.*
Table 4. Allelic crosses of *mcca* and *mccb* mutants.

<table>
<thead>
<tr>
<th>Crosses (♀ x ♂)</th>
<th>Number of crosses</th>
<th>F1 Genotype</th>
<th>Number of plants in each category</th>
<th>Chi-square</th>
<th>p-value of Chi-square test</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcca-1 x mcca-2</td>
<td>2</td>
<td>MCCA/MCCA</td>
<td>20</td>
<td>0.27</td>
<td>0.966</td>
<td>Failed to reject H₀&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-1/MCCA</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-2/MCCA</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-1/mcca-2</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcca-2 x mcca-1</td>
<td>2</td>
<td>MCCA/MCCA</td>
<td>7</td>
<td>2.00</td>
<td>0.572</td>
<td>Failed to reject H₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-1/MCCA</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-2/MCCA</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-1/mcca-2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcca-2 x mcca-3</td>
<td>2</td>
<td>MCCA/MCCA</td>
<td>17</td>
<td>5.78</td>
<td>0.123</td>
<td>Failed to reject H₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-2/MCCA</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-3/MCCA</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-2/mcca-3</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcca-3 x mcca-2</td>
<td>3</td>
<td>MCCA/MCCA</td>
<td>8</td>
<td>1.73</td>
<td>0.630</td>
<td>Failed to reject H₀</td>
</tr>
<tr>
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<td>mcca-2/MCCA</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-3/MCCA</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mcca-2/mcca-3</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mccb-1 x mccb-2</td>
<td>2</td>
<td>MCCB/MCCB</td>
<td>6</td>
<td>2.37</td>
<td>0.499</td>
<td>Failed to reject H₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-1/MCCB</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-2/MCCB</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-1/mccb-2</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mccb-2 x mccb-1</td>
<td>2</td>
<td>MCCB/MCCB</td>
<td>8</td>
<td>5.04</td>
<td>0.169</td>
<td>Failed to reject H₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-1/MCCB</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-2/MCCB</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mccb-1/mccb-2</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The H₀ for all Chi-square tests is proposed as: wild-types: heterozygous mutants: heterozygous mutants: heteroallelic mutants = 1:1:1:1.
Table 5. Germination rate comparison between the seeds of heterozygous mutant parent and the seeds of homozygous mutant parent from *mcca-1*, *mcca-2* and *mccb-1* alleles.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of parent plants</th>
<th>Total seeds</th>
<th>Total germinate</th>
<th>Germination rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mcca-1/MCCA</em></td>
<td>5</td>
<td>664</td>
<td>606</td>
<td>91%</td>
</tr>
<tr>
<td><em>mcca-1/mcca-1</em></td>
<td>5</td>
<td>582</td>
<td>13</td>
<td>2%</td>
</tr>
<tr>
<td><em>mcca-2/MCCA</em></td>
<td>2</td>
<td>239</td>
<td>228</td>
<td>95%</td>
</tr>
<tr>
<td><em>mcca-2/mcca-2</em></td>
<td>2</td>
<td>232</td>
<td>8</td>
<td>3%</td>
</tr>
<tr>
<td><em>mccb-1/MCCB</em></td>
<td>3</td>
<td>389</td>
<td>305</td>
<td>78%</td>
</tr>
<tr>
<td><em>mccb-1/mccb-1</em></td>
<td>3</td>
<td>383</td>
<td>18</td>
<td>5%</td>
</tr>
</tbody>
</table>
Table 6. Germination rate of seeds from the germinated parent plants.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of parent plants</th>
<th>Total seeds</th>
<th>Total germinate</th>
<th>Germination rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCCA/MCCA</td>
<td>2</td>
<td>160</td>
<td>151</td>
<td>93.7%</td>
</tr>
<tr>
<td>mcca-1/mcca-1</td>
<td>2</td>
<td>171</td>
<td>33</td>
<td>16.2%</td>
</tr>
<tr>
<td>MCCA/MCCA</td>
<td>2</td>
<td>137</td>
<td>132</td>
<td>95.8%</td>
</tr>
<tr>
<td>mcca-2/mcca-2</td>
<td>2</td>
<td>207</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCCB/MCCB</td>
<td>2</td>
<td>91</td>
<td>59</td>
<td>65.7%</td>
</tr>
<tr>
<td>mccb-1/mccb-1</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCCB/MCCB</td>
<td>2</td>
<td>99</td>
<td>78</td>
<td>80.4%</td>
</tr>
<tr>
<td>mccb-2/mccb-2</td>
<td>2</td>
<td>96</td>
<td>0</td>
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</tr>
</tbody>
</table>
Table 7. The list of acyl-CoA analyzed and their molecular ions, characteristic ions and detection limit. HMG, 3-hydroxy-3-methyl-glutaryl-CoA. IB-CoA, isobutyryl-CoA. IV-CoA, isovaleryl-CoA. MC, 3-methylcrotonyl-CoA. ISTD, internal standard.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular ion [m + H]</th>
<th>Characteristic daughter ion [m + H] – 507&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Detection limit (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>811</td>
<td>303</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>2-Butenoyl-CoA (ISTD)</td>
<td>837</td>
<td>329</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>912</td>
<td>405</td>
<td>10</td>
</tr>
<tr>
<td>IB-CoA</td>
<td>839</td>
<td>331</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>IV-CoA</td>
<td>853</td>
<td>345</td>
<td>5.0</td>
</tr>
<tr>
<td>MC-CoA</td>
<td>851</td>
<td>343</td>
<td>5.0</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>825</td>
<td>317</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> 507 is the ion that has been lost from the fragmentation.
Table 8. Comparisons among samples from amino acid analysis.
Student’s *t* tests were carried out to test the indicated comparisons. WT, wild-type. Mu, homozygous mutant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Comparison</th>
<th>p-value</th>
<th>Leu</th>
<th>Total amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>No</td>
<td>WT</td>
<td>Darkness</td>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<td></td>
</tr>
<tr>
<td>Mu</td>
<td>No</td>
<td>Mu</td>
<td>Darkness</td>
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<td>0.0003</td>
<td>0.0004</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>MCCB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>No</td>
<td>WT</td>
<td>Darkness</td>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mu</td>
<td>No</td>
<td>Mu</td>
<td>Darkness</td>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCCA</td>
<td></td>
<td></td>
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<td>Mu</td>
<td>No</td>
<td></td>
<td>0.2114</td>
<td>0.0545</td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>MCCB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>No</td>
<td>Mu</td>
<td>No</td>
<td></td>
<td>0.0291</td>
<td>0.0634</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>MCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Darkness</td>
<td>Mu</td>
<td>Darkness</td>
<td></td>
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<td>0.0240</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCCB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Darkness</td>
<td>Mu</td>
<td>Darkness</td>
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<td>&lt;.0001</td>
<td>0.0459</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* The gene on which the mutation resides.

*b* The p-values that are smaller than 0.05 are in boldface.
Table 9. Correlation between leucine and other amino acid.
Amino acids were listed in a descending order of the Pearson’s correlation coefficient with Leu. Pearson’s correlation coefficient and p-value were calculated by SAS.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Pearson's correlation coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILE</td>
<td>0.95</td>
<td>&lt;.0001a</td>
</tr>
<tr>
<td>VAL</td>
<td>0.85</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>MET</td>
<td>0.83</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TRP</td>
<td>0.79</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>HIS</td>
<td>0.79</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>PHE</td>
<td>0.78</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TYR</td>
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<td>&lt;.0001</td>
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<tr>
<td>ASN</td>
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<td>&lt;.0001</td>
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<tr>
<td>LYS</td>
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<td>&lt;.0001</td>
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<tr>
<td>SER</td>
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<td>&lt;.0001</td>
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<td>-0.07</td>
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</tr>
<tr>
<td>PRO</td>
<td>-0.09</td>
<td>0.4381</td>
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<tr>
<td>ALA</td>
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<tr>
<td>GLU</td>
<td>-0.24</td>
<td>0.0395</td>
</tr>
<tr>
<td>ASP</td>
<td>-0.26</td>
<td>0.0252</td>
</tr>
</tbody>
</table>

*a The p-values that are smaller than 0.05 are in boldface.
Table 10. Comparisons among samples from acyl-CoA analysis.

Student’s t tests were applied to acyl-CoA concentrations with the comparisons indicated in the table. WT, wild-type. Mu, homozygous mutant. AC, acetyl-CoA. HMG, 3-hydroxy-3-methyl-glutaryl-CoA. IB, isobutyryl-CoA. IV, isovaleryl-CoA. MC, 3-methylcrotonyl-CoA. PC, propionyl-CoA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Treatment</td>
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<tr>
<td>MCCA</td>
<td>WT</td>
<td>No</td>
</tr>
<tr>
<td>MCCB</td>
<td>WT</td>
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</tr>
<tr>
<td>MCCA</td>
<td>Mu</td>
<td>No</td>
</tr>
<tr>
<td>MCCB</td>
<td>Mu</td>
<td>No</td>
</tr>
<tr>
<td>MCCA</td>
<td>WT</td>
<td>No</td>
</tr>
<tr>
<td>MCCB</td>
<td>WT</td>
<td>No</td>
</tr>
<tr>
<td>MCCA</td>
<td>WT</td>
<td>Darkness</td>
</tr>
<tr>
<td>MCCB</td>
<td>WT</td>
<td>Darkness</td>
</tr>
</tbody>
</table>

*a* The gene on which the mutation resides.

*b* The p-values that are smaller than 0.05 are in boldface.
CHAPTER III BIOTIN CARBOXYL CARRIER PROTEIN-LIKE PROTEINS IN ARABIDOPSIS

A manuscript to be submitted to Plant Physiology

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¹Department of Biochemistry, Biophysics and Molecular Biology and ²Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011

ABSTRACT

Biotin-containing enzymes are involved in metabolic reactions that are essential for normal cellular functions in all organisms. In plants, four biotin-containing enzymes have been identified to date; they are homomeric acetyl-CoA carboxylase (hmACCcase), heteromeric acetyl-CoA carboxylase (htACCcase), 3-methylcrotonyl-CoA carboxylase (MCCase), and geranoyl-CoA carboxylase (GCCase). Lipoic acid, another enzyme cofactor, plays a pivotal role in energy metabolism. Lipoic acid is required for enzyme activities in multienzyme complexes, i.e., α-keto acid dehydrogenase complexes and glycine decarboxylase complex. Using bioinformatic approaches, we found that three novel Arabidopsis proteins may represent novel biotin- or lipoate-containing proteins in plants. These proteins were named biotin carboxyl carrier protein-like (BCCPL) proteins and they
share high amino acid sequence similarities. Immunological analysis demonstrates that BCCPL proteins are indeed expressed in plants. However, affinity chromatography purification indicates that these proteins are not biotinylated, nor lipopectylated in plants. We have recovered and characterized T-DNA knockout alleles of BCCPL genes, yet no observable phenotypic changes were found. The biochemical and physiological functions of BCCPLs remains unclear.

INTRODUCTION

Biotin and Biotin-Containing Proteins

Biotin, also known as Vitamin H, is an essential water-soluble molecule required by all organisms for a series of metabolic processes that are thought to be indispensable to normal cellular functions, including fatty acid biosynthesis, amino acid metabolism, gluconeogenesis, lipogenesis and secondary metabolism. Biotin can be synthesized in plants and a subset of microbes in the biosphere. Many archaea, bacteria, fungi and all animals, including humans, must uptake biotin from the environment or diets for normal growth and development. On biotin-containing proteins, biotin carboxyl group is covalently bound to the ε amino group of a specific lysine (Lys) residue located within a conserved -(A/I/V)MK(L/M/A/T)-biotinylatation motif via an amide linkage (Chapman-Smith and Cronan, 1999). This covalent attachment is catalyzed by holocarboxylase synthetase, also known as biotin protein ligase (Chapman-Smith and Cronan, 1999).
Based on the chemical nature of the substrates, biotin-containing enzymes can be divided into three classes: carboxylases, decarboxylases, and transcarboxylases (Moss and Lane, 1971). Although each enzyme catalyzes distinct metabolic reactions, a common biochemical mechanism is shared by all biotin-containing enzymes. Biotin prosthetic group plays a role as an intermediate carrier of the activated carboxyl group that is being transferred from donor substrate to acceptor substrate (Moss and Lane, 1971). The overall reaction catalyzed by biotin-containing enzymes is carried out in two steps. The first step is the carboxylation of biotin on the enzyme and the second step is the transfer of the carboxyl group from biotin-containing enzyme to the acceptor substrate (Knowles, 1989).

All biotin-containing enzymes have three distinctive functional domains: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyl transferase (CT). BC domain is responsible for catalyzing the first step reaction, and CT domain is required for the second step. BCCP, a structural domain, provides the conserved Lys residue to which the biotin prosthetic group is attached and is thought to swing between the BC and the CT domains. To date, four biotin-containing enzymes have been characterized in plants: homomeric acetyl-CoA carboxylase (hmACCase) (Harwood, 1988; Konishi and Sasaki, 1994; Yanai et al., 1995; Konishi et al., 1996), heteromeric acetyl-CoA carboxylase (htACCase) (Sasaki et al., 1993; Konishi and Sasaki, 1994; Choi et al., 1995; Konishi et al., 1996), 3-methylcrotonyl-CoA carboxylase (MCCase) (Alban et al., 1993; Chen et al., 1993; Diez et al., 1994; Song et al., 1994; Wang et al., 1994; Weaver et al., 1995; Anderson et al., 1998; McKean et al., 2000), and geranoyl-CoA carboxylase (GCCase) (Guan et al., 1999). Also, plants contain a specific seed storage biotin-protein (SBP) that is non-catalytic and thought to play a role in storing biotin (Duval et al., 1994). Interestingly, although biotin is
still attached to a Lys residue, the biotinylation motif of SBP differs markedly from that of biotin enzymes (Duval et al., 1994). In addition, there are indications that additional biotin-containing enzymes, i.e., propionyl-CoA carboxylase and pyruvate carboxylase, may be present in the plant kingdom (Wurtele and Nikolau, 1990). Yet the actual proteins have not been identified, which raises the possibility that plants may have novel biotin-containing proteins.

**Lipoic Acid and Lipoylate-Containing Proteins**

Lipoic acid, another enzyme cofactor, plays a pivotal role in energy metabolism. It was discovered in the 1930s as a growth factor of certain bacteria, and first isolated by Reed and coworkers in the 1950s (Reed et al., 1951). Lipoic acid is required for enzyme activities in multienzyme complexes which are known as α-keto acid dehydrogenase complexes (Reed et al., 1951; Reed, 1998) and glycine decarboxylase complex (Douce et al., 1994; Pares et al., 1994). Like biotin, the carboxyl group of lipoic acid is covalently linked to the ε amino group of a specific Lys through an amide bond. This Lys residue is located in the -DKA- motif which is conserved in all lipoylate-containing proteins (Reed and Hackert, 1966; Reche et al., 1998). Similar to biotin prosthetic group which serves as an intermediate carrier of carboxyl group, the lipooyl domain plays a role as an acyl group carrier in α-keto acid dehydrogenase complexes or as an aminomethyl group carrier in glycine decarboxylase complex (Reche and Perham, 1999).

Three types of complexes, pyruvate dehydrogenase complex (PDC), α-ketoglutarate dehydrogenase complex (KGDC) and branched-chain keto acid dehydrogenase complex (BCKDC), constitute the α-keto acid dehydrogenase complex family, which is among the
largest nonviral protein assemblies (Mooney et al., 2002). All complexes consist of three components: α-keto acid dehydrogenase/decarboxylase (E1), dihydrolipoyl acyltransferase (E2), and dihydrolipoyl dehydrogenase (E3). Lipoylated E2 is the core component of the complexes, with E1 and E3 non-covalently attached to it (Reed and Hackert, 1990; Berg and de Kok, 1997). Plant cells uniquely contain two forms of PDCs, which are mitochondrial PDC (mtPDC) and plastidial PDC (ptPDC) (Reid et al., 1977; Mooney et al., 1999). mtPDC utilizes pyruvate to generate acetyl-CoA which links glycolysis to the Krebs cycle, whereas the ptPDC catalyzes the same reaction but acetyl-CoA produced is used for de novo fatty acid biosynthesis in plastids (Mooney et al., 2002). The KGDC catalyzes oxidative decarboxylation of α-ketoglutarate to produce succinyl-CoA, CO₂, and NADH. This reaction is located in mitochondria as part of the Krebs cycle (Millar et al., 1999). The BCKDC is involved in catabolism of branched-chain amino acids (BCAAs), i.e., Leu, isoleucine (Ile), and valine (Val). BCKDC catalyzes the oxidative decarboxylation of branched-chain α-keto acids which were generated by transamination reactions (Harper et al., 1984). In plants, it has been suggest that BCKDC activities present in both mitochondria and peroxisomes (Gerbling and Gerhardt, 1989; Anderson et al., 1998), providing evidence that BCAAs catabolism might occur in two cell compartments.

In mitochondria, the glycine decarboxylase complex (GDC) and the serine hydroxymethyltranserase catalyze two sequential reactions, which convert two glycine molecules into one each of serine, CO₂ and NH₃, with the reduction of NAD⁺ to NADH (Oliver, 1994). These reactions are considered to interconnect the metabolism of one-, two- and three-carbon compounds and are essential for photorespiration (Kikuchi, 1973; Oliver, 1994). The GDC consists of four different components, the P-, H-, T- and L-proteins. H-
protein is the lipoylate-containing protein, and the linkage between the lipoyl moiety to H-protein provides a flexible arm which commutes successively to other three proteins (Douce et al., 1994; Oliver, 1994; Douce et al., 2001).

In this paper, three novel Arabidopsis proteins which share high sequence similarity with the BCCP subunit of the Arabidopsis htACCase were identified and named as biotin carboxyl carrier protein-like (BCCPL) proteins. These proteins may represent novel biotin-containing or lipoylate-containing proteins in plants. cDNA of BCCPL genes were expressed in Escherichia coli and polyclonal antisera against BCCPL proteins were generated. Immunological analysis demonstrated that these proteins are indeed expressed in plants, but they are not biotinylated or lipoylated. Plants carrying mutations in each of these BCCPL genes appear to grow normally, as well as a double mutant of two BCCPL genes. To date the biochemical and physiological functions of these proteins are still not clear. To further investigate BCCPL proteins, double and triple mutants are being generated, which may give new insights into the physiological function of these genes.

RESULTS

Identification of BCCPL Proteins from GenBank and Bioinformatic Analysis on BCCPLs

We undertook a bioinformatic approach to find novel biotin-containing proteins in Arabidopsis. We used the amino acid sequence of CAC1A (At5g16390, also called BCCP1), which is the BCCP subunit of the Arabidopsis htACCase, to perform “BLASTP” at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Besides CAC1B (At5g15530, also called BCCP2), a paralog of CAC1A, there are three other
novel proteins showing high similarity to CAC1A. We named these proteins BCCPL which stands for BCCP-like proteins. The genes encoding these three proteins are At1g52670 (BCCPL1), At3g15690 (BCCPL2) and At3g56130 (BCCPL3). It is also indicated in GenBank that the mRNAs of BCCPL2 and BCCPL3 might undergo alternative splicing. In this paper we only consider the longest transcripts of both genes without further notification.

BCCPL proteins are annotated in GenBank as “biotin carboxyl carrier protein of acetyl-CoA carboxylase-related” or “biotin/lipoyl attachment domain-containing protein”, suggesting that these proteins might be biotinylated and/or lipoylated. A rooted phylogenetic tree was generated by PHYLIP (Felsenstein, 1989) using amino acid sequences of BCCPL proteins, biotin-containing proteins including CAC1A, CAC1B, MCCA (At1g03090, the biotinylated subunit of MCCase) (Weaver et al., 1995), and ACC1 (At1g36160, hmACCCase) (Baud et al., 2003), and lipoylate-containing proteins including LTA1(At3g06850, E2 of BCKDC) (Mooney et al., 1998), LTA2 (At3g25860, E2 of ptPDC) (Mooney et al., 1999), and LTA3 (At3g52200, E2a of mtPDC) (Heazlewood et al., 2004; Taylor et al., 2004; Ewald et al., 2007) (Figure 1). The phylogenetic tree shows that BCCPL proteins are clustered closer to biotin-containing proteins rather than lipoylate-containing proteins, suggesting that the function of BCCPL proteins is more likely to be close to biotin-containing proteins.

The amino acid sequences of BCCPL proteins share high similarity with each other, especially between BCCPL1 and BCCPL2 (Table1). The theoretical molecular weight of full length BCCPL proteins is between 28 and 30 kDa (http://www.expasy.ch/tools/pi_tool.html). The subcellular localizations of these proteins are mitochondria or chloroplast, which is predicted by bioinformatic softwares (TargetP [http://www.cbs.dtu.dk/services/TargetP/], Predotar [http://urgi.versailles.inra.fr/predotar/predotar.html] and PSORT
The predicted molecular weight of the mature BCCPL proteins without the transit peptide is about 23 kDa.

Both biotin-containing proteins and lipoylate-containing proteins have a conserved biotinylation or lipoylation motif, respectively, and these motifs are well conserved. To search the possible motifs on BCCPL proteins, multiple sequence alignment was conducted using CLUSTAL W (Thompson et al., 1994) on biotin-containing proteins with BCCPLs, and lipoylate-containing proteins with BCCPLs, respectively. Figure 2 shows alignment of amino acid sequences of CAC1A, CAC1B and three BCCPL proteins. The result indicates that the BCCPLs do not contain the -(A/I/V)MK(L/M/A/T) biotinylation motif, which is conserved in all biotinylated proteins, except the seed SBP (Samols et al., 1988; Duval et al., 1994; Hsing et al., 1998). Moreover, we have not found the -DKA- motif on BCCPL proteins (data not shown). However, another Lys residue is conserved among BCCPLs. The amino acids around this Lys, -(I/V)(V/L)K(I/L)L-, are also rich in non-polar amino acids, like the amino acids around the Lys in the tradition biotinylation motif. Based on these information, we hypothesized these BCCPLs are biotinylated proteins, with a novel biotinylation site.

Characterization of T-DNA Tagged Mutants in BCCPL Genes

To distinguish between and identify BCCPL proteins in planta and to study the physiological function of these proteins, we used a reverse genetic approach to isolate and recover loss-of-function mutants at each locus. Five T-DNA insertion mutant lines, i.e., bccpl1-1 (SALK_021108), bccpl1-2 (SALK_121810), bccpl2-1 (GABI_170E12), bccpl3-1 (SALK_000817), and bccpl3-2 (SALK_150657), were found by searching the sequence-indexed Arabidopsis insertion database at The Salk Institute Genomic Analysis Laboratory.
Seeds obtained from different collections were planted and genomic DNA was extracted from rosette leaves of individual progeny plants and subjected to PCR-based genotyping. PCR products were analyzed by gel electrophoresis and verified by sequencing (data not shown). For each mutant allele, we were able to recover all three categories of progeny, i.e., wild-type homozygous, mutant homozygous, and heterozygous. The molecular structures of these alleles were determined by aligning the sequences of the PCR products with the Arabidopsis genomic sequence (Figure 3A).

Both bccpl1-1 and bccpl1-2 alleles carry head-to-head chimeric copies of the T-DNA insertion, which is associated with 15-bp and 1-bp deletion in exon 7 and exon 6 of the BCCPL1 gene, respectively (Figure 3A). The bccpl-2 allele carries a single T-DNA insertion in the first intron of the BCCPL2 gene, which is associated with a 55-bp deletion (Figure 3A). Noticeably, the bccpl2-1 also carries a second T-DNA insertion in the genome at the At1g78920 locus, as indicated in the insertion database (http://www.gabi-kat.de/). From progeny of these plants we isolated individuals that are heterozygous for bccpl2-1 allele and homozygous for wild-type allele at the At1g78920 locus. Plants with this genotype were outcrossed with wild-type Columbia plants. The resulting progeny carrying only the bccpl2-1 allele were used for further analysis. Both bccpl3-1 and bccpl3-2 alleles carry a single T-DNA insertion in intron 4 and intron 6, and they are associated with 11-bp and 20-bp deletion, respectively.
Are becpl1, becpl2, and becpl3 Null Alleles?

To test whether the mutant alleles fully disrupt the expression of BCCPL genes, investigations on the accumulation of respective mRNAs were conducted. First, reverse transcriptase mediated PCR (RT-PCR) was conducted to detect the expression of BCCPL mRNAs. RNA was isolated from the aerial parts (including rosette leaves, cauline leaves, stems, buds, flowers and siliques) at 40-50 days after planting (DAP) or only siliques of plants that were homozygous either for the wild-type or for the mutant alleles. Primers were designed to detect the transcripts that span the position of the T-DNA insertions (Figures 3A, 3B and Table 3). PCR products were analyzed by agarose gel electrophoresis and sequenced; sequences were aligned against mRNA sequences of BCCPL genes to confirm the products. In all cases, transcripts of BCCPL1, BCCPL2 or BCCPL3 were not detected in the corresponding homozygous mutants, but they are readily detectable in all RNA samples isolated from wild-type plants. We conclude that T-DNA insertions have disrupted the accumulation full length BCCPL mRNAs in corresponding mutants.

Cloning and Expression of Arabidopsis BCCPL cDNA in E. coli

To evaluate whether the expression of BCCPL proteins has been blocked by the respective mutations and to identify each BCCPL protein in planta, we first expressed each Arabidopsis BCCPL protein in E. coli and generated antisera against each protein.

EST cDNAs clones of BCCPL1, BCCPL2 and BCCPL3 were obtained from ABRC (Arabidopsis Biological Resource Center, Columbus, OH) or RIKEN BioResource Center (Tokyo, Japan). The cDNAs were fully sequenced to confirm the amino acid sequence of the BCCPL proteins. Then the cDNAs were cloned into pET-30a/b vectors (Novagen, Madison,
BCCPL1 cDNA M53C9 (GenBank Accession number BE524711) was cloned into pET-30b vector using restriction enzymes EcoRI and XhoI. BCCPL2 cDNA 224O9 (GenBank Accession number N65015) was cloned into pET-30b vector using restriction enzymes NcoI and BamHI. BCCPL3 cDNA sequence was first amplified from RAFL04-09-B10 (GenBank accession number AV821358) by PCR using primers At3g56130-1 and At3g56130-2 (Table 3). The PCR product was then cloned into pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA) and later subcloned into pET-30a vector using restriction enzymes NcoI and SacI. All restriction enzymes are either from Promega (Madison, WI) or Invitrogen. These three constructs were named pET-BCCPL1, pET-BCCPL2 and pET-BCCPL3. For each clone, recombinant protein was expressed in E. coli strain BL21. SDS-PAGE analyses were applied to protein extracts from the recombinant strains, and gels were either stained with Coomassie Blue or analyzed by western blots to detect S-tag of the recombinant proteins. As Figure 4 demonstrates the cDNAs were successfully expressed in E. coli. The molecular weight of all three recombinant proteins is approximately 35-37 kDa, consistent with the molecular weight predicted from the cDNA sequence (28-30 kDa) plus the upstream tags provided by the vector (~5 kDa).

Cross-Reactivity of Antisera against BCCPLs

To generate reagents for analysis of the BCCPLs expression in plants, antisera against BCCPLs were produced by immunizing rabbits with recombinant BCCPL1, BCCPL2 and BCCPL3 proteins. Because these proteins share high amino acid sequence similarities, there was a concern that the antiserum generated against each BCCPL might cross-react with other two BCCPLs. Western blot analyses of the protein extract from E. coli strain overexpressing
recombinant BCCPL1 were conducted, in which all three antisera were used to probe the membrane to determine the cross-reactivity (Figure 5). Similar experiments were performed for BCCPL2 and BCCPL3. The results show that BCCPL1 antiserum reacts with all BCCPLs; BCCPL2 antiserum strongly reacts with BCCPL2, and can also react with BCCPL1 but barely reacts with BCCPL3; BCCPL3 antiserum is the most specific antiserum of the three, and only strongly reacts with BCCPL3 itself. Due to the non-specificity of BCCPL1 antiserum and the reactivity of BCCPL2 antiserum to BCCPL1 and BCCPL2, we used BCCPL2 antiserum to detect both BCCPL1 and BCCPL2 proteins in the experiments henceforth.

Mutations Block the Expression of BCCPL Proteins

Protein extracts from wild-type and homozygous mutant siblings of \textit{bccpl1-1}, \textit{bccpl1-2}, \textit{bccpl2-1}, \textit{bccpl3-1} and \textit{bccpl3-2} were subjected to western blot analysis with antiserum against BCCPL proteins (Figure 3C). In all cases tested, a single protein band which is detectable in extracts from wild-type plants is missing in extracts from homozygous mutant plants, indicating that the T-DNA insertions eliminate the expression of BCCPL proteins and thus identifying each BCCPL protein, respectively.

Based on RT-PCR and western blot analysis, we demonstrated: 1) all three BCCPL proteins are expressed in planta; and 2) the T-DNA mutations in the \textit{BCCPL} genes resulted in mRNA and protein null alleles.

Identification of BCCPL Proteins in Plants

Because the BCCPL proteins share similar molecular weight and high amino acid sequence similarities, initial investigations on the identification of BCCPL proteins using
protein extracts from wild-type Columbia plants were rather ambiguous (data not shown). With the help of the mutants used in the western blot analyses, we were able to discover the differences between wild-type and mutant plants, and the comparisons enabled us to identify and distinguish each BCCPL protein in planta.

In summary, BCCPL2 antiserum detects both BCCPL1 and BCCPL2 in plant. As shown by western blot analysis, two closely migrated proteins (approximately 29 kDa) represent BCCPL1 and BCCPL2; BCCPL1 is the one with the higher molecular weight, while BCCPL2 is the one with the lower molecular weight (Figure 3C). This is in agreement with that the theoretical molecular weight of BCCPL1 is 1.4 kDa larger than that BCCPL2. BCCPL3 is specifically detected by BCCPL3 antiserum at approximately 29 kDa (Figure 3C).

**Biotinylation Status of the Recombinant BCCPLs in E. coli**

To detect the biotinylated proteins in extracts from *E. coli* strains expressing BCCPL proteins, western blot analysis using streptavidin conjugated with horseradish peroxidase (HRP) was conducted (Figure 6). In *E. coli*, the sole biotinylated protein is the BCCP subunit (also called AccB in *E. coli*) of the htACCase (Alberts and Vagelos, 1968; Cronan, 1989). The molecular weight of BCCP is 22.5 kDa (Li and Cronan, 1992). This protein could be detected as a 23 kDa band and a presumed proteolytic degradation product (18 kDa) (Figure 6). When the membrane was exposed to film for longer exposures, we could also detect a very weak protein band at about 37 kDa in extracts from *E. coli* expressing pET-BCCPL constructs, which was absent in the control cells. Thus, although we could readily detect the endogenous *E. coli* AccB protein, the recombinant BCCPLs were barely detected even for extremely long exposures. Considering these proteins were overexpressed in *E. coli* (Figure
4), the weak band intensities detected by western blot analysis indicate that the BCCPLs are very poorly biotinylated.

**BCCPL Proteins Are Not Biotinylated in Plant**

BCCPL proteins contain a conserved Lys-containing motif, -(I/V)(V/L)K(I/L)L-, which we propose to be a novel biotinylation site of these proteins. Because the results in *E. coli* demonstrate that the BCCPL proteins are very poorly biotinylated, one possible explanation would be that the holocarboxylase synthetase (Chapman-Smith and Cronan, 1999) of *E. coli* failed to recognize the novel biotinylation site of BCCPLs. In fact, it has been shown that the novel biotinylation site of soybean SBP is not biotinylated when recombinant SBP is expressed in *E. coli* (Hsing et al., 1998). Therefore, we raised the question that BCCPL proteins are biotinylated in planta.

To address this question, affinity chromatography purification of biotin-containing proteins was carried out. We chose 40-50 DAP wild-type Columbia plants to conduct the experiment, because all BCCPL proteins are expressed in aerial parts of wild-type plants at this growth stage as demonstrated in Figure 3. Protein extracts were first incubated with avidin-agarose gel to purify the biotin-containing proteins. After incubation, avidin-agarose gel (to which the biotin-containing proteins were already bound) was separated from the non-binding proteins. Finally the biotinylated proteins were released from avidin-agarose gel by boiling with SDS-containing buffer. Three protein samples: the initial plant extracts, the non-bound proteins and the avidin-bound proteins, i.e., biotinylated proteins, were subjected to SDS-PAGE and subsequent western blot analysis. As shown in Figure 7, BCCPL proteins could be detected in the initial plant extracts and the non-bound protein fraction, but they
could not be detected in the purified biotinylated protein fraction. In the parallel analysis, the biotinylated MCC subunit of MCCase as positive control was present in the biotinylated protein fraction. This result indicates the BCCPL proteins are not biotinylated in plants, at least in the tissue we tested.

**BCCPL Proteins Are Not Lipoylated**

Because the BCCPL proteins are not biotinylated in plants, the function of these proteins remains unknown. As mentioned earlier, there is possibility that these proteins might be lipoylated based on amino acid sequences. Therefore, we conducted immunological analysis using a lipoic acid antibody (Ewald et al., 2007) to test lipoylation status of these proteins in planta.

In this analysis we used extracts from aerial parts of 40-50 DAP plants that are wild-type, bccpl1-1 bccpl3-1 double homozygous mutant (see “bccpl1-1 bccpl3-1 Double Mutant” for detailed description), and bccpl2-1 single mutant. If these proteins are indeed lipoylated, we would expect a difference in the pattern of proteins detected between wild-type and mutant extracts at their respective molecular weight. However, we could not detect any lipoylated proteins at about 29 kDa, the molecular weights of BCCPL proteins, even in wild-type plants (Figure 8). In contrast, there are five lipoylated proteins that were detected in both wild-type and mutant plants at other molecular weight range, and there is no change in the patterns of these lipoylate-containing proteins in the extracts from wild-type and mutant plants. Based on antigenicity and apparent molecular masses (Taylor et al., 2004; Ewald et al., 2007), we identified these proteins as E2a of mtPDC (LTA3,At3g52200) at 82 kDa, E2b of mtPDC (At3g13930) at 65 kDa, E2 of KGDC (At5g55070) at 52 kDa, E2 of BCKDC (LTA1,
At3g06850) at 51 kDa and H-protein of GDC (At2g35370) at 14 kDa (Oliver and Raman, 1995; Friso et al., 2004).

**The Search for Phenotypes of bccpl Mutants**

As the first step to search for the phenotypes of bccpl mutant, we carried out segregation analysis to test whether gamete transmission is affected mutations in BCCPL genes. Heterozygous mutant plants of each allele were planted and allowed to self-pollinate, and progeny seeds from each plant were collected individually and planted after breaking dormancy. Segregation ratio of each allele was determined by PCR-based genotyping the resulting seedlings (Table 4). Based on Chi-square test, segregation of all five mutant alleles failed to reject the null hypothesis, which is the normal Mendelian segregation for a recessive allele (1:2:1). This result indicates that blocking the expression of BCCPL proteins does not affect the viability of the plants.

Moreover, under normal growth conditions, we have not found any observable phenotypes on these homozygous bccpl mutants. The physiological criterions, including the germination rate, the physical appearance, growth rate, and seed yield of bccpl mutants are similar to that of the wild-type sibling plants.

**bccpl1-1 bccpl3-1 Double Mutant**

To further investigate the physiological function of BCCPL proteins, we have begun to generate stocks that carry double mutant combinations of bccpls. The ultimate goal is to generate a triple mutant stock. Plants heterozygous for the bccpl1-1 and bccpl3-1 alleles were crossed and F1 seeds were collected. After breaking dormancy, F1 seeds were planted, each F1 plant was genotyped by PCR to ensure the cross was successful, and the plants were
allowed to self-pollinate to generate the F2 stock. These F2 seeds were planted, plants that were homozygous wild-type or mutant at both alleles were identified by PCR-based genotyping. Wild-type and \textit{bccpl1-1 bccpl3-1} double mutant segregating from one family were used for further analysis. However, to date no observable phenotype has been found on the \textit{bccpl1-1 bccpl3-1} double mutant.

\textbf{DISCUSSION}

\textbf{Bioinformatic Hypothesis Is Not Always Correct}

When the Arabidopsis genome was completely sequenced, 69\% of the genes were assigned a likely function based on comparing their sequences with those of other eukaryotes. Yet only 9\% of these genes have been experimentally characterized (Wigge and Weigel, 2001). In this paper, our initial hypothesis was relying on the sequencing based bioinformatic prediction. However, our results disprove the hypothesis and provide an example that the computer prediction for gene functions is not always accurate.

In plants four biotin-containing enzymes have been characterized. They are hmACCase, htACCase, MCCase, GCCase. Moreover, there are possibilities that additional biotin-containing enzymes, i.e., propionyl-CoA carboxylase and pyruvate carboxylase, exist in plants (Wurtele and Nikolau, 1990). In this study, we identified three BCCPL proteins in Arabidopsis based on sequence similarities. We hypothesized that these proteins could be novel biotin-containing proteins. However, using immunological analysis and affinity chromatography purification we demonstrated these three proteins are not biotinylated in plants. Therefore, the initial hypothesis that the proteins may represent BCCP subunit of novel biotin-containing enzymes was incorrect. Further, we showed that these proteins are
not lipoylated either. Hence, the results we obtained provide an open conclusion that the BCCPL proteins are neither biotinylated, nor lipoylated, and their biochemical functions are still unknown.

Though the BCCPLs are not biotinylated or lipoylated in plants, it is still interesting to understand the significance for these proteins to possess such a domain that looks a lot like a biotinylation domain. One possibility is that these proteins may have regulatory roles for other BCCP proteins in plants. For example, they might be competitors for other BCCP proteins when the corresponding heteromeric biotinylated enzyme assembles \textit{in vivo}, and provide a means of controlling the activity of the enzyme post-translationally.

Molecular Weight of BCCPL Proteins Indicates the Subcellular Localization

The molecular weight of the BCCPL proteins (28-30 kDa) were first predicted using full length cDNAs. Yet it has been suggested that these proteins might have transit peptide which can lead them to subcellular compartments such as chloroplast or mitochondria with the mature protein molecular weight reduced to approximately 23 kDa. However, we have demonstrated that the molecular weight of all BCCPL proteins were approximately 29 kDa in planta. This is in agreement with the prediction based on full length \textit{BCCPL} cDNAs, which indicates that BCCPL proteins may not be cleaved under physiological conditions. It also suggests that these proteins might be targeted to cytosol. However, a more direct approach, such as isolation of cellular compartments, needs to be conducted in order to confirm the subcellular localization of these proteins.
Redundancy of BCCPL Proteins

To understand the physiological function of the BCCPL proteins, we isolated and recovered homozygous mutants of $BCCPL1$, $BCCPL2$ and $BCCPL3$ genes. We have not observed any morphological phenotypes on these mutants. Also, double mutant of $BCCPL1$ and $BCCPL3$ genes grow normally. Based on these findings and high sequence similarity shared by BCCPLs, we postulate that these proteins may have redundant functions. Blocking the expression of one or two genes may not fully disrupt the function of the three genes as a whole. The future work would be toward generating other possible double mutant combinations and a triple mutant of these three genes in order to further investigate the function of the BCCPL proteins.

MATERIALS AND METHODS

Plant Materials

Seed stocks of *Arabidopsis thaliana* wild-type (ecotype Columbia) and T3 T-DNA tagged mutant lines carrying $bccpl1$-1 (SALK_021108), $bccpl1$-2 (SALK_121810), $bccpl3$-1 (SALK_000817) and $bccpl3$-2 (SALK_150657) alleles (Alonso et al., 2003) were obtained from ABRC (Arabidopsis Biological Resource Center, Columbus, OH). T2 T-DNA tagged seed stocks carrying the $bccpl2$-1 (GABI_170E12, ecotype Columbia) allele was purchased from GABI-Kat (Rosso et al., 2003).

Growth Conditions

Seeds were surface-sterilized by treating with 75% (v/v) ethanol for 1 min, and followed by a 10 min treatment with 50% (v/v) bleach containing 0.1% (v/v) Tween-20. After 3
washes with sterile water, seeds were placed in Petri dish (BD Labware, Franklin Lakes, NJ) on Murashige and Skoog (MS) agar solid media (Sigma-Aldrich, St. Louis, MO) containing 1× multivitamin (Sigma-Aldrich) and 1% sucrose. These dishes were first placed at 4 ºC for 2 days in order to break dormancy, and were then transferred to a growth room which was maintained at 22°C under constant illumination provided by 40 W Sylvania cold-white fluorescent light bulbs at white light irradiation of 150 µmol m⁻² s⁻¹. If needed, seedlings were gently transferred in LC1 Sunshine Mix soil (Sun Gro Horticulture, Canada) at 10-12 DAP (including the first 2 days in 4 ºC).

**PCR-Based Genotyping**

Two sets PCR analyses were conducted to identify the genomic DNA sequences flanking T-DNA insertions. The first set of reaction was specific to the wild-type allele and used a pair of gene-specific primers that span the annotated position of the T-DNA insertions present in the mutant allele. Positive result of this PCR means the plant being tested has a wild-type allele in its genome. The second set of reactions was composed of two PCRs, which lead to the identification of the borders of the insertions. Each reaction of this set used one gene-specific primer in combination with a primer that is complementary to the T-DNA (left border or right border sequences). Positive results of this set of PCRs mean that the plant being tested has a mutant allele. Moreover, if only the first set was positive, the plant was categorized as homozygous for the wild-type allele, and if only the second set of reactions was positive, the plant was categorized as homozygous for the mutant allele. If both sets were positive, the plant was categorized as heterozygote. Primer sequences are shown in Table 3.
cDNA of BCCPL genes

cDNA clones containing full length cDNA of BCCPL1 (M53C9, GenBank accession number BE524711) and BCCPL2 (224O9, GenBank accession number N65015) were obtained from ABRC. cDNA clone for BCCPL3 (M26B11, GenBank accession number BE522468) was also from ABRC, but we found this cDNA contains introns of the gene in the initial sequencing validation experiment. Thus, we abandoned it. We then obtained another cDNA clone of BCCPL3 (RAFL04-09-B10, GenBank accession number AF378871) from RIKEN BioResource Center and used it in this study.

Expression and Extraction of Proteins in E. coli

Single colony of E. coli BL21 cells transformed with expression vector was picked from LB plates and then grown with shaking overnight at 37°C in 10 mL LB liquid media containing the appropriate antibiotic. Two mL of the overnight culture was added into 200 mL fresh antibiotic-containing LB media and grown under same condition. When the cell culture reached OD600 ≈ 0.6, expression of the recombinant protein was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After five hours induction, E. coli cells were collected by centrifugation at 2000g for 10 min. The cell pellet was resuspended in 5 mL 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2% (w/v) SDS. The resulting mixture was sonicated to break the cell wall and then centrifuged at 4000g. The supernatant was boiled at 100°C for 15 min. After clarifying the mixture with another centrifugation at 4000g, the final supernatant was flash-frozen in liquid N₂ and stored at -70°C until further analysis.
**Generation of Antisera against BCCPLs**

The constructs expressing recombinant BCCPL proteins were overexpressed in *E. coli* BL21 cells. Proteins were extracted from *E. coli* cell pellet and subjected to 15% SDS-PAGE. Protein bands corresponding to each BCCPL were sliced from the gel and lyophilized. Antisera against these proteins were generated by immunizing New Zealand White female rabbits following the procedures described previously (Ke et al., 2000).

**RNA Extraction and RT-PCR Analysis**

About 0.1 g plant tissues were flash-frozen in liquid N$_2$ and stored at -70°C. The frozen tissue was ground into fine powder with a Kontes pellet pestle (Fisher Scientific, Pittsburgh, PA) in eppendorf tubes. Total RNA was extracted with RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). For RNA extraction from siliques, 100 µL 0.1 g/mL polyethylene glycol (PEG) was added to 450 µL RLC buffer provided by the kit. DNase I (Invitrogen) was used to remove trace amount genomic DNA. The RNA quality was evaluated by agarose gel electrophoresis and the quantity was measured by absorbance at 260 nm and 280 nm. One µg of isolated RNA was used to synthesize first-strand cDNA using the SuperScript™ First-Strand Synthesis System (Invitrogen) with random hexamer primers provided in the kit. With 18S rRNA as the reference gene, cDNAs were relatively quantified using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) by comparing the intensity of the PCR products after electrophoresis and stained with ethidium bromide. Equal amount of cDNAs were used for further PCRs with gene-specific primers.
**Protein Extraction from Plant Material**

The plant protein extraction procedures under non-denaturing condition were modified from Che et al. (2002). The extraction buffer contained 0.3% (v/v) Protease Inhibitor Cocktail for plant cell extracts (Sigma-Aldrich). For protein extraction under denaturing condition, a method described in Wang et al. (1995) was followed. Protein concentration was determined by Bradford method (Bradford, 1976) with BioRad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

**Protein Electrophoresis and Western Blot Analysis**

Proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984). Linear gradient gels were purchased from Bio-Rad. SDS-PAGE of different percentage polyacrylamide were performed, i.e., Figure 3C and Figure 7, 10-20% linear gradient of polyacrylamide; Figure 4 and Figure 6, 15% of polyacrylamide; Figure 5, 20% of polyacrylamide; Figure 8, 4-20% linear gradient of polyacrylamide.

Procedures of western blot for detecting S-tag are described in the manual of Novagen S·Tag System. S-protein AP (alkaline phosphatase) conjugate was obtained from Novagen. AP conjugate substrate kit (Bio-Rad) was used for detection.

Membranes for western blot analysis using antisera against BCCPLs, MCCA (Weaver et al., 1995) or anti-lipoic acid rabbit polyclonal antibody (anti-LA, EMD, San Diego, CA) were first blocked for 1 h in 5% (w/v) Instant Nonfat Dry Milk (Nestle, Vevey, Switzerland) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and then were incubated with respective antiserum diluted 1:5,000 (BCCPL antisera and anti-LA) or
1:10,000 (MCCA antiserum) in 5% milk in TBST for 1 h. Following three times rinses with TBST, the membrane was incubated for 1 h with horseradish peroxidase (HRP) linked anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, United Kingdom), diluted 1:5,000 (for detecting BCCPLs and lipoic acid) or 1:7,500 (for detecting MCCA) in 5% milk in TBST for 1 h. After washing four times with TBST, BCCPL proteins were detected by ECL Chemiluminescent Detection System (Amersham Biosciences) on X-ray films (Kodak, Rochester, New York).

For western blot analysis using Streptavidin HRP conjugate (Amersham Biosciences), membranes were first blocked in 3% bovine serum albumin (BSA) in TBST, and then incubated with Streptavidin-HRP, diluted 1:1,000. The detection of streptavidin-HRP is the same as the detection of anti-rabbit IgG HRP conjugate.

**Purification of Biotin-Containing Polypeptides**

Aerial parts of Arabidopsis Columbia ecotype were collected at 40-50 DAP and immediately frozen in liquid N₂. Proteins were extracted under denaturing condition, and extracts were stored until usage. Avidin-agarose gel was purchased from Pierce (Rockford, IL). For every gram of plant extract, about 0.8 mL avidin-agarose gel was used. The gel was first collected by centrifugation at 5000g. After removing the supernatant, the gel was dissolved in dilution buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA) and centrifuged at 5000g again. After repeat this step three times, the gel is ready for chromatography. Biotin-containing polypeptides were then purified following the procedures described previously (Wang et al., 1995).
REFERENCES


Konishi, T., Shinohara, K., Yamada, K., and Sasaki, Y. (1996). Acetyl-CoA carboxylase in higher plants: most plants other than gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. Plant Cell Physiol 37, 117-122.


Figure 1. The rooted phylogenetic tree of BCCPL proteins, representatives of biotin-containing proteins and lipoylate-containing proteins in Arabidopsis.

MCCA (At1g03090), the biotinylated subunit of 3-methylcrotonyl-CoA carboxylase. ACC1 (At1g36160), homomeric acetyl-CoA carboxylase 1. CAC1A (At5g16390), BCCP1 subunit of heteromeric acetyl-CoA carboxylase. CAC1B (At5g15530), BCCP2 subunit of heteromeric acetyl-CoA carboxylase. LTA1 (At3g06850), dihydrolipoyl branched-chain acyltransferase (E2) of mitochondrial branched-chain keto acid dehydrogenase complex. LTA2 (At3g25860), dihydrolipoamide acyltransferase (E2) of pastidial pyruvate dehydrogenase complex. LTA3 (At3g52200), dihydrolipoamide acyltransferase (E2) of mitochondrial pyruvate dehydrogenase complex.
**Figure 2.** Multiple sequence alignment of CAC1A, CAC1B, and BCCPLs using amino acid sequences.

Identical amino acid residues are black-boxed. Conserved residues are gray-boxed. The biotinylation motif of CAC1A and CAC1B is red-boxed and the biotin attached Lys residue is labeled with an asterisk. The proposed biotinylation motif of BCCPLs is red-boxed and the Lys residue is labeled with a diamond.
Figure 3. Molecular characterization of bccpl mutants.
Figure 3. (Continued).

(A) Schematic representation of the molecular structure of the BCCPL1, BCCPL2 and BCCPL3 loci in the mutant alleles. Solid boxes represent the exons and the open boxes represent the 5’ and 3’ UTRs. Thick lines represent the introns. The triangles represent the T-DNA insertions (the T-DNA insertions are not to scale). The vertical lines linking the triangles to the genes show the position of insertions. Deletions associated with bccpl1-1, bccpl2-1, bccpl3-1 and bccpl3-2 alleles are 15-bp, 1-bp, 55-bp, 11-bp and 20-bp respectively. Arrows under each gene structure show the positions and direction of 5’ and 3’ primers used for RT-PCR. The dashed line in some of these primers indicates that the primer sequence is composed of sequences from two adjacent exons without the sequence of the intervening intron. Arrows on the T-DNA insertions represent the primers used to identify the T-DNA borders.

(B) RT-PCR analysis on mutants carrying bccpl1-1, bccpl1-2, bccpl2-1, bccpl3-1 and bccpl3-2 alleles. RNA was isolated from aerial parts of 40-45 DAP plants (all alleles except bccpl1-2) or siliques (bccpl1-2) of siblings that are either wild-type (WT) or homozygous mutant (Mu) plants. Primers used in RT-PCR for BCCPL transcripts are indicated in (A). Primers used for 18s rRNA are 18SFW and 18SRV. Sequences of primers are shown in Table 3.

(C) Western blot analysis of BCCPL1, BCCPL2 and BCCPL3 in bccpl1-1, bccpl1-2, bccpl2-1, bccpl3-1 and bccpl3-2 alleles. Aliquots of protein extracts containing 10 µg total protein from aerial parts of 40-50 DAP plants (lanes 1-2 and 5-6), siliques (lanes 3-4) or from 18-day-old seedlings (lanes 7-10) were subject to SDS-PAGE. After electrophoresis, gels were either stained with Coomassie Blue (lower panels) to show equal loading, or transferred to nitrocellulose membranes (upper panels). Western blot analysis was conducted using BCCPL2 antiserum (lanes 1-6) or BCCPL3 antiserum (lanes 7-10). Arrows indicate the position of BCCPL proteins.
Figure 4. Expression of Arabidopsis BCCPL proteins in *E. coli*.

For both (A) and (B), lane 1, protein molecular weight standards (Bio-Rad); lanes 2 and 4, protein extracts from *E. coli* transformed with pET-30b as negative controls; lane 3, protein extracts from *E. coli* transformed with pET-BCCPL1; lane 5, protein extracts from *E. coli* transformed with pET-BCCPL2; lane 6, protein extracts from *E. coli* transformed with pET-30a as negative control; lane 7, protein extracts *E. coli* transformed with pET-BCCPL3. The arrow indicates the position of the recombinant proteins.

(A) Coomassie Blue stained gels after SDS-PAGE separation.

(B) Western blot analysis to detect S-tag of recombinant proteins.
Figure 5. Cross-reactivity of antiserum against BCCPL1, BCCPL2 and BCCPL3 recombinant proteins. Equal amounts (~0.07 µg) of proteins extracted from *E. coli* cells carrying pET-BCCPL1 (lanes 1, 4 and 7), pET-BCCPL2 (lanes 2, 5 and 8) and pET-BCCP3 (lanes 3, 6 and 9) were separated by SDS-PAGE, and subjected to western blot analysis using BCCPL1, BCCPL2 and BCCPL3 antisera, respectively.
Figure 6. Biotinylation status of recombinant Arabidopsis BCCPLs in *E. coli*.

Equal amount of protein extracted from *E. coli* were separated by SDS-PAGE and the biotinylation status of BCCPLs was detected by subsequent western blot using streptavidin-HRP with 30-second exposure (A) and overexposure (B). Lane 1, protein extracts from *E. coli* transformed with pET-30a as negative control; lane 2, protein extracts from *E. coli* transformed with pET-BCCPL1; lane 3, protein extracts from *E. coli* transformed with pET-BCCPL2; lane 4, protein extracts from *E. coli* transformed with pET-BCCPL3. The arrow indicates the position of recombinant BCCPLs.
**Figure 7.** Avidin affinity chromatography purification of biotinylated proteins.

Proteins were extracted from aerial parts of wild-type Arabidopsis plants at 40-50 DAP. Biotin-containing proteins were purified by affinity chromatography using avidin-agarose. Protein aliquots from initial protein extract (lanes 1, 4 and 7), non-bound proteins (lanes 2, 5 and 8) and avidin-bound (biotin-containing) proteins (lanes 3, 6 and 9) were separated by SDS-PAGE and subjected to subsequent western blot analysis using BCCPL2 antiserum (lanes 1-3), BCCPL3 antiserum (lanes 4-6) and MCCA antiserum (lanes 7-9). MCCA (80kDa) is the biotin-containing subunit of MCCase and was used as the positive control. BCCPL2 antiserum was used to detect both BCCPL1 and BCCPL2. BCCPLs are indicated by arrows.
Figure 8. BCCPL proteins are not lipoylated in plants.

Protein extracts from 40-50 DAP plants were separated by SDS-PAGE and subject to western blot analysis using BCCPL2 antiserum, BCCPL3 antiserum and antibody against lipoic acid (anti-LA, EMD). Lanes 1, 3, 5, 7 and 9 are extracts from wild-type siblings, lanes 2, 6 and 8 are extracts from bccpl1-1 bccpl3-1 double mutant, and lanes 4 and 10 are extracts from bccpl2-1 mutant. The known lipoylated proteins are indicated based on Taylor et al. (2004) and Ewald et al. (2007), and they are E2a of mtPDH (LTA3,At3g52200) at 82 kDa, E2b of mtPDH (At3g13930) at 65 kDa, E2 of KGDC (At5g55070) at 52 kDa, E2 of BCKDC (LTA1, At3g06850) at 51 kDa and H-protein of GDC (At2g35370) at 14 kDa (Srinivasan and Oliver, 1995; Friso et al., 2004; Taylor et al., 2004; Ewald et al., 2007).
Table 1. Amino acid sequence similarity among BCCPL proteins.

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<tr>
<th>Comparison</th>
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<td>BCCPL1 vs. BCCPL3</td>
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<td>54%</td>
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<tr>
<td>BCCPL2 vs. BCCPL3</td>
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<td>55%</td>
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Table 2. Bioinformatic predictions of the subcellular localization of BCCPL proteins.
Subcellular localization of BCCPL proteins were predicted using online softwares TargetP, Predator and PSORT. The scores indicate the possibilities associated with the predictions. Each software uses different algorism, thus the predictions between software is not recommended. Among these results, the prediction with highest score is in boldface. The numbers in the parentheses represent the length of the transit peptide predicted by TargetP.

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<th>PSORT</th>
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<td>Mitochondrial matrix space</td>
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<td>Chloroplast/Plastid (54 aa)</td>
<td>Chloroplast/Plastid</td>
<td>Microbody (peroxisome)</td>
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<td>Chloroplast/Plastid (56 aa)</td>
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Table 3. Primers for cloning, characterizing the mutant lines and RT-PCR.

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Table 3. (Continued).

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Table 4. Segregation analysis on *bccpl* mutant alleles.

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<th>p-value of Chi-square test</th>
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<sup>a</sup>The H₀ for all Chi-square tests is proposed as: wild-types: heterozygous mutants: homozygous mutants = 1:2:1.
CHAPTER IV GENERAL CONCLUSIONS

This dissertation describes and discusses two studies that are both part of the biotin metabolic network. The first study is the functional analysis of 3-methylcrotonyl-CoA carboxylase (MCCase) in Arabidopsis. The second study is the characterization of three Arabidopsis genes that code for biotin carboxyl carrier protein-like (BCCPL) proteins. This chapter will summarize the two studies respectively.

FUNCTIONAL ANALYSIS OF 3-METHYL CROTONYL-COA CARBOXYLASE

MCCase is involved in leucine (Leu) catabolism in mitochondria. To study the physiology function of MCCase and the carbon flux controlled by MCCase in plants, reverse genetic approach was used to isolate and recover loss-of-function mutants in both subunits of Arabidopsis MCCase, i.e., MCCA and MCCB. Five T-DNA or transposon lines (three for MCCA gene and two for MCCB gene), have been characterized. Homozygous wild-type, heterozygous, and homozygous mutant plants were recovered. Reverse transcriptase mediated PCR (RT-PCR), western blot analyses and enzyme activity analysis demonstrated that the expression of MCCA and MCCB genes is eliminated in all mutant alleles.

In this paper, we confirmed that MCCA undergoes alternative splicing generating two types of transcripts, MCCA-L (the longer transcript) and MCCA-S (the shorter transcript), which vary in one exon. Relative amount of the two transcripts was quantified using real-
time RT-PCR analysis. *MCCA-L* is more abundant than *MCCA-S* during early growth of plants.

By comparing the accumulation of mRNAs and proteins of MCCA and MCCB subunit in wild-type and homozygous mutant plants, we have found that eliminating expression of the *MCCA* gene, does not affect the accumulation of MCCB subunit, and *vice versa*. Although previous studies have shown that *MCCA* and *MCCB* genes are temporally and spatially coordinately regulated, our results indicate that degradation/turnover of one subunit is not affected by the abundance of the other subunit.

To test whether gamete transmission is affected in the MCCase mutants, segregation analysis was conducted using progeny of heterozygous plants. *mcca-1*, *mcca-2* and *mccb-1* alleles, exhibit normal Mendelian segregation for a recessive allele. *mcca-3* and *mccb-2* alleles showed non-Mendelian segregation in the initial tests. A possible model to explain the different results is that *mcca-3* and *mccb-2* alleles might carry a background mutation in the genome. In fact, the segregation analysis on heteroallelic mutant carrying *mcca-2/mcca-3*, and *mccb-1/mccb-2*, respectively, demonstrates that the transmission of these mutants is consistent with the normal Mendelian inheritance, which supports the model. Therefore, gamete transmission of MCCase mutants is not affect by blocking the MCCase expression.

Although the vegetative growth of *mcca* and *mccb* mutants is quite normal as compared to wild-type plants, the reproductive growth is severely affected. Many siliques on the mutants do not elongate to the length of the wild-type siliques. When we opened the mutant siliques at 8 DAF, we found many aborted embryos besides normal-looking embryos. Moreover, even the normal-looking embryos showed various developmental stages, not as wild-type embryos which uniformly developed. Though individual mutant seeds are bigger
and heavier than wild-type seeds, the total seed yields of these mutants are markedly reduced.
Most importantly, the germination rates of the mutants are significantly lower than that of
wild-type plants. Interestingly, this germination defect phenotype is only expressed when
homozygous or heteroallelic mutant seeds are collect from a parent plant that is itself
homozygous or heteroallelic mutant, but not expressed when collected from a parent plant
that is heterozygous. These observations indicate that the maternal mitochondrial Leu
catabolism is required for normal seed development.

To investigate the carbon flux controlled by MCCase, we conducted targeted metabolite
profiling analysis. The metabolites we analyzed were amino acids and selected acyl-CoA
intermediates of mitochondrial and peroxisomal Leu catabolism pathways. The rationale was
to expose plants to an environment which requires high MCCase activity (using light
deprivation conditions), but at the same time remove MCCase (using the MCCase mutants),
and measure the metabolic response of these plants.

When grown under constant illumination, the concentration of 3-methylcrotonyl-CoA
(MC-CoA, the substrate of MCCase) is increased in *mcca* or *mccb* mutants, while
concentrations of Leu and acyl-CoA intermediates of mitochondrial and peroxisomal
pathways are similar in wild-type and mutants. This observation demonstrates that MCCase
mutants disrupt the mitochondrial Leu catabolism pathway. The fact that the overall Leu
accumulation is not affected indicates that under normal condition plants only require basal
level of Leu catabolism, which can be conducted either in mitochondria or peroxisomes.

When illumination is withdrawn for prolonged time, we found that concentrations of
common acyl-CoA intermediates of both Leu catabolism pathways, i.e., isovaleryl-CoA (IV-
CoA) and MC-CoA are increased more than 2 folds in *mcca* and *mccb* mutants, as well as in
wild-type plants, but the intermediates that are specific to peroxisomal Leu catabolism pathway, i.e., isobutyryl-CoA (IB-CoA) and propionyl-CoA are not increased correspondingly, indicating that only the mitochondrial Leu catabolism pathway is induced. Furthermore, Leu concentration is dramatically increased in wild-type plants under dark treatment, and MCCase mutants amplified this increase to an even higher level. However, we have not seen any significant increase of IB-CoA and propionyl-CoA in MCCase mutants, indicating the peroxisomal pathway does not respond to the block of mitochondrial Leu catabolism pathway. In consequence, the higher demand for Leu catabolism, the block of the mitochondrial Leu catabolism pathway, and no compensation from peroxisomal pathway, result the plant in hyper accumulation of Leu. Therefore, we conclude that physiological roles of the mitochondrial and peroxisomal Leu catabolism pathways are different and the regulation of these pathways is independent under sugar starvation conditions.

In summary, this study demonstrates that MCCase is required for normal reproductive growth in plants; it also suggested a non-redundant relationship between the two Leu catabolism pathways under sugar starvation conditions. The research here provides new insights into the role of catabolic processes in growth and development, an area of plant biology that is poorly understood.

CHARACTERIZATION OF BIONTIN CARBOXYL CARRIER PROTEIN-LIKE PROTEINS

As a first step to find novel biotinylated proteins, we undertook a bioinformatic approach. Three novel Arabidopsis proteins which share high sequence similarity with the biotin carboxyl carrier protein (BCCP) subunit of heteromeric acetyl-CoA carboxylase were
identified using “BLASTP” and these proteins were named as biotin carboxyl carrier protein-like (BCCPL) proteins. Based on annotation in GenBank, these proteins may also contain lipooyl attachment domain. However, multiple sequence alignment did not identify the normal biotinylation or lipoylation motif on these proteins. Yet a conserved the lysine-containing motif which is similar to the biotinylation motif was found on BCCPL proteins and was hypothesized as a novel biotinylation motif.

Full length cDNAs of BCCPL genes were expressed in E. coli, the recombinant BCCPL proteins were found to be very poorly biotinylated. To test whether these proteins are biotinylated in planta, we first confirmed that these proteins are indeed expressed in plants by using immunological analysis. As indicated by affinity chromatography purification, the BCCPL proteins are not biotinylated in plants. Moreover, using antibody which specific reacts with lipoic acid, we have demonstrated that these proteins are not lipoylated in plants either. Thus the biochemical function of these proteins remains unclear.

To study the physiological function of the BCCPL proteins, five T-DNA knockout lines, i.e., two for BCCPL1, one for BCCPL2, and two for BCCPL3, were isolated and recovered. RT-PCR and western blot analyses demonstrate that the expression of the respective BCCPL gene was disrupted. Segregation analysis indicates that the gamete transmission was not affected in the mutant lines. The growth of these bccpl mutants appears normal and we have not found observable phenotypes. Based on high sequence similarities shared among the BCCPL proteins, we proposed that the functions of these proteins might be redundant. As the next step to investigate these proteins, we are in the process of generating double mutant combinations and triple mutant of BCCPL proteins. bccpl1-1 bccpl3-1 is the only available
double mutant to date, and the growth of this mutant is still normal as compared to wild-type plants.

In summary, this study is an example characterization of proteins with unknown functions under the direction of bioinformatics based hypothesis. Unfortunately, in this case, the experimental data disproved the hypothesis, and the research is resulted in an open conclusion, that is, the BCCPL proteins are not biotinylated or lipoylated in planta, and the function of BCCPL proteins remains unclear.
APPENDIX I THE PHENOTYPIC CHANGES OF NECTARY GLANDS IN MCCASE MUTANTS

A

WT

B

Mu

C

WT

D

Mu

E

WT

F

Mu

G

WT

H

Mu
Appendix I. The phenotypic changes of nectary glands in MCCase mutants (Continued).

(A) and (B) Stereo micrographs of Toluidine Blue O stained nectaries from wild-type (WT) and mutant (Mu) plants of *mcca*-2 allele. Flowers were collected from wild-type or homozygous mutant plants when they just opened.

(C) to (F) Stereo micrographs of periodic acid–Schiff’s reagent (PAS)-stained nectaries from wild-type or mutant plants of *mcca*-1 allele. Flowers were collected from wild-type or homozygous mutant plants when they just opened. Water-insoluble starch granules are stained as dark particles. Stomata are indicated by arrows.

(G) and (H) Transmission electron micrographs of nectary cells from wild-type and *mcca* mutant plants.

In, lateral nectary gland. mn, medial nectary gland. mt, mitochondria.
Methods for Appendix I

For PAS observations, isolated nectaries by removing sepals and petals from the flowers were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 6.8, at 4°C for 3 h. The tissue was then washed with cold buffer and deionized water, dehydrated in an ethanol series to 100% ethanol, and infiltrated in a 3:1, 1:1, and 1:3 mixture of 100% ethanol: LR White Resin (http://www.emsdiasum.com). Infiltration was followed by pure resin. Specimens were embedded in resin in covered aluminum trays to eliminate oxygen, and they were polymerized at 55°C for 24 h. Sections were cut on a Leica-Reichert Ultracut S ultra microtome (http://www.leica-microsystems.com) using glass knives and mounted on Probe-On Plus slides (http://www.fisherscientific.com). One µm thick sections were treated with the PAS to localize water-insoluble polysaccharides.

For Toluidine Blue O staining observations, isolated nectaries were initially fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C overnight. The tissue was then washed with cold buffer, post-fixed in 1% osmium tetroxide in the same buffer at 4°C for 1 h, washed with buffer and deionized water, and en bloc stained with 5% aqueous uranyl acetate in the dark. Then the tissue was rinsed three times with deionized water and dehydrated through a graded ethanol series (50, 70, 85, 95, and 100%; 30 min per step), followed by two changes of ultra pure 100% ethanol, and transferred to pure acetone. Then the tissue was infiltrated in a series of 3:1, 1:1, 1:3 acetone: Spurr’s resin (v/v). Specimens were then transferred into pure resin mixture overnight, and finally embedded in aluminum trays, and polymerized at 70°C. Specimens embedded in Spurr resin were sectioned using glass knives. One µm thick sections were stained with 1%
(w/v) Toluidine Blue O in 1% (w/v) sodium borate (pH 11.0). Stereo microscopic observations were made on a Zeiss Axioplan compound microscope with a Zeiss AxioCamHRc digital camera (Carl Zeiss, Inc., Thornwood, NY, USA). The same samples were used for stereo microscopy (Toluidine Blue O staining technique) and transmission electron microscopy (TEM).

For TEM observations, thin Spurr resin sections (60 ± 90 nm) were cut using a Diatome diamond knife (www.emsdiasum.com) and mounted on Formvar-coated slotted grids then stained with 5% aqueous uranyl acetate for 30 min and in lead citrate for 45 min. Sections were observed with a JEOL 1200 EX-II STEM at 80 kV (www.jeol.com) and images captured with an SIS Mega View III camera and software (www.soft-imaging.net/en/3.htm).
APPENDIX II EFFECT OF MCCASE MUTATIONS ON AMINO ACID ACCUMULATION IN INFLORENCES TIPS

Sibling plants that are wild-type, heterozygous mutant and homozygous mutant of mcca-1, mcca-2, mccb-1 and mccb-2 alleles were grown on MS agar media for 10 days before transferring to soil. At 40-50 DAP, inflorescences tips including buds, flowers, siliques younger than 3 DAF and stems were collected. Two samples were collected from each plant. Two plants were used for each genotype per mutant allele. The tissue preparation was independently repeated once as biological replication. These two batches of samples were processed at the same time. Free amino acids (not from proteins) were extracted and subjected to GC-FID analysis. Number of the plants analyzed is indicated. Bars represent ± SE.
Appendix II. Effect of MCCase mutations on amino acid accumulation in infloresences tips (Continued).

(A) Total amino acid concentration. Calculation based on sum of all amino acids (Arg and Cys are not included). Sample sizes were indicated. FW, fresh weight.

(B) Free Leu concentration. Sample sizes are same as (A).
APPENDIX III EFFECT OF MCCA OR MCCB MUTATIONS ON MCCB OR MCCA SUBUNIT ORGANIZATION

MCCA antiserum

MCCB antiserum

Coomassie Blue Staining
Appendix III. Effect of $mcca$ or $mccb$ mutations on MCCB or MCCA subunit organization (Continued).
Aliquots of protein extract from 12 DAP seedlings containing 20 µg protein were subjected to native PAGE (4-20% linear gradient of polyacrylamide) at 170 v for 7.5 h (top two panels) or SDS-PAGE (10% of polyacrylamide) (bottom panel). After electrophoresis, gels were either transferred to nitrocellulose membranes (top two panels), or stained with Coomassie Blue (bottom panel). Western blot analysis was conducted using MCCA antiserum (top panel) or MCCB antiserum (middle panel). MCCase is indicated by arrows. MCCB subunit is indicated by triangle. WT, wild-type. Mu, mutant.
APPENDIX IV ADDITIONAL MUTANTS

$bccpl2-2$ (SAIL_611_D08) and $bccpl2-3$ (SALK_009735) were isolated and recovered from Syngenta Arabidopsis Insertion Library (SAIL) collection and SALK collection, respectively. The left border of $bccpl2-2$ was identified using SAILLB primer (5’-GCC TTTTCAGAAATGGATAAATAGCCTTGCTTCC-3’) and an gene-specific primer D08-RP (5’-CCCCAAAGTTACAACCTTTCGATG-3’). The right border of the T-DNA could not be identified. Using primers LP5 (5’-ACATATCTCTCCAAGTTCATCAAGGTCAG-3’) and RP5 (5’-GAAATTACCAAAGTCTAAGCCACCAACTC-3’), we have demonstrated that the upstream genomic sequence of $At3g15680$ is deleted in the homozygous mutant of $bccpl2-2$. Due to this deletion, the $bccpl2-2$ allele was not used for further investigation. $bccpl2-3$ allele carries head-to-head chimeric copies of the T-DNA insertion. Because the T-DNA insertion of $bccpl2-3$ is localized downstream of the last exon of $BCCPL-2$, this allele was not used for further investigation.
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