The Role of Biotin in Regulating 3-Methylcrotonyl-Coenzyme A Carboxylase Expression in Arabidopsis[1]

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As a catalytic cofactor, biotin has a critical role in the enzymological mechanism of a number of enzymes that are essential in both catabolic and anabolic metabolic processes. In this study we demonstrate that biotin has additional non-catalytic functions in regulating gene expression in plants, which are biotin autotrophic organisms. Biotin controls expression of the biotin-containing enzyme, methylcrotonyl-coenzyme A (CoA) carboxylase by modulating the transcriptional, translational and/or posttranslational regulation of the expression of this enzyme. The *bio1* mutant of Arabidopsis, which is blocked in the de novo biosynthesis of biotin, was used to experimentally alter the biotin status of this organism. In response to the *bio1*-associated depletion of biotin, the normally biotinylated A-subunit of methylcrotonyl-CoA carboxylase (MCCase) accumulates in its inactive apo-form, and both MCCase subunits hyperaccumulate. This hyperaccumulation occurs because the translation of each subunit mRNA is enhanced and/or because the each protein subunit becomes more stable. In addition, biotin affects the accumulation of distinct charge isoforms of MCCase. In contrast, in response to metabolic signals arising from the alteration in the carbon status of the organism, biotin modulates the transcription of the MCCase genes. These experiments reveal that in addition to its catalytic role as an enzyme cofactor, biotin has multiple roles in regulating gene expression.

Biotin is a water-soluble vitamin biosynthesized by plants, some fungi, and most bacteria and is required by all living organisms for normal cellular functions and growth. Extensive genetic and biochemical studies of prokaryotic organisms have established that biotin is biosynthesized from pimeloyl-CoA and Ala via a four-reaction biosynthetic pathway (DeMoll, 1996). Less extensive studies indicate that plants biosynthesize biotin via an analogous pathway (Shellhammer and Meinke, 1990; Weaver et al., 1995; Patton et al., 1996; Patton et al., 1998; Alban et al., 2000). Of the four enzymes required for biotin biosynthesis, only the one catalyzing the terminal reaction has been molecularly characterized in plants. This enzyme, called biotin synthase, is encoded by the *BIO2* gene of Arabidopsis and is a mitochondrial protein (Weaver et al., 1995; Patton et al., 1996; Baldet et al., 1997; Patton et al., 1998). Hence, in plants, biotin is biosynthesized in the mitochondria.

Biotin acts as a coenzyme, covalently bound to a Lys residue of a group of enzymes that catalyze carboxylation, decarboxylation or transcarboxylation reactions (Moss and Lane, 1971). The reactions catalyzed by these enzymes are involved in diverse metabolic processes including lipogenesis (acetyl-CoA carboxylase [ACCase]), gluconeogenesis (pyruvate carboxylase), and amino acid metabolism (methylcrotonyl-CoA carboxylase [MCCase] and propionyl-CoA carboxylase). These enzymes share a common biochemical reaction mechanism, in which the biotin prosthetic group acts as an intermediate carrier of the carboxyl group that is used as the substrate in the reaction. The carboxyl group specifically is first transferred from the donor substrate ($D\text{-}CO_2^-$) to the enzyme-bound biotin (reaction 1) and then to the final acceptor substrate (A; reaction 2).

$$\text{ENZ-BIOTIN} + \text{D-}CO_2^- \rightarrow \text{ENZ-BIOTIN-}CO_2^- + \text{D} \quad (1)$$

$$\text{ENZ-BIOTIN-}CO_2^- + \text{A} \rightarrow \text{ENZ-BIOTIN} + \text{A-}CO_2^- \quad (2)$$

$$HCO_3^- + \text{ATP} + \text{ACCEPTOR} \rightarrow \text{ADP} + \text{P} + \text{ACCEPTOR-}CO_2^- \quad (3)$$

Different organisms contain different complements of biotin-containing proteins. Bacteria and archaea have one to three biotin-containing proteins; for example, *Escherichia coli* contains only the biotin carboxyl carrier subunit of ACCase. Eukaryotic organisms have four or five such proteins. For example, animals contain ACCase, MCCase, pyruvate carboxylase, and propionyl-CoA carboxylase; Brewer's yeast (*Saccharomyces cerevisiae*), contains ACCase, pyruvate carboxylase, and urea carboxylase; and

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plants contain two isozymes of ACCase (Sasaki et al., 1993; Yanai et al., 1995; Ke et al., 2000), MCCase (Alban et al., 1993; Chen et al., 1993; Diez et al., 1994; Song et al., 1994; Wang et al., 1994; McKean et al., 2000), geranoyl-CoA carboxylase (Guan et al., 1999), and a seed-specific biotin protein that may function to store biotin (Duval et al., 1994; Hsing et al., 1998).

In addition to its catalytic function as an enzyme-bound prosthetic group, biotin may have a role in regulating gene expression. For example, in bacteria, biotin biosynthesis is regulated by the biotin status of the organism, and the activated form of biotin, biotinyl-AMP, acts as a corepressor to control the transcription of the biotin-biosynthetic operon (*bio*) and thus regulate biotin content of the cell (Cronan, 1989). A nonenzymological function(s) for biotin has also been reported in animals. For example, biotin affects the expression of several genes, including those coding for glucokinase (Chauhan and Dakshinamurti, 1991), phospho*enol*pyruvate carboxykinase (Dakshinamurti and Li, 1994), holocarboxylase synthetase, ACCase, and propionyl-CoA carboxylase (Solorzano-Vargas et al., 2002). In addition, several studies have demonstrated that enhanced dietary biotin intake has beneficial effects on the growth of farm animals, which appear to be independent of biotin's cofactor function (Whitehead et al., 1976; Whitehead and Bannister, 1981; Lischer et al., 2002). Although mechanistically not well understood, these latter observations indicate that biotin may have more extensive roles in biology than its enzymological function as an enzyme cofactor.

We report here that in Arabidopsis, a the eukaryotic biotin autotrophic organism biotin regulates the expression of MCCase subunit genes via complex mechanisms that are independent of the role of the molecule as a cofactor in the carboxylation reaction catalyzed by this enzyme.

RESULTS

The Effects of Biotin Depletion on MCCase Expression

To ascertain the effect of biotin on gene expression, tissues that contain low endogenous levels of biotin, optimally no biotin, are needed. Although it is simple to experimentally manipulate biotin levels in biotin heterotrophic organisms, this is more problematic in biotin autotrophs such as plants and bacteria. Because the *bio1* mutant of Arabidopsis cannot biosynthesize biotin (Shellhammer and Meinke, 1990), it is ideally suited for investigations into the effect of biotin. The *BIO1* gene is thought to encode for 7,8-diaminopelargonic acid aminotransferase, the second enzyme required in the conversion of pimeloyl-CoA and Ala to biotin (Patton et al., 1996). Plants homozygous for the *bio1* mutation show an embryonic-lethal phenotype, which can be rescued by the exogenous supply of biotin (Shellhammer and Meinke, 1990). Hence, homozygous *bio1* seeds germinate and grow on maternally supplied biotin. When this store of biotin is depleted, *bio1* seedlings stop growing (at the cotyledon stage), but these seedlings can be rescued with exogenously provided biotin. From such biotin-rescued plants, homozygous *bio1* plants can be grown, and seeds of this genotype can be recovered (Shellhammer and Meinke, 1990).

As an initial step to ascertain the effect of biotin depletion in the *bio1* mutant, MCCase activity was compared between *bio1* and wild-type Arabidopsis seedlings. As shown in Figure 1, MCCase activity increases during seedling development, peaking at 8 d after planting (DAP) in *bio1* seedlings and at 10 DAP in wild-type seedlings. Within 2 d after this peak, MCCase activity declines to lower levels, but in the *bio1* mutant, this activity is 3-fold lower than in the wild-type. The addition of exogenous biotin to *bio1* seedlings does not alter the pattern of MCCase expression, but elevates MCCase activity even above wild-type levels. These data indicate that in the *bio1* plants, maternally derived biotin is depleted from MCCase by 10 DAP. Consistent with this conclusion, parallel western-blot analyses with streptavidin indicates that the biotin content on the MCC-A subunit is reduced as biotin is depleted from the *bio1* seedlings; but when exogenous biotin is provided to these seedlings, the biotin content on this subunit increases (Fig. 2A).

In contrast, immunological detection of MCC-A indicates that the accumulation of this subunit is induced as biotin is depleted (Fig. 2B). This induction is paralleled by a similar increase in the accumulation of the non-biotinylated MCC-B subunit (Fig. 2C). Thus, in response to biotin depletion, the accumulation of both the MCC-A and MCC-B subunits is induced 5- to 10-fold, but the MCC-A subunit accumulates in the non-biotinylated apo-form.

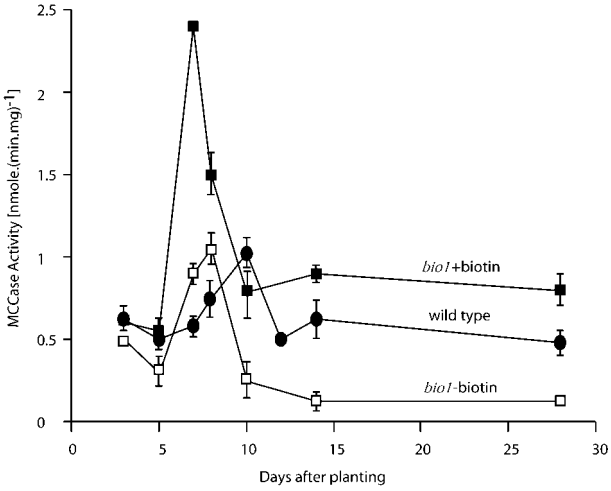

Figure 1. The effect of plant growth on MCCase activity. MCCase specific activity was determined in extracts of wild-type or *bio1* mutant seedlings between 3 and 28 d after sowing. Seedlings were grown either with or without the exogenous addition of 1 mM biotin. Data are the mean ± SE from four replicates.

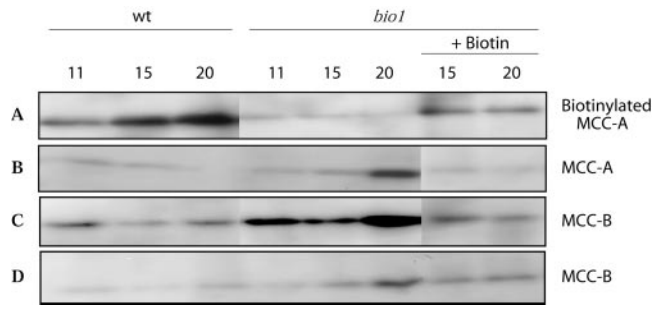

Figure 2. The effect of biotin on the biotinylation status and accumulation of the MCCase subunits. Protein extracts were prepared from seedlings (A–C) or excised cotyledons (D) of wild-type and *bio1* Arabidopsis seedlings at the indicated DAP. Aliquots of extracts containing equal amounts of protein (150 μg) were subjected to SDS-PAGE, followed by western-blot analysis with either ^{125}I-streptavidin to detect the biotinylated MCC-A subunit (A) or immunological detection with antibodies to MCC-A (B) or MCC-B (C and D). Where indicated, exogenous biotin (0.25 mM) was provided to the *bio1* seedlings 2 d before harvest. The data presented were gathered from a single experiment; five replicates of this experiment, with two different batches of *bio1* seeds, gave similar results.

Because *bio1* mutant seedlings are developmentally arrested at the cotyledon stage, whereas wild-type plants develop to the 4-leaf stage by 20 DAP, it was necessary to ensure that the observed induction of MCC-A and MCC-B accumulation was directly due to biotin depletion and not to the developmental arrest of the seedling. This question was addressed by comparing the accumulation of these subunits in the cotyledons of wild-type and *bio1* seedlings, which are morphologically indistinguishable between these two genotypes. These analyses indicate that the accumulation of the MCC-A (data not shown) and MCC-B (Fig. 2D) subunits are induced in the cotyledons of *bio1* seedlings relative to the cotyledons of wild-type seedlings. Furthermore, the induction of the accumulation of the MCC-A and MCC-B subunits is reversed when exogenous biotin is provided to the seedlings (Fig. 2, B–D), indicating that MCC-A and MCC-B accumulation is a direct consequence of biotin depletion. These findings demonstrate for the first time, to our knowledge, that biotin plays a role in the regulation of MCCase gene expression. Specifically, the accumulation of MCC-A and MCC-B proteins is inversely related to the biotin content of the Arabidopsis seedling.

Biotin Regulation of MCCase Expression Is Controlled at the Translational and/or Posttranslational Level

To further investigate the mechanism by which biotin depletion enhances the accumulation of the MCCase subunits, we compared the accumulation of the *MCC-A* and *MCC-B* mRNAs between wild-type and *bio1* plants. As shown in Figure 3A, the abundance of the *MCC-A* and *MCC-B* mRNAs is similar in both wild-type and *bio1* plants at 20 DAP. Furthermore, *MCC-A*- and *MCC-B*-promoter-mediated *GUS* expression is similar or slightly reduced when these transgenes are in the *bio1* background as compared with the wild-type genetic background (Fig. 3, C and D).

We interpret these results to indicate that the enhanced accumulation of MCC-A and MCC-B proteins in response to biotin depletion is not caused by enhanced transcription of the respective genes or increased accumulation of the respective mRNAs. Instead, the enhanced accumulation of the MCC-A and MCC-B proteins is due to either enhanced translation of the respective mRNAs or reduced turnover of these proteins.

Biotin Is Required for Metabolic Control of MCCase Gene Expression

In Arabidopsis, the expression of *MCC-A* and *MCC-B* genes respond to the carbon status of the organism (Che et al., 2002). Carbon starvation specifically increases MCCase activity, and the accumulation of MCC-A and MCC-B mRNAs, proteins, and this is the consequence of increased transcription of the *MCC-A* and *MCC-B* genes (Che et al., 2002). This conclusion was based upon experiments in which the carbon status of Arabidopsis seedlings was manipulated by altering the illumination of seedlings or by growing seedlings in a CO_2-free atmosphere. Darkness and CO_2 deprivation induce the expression of

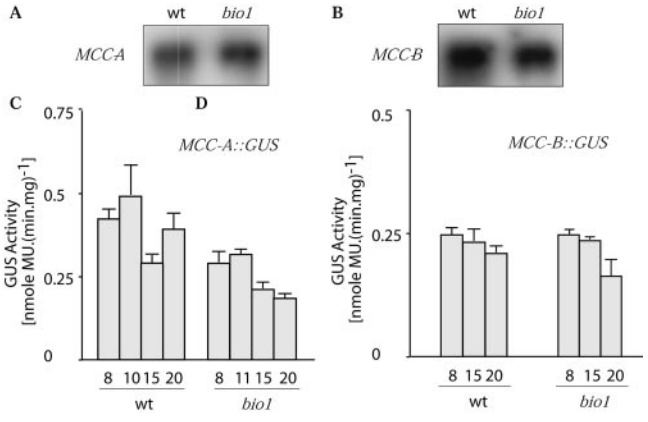

Figure 3. The effect of biotin-depletion on MCCase gene transcription. Northern-blot analysis of *MCC-A* (A) and *MCC-B* (B) mRNA accumulation in wild-type and *bio1* Arabidopsis seedlings. RNA was isolated from wild-type and *bio1* seedlings grown to 20 DAP in the absence of exogenous biotin. Equal amounts of isolated RNA (50 μg) were subjected to electrophoresis in formaldehyde-containing agarose gels, and *MCC-A* or *MCC-B* mRNAs were detected by hybridization with respective ^{32}P-labeled probes. Reporter gene expression studies of the *MCC-A* and *MCC-B* genes. GUS activity was determined in protein extracts from transgenic Arabidopsis seedlings of either wild-type (wt) or *bio1* genetic background and carrying an *MCC-A*::*GUS* (C) or *MCC-B*::*GUS* (D) reporter transgene. Seedlings were grown without exogenous biotin to the indicated DAP. Data are the means ± SE from three replicates.

MCC-A::*GUS* and *MCC-B*::*GUS* transgenes. To test whether biotin plays any role in this regulation, wild-type or *bio1* seedlings carrying either the *MCC-A*::*GUS* or *MCC-B*::*GUS* transgenes were grown under constant illumination for 13 d and were then either maintained in continuous illumination or transferred to darkness for 2 additional d. In parallel experiments, 13-d-old seedlings grown under continuous illumination were transferred to a CO_2-free atmosphere for 2 additional d without any change in the illumination status. As shown in Figure 4, A and B, in the wild-type background, these environmental manipulations (darkness or CO_2-free atmosphere) induced the expression of the *MCC-A*::*GUS* and *MCC-B*::*GUS* transgenes 10- to 20-fold. However, in the *bio1* genetic background, this induction is suppressed. That this failure to induce *MCC-A*- and *MCC-B*-mediated GUS activity in the *bio1* mutant background is due to the lowered biotin status of the seedlings is evidenced by the fact that the addition of exogenous biotin partially reverses the suppression. These results indicate that biotin is required for the metabolic regulation of the transcription of the MCCase subunit genes.

To further characterize this interaction between biotin and carbon status in the regulation of MCCase gene transcription, we studied the timing of the induction of the *MCC-A*- and *MCC-B*-mediated GUS expression in response to light deprivation. In this experiment, seedlings containing the *MCC-A*::*GUS* and *MCC-B*::*GUS* transgenes, carried in either the wild-type or *bio1* mutant background, were grown under continuous illumination for 6, 9, 13, and 18 d, and then transferred to complete darkness for an additional 2 d of growth, at which stage GUS activity was determined. As shown in Figure 5, A and B, at 8 DAP, *MCC-A*- and *MCC-B*-mediated GUS activity is similarly induced by darkness in both *bio1* and wild-type seedlings. However, by 11 DAP the induction of the transgenes in the *bio1* plants declines, and by 15 DAP, they are at 15% to 20% of the wild-type levels. This reduction in the ability of the *MCC-A*::*GUS* and *MCC-B-GUS* transgenes to be dark-induced at 11 DAP, coincides with the timing of the loss of biotin from the MCC-A subunit (Figs. 1 and 2), consistent with a role for biotin in regulating MCCase gene transcription.

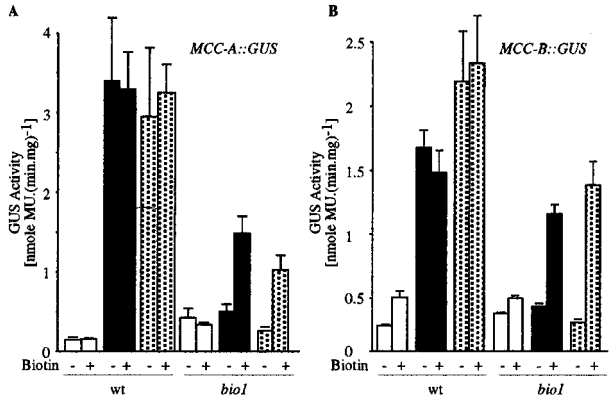

Figure 4. The effect of biotin-depletion on the metabolic regulation of *MCC-A* and *MCC-B* gene transcription. GUS activity was determined in protein extracts from wild-type (wt) or *bio1* Arabidopsis seedling carrying an *MCC-A*::*GUS* (A) or *MCC-B*::*GUS* (B) reporter transgene. Seedlings were grown to 13 DAP on Murashige and Skoog agar medium without biotin, followed by 2 d of additional growth either in the absence (−) or presence (+) of exogenous biotin. In these last 2 d of growth, seedlings were grown either under constant illumination (white bars), or transferred to darkness (black bars), or CO_2-free air (dotted bars). Data are the means ± SE from three replicates.

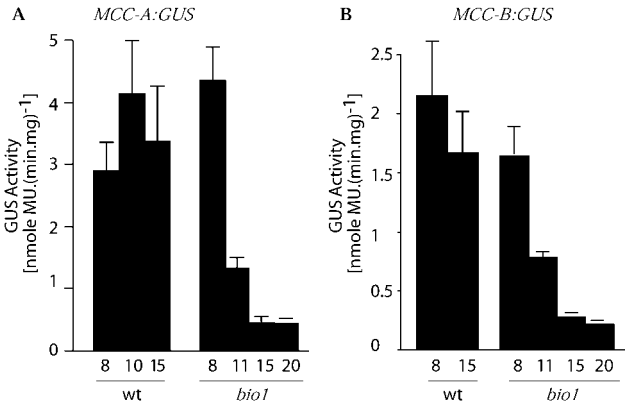

Figure 5. Time course of the biotin dependence of the metabolic regulation of *MCC-A* and *MCC-B* gene transcription. GUS activity was determined in protein extracts from wild-type (wt) or *bio1* Arabidopsis seedlings carrying an *MCC-A*::*GUS* (A) or *MCC-B*::*GUS* (B) reporter transgene. Seedlings were grown in the absence of exogenous biotin to the indicated DAP. These seedlings were maintained in constant illumination until the last 2 d of growth, at which stage they were transferred to total darkness. Data are the means ± SE from three replicates.

The Effect of Biotin on MCCase Subunit Stoichiometry

All MCCases investigated to date (from bacterial, plant, and animal sources) are composed of two subunits, a larger biotinylated subunit of about 80 kD (MCC-A) and a smaller non-biotinylated subunit of about 60 kD (MCC-B; Schiele et al., 1975; Lau et al., 1980; Wurtele and Nikolau, 2000). However, two types of MCCase differing in their subunit stoichiometries and hence molecular weights have been reported. The MCCase from animals (Lau et al., 1980), carrot (*Daucus carota*; Chen et al., 1993), maize (*Zea mays*; Diez et al., 1994), soybean (*Glycine max*; Song, 1993), and tomato (*Lycopersicon esculentum*; Wang, 1993) appear to have an A_6B_6 quaternary structure, with a molecular mass of about 850 kD. In contrast, MCCase from bacteria (Schiele et al., 1975), pea (*Pisum sativum*), and potato (*Solanum tuberosum*; Alban et al., 1993) appear to have an A_4B_4 quaternary structure, with a molecular mass of about 530 kD. Because the biotinylation of MCCase is an important mecha-

nism for regulating this enzyme (Wang et al., 1995), we considered the possibility that the apparent differences in the quaternary structure of MCCase from different sources may reflect the biotinylation status of the enzyme. To test this hypothesis, we determined the M_r of the MCCase complexes from wild-type and the biotin-depleted *bio1* mutant Arabidopsis seedlings and compared them with the soybean and pea MCCase.

The M_r of MCCase was determined by subjecting extracts to exhaustive electrophoresis in gels consisting of a 5% to 30% (w/v) linear gradient of polyacrylamide (Hedrick and Smith, 1968; Diez et al., 1994). Under these conditions, migration of proteins becomes limited when the sieve size of the gel pores is similar to the Stokes radius of the protein (which is proportional to the protein's M_r). Thus, migration in this electrophoresis system is inversely proportional to the M_r of the protein. Hence, by comparing the migration of MCCase with the migration of standard proteins, it was possible to determine the apparent M_r of MCCase. As shown in Figure 6A, the molecular mass of MCCase in pea and Arabidopsis is about 500 kD, whereas soybean MCCase is about 900 kD. Furthermore, biotin-depleted MCCase from Arabidopsis *bio1* seedlings migrated identically to the enzyme from wild-type seedlings, i.e. with a molecular mass of about 500 kD.

These data indicate that the subunit stoichiometry of MCCase is unaffected by the biotinylation status of the enzyme. Furthermore, these results confirm the earlier studies, which implied that MCCase from different plant species have different subunit stoichiometries. Namely, soybean MCCase appears to have an A_6B_6 configuration, whereas the pea and Arabidopsis MCCase has an A_4B_4 configuration. However, the physiological significance of this difference is still unclear.

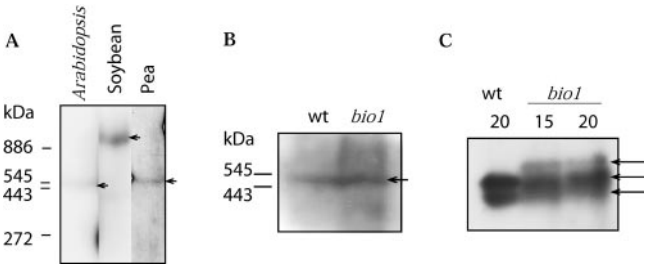

Figure 6. Electrophoretic characterization of MCCase. Protein extracts from Arabidopsis, soybean, and pea seedlings (A) and wild-type (wt) and *bio1* mutant Arabidopsis seedlings (B) were subjected to exhaustive electrophoresis (for 14,400 V h^{-1}) in gels composed of a linear gradient of 5% to 30% polyacrylamide according to the method of Hedrick and Smith (1968). After western blotting, MCCase was immunologically detected by reacting the membranes with anti-MCC-B serum (identical results were obtained with anti-MCC-A serum; data not shown). The native molecular mass of MCCase was determined by comparing its migration to standard proteins (apoferritin dimer, 886 kD; apoferritin monomer, 443 kD; urease dimer, 545 kD; and urease monomer, 272 kD). The position of MCCase is indicated by arrows. C, Analysis of MCCase charge isoforms. Aliquots of protein extracts from wild-type (wt) and *bio1* Arabidopsis seedlings at the indicated DAP, containing equal amounts of MCCase activity, were subjected to electrophoresis at 70 V for 17 h in a linear 5% to 20% gradient polyacrylamide gel (Lambin and Fine, 1979). After western blotting, MCCase was immunologically detected by reacting the membranes with anti-MCC-B serum (identical results were obtained with anti-MCC-A serum; data not shown).

The Effect of Biotin on the Formation of MCCase Charge Isoforms

Fractionation of Arabidopsis extracts by non-denaturing PAGE identifies two distinct forms of MCCase that migrate at different rates during electrophoresis (Fig. 6C). The electrophoresis system used in this study (Lambin and Fine, 1979) separates proteins both on the basis of charge and size. Knowing that there is no size heterogeneity in the MCCase present in these extracts (see Fig. 6, A and B), we conclude that these electrophoretically separable forms of MCCase represent charge isoforms of this enzyme. These charge isoforms of MCCase were also detectable by isoelectric focusing of Arabidopsis extracts (data not shown). Furthermore, previous analyses indicated that charge isoforms of MCCase occur in soybean (Song, 1993).

To investigate whether changes in the biotinylation status of MCCase affects the accumulation of these charge isoforms, we performed non-denaturing PAGE of seedling extracts from wild-type and biotin-depleted, *bio1* plants. MCCase was detected by western-blot analysis of the resulting gels using anti-MCC-B serum. As shown in Figure 6C, two MCCase bands accumulate in extracts from wild-type seedlings, but an additional band is detected in extracts from *bio1* seedlings. The origin of these MCCase charge isoforms is not clear. Because the two MCCase subunits are each encoded by a single gene in Arabidopsis (Weaver et al., 1995; McKean et al., 2000), the charge isoforms cannot represent products of different members of a gene family. However, there is evidence that the *MCC-A* gene can generate two alternatively spliced mRNAs (Che et al., 2002), which could generate isoforms of MCCase. Two *MCC-A* cDNAs have specifically been identified (GenBank accession nos. U12536 and AY070723) that differ from each other by the insertion of a 62-nucleotide sequence, which would be the result of alternative splicing of the initial transcript. An alternative explanation is that the charge isoforms are a direct effect of biotinylation. Namely, apo- and holo-MCC-A would be expected to differ from each other by a single charge as the Lys residue that becomes biotinylated contributes a single positive charge to apo-MCC-A, which is eliminated upon biotinylation and conversion to holo-MCC-A. Hence, MCCase that contains apo-MCC-A subunits would migrate more slowly during electrophoresis due to the increased positive charge associated with this form of the enzyme. The

change in electrophoresis pattern of MCCase isoforms associated with the change in the biotinylation status of the enzyme is consistent with this explanation.

DISCUSSION

The biochemical function of biotin as an enzyme-bound cofactor was established with the purification and characterization of such biotin-containing enzymes as MCCase, ACCase, and propionyl-CoA carboxylase. Each of these enzymes catalyze reactions that are critical in primary metabolism. For example, MCCase is part of the mitochondrial Leu catabolic pathway, a function that in humans is essential for growth and development. To date, three mechanisms have been reported to regulate MCCase expression. First, during the development of Arabidopsis, the *MCC-A* and *MCC-B* mRNAs show a coordinated programmed accumulation pattern (McKean et al., 2000) that probably reflects the metabolic role of MCCase in Leu (Anderson et al., 1998) and possibly cytosolic mevalonate-derived isoprenoid metabolism (Nes and Bach, 1985). Second, a complex interplay between environmental and metabolic signals mediates the transcription of the Arabidopsis *MCC-A* and *MCC-B* genes (Che et al., 2002). This sensitive regulation of MCCase expression probably reflects the physiological demands for MCCase function in Leu catabolism in response to changes in the carbon status of the organism. The transcription of the MCCase subunit genes is induced when the carbon status of the plant is lowered, and this response appears to be mediated via a sugar-signaling pathway (Che et al., 2002). Finally, in tomato, MCCase activity in roots and leaves is regulated by differential biotinylation of the MCC-A subunit (Wang et al., 1995). Specifically, whereas the MCC-A subunit accumulates to near equal levels in both roots and leaves, leaves express only 10% of the MCCase activity found in roots, and this difference is due to the lower biotinylation status of the MCC-A subunit in the leaf.

The studies reported herein indicate that as is the case for another cofactor, vitamin A (Truckenmiller et al., 2001; Chang et al., 2002), biotin has additional biological roles, namely in regulating gene expression. We have used the *bio1* mutant of Arabidopsis, which is blocked in the de novo biosynthesis of biotin, to elucidate the role of biotin in regulating MCCase expression. In response to biotin depletion (due to the *bio1* mutation), the MCC-A subunit accumulates in its inactive apo-form, and the accumulation of both MCCase subunits is induced. This induction occurs either because the translation of each subunit mRNA is enhanced or because the turnover of each subunit protein is reduced. Because the accumulation of both MCCase subunits is similarly induced, the mechanism(s) that controls the expression of these subunits probably coordinately affects both subunits. In addition, biotin is required for the two MCCase subunit genes to respond to metabolic signals. Specifically, in environmental conditions that reduce the carbon status of seedlings (deprivation of CO_2 or deprivation of illumination), transcription of the MCCase genes is normally induced (Che et al., 2002). However, this induction in gene transcription fails to occur in seedlings that are depleted of biotin. Finally, MCCase can accumulate as distinct isoforms that are separable by electrophoresis, and biotin influences the distribution of these isozymes. These charge isoforms may be due to alternatively spliced *MCC-A* mRNAs and/or to incomplete biotinylation, which leads to the accumulation of the differently charged apo-form of MCC-A.

It is interesting to consider how this complex regulation of MCCase expression by biotin is mediated. For example, does the organism sense the accumulation of the apo-subunit directly and alter MCCase expression, or does it respond to a decreased concentration of biotin, or does the organism detect the block in the metabolic function associated with MCCase (i.e. Leu catabolism) and alter MCCase expression? Although these questions cannot be addressed by the data presented herein, ongoing studies of *MCC-A* expression in an Arabidopsis *mcc-B* knockout mutant indicate that the organism is responding either to changes in the biotinylation status of MCCase or changes in the biotin status of the organism per se (P. Che and B.J. Nikolau, unpublished data). Namely, when Leu catabolism is blocked due to the *mcc-B* mutation, the accumulation of the MCC-A subunit is not induced, and the metabolic induction of *MCC-A* transcription still occurs. Hence, the experiments reported herein reveal that, in addition to its catalytic role as an enzyme cofactor, biotin may have a role in regulating MCCase gene expression. Therefore, previous findings, which indicate that biotin concentration differs among different cellular and subcellular compartments of a plant (Shellhammer and Meinke, 1990; Baldet et al., 1993; Wang et al., 1995) may manifest different patterns of gene expression among these compartments.

Non-catalytic roles for biotin have previously been reported in bacteria, where biotin directly affects the transcription of the *bio* operon and thus autoregulates its own biosynthesis (Cronan, 1989). In this capacity, the activated form of biotin, biotinyl-AMP, binds to holocarboxylase synthetase and induces functional changes in that protein, which enable it to bind to the *bio* operator and to suppress the transcription of the *bio* operon. In addition, in animals, the expression of a number of genes is enhanced by biotin. Specifically, in rat, biotin induces the transcription of the glucokinase (Chauhan and Dakshinamurti, 1991) and phospho*enol*pyruvate carboxykinase (Dakshinamurti and Li, 1994) genes. Furthermore, in multiple carboxylase deficiency syndromes of humans, holocarboxylase synthase is required for the biotin-dependent induction of the accumulation of mRNAs coding for

holocarboxylase synthase, and biotin subunits of ACCase and propionyl-CoA carboxylase (Solorzano-Vargas et al., 2002). Although the mechanism by which biotin affects gene expression is unclear, the intriguing observation that biotinidase can catalyze the biotinylation of histones offers the possibility that this modification of histones may affect gene transcription (Hymes et al., 1995; Stanley et al., 2001).

Our characterization of the biotin-mediated regulation of MCCase expression demonstrates a noncatalytic function of biotin in plants, which are biotin autotrophic organisms. Furthermore, these studies indicate that biotin cannot only regulate gene expression by modulating transcription (as occurs in bacteria and animals), but also mediates regulation of gene expression at the translational and/or posttranslational level. These regulatory functions appear not to be confined only to MCCase, but may be part of a more complex regulatory pathway for controlling the biotin metabolic network. For example, we have found that the transcription of one of the two genes that code for the biotin subunit of the chloroplastic ACCase is induced by biotin (Che, 2000; P. Che and B.J. Nikolau, unpublished data).

MATERIALS AND METHODS

Plant Genetic Stocks and Plant Growth Conditions

The Arabidopsis *bio1* mutant genetic stock (Shellhammer and Meinke, 1990) in the Columbia ecotype background was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The *MCC-A*::*GUS* and *MCC-B*::*GUS* transgenic stocks were generated in the Columbia ecotype, and the transgenes were made homozygous by propagating stocks to the T3 generation (Che et al., 2002). The *MCC-A*::*GUS* and *MCC-B*::*GUS* transgenes were moved into the *bio1*-mutant background by intercrossing. Homozygotes for each of the *MCC-A*::*GUS*, *MCC-B*::*GUS* transgenes (scored by the tightly linked KAN^R gene) in a homozygous mutant *bio1* background (scored by the biotin-requirement phenotype) were identified at the F_3 generation. Unless otherwise stated, plants were grown on Murashige and Skoog media either with or without biotin, in a controlled growth-room maintained at 22°C under continuous white light irradiation at 150 μmol m^{-2} s^{-1} provided by 40-W cold-white fluorescent bulbs.

Extraction and Analysis of Proteins

Arabidopsis protein extracts were prepared from 0.1 to 0.3 g of tissue using 3 volumes of 0.1 M HEPES-KOH, pH 7.0, 20 mM 2-mercaptoethanol, 0.1 mg mL^{-1} phenylmethylsulfonyl fluoride, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v) glycerol, as described by Diez et al. (1994). Proteins were fractionated by electrophoresis in polyacrylamide gels either after denaturation with SDS (Laemmli, 1970), or in a non-denatured state (Hedrick and Smith, 1968; Lambin and Fine, 1979). Gels were either stained with Coomassie Brilliant Blue or subjected to western-blot analysis. The two MCCase subunits (MCC-A and MCC-B) were immunologically detected with subunit-specific antisera (McKean et al., 2000; Che et al., 2002). The biotin-containing proteins were detected by using ^{125}I-streptavidin (Nikolau et al., 1985). Relative intensities of protein bands detected by western analysis were quantified by exposing membranes to a phosphor screen (Molecular Dynamics, Sunnyvale, CA), and the radioactivity associated with each band was quantified with a Storm 840 PhosphorImager (Molecular Dynamics).

Assays

MCCase activity was determined as the rate of methylcrotonyl-CoA-dependent incorporation of radioactivity from $H^{14}CO_3^-$ into an acid-stable product (Wurtele and Nikolau, 1990). GUS activity was determined in extracts with a fluorometric assay essentially as described by Jefferson (1987). Protein concentrations were determined by the Bradford (1976) method. All of the experiments were repeated three times using three independently transformed plant lines.

Isolation of RNA and Hybridization Analysis

RNA was isolated by the method of Logemann et al. (1987). Twenty micrograms of each RNA sample was subjected to electrophoresis in a 1.4% (w/v) agarose gel containing formaldehyde, and the RNA was blotted to nylon membrane by capillary transfer using 25 mM sodium phosphate buffer, pH 7.0. After baking the membrane at 90°C for 60 min, it was hybridized with ^{32}P-labeled *MCC-A* or *MCC-B* cDNA fragments (Weaver et al., 1995; McKean et al., 2000). The blots were washed once with 1× SSC and 0.1% (w/v) SDS at 60°C for 15 min followed by 0.25× SSC and 0.1% (w/v) SDS at 60°C for 30 min.

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