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Biotin Synthase from *Arabidopsis thaliana*

cDNA Isolation and Characterization of Gene Expression

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The full-length *BIO2* cDNA from *Arabidopsis thaliana* was isolated using an expressed sequence tag that was homologous to the *Escherichia coli* biotin synthase gene (*BioB*). Comparisons of the deduced amino acid sequence from *BIO2* with bacterial and yeast biotin synthase homologs revealed a high degree of sequence similarity. The amino terminus of the predicted BIO2 protein contains a stretch of hydrophobic residues similar in composition to transit peptide sequences. *BIO2* is a single-copy nuclear gene in Arabidopsis that is expressed at high levels in the tissues of immature plants. Expression of *BIO2* was higher in the light relative to dark and was induced 5-fold during biotin-limited conditions. These results demonstrate that expression of at least one gene in this pathway is regulated in response to developmental, environmental, and biochemical stimuli.

Biotin is an essential, water-soluble vitamin found in virtually all living cells (Dakshinamurti et al., 1985). Microorganisms, plants, and a few fungi make their own biotin, animals require trace amounts in their diets, and most fungi must obtain biotin from their environment. Biotin is an enzyme cofactor that functions as a carrier of CO_2 during carboxylation, transcarboxylation, and decarboxylation reactions (Knowles, 1989). CO_2 binds to biotin at the 1′ N atom of the imidazole ring, forming carboxybiotin, which is covalently attached to a Lys residue in the enzyme. Many organisms contain biotinidase, an enzyme that facilitates recovery and recycling of biotin following proteolytic degradation of biotin-containing enzymes, by cleaving the covalent bond between biotin and carboxylase to release free biotin (Wolf et al., 1975).

The genetics and biochemistry of biotin biosynthesis and its regulation have been extensively studied in microorganisms by a number of researchers (del Campillo-Campbell et al., 1967; Pai, 1968; Eisenberg, 1973). At least six genes that play a direct role in the synthesis of biotin, five of which reside in a bidirectionally transcribed operon, have been identified in *Escherichia coli*. The *bio* operon is negatively regulated at the operator sequence by biotin bound to the product of the *birA* gene. The BirA protein is both a ligase that attaches biotin to carboxylases and a repressor that suppresses transcription of the *bio* operon under high-biotin conditions. Detailed mutational analysis has led to a comprehensive understanding of the biotin biosynthetic pathway in *E. coli* (Rolfe and Eisenberg, 1968; Cleary and Campbell, 1972). The first data suggesting that plants synthesize biotin using the same biosynthetic route as *E. coli* came from a study of a biotin-auxotrophic mutant of *Arabidopsis thaliana* called *bio1* (Shellhammer, 1991). The *bio1* mutant was initially identified by testing the growth response of arrested embryos from different lethal mutants on basal and enriched media (Baus et al., 1985). Embryos from mutant 122G-E, later renamed *bio1*, grew specifically in response to supplemented biotin and could develop into phenotypically normal plants in soil as long as biotin supplements were continued (Schneider et al., 1989). Based on feeding studies, Shellhammer and Meinke (1990) suggested that *bio1* was defective in the conversion of 7-keto-8-aminopelargonic acid to 7,8-diaminopelargonic acid, the enzymatic function of the BioA protein of *E. coli* (see Fig. 1). We have recently shown that expression of the *E. coli bioA* gene in homozygous *bio1/bio1* plants eliminates the auxotrophic phenotype (Patton et al., 1996), demonstrating that previous predictions about the defect in *bio1* were correct.

The final step of biotin biosynthesis has recently received a great deal of attention. This reaction involves the addition of a sulfur atom between two unactivated carbon atoms adjacent to the imidazole ring of dethiobiotin (Fig. 1). Until recently it was thought that in *E. coli* this ring-closure step was catalyzed solely by the product of the *BioB* gene. Accumulating evidence now suggests that this step is catalyzed by at least three separate protein components: the *BioB* gene product, flavodoxin, and an unidentified protein (Ifuku et al., 1994; Birch et al., 1995). An apparent intermediate in this reaction, 9-mercaptodethiobiotin, has been isolated from plants and has been shown to support the growth of *E. coli* mutants defective in the *BioB* gene (Baldet et al., 1993a). Therefore, 9-mercaptodethiobiotin may represent a true intermediate in the pathway, so that two enzymatic steps are required for the conversion of dethiobiotin to biotin. Alternatively, 9-mercaptodethiobiotin may represent a transition-state intermediate that easily disassociates from the biotin synthase reaction complex. Thus, the most recently proposed pathway for biotin biosynthesis in plants (Baldet et al., 1993a) has 9-mercaptodethiobiotin as an intermediate (Fig. 1), but it is still unclear how the final synthetic steps proceed.

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Abbreviation: EST, expressed sequence tag.

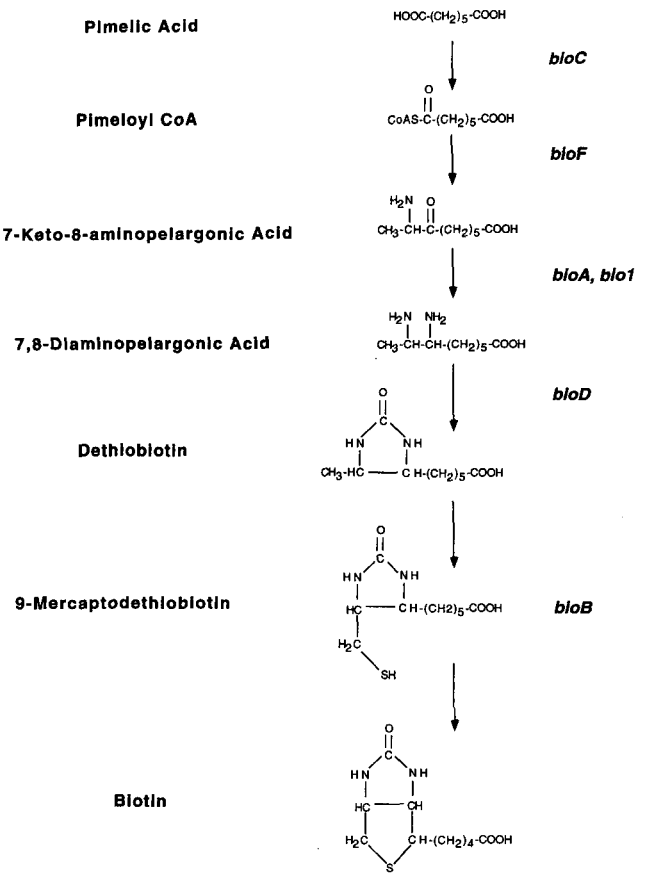

Figure 1. Biotin biosynthetic pathway in plants. Positions where mutations block biotin biosynthesis in *E. coli* (*bioC, bioF, bioA, bioD,* and *bioB*) and Arabidopsis (*bio1*) are shown. Adapted from Baldet et al. (1993b).

Biotin content in plant tissues varies widely between different species (Shellhammer and Meinke, 1990; Baldet et al., 1993b; Mozafar, 1993). For instance, even though total biotin concentrations are similar between pea and Arabidopsis, the partitioning between free and bound biotin varies widely between these two species. In pea leaves over 80% of the biotin is unbound and found mostly in the cytoplasm (Baldet, 1993b), whereas in Arabidopsis the bulk of biotin is bound to proteins, most of which are likely to reside in the chloroplast and mitochondria (Shellhammer, 1991). Regulation of the synthesis and loading of biotin on carboxylases in plants is poorly understood.

In this paper we describe the isolation of a full-length cDNA corresponding to a plant biotin biosynthetic gene, which we call *BIO2*, and show that this cDNA encodes a functional protein that complements a *bioB* defect in *E. coli*. We also demonstrate that *BIO2* is a single-copy gene in Arabidopsis and its expression is temporally and spatially regulated by such factors as development, light, and biotin status. Inspection of the Arabidopsis BIO2 protein sequence and functional analysis of 5′ deletions of the cloned cDNA leads us to believe that biotin biosynthesis may occur in the chloroplast or mitochondria rather than in the cytoplasm.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* seeds of the Columbia ecotype were obtained from Lehle Seeds (Round Rock, TX). Homozygous mutant *bio1/bio1* seeds were obtained from David Meinke (Oklahoma State University, Stillwater). Columbia plants used for DNA and tissue-specific RNA analysis were sown in MetroMix 360 (Grace Sierra, Milpitas, CA) in 3-inch pots and grown at 23 ± 4°C under 8-h light/16-h dark cycles. Seedlings used for light/dark experiments were grown under 12-h light (150 μE m^{-2} s^{-1})/12-h dark conditions in a growth chamber at 20 ± 2°C with 50% RH. Duplicate samples were taken 3.5, 6, and 11.5 h into the light cycle, and 6 and 11.5 h into the dark cycle. Columbia and *bio1/bio1* plants used to analyze gene expression in response to biotin status were germinated and grown for 14 d on agar medium with or without 10 nM biotin and then transferred to liquid medium as described previously (Guyer at al., 1995). Plants were grown for 14 d in liquid medium supplemented with or without 100 nM biotin and then transferred to fresh medium with or without 100 nM biotin and grown for another 14 d. Samples were harvested in triplicate 28 d after the transfer of seedlings to the liquid medium.

Isolation of the Full-Length *BIO2* cDNA

A keyword search of the National Agricultural Library Database identified an Arabidopsis EST clone that contained an open reading frame homologous to the *Escherichia coli BioB* gene. This clone, 86E12 (GenBank accession no. T20529) was obtained from the Arabidopsis Stock Center (The Ohio State University, Columbus) and sequenced. The open reading frame of 86E12 started at the 5′ terminus of the cDNA, and the position at which homology started with the *E. coli BioB* gene suggested that 86E12 was less than full-length. To obtain a full-length clone we radioactively labeled the insert from 86E12 and used it as a probe to screen an Arabidopsis (Columbia) leaf cDNA library in bacteriophage (λ ZAPII, Stratagene). Plaque screening and in vivo excision were performed according to the manufacturer's instructions. Five clones were purified to homogeneity out of a total of 250,000 plaques screened. Inserts were sized on agarose gels following restriction digests with *Eco*RI and *Xho*I. The clone containing the longest insert (pMP101) was sequenced with an automated sequencer (ABI373, Applied Biosystems) using fluorescent dye terminators. The GenBank accession number for this sequence is U31806.

Functional Complementation of *E. coli* bioB Mutant

Deletions of the *BIO2* cDNA were created using PCR with pMP101 as a template and specific primers designed to remove varying numbers of residues from the amino terminus of the BIO2 gene product. PCR products were ligated into the vector (PCRII, Invitrogen, San Diego, CA) and sequenced to ensure that no artifacts were generated during amplification. *Eco*RI inserts were ligated into the

pKK388 expression vector (Clontech, Palo Alto, CA) giving an in-frame fusion that resulted in proteins with five amino acid residues from the vector fused to the desired product. Sequence-verified plasmids were then used to transform the *bioB105 E. coli* mutant obtained from the *E. coli* genetic stock center (Yale University, New Haven, CT). Aliquots from the transformation mixture were washed with SOC medium (Maniatis et al., 1982) containing 4 μg/mL streptavidin (GIBCO-BRL) to remove residual biotin. They were then plated on Luria broth selection medium containing 100 μg/mL ampicillin, with or without 1 mM isopropyl β-D-thiogalactoside, and with or without 1 μg/mL streptavidin. To determine the effect of the deletions on the function of the resulting BIO2 proteins, growth rates of complemented *bioB105* cells were measured at 28°C in biotin-free (streptavidin-containing) Luria broth. Empty pKK388 vector was used as a negative control in these experiments.

Sequence Comparisons

Pairwise comparisons between the predicted BIO2 protein and the five additional protein sequences in the GenBank database similar to BIO2 were made with the computer program GAP (GCG Package, Genetics Computer Group, Madison, WI; Devereux et al., 1984), which uses the algorithm of Needleman and Wunsch (1970) to maximize the number of matches and minimize the number of gaps between two sequences. Optimal alignments of all six BioB homologs were made with the program PileUp (GCG Package), which creates a multiple sequence alignment from a group of related sequences using a simplification of the progressive alignment method of Feng and Doolittle (1987).

Southern Blot Analysis

Approximately 1 μg of genomic DNA was digested with 20 units of restriction enzyme for 2 h, electrophoretically separated in 0.75% agarose, and alkaline blotted to membranes (Hybond N^+, Amersham). To determine *BIO2* copy number in the Arabidopsis genome, blots were probed with the 877-bp insert from EST 86E12. Probes labeled by the random priming method (Feinberg and Vogelstein, 1983) were purified using push columns (NucTrap, Stratagene). Genomic DNA isolation, blot hybridization, and blot washing were carried out as described by Reiter et al. (1992).

Northern Blot Analysis

Tissue harvested for RNA isolation was frozen in liquid N_2 and stored at −80°C until use. Total RNA was isolated using the phenol extraction method of Lagrimini et al. (1987) and quantified by measuring UV A_{260}. Samples containing 2 μg of total RNA were separated on formaldehyde electrophoresis gels and blotted to membranes (GeneScreenPlus, NEN). Blots were hybridized to radioactively labeled probes and washed as described by Ausubel et al. (1987). Quantification of signals was carried out using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the signals were visualized by exposing blots to film overnight. Equal loading was achieved by quantification of RNA extracts with UV absorbance and ensured by ethidium bromide staining of samples in the loading buffer and visualization of UV-excited fluorescence.

RESULTS AND DISCUSSION

The *BIO2* Gene of Arabidopsis

We isolated a full-length *BIO2* cDNA from Arabidopsis using an Arabidopsis EST homologous to the *E. coli BioB* gene as a probe. Our *BIO2* cDNA clone (Fig. 2) is nearly identical in sequence to the *BIO2* cDNA clones isolated by Weaver et al. (1996) and Baldet et al. in GenBank (accession no. L34413). Our *BIO2* cDNA has an additional 125 bp at

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   1  GGCACGAGCTCATTTCTTCTTCTTCTTTTTCCACATTTTCTGATTAGCAGATCAAAT   60
                                                          M
  61  GATGCTTGTTCGATCTGTATTTCGATCTCAGTTGCGACCCTCTGTCTCGGGTGGTCTGCA  120
      M  L  V  R  S  V  F  R  S  Q  L  R  P  S  V  S  G  G  L  Q
 121  ATCTGCTTCTTGCTATTCTTCATTATCTGCTGCTTCAGCTGAAGCTGAGAGGACTATCAG  180
      S  A  S  C  Y  S  S  L  S  A  A  S  A  E  A  E  R  T  I  R
 181  AGAAGGTCCCAGAAACGATTGGAGTAGAGATGAAATCAAGTCTGTTTATGATTCTCCTCT  240
      E  G  P  R  N  D  W  S  R  D  E  I  K  S  V  Y  D  S  P  L
 241  TCTTGACCTCCTCTTCCATGGAGCTCAGGTTCATAGACATGTTCATAACTTCAGGGAGGT  300
      L  D  L  L  F  H  G  A  Q  V  H  R  H  V  H  N  F  R  E  V
 301  ACAACAATGTACCCTCCTCTCCATAAAGACTGGTGGCTGTAGTGAAGACTGTTCATATTG  360
      Q  Q  C  T  L  L  S  I  K  T  G  G  C  S  E  D  C  S  Y  C
 361  TCCTCAGTCTTCGAGATATAGCACTGGAGTTAAGGCACAAAGACTCATGTCTAAGGACGC  420
      P  Q  S  S  R  Y  S  T  G  V  K  A  Q  R  L  M  S  K  D  A
 421  TGTCATTGATGCTGCTAAGAAGGCAAAAGAAGCTGGGAGCACACGTTTTTGCATGGGTGC  480
      V  I  D  A  A  K  K  A  K  E  A  G  S  T  R  F  C  M  G  A
 481  TGCTTGGCGAGATACAATTGGACGGAAAACCAACTTCAGCCAGATTCTTGAATACATCAA  540
      A  W  R  D  T  I  G  R  K  T  N  F  S  Q  I  L  E  Y  I  K
 541  AGAAATAAGAGGCATGGGGATGGAAGTTTGCTGCACCTTAGGCATGATTGAGAAACAACA  600
      E  I  R  G  M  G  M  E  V  C  C  T  L  G  M  I  E  K  Q  Q
 601  AGCACTAGAGCTAAAGAAGGCTGGCCTCACTGCTTATAACCACAATCTTGATACTTCAAG  660
      A  L  E  L  K  K  A  G  L  T  A  Y  N  H  N  L  D  T  S  R
 661  AGAGTACTACCCAAACGTCATCACTACTAGAAGTTATGACGATCGCCTTGAAACTCTTAG  720
      E  Y  Y  P  N  V  I  T  T  R  S  Y  D  D  R  L  E  T  L  S
 721  CCATGTTCGTGATGCTGGAATCAACGTTTGTTCAGGAGGAATCATAGGGCTTGGTGAGGC  780
      H  V  R  D  A  G  I  N  V  C  S  G  G  I  I  G  L  G  E  A
 781  AGAGGAAGACAGAATAGGTTTATTACACACGCTGGCAACACTTCCTTCTCACCCTGAGAG  840
      E  E  D  R  I  G  L  L  H  T  L  A  T  L  P  S  H  P  E  S
 841  TGTTCCCATTAATGCTCTACTTGCAGTGAAAGGCACTCCTCTTGAAGACCAGAAGCCAGT  900
      V  P  I  N  A  L  L  A  V  K  G  T  P  L  E  D  Q  K  P  V
 901  TGAGATATGGGAGATGATCAGGATGATTGGAACCGCACGTATTGTAATGCCAAAAGCGAT  960
      E  I  W  E  M  I  R  M  I  G  T  A  R  I  V  M  P  K  A  M
 961  GGTGAGACTGTCTGCTGGTAGAGTCCGGTTCTCAATGTCCGAACAAGCTCTCTGTTTCCT 102C
      V  R  L  S  A  G  R  V  R  F  S  M  S  E  Q  A  L  C  F  L
1021  TGCTGGTGCAAACTCTATCTTCACCGGAGAGAAGCTTTTAACCACACCAAACAATGATTT 108C
      A  G  A  N  S  I  F  T  G  E  K  L  L  T  T  P  N  N  D  F
1081  TGACGCTGACCAGCTCATGTTCAAGACATTAGGCCTCATTCCTAAACCGCCAAGTTTCTC 114C
      D  A  D  Q  L  M  F  K  T  L  G  L  I  P  K  P  P  S  F  S
1141  TGGAGATGATTCTGAATCAGAAAACTGCGAGAAAGTTGCTTCCGCTTCTCACTAATATCA 120C
      G  D  D  S  E  S  E  N  C  E  K  V  A  S  A  S  H  *
1201  TTATCCACTTTTTTTTTTGTTTGGAGTCGGGACACTATAGAGCAGTCCCTTTTACTATGT 126C
1261  AGCATGGTTTGACGATTTTGTGATATCATTTTTCGTTAATCGTTATTCGAAGATGTCTAG 132C
1321  ATTTCTCATCTGAAAAAAAAAAAAAAAAAAA 1351
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Figure 2. Sequence of full-length *BIO2* cDNA. Base 1 starts immediately after the *Eco*RI site in pBluescript IISK+, and base 1351 immediately precedes the *Xho*I site. Deduced BIO2 protein is shown below the DNA sequence using the standard one-letter abbreviation. The GenBank accession no. for this sequence is U31806.

the 5′ end, 59 extra bp at the 3′ end, and lacks the C residue at base 1085 reported in GenBank accession no. L34413.

For the sake of clarity, in the remainder of this paper we will refer to the product of the Arabidopsis *BIO2* gene as the BIO2 protein and not biotin synthase. This is due to uncertainty about which enzymatic step is catalyzed by this protein.

DNA gel-blot analysis (Fig. 3) showed that *BIO2* is a single-copy gene in Arabidopsis. The deduced BIO2 protein from Arabidopsis showed significant identity to the published bacterial and yeast BioB proteins, with values ranging from 32 to 53%. The range of homology increased to 55 to 68% with inclusion of conservative substitutions (data not shown).

Alignment of the BioB homologs revealed several stretches of conserved residues that may be important for the function of this enzyme (Fig. 4). The conserved Cys residues found at positions 106, 110, 113, 151, 185, and 245 in the Arabidopsis BIO2 protein are consistent with the finding that the BioB protein from *E. coli* is a [2Fe-2S] cluster protein (Sanyal et al., 1994). These Cys residues may play a role in coordinating the Fe atoms in the Fe-S cluster. One striking difference between the proteins is the presence of additional hydrophobic residues at the amino terminus of the Arabidopsis BIO2 protein, which is suggestive of an organelle-localized enzyme (Fig. 4). This finding was unexpected because previous studies had suggested that biotin biosynthesis occurs in the cytosol (Shellhammer, 1991; Baldet et al., 1993a). Import studies with the BIO2 protein or immunolocalization with antibodies to the BIO2 protein will be required to determine which subcellular compartment contains the mature BIO2 protein.

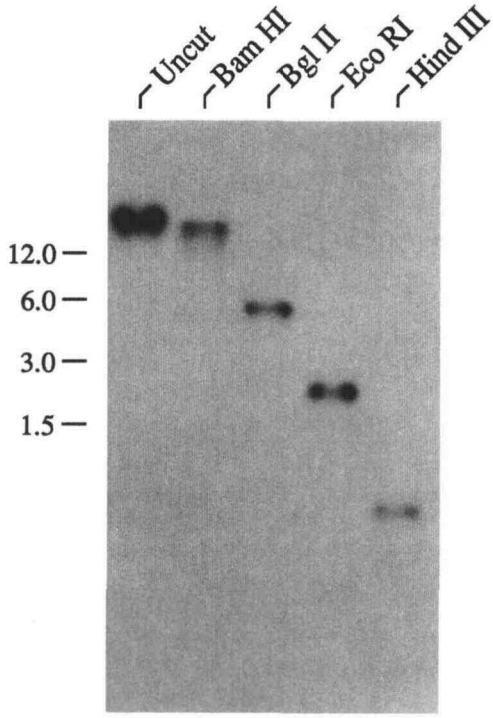

Figure 3. Gel blot of Arabidopsis genomic DNA probed with the BIO2 cDNA. Size standards are shown on the left in kb. Single bands in each lane indicate that *BIO2* is a single-copy gene in the Arabidopsis nuclear genome.

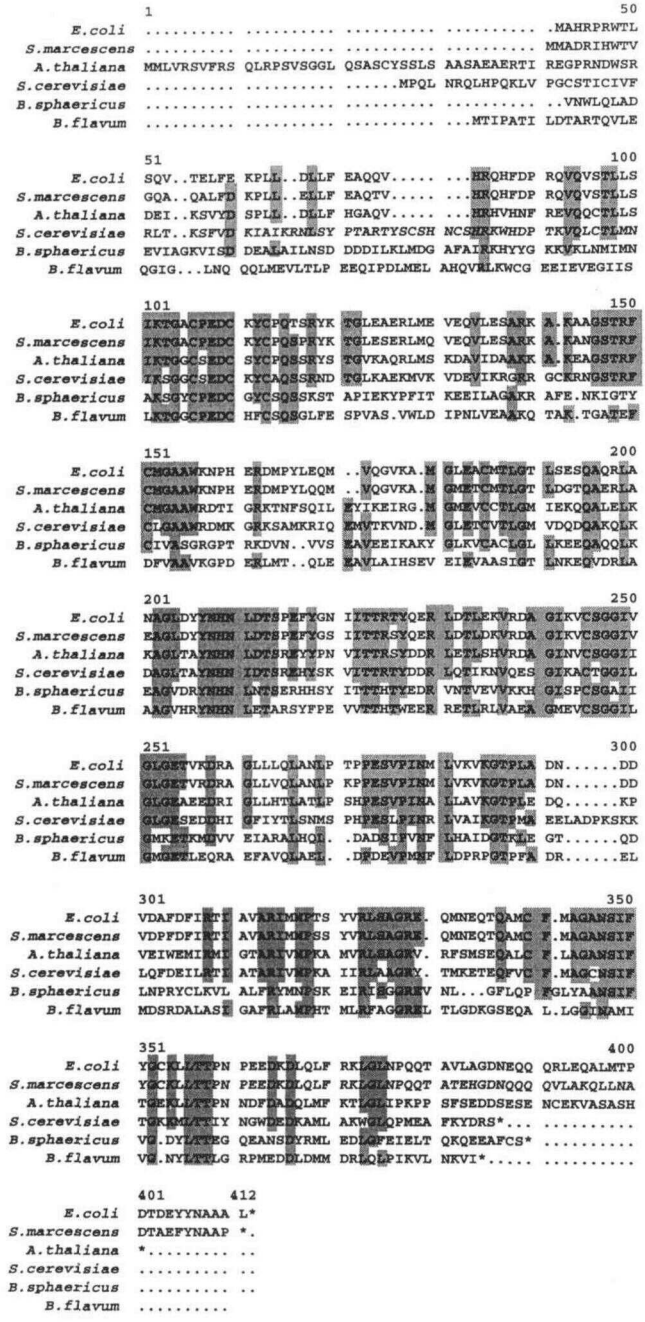

Figure 4. Alignment of *bioB* homologs created by the program PileUp (GCG Package). The positions where four or more identical residues occur are highlighted.

The Arabidopsis *BIO2* Gene Functionally Complements an *E. coli bioB* Mutant

Mutant *bioB105* cells of *E. coli* formed slow-growing colonies on biotin-free medium at 37°C when transformed with plasmids carrying the full-length *BIO2* gene from Arabidopsis (data not shown). The growth rate of these

cells increased at 28°C, normally a suboptimal temperature for *E. coli*. As expected, no colonies formed when *bioB105* cells were transformed with empty vector and plated on biotin-free medium. These findings suggest that the plant enzyme is either unstable at higher temperatures or that the addition of five residues as a protein fusion from the vector interferes with catalytic activity of the BIO2 protein at 37°C. Addition of isopropyl β-D-thiogalactoside, which induces expression of the *BIO2* insert in pKK388, also had an inhibitory effect on the growth of these cells (data not shown). The same inhibitory effect is seen in wild-type *E. coli* cells that harbor on a high-copy plasmid either the wild-type *BioB* gene or any of several *bioB* mutant forms with single amino acid substitutions (Ifuku et al., 1995). At this time it is not clear what the precise relationship is between *BioB* copy number and growth inhibition in *E. coli*.

Deletions of up to 40 amino-terminal residues in the BIO2 protein also resulted in functional complementation of *bioB105* cells (Fig. 5), so these residues are clearly not required for catalytic activity of the BIO2 protein. In fact, growth of mutant *bioB105* cells was enhanced by expression of some of the deletions, compared with the full-length clone (Fig. 5). These results provide additional evidence that the amino-terminal portion of BIO2 may play a role in localizing the BIO2 protein to a subcellular compartment.

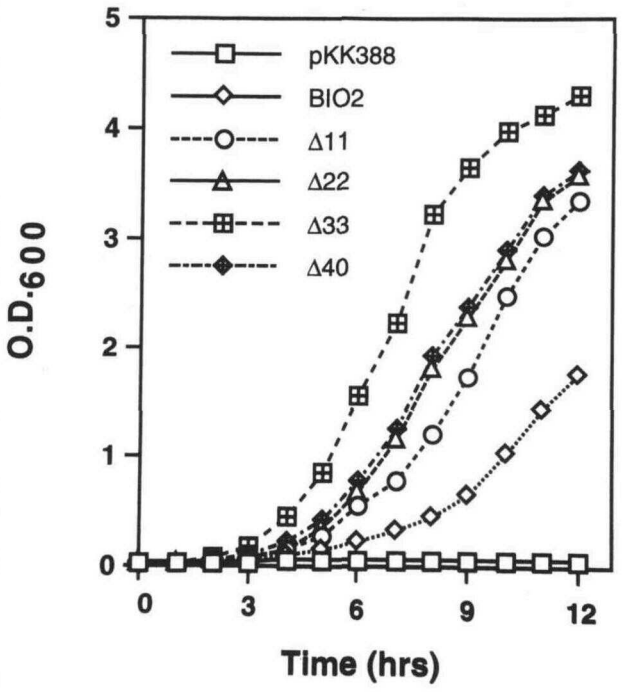

Figure 5. Effect of amino terminal deletions of BIO2 on growth-promoting activity in *bioB105* of *E. coli*. Plasmids were constructed as described in "Materials and Methods." pKK338 is the empty vector; BIO2 contains the full-length BIO2 protein minus the two Met residues; and Δ11, Δ22, Δ33, and Δ40 have amino acid deletions of the indicated length. O.D.$_{600}$, A_{600}.

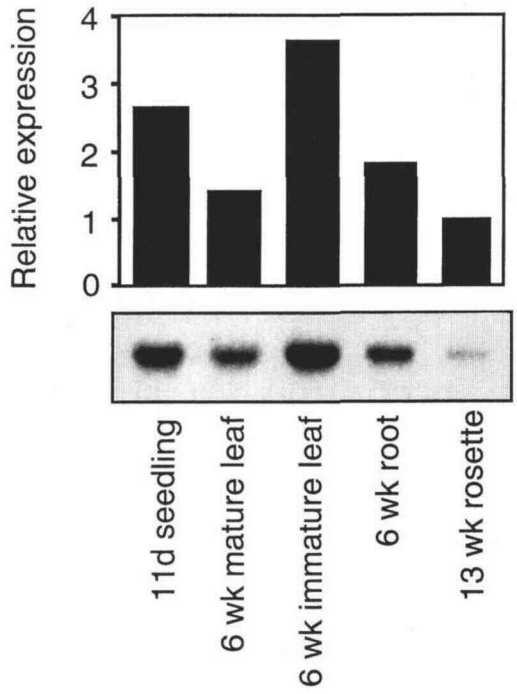

Figure 6. Organ-specific expression of *BIO2*. Signal strength of *BIO2*-hybridizing bands in the RNA gel blot shown in the lower panel was quantified with a PhosphorImager (Molecular Dynamics) and used to calculate the relative expression levels shown in the upper panel. The age and type of tissue sampled is listed.

BIO2 Expression Is Developmentally Regulated

To understand the regulation of *BIO2* gene expression, we analyzed RNA from various tissues by probing RNA gel blots with the *BIO2* cDNA. We first examined levels of *BIO2* mRNA in tissue samples taken from plants at different ages (Fig. 6). *BIO2* expression seems to inversely correlate with age of the tissue. In the tissues tested, *BIO2* was most highly expressed in immature leaves and young seedlings, with moderate expression seen in roots, and the lowest level of expression in older leaves from mature plants. We would expect that biotin demand is higher in young tissues because of the increased anabolism associated with dividing and expanding cells. These young tissues have been shown to contain large amounts of free biotin (Shellhammer, 1991; Duval et al., 1994b), as might be expected in tissues with increased synthesis. A more extensive, time-course analysis of RNA isolated from different tissues will be required to fully understand the temporal and spatial regulation of the *BIO2* gene in Arabidopsis.

Expression of *BIO2* Changes during the Light/Dark Cycle

At least one biotin-requiring carboxylase, acetyl CoA carboxylase, has been shown to be chloroplast-localized (Choi et al., 1995). Furthermore, biotin requirements might change in light rather than in dark conditions. Therefore, we tested expression of *BIO2* in seedlings during the light/dark cycle to see if *BIO2* expression is light-regulated. In three separate experiments we found that accumulation of *BIO2* mRNA is consistently highest at the end of the light

cycle and lowest at the end of the dark cycle (Fig. 7). The magnitude of the change is not dramatic, but the trend is reproducible and consistent with regulation by light or circadian rhythm. Alternatively, biotin demand could change during the day/night cycle. Regulation of steady-state mRNA levels could occur either at synthesis or through degradation. Further analysis will be required to understand how *BIO2* expression is regulated in response to light.

BIO2 Expression Is Regulated by Cellular Biotin Concentrations

To study the effects of biotin starvation on expression of *BIO2*, we used the *bio1* auxotroph of Arabidopsis. We germinated Columbia and *bio1/bio1* seeds on minimal and biotin-supplemented media, then shifted the seedlings to biotin-free media. We found that supplemental biotin at 100 nM final concentration had little effect on expression of the *BIO2* gene in wild-type plants (Fig. 8B, compare lanes 1 and 2). Growth of Columbia plants in vast excesses of biotin (up to 1 mM) also had no effect on *BIO2* expression (data not shown). Homozygous *bio1/bio1* plants grown in biotin-free medium for 2 weeks had greater than 5-fold more *BIO2* message than Columbia plants grown under the same conditions (Fig. 8B, compare lanes 3 and 5). In the same experiment, *bio1/bio1* plants grown in the presence of biotin for the final 2 weeks had 3-fold less *BIO2* message relative to *bio1/bio1* plants grown in the absence of biotin. Thus, expression of *BIO2* appears to be induced under biotin-limiting conditions. Conceivably, the activity encoded by *BIO2* is limiting, and its induction in a low-biotin environment can lead to increased biosynthesis. Alternatively, expression of the entire pathway may be increased under biotin-limiting conditions.

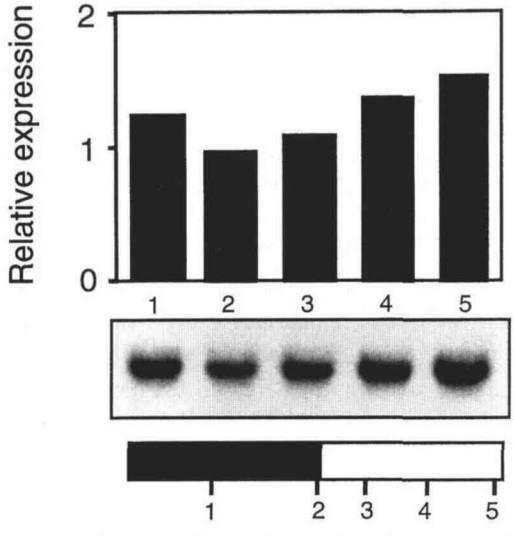

Figure 7. Expression of *BIO2* during the light/dark cycle. The horizontal bar represents one 24-h period, with 12 h of dark from 6:00 PM to 6:00 AM (black bar) and 12 h of light from 6:00 AM to 6:00 PM (white bar). Numbers along the horizontal axis represent when the five different samples were taken during the light/dark cycle; 1 = 12:00 midnight, 2 = 5:30 AM, 3 = 9:30 AM, 4 = 12:00 noon, and 5 = 5:30 PM. Signal strength of BIO2-hybridizing bands in the RNA gel blot shown in the lower panel was quantified with a PhosphorImager (Molecular Dynamics) and used to calculate the relative expression levels shown in the upper panel.

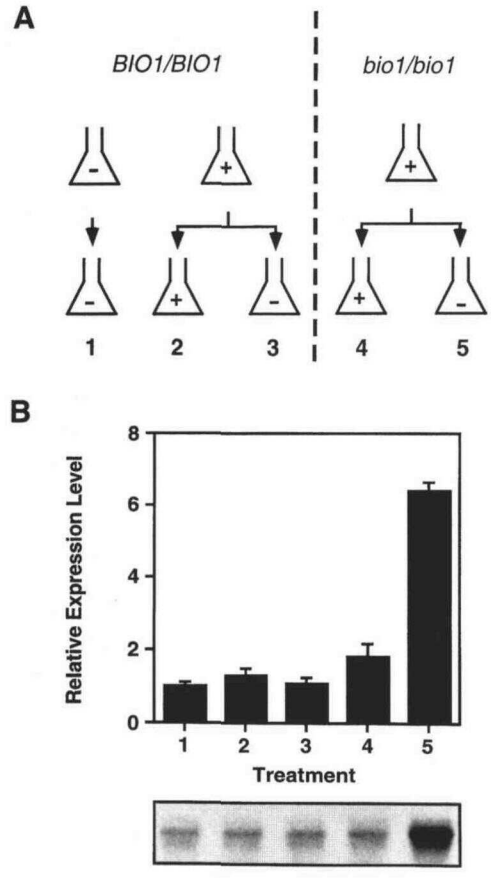

Figure 8. Expression of *BIO2* under different biotin conditions. A, Schematic diagram depicting experimental conditions described in "Materials and Methods." The left half of the panel (*BIO1/BIO1*) represents conditions under which wild-type Columbia plants were grown, and the right half is for mutant (*bio1/bio1*) plants. All seeds were germinated and grown for 2 weeks on agar plates with biotin (treatments 2, 3, 4, and 5) or without biotin (treatment 1), and then transferred and grown for 2 additional weeks in culture flasks with liquid medium supplemented with 100 nM biotin ([+] treatments 2, 3, 4, and 5) or no biotin ([−] treatment 1). The plants were then transferred to fresh medium with 100 nM biotin ([+] treatments 2 and 4) or no biotin ([−] treatments 1, 3, and 5). B, Typical gel blot of RNA samples isolated from tissue harvested at 6 weeks from the five treatments described in A and probed with the full-length BIO2 cDNA. Relative expression levels (+1 SD) shown in the bar graph were determined using three independent samples from each treatment.

Complex Biotin Biosynthetic Gene Regulation

These initial observations on *BIO2* gene expression suggest that regulation of this gene in plants may be complex. At least three different stimuli have an effect on the steady-state levels of *BIO2* mRNA: development, light or circadian period, and biotin starvation. Further analysis of the promoter sequence may reveal sites where some of the regu-

latory elements control *BIO2* expression. Promoter elements that play a role in directing either developmental or light-regulated expression have already been identified in a number of plant genes (Kuhlemeier et al., 1987; Guerrero et al., 1990; de Pater et al., 1993). Transgenic plants carrying reporter genes fused to the various portions of the *BIO2* promoter may help to identify regions that play a role in regulating *BIO2* expression.

Regulation of many anabolic pathways in microorganisms is simplified due to the organization of genes into an operon. In many cases flux through the pathway is regulated by levels of the end product of the pathway, which works either by modulating gene expression through factors acting at the operator sequence, or by direct allosteric inhibition of a pathway enzyme. Thus, it is unlikely that plants contain the equivalent of the repressor function of the BirA protein found in *E. coli*. A *BirA* mutant of *E. coli* was recently used to clone mammalian cDNAs that encode the biotin ligase activity, the other function of BirA (Leondelrio et al., 1995). As expected, the protein encoded by these mammalian cDNAs had no repressor function in *E. coli*.

Cloning of additional plant genes from the biotin biosynthetic pathway will be required before we have a comprehensive understanding of how biotin synthesis is regulated in plants. Toward this end, one other gene from the pathway, *BIO1*, has been identified by mutation in Arabidopsis (Schneider et al., 1989). The *bio1* mutation has been mapped to within 0.5 centimorgan of a molecular marker on chromosome 5 (Patton et al., 1991). Thus, isolation of *BIO1* should be possible soon using a map-based cloning approach or through the identification of an EST with a sequence similar to that of *bioA*.

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