ANALYSIS OF A BIOTIN AUXOTROPH
OF *ARABIDOPSIS THALIANA*

By

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Bachelor of Science
in Arts and Sciences

Oklahoma State University

Stillwater, Oklahoma

1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1991

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Thesis Approved

Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

I thank Linda Franzmann for teaching me the various techniques routinely used in David Meinke's laboratory David Patton's advice and assistance were also greatly appreciated throughout the past few years in graduate school I thank Tama Schneider-Barfield, Leigh Mickelson, and Karl Hansen for assistance with tissue culture, seed dissections, and thin-layer chromatography analyses The advice and guidance of my advisor, Dr David Meinke, and my graduate committee members, Drs James Ownby, Arnon Rikin, Alan Harker, and Ulrich Melcher, were greatly appreciated as was their assistance with writing and reviewing this dissertation Most of all, the friendship of everyone in David's lab was of great value to me in addition to their advice and technical expertise

I also thank Drs Bob Baxter and Nicholas Shaw for independently providing 7,8-diaminopelargonic acid The biotin precursor 7-keto-8-aminopelargonic acid and *E* *coli* *bioA* strain 109 were kindly provided by Dr Max Eisenberg The assistance of Drs Michael McInerney and David Nagle with growing 100 L of *E* *coli* *bioA* was also greatly appreciated Chemical structures in Figure 6 were drawn with the assistance of Dr Richard Bunce using the Macintosh ChemDraw program

Finally I would like to express my love and appreciation to my wife Valerie, her patience and support were a constant motivation behind the pursuit of this degree I would like to dedicate this dissertation to my son Nolan who reminds me every day what things in life are really

important

Portions of Chapter II were taken from a grant proposal written by David Meinke with input from myself Most of the research described in Chapter III has been published as a paper written by David Meinke with my assistance Some of the work described in Chapter IV has recently been submitted for publication as a communication written by David Meinke and myself The research described in this dissertation was supported by NSF Grant No DCB86-02804 and USDA Grant No 88-37261-3708 to David Meinke and a Presidential Fellowship from the Oklahoma State University Center for Water Research

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NOMENCLATURE

°C	degrees Celsius
cDNA	complementary DNA
μCi	microCurie
cM	centiMorgan
cm	centimeter
d	day
2,4-D	2,4-dichlorophenoxyacetic acid
DAP	7,8-diaminopelargonic acid
DPM	disintegrations per minute
EMS	ethyl methanesulfonate
FW	fresh weight
g	gram (10 g = 10 grams) or force of gravity (10g = 10 times the force of gravity)
μg	microgram
h	hour
KAP	7-keto-8-aminopelargonic acid
kD	kiloDalton
l	liter
μl	microliter
μm	micrometer
M	molar
μM	micromolar
M_2	second generation produced following mutagenesis

MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
NAA	1-naphthaleneacetic acid
ng	nanogram
PBS	phosphate-buffered saline
pg	picogram
psi	pounds per square inch
RFLP	restriction fragment length polymorphism
s	second
SD	standard deviation
T-DNA	transfer-DNA
TLC	thin-layer chromatography
v	volume
W	Watt
w	weight
YAC	yeast artificial chromosome

CHAPTER I

INTRODUCTION

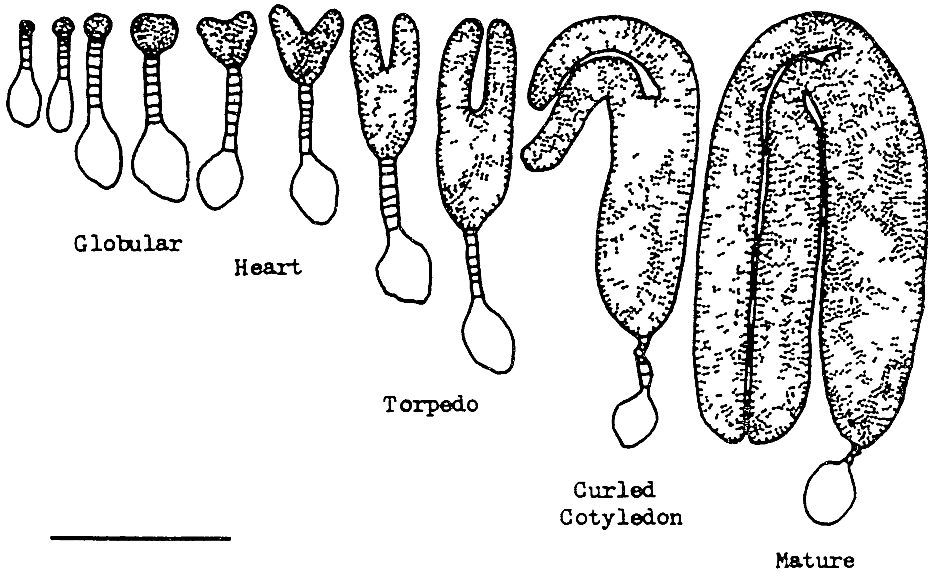
The genetic control of plant embryo development has been approached in part through the isolation and characterization of recessive embryo-lethal mutants (Meinke, 1986) The most extensive studies have dealt with defective-kernel mutants of maize (Sheridan, 1988) and embryo-lethal mutants of Arabidopsis (Meinke, 1991) The drawing of Arabidopsis thaliana (L) Heynh presented in Figure 1 illustrates many of the morphological characteristics of this small, flowering plant Some of these features include a basal rosette, small flowers, and fruits called siliques This plant has emerged as a model system for basic research in plant biology because of its small size, short generation time, ability to produce large numbers of seeds from one plant, small genome, susceptibility to Agrobacterium-mediated transformation, and availability of well-established linkage maps (Koornneef et al , 1983, Meinke and Sussex, 1979a, Meyerowitz, 1989, Meyerowitz and Pruitt, 1985, Patton et al , 1991, Rédei, 1970, 1975a,b) These characteristics make Arabidopsis a useful tool for studying classical and molecular aspects of plant genetics, development, physiology, and biochemistry

Normal embryo development in Arabidopsis is illustrated in Figure 2 This pathway is similar to that described for Capsella, which has been extensively used for descriptive analyses and embryo culture

Figure 1 Drawing of Arabidopsis thaliana (L) Heynh Illustrated are the mature plant with basal rosette, main stem with terminal inflorescence and lateral branches (A), epidermal hairs (trichomes) on the surface of leaves (B), flower at pollination (C), petal (D), stamens (E, F), compound pistil (G), mature silique splitting along both sides of the central septum to reveal two rows of seeds (H), and mature seed (I) Drawing reprinted from Ross-Craig (1948)

C
x8
D
x8
E
x8
F
x8
G
x12
B
x12
A
x1
H
x4
I
x24

Figure 2 Normal embryo development in _Arabidopsis_ _thaliana_ The zygote divides to form an embryo which is composed of a filamentous suspensor (clear) and a terminal embryo proper (stippled) The embryo proper then becomes green at an early torpedo stage of development Scale bar = 250 μm Figure adapted from Raghavan (1976) and taken from Meinke (1986)


Globular
Heart
Torpedo
Curled
Cotyledon
Mature

(Monnier, 1976, Raghavan and Torrey, 1963, Schulz and Jensen, 1968a,b,c) Embryo-lethal mutants of Arabidopsis have been isolated that abort at various stages of this developmental pathway (Meinke, 1982, 1985, 1986, 1991, Meinke et al , 1985, Meinke and Sussex, 1979a,b) These mutants have been characterized using a number of different approaches ranging from descriptive analyses to molecular studies aimed at isolating genes involved in plant embryo development (Baus et al , 1986, Errampalli et al , 1991, Franzmann et al , 1989, Patton et al , 1991, Patton and Meinke, 1988, 1990, Schneider et al , 1989) Analysis of mutant embryos in culture is one approach that has led to a better understanding of the underlying biochemical defect in at least one of these mutants, the biotin auxotroph, described in this dissertation

The biol auxotroph of Arabidopsis is part of a collection of recessive embryo-lethal mutants isolated following seed mutagenesis with EMS (Meinke, 1985) Heterozygous (BIO1/biol) plants are phenotypically normal but produce siliques with 25% aborted seeds following self-pollination The lethal phase of biol/biol embryos extends from the globular to mature stage of embryo development Arrested embryos are much paler than normal and often have a reduced hypocotyl and distorted cotyledons (Figure 3) Segregation ratios are consistent with lethality caused by a single recessive factor This mutant was originally shown by Baus et al (1986) to be rescued on media supplemented with amino acids, nucleosides, and vitamins but not on basal media (Table I) Mutant biol embryos were subsequently shown by Schneider et al (1989) to produce phenotypically normal plants when cultured in the presence of either biotin or desthiobiotin (Table II), the immediate biosynthetic

Figure 3 Morphology of biol arrested embryos The frequencies of these mutant phenotypes in a random sample of 254 arrested embryos were 10 3% A-B, 10 7% C-D, 39 5% E-F, 17 4% G-I, 10 2% J-K, 0 4% L, and 11 5% not readily classified Scale bar = 100 µm Drawing and data from Baus et al (1986)

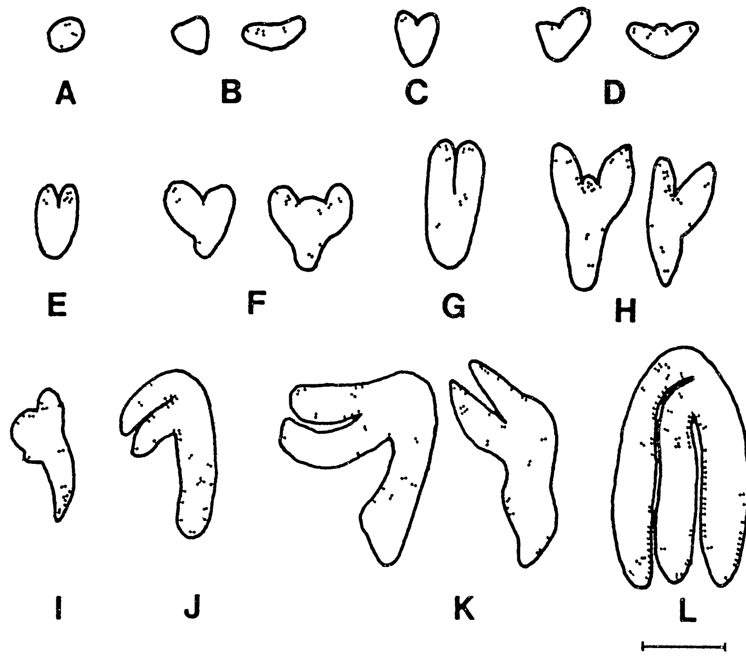

A
B
C
D
E
F
G
H
I
J
K
L

TABLE I

RESPONSE OF ARRESTED EMBRYOS FROM 17 EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA ON BASAL AND ENRICHED MEDIA[a]

Mutant Name		Nutrient Medium		
Old	New	Basal[b]	Enriched[c]	Lethal Phase
111B-5E	emb13	-	-	Early globular
95A-2B	emb14	o	o	Globular
123B	emb15	-	-	Globular
57B-4C	emb16	-	-	Globular
109A-1B	emb17	-	-	Globular
109F-5D	emb18	o	-	Globular
112G-1A	emb19	-	-	Globular/heart
117N-1B1	emb20-1	-	-	Globular/heart
87A	emb20-2	-	-	Globular/heart
129AX2-A	emb21	-	-	Globular/heart
115D-4A	emb22	-	-	Green blimp
126E-B	emb23	+	+	Globular/linear
109F-1C	emb24	+	+	Globular/linear
115J-4A	emb25	+	+	Globular/mature
122G-E	bio1	-	+	Globular/mature
115H-1A	emb28	+	+	Globular/mature
112A-2A	emb30	+	+	Fused cotyledon

[a]The response of mutant embryos in culture was classified as either no growth (o), slight callus (-), or extensive callus (+) Data from Baus et al (1986)

[b]Basal medium for callus formation contained the inorganic salts of Murashige and Skoog (1962), 3% (w/v) glucose, 0 8% (w/v) Difco purified agar, 0 55 mM inositol, 5 μM thiamine hydrochloride, 0 5 mg/l kinetin and 2 mg/l of either 2,4-D or NAA (Baus et al , 1986)

[c]Enriched medium for callus formation consisted of basal medium supplemented with 0 2 mM each of 20 L-amino acids, 0 1 mM each of 5 nucleosides, 0 5 μM each of 7 vitamins (p-aminobenzoic acid, biotin, folic acid, nicotinamide, Ca-pantothenate, pyridoxine hydrochloride, and riboflavin-5'-phosphate), 50 μM choline chloride, and 0 5 mM each of DL-malic and citric acids (Baus et al , 1986)

TABLE II

RESPONSE OF BIO1 ARRESTED EMBRYOS IN CULTURE[a]

Medium Supplement	Concentration (μM)	Response
Biotin	≥ 0 01	Normal plants
	0 001	Green callus
	≤ 0 0001	No growth
Desthiobiotin	≥ 0 01	Normal plants
	0 001	Pale callus
	≤ 0 0001	No growth
Pimelic acid	≤ 250	No growth
Pimelic acid + alanine	250 + 250	No growth
Pelargonic acid	≤ 50	No growth
Malonic acid	≤ 1000	No growth

[a]Arrested embryos were removed from aborted seeds and cultured on a medium containing the inorganic salts of Murashige and Skoog (1962), B5 vitamins (Gamborg et al , 1968), 3% (w/v) sucrose, 0 8% (w/v) Difco purified agar, 1 0 mg/l 6-benzylaminopurine, 0 1 mg/l NAA, and the indicated amount of supplement (Schneider, et al , 1989) Wild-type cotyledon segments cultured as described by Patton and Meinke (1988) on each medium produced normal shoots or callus tissue Data from Schneider et al (1989)

precursor of biotin in bacteria (Eisenberg, 1973) Arrested embryos from other mutants with a wide range of lethal phases did not exhibit this biotin requirement for growth in culture (Baus et al , 1986, Franzmann et al , 1989) Mutant bio1 plants rescued in culture and subsequently transplanted to soil produced phenotypically normal seeds when supplemented with biotin but became chlorotic and failed to produce fertile flowers in the absence of biotin (Schneider et al , 1989)

This dissertation describes in detail some different approaches taken in the analysis of this biotin auxotroph The results obtained have led to a model to explain the bio1 defect, and have provided a better understanding of biotin synthesis and utilization, the genetic control of embryo development, and the effect of a metabolic defect on embryo development in plants The current model for describing biotin synthesis in plants and the nature of the specific defect in bio1 is as follows (a) the pathway for biotin synthesis is conserved in plants and microorganisms, (b) the lesion in bio1 disrupts the conversion of KAP to DAP in this pathway, and (c) bio1 fails to accumulate detectable levels of KAP Chapter II is a review of the literature describing the past and present knowledge of biotin, plant auxotrophs, and developmental mutants Sections of this chapter describing plant auxotrophs and developmental mutants contain material taken from a grant proposal written by David Meinke with my input Chapter III consists primarily of a paper written by David Meinke with assistance from myself that was published in Plant Physiology (Shellhammer and Meinke, 1990) This chapter describes the results of biotin assays conducted on mutant and wild-type tissues to determine if bio1 contained reduced levels of biotin Chapter IV describes work conducted to determine the specific

biochemical defect in <u>bio1</u> Sections of this chapter have been recently submitted to Plant Physiology as a communication written jointly by David Meinke and myself The analysis of biotin synthesis in subcellular fractions of wild-type *Arabidopsis* plants is discussed in Chapter V These chapters are followed by a list of references and an appendix The appendix is a detailed protocol for the microbiological assay of biotin using the biotin-requiring bacterium *Lactobacillus plantarum*

CHAPTER II

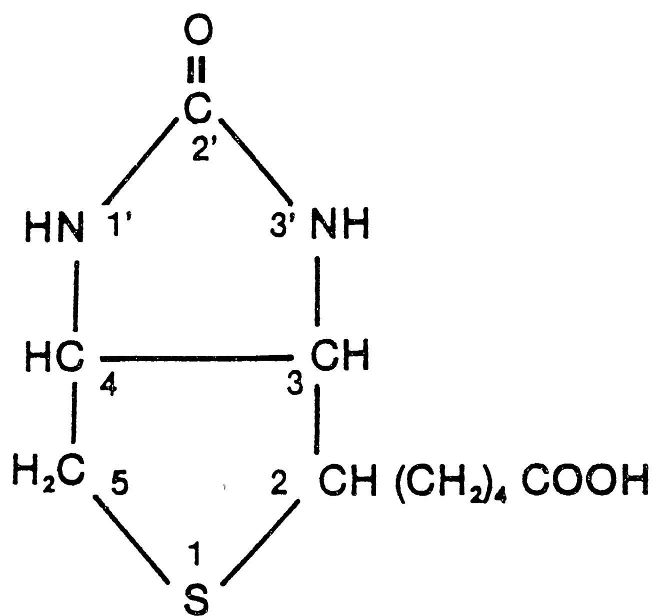
LITERATURE REVIEW[1]

Biotin

The study of biotin has progressed sporadically since its discovery in the early 1900's as a yeast growth factor and essential nutrient for all organisms (Eisenberg, 1973) Early studies dealt with the isolation, purification, structure determination, and chemical synthesis of biotin The first crystalline preparation of biotin was not isolated until 1936 (Kogl and Tonnis), and it took several years to determine the correct chemical structure of this compound and achieve its chemical synthesis (du Vigneaud et al , 1942b, Harris et al , 1943, 1944, Hofmann et al , 1941, 1942, Melville et al , 1942) Several more years passed before the biological function of biotin as a cofactor in carboxylation reactions was determined (Knappe et al , 1961, Lynen et al , 1961, Wakil and Gibson, 1960, Wakil et al , 1958, Wood et al , 1963) Biotin is a cofactor that binds covalently to carboxylases to facilitate the transfer of CO_2 during carboxylation, decarboxylation, and transcarboxylation reactions (Eisenberg, 1973, Dakshinamurti and Bhagavan, 1985, Wood and Barden, 1977) The naturally occurring isomer of biotin, as shown in Figure 4, contains a ureido group in a five-membered ring fused with a tetrahydrothiophene ring possessing a five-

[1]Portions of this chapter were taken from a grant proposal written by David Meinke

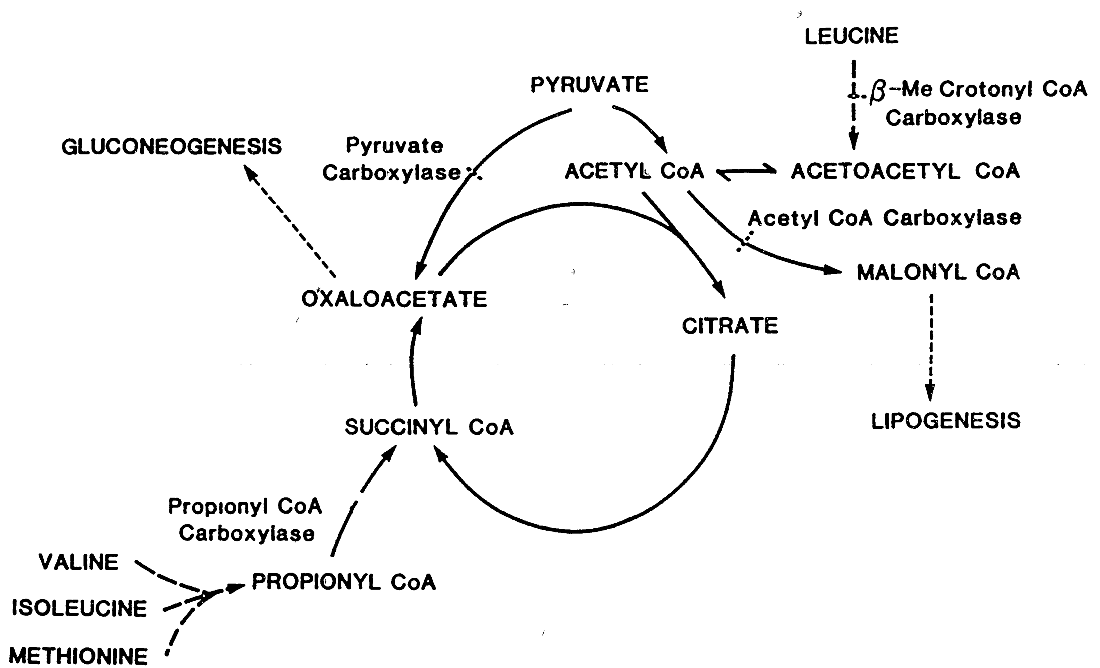
Figure 4 Structure of biotin Biotin binds to carboxylases through a linkage between the carboxyl group of the acid side chain of biotin and the ϵ-amino group of a lysine residue in the enzyme During carboxylation reactions the transported CO_2 group is attached to the 1'-nitrogen atom of enzyme-bound biotin Drawing adapted from Schneider et al (1989)


O
C
2'
HN 1'
3' NH
HC
4
3
CH
H2C 5
2 CH (CH2)4 COOH
1
S

carbon acid side chain Biotin binds to carboxylases through a linkage between the carboxyl group of the acid side chain and a lysine residue of the enzyme The active form of biotin (1'-N-carboxybiotin) contains CO_2 bound to the 1' nitrogen atom Examples of biotin-dependent carboxylases in cellular metabolism are summarized in Figure 5 The most thoroughly studied biotin-dependent enzyme in higher plants is acetyl-CoA carboxylase, which is the key enzyme in the initiation of fatty acid biosynthesis (Charles et al , 1986, Lane et al , 1974, Nikolau et al , 1984, 1985) Other enzymes that require biotin for normal function have also been identified in plants These include propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, and pyruvate carboxylase (Hoffman et al , 1987, Wurtele and Nikolau, 1990)

Elucidation of the pathway for biotin synthesis lagged behind the knowledge of its mode of action primarily because of the trace amounts of biotin required for cellular metabolism A further problem encountered was the limited availability of putative, chemically-synthesized intermediates (Eisenberg, 1973) In the 1960's, the isolation and characterization of biotin auxotrophs provided the driving force behind the elucidation of the pathway for biotin synthesis in microorganisms (Eisenberg, 1973) Early studies on biotin biosynthesis centered around the precursor-product relationship between pimelic acid, desthiobiotin, and biotin Pimelic acid was initially shown to be a growth factor for certain strains of *Corynebacterium diphtheriae* (Mueller, 1937a,b,c) and was later hypothesized to be a biotin precursor because it was a product of biotin degradation in microorganisms (du Vigneaud et al , 1942a) Desthiobiotin was found to support the growth of the biotin-requiring yeast *Saccharomyces cerevisiae* and shown to be

Figure 5 Function of biotin-dependent carboxylases in cellular metabolism The most well-characterized carboxylase in higher plants is acetyl-CoA carboxylase which is the key enzyme in the initiation of fatty acid biosynthesis Figure taken from Dakshinamurti et al (1985)


LEUCINE
β-Me Crotonyl CoA
Carboxylase
PYRUVATE
GLUCONEOGENESIS
Pyruvate
Carboxylase
ACETYL CoA
ACETOACETYL CoA
Acetyl CoA Carboxylase
MALONYL CoA
OXALOACETATE
CITRATE
SUCCINYL CoA
LIPOGENESIS
Propionyl CoA
Carboxylase
VALINE
PROPIONYL CoA
ISOLEUCINE
METHIONINE

converted into biotin using multiplying yeast cells (Dittmer et al , 1944) An X-ray-induced mutation in Penicillium chrysogenum resulted in a biotin auxotroph which excreted large amounts of desthiobiotin into the medium when grown in the presence of biotin (Tatum, 1945) The amount of biotin excreted increased when pimelic acid was included in the growth medium As of 1945, information on the biosynthetic pathway for biotin was limited and certain steps remained to be elucidated The following pathway represented the extent of the knowledge of biotin synthesis for several years pimelic acid → desthiobiotin → biotin

The isolation and analysis of biotin auxotrophs of Escherichia coli finally led to the elucidation of the remaining steps of biotin synthesis (del Campillo-Campbell et al , 1967, Eisenberg, 1973, Pai, 1969b) The order of E coli bio genes was determined by transduction experiments Five bio genes (A, B, C, D, and F) were found to be located within an operon near the λ attachment site on the bacterial chromosome (Cleary and Campbell, 1972, Rolfe, 1970) Biotin auxotrophs of E coli were classified into four nutritional groups based on the ability of one class of auxotroph to rescue another by excretion of a specific biotin vitamer (a biotin analog or precursor capable of supporting the growth of biotin auxotrophs) Such crossfeeding experiments were essential in the early analysis of biotin synthesis in microorganisms and were useful techniques in the study of other biosynthetic pathways Biotin auxotrophs of E coli were also isolated by Rolfe and Eisenberg (1968) and classified into four groups based on chromatographic properties of the vitamer each excreted Pai (1969b) also isolated four groups of E coli biotin auxotrophs and proposed a biosynthetic pathway based on crossfeeding experiments, responses to

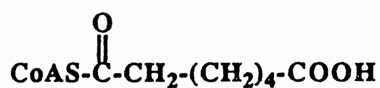
chemically-synthesized biotin vitamers, and the identity of excreted vitamers

The use of cell-free extracts of E coli and Bacillus sphaericus strains eventually led to enzymatic characterization of the steps required for synthesis of desthiobiotin from pimelic acid (Eisenberg, 1973, 1985, 1987, Gloeckler et al , 1990, Izumi et al , 1972, 1979, 1980, 1981) Conversion of desthiobiotin into biotin however is still not fully understood Some questions have been raised concerning the role of pimelic acid in biotin synthesis, primarily because of feeding experiments with organisms such as E coli that do not produce increased levels of biotin in the presence of supplemental pimelic acid (Pai and Lichstein, 1965) This observation was inconclusive because pimelic acid is not taken up by E coli (Pai and McLaughlin, 1969) and is not converted into detectable levels of pimeloyl-CoA in cell-free extracts of E coli (Gloeckler et al , 1990, Izumi et al , 1981) Questions then arose concerning the synthesis of pimeloyl-CoA Izumi et al (1972, 1979) indirectly demonstrated synthesis of pimeloyl-CoA from pimelic acid in B sphaericus cell-free extracts by coupling this reaction with the synthesis of KAP Direct conversion of pimelic acid into pimeloyl-CoA was not observed, possibly because of the insensitivity of methods available for detection of pimeloyl-CoA and the presence of pimeloyl-CoA deacylase (Eisenberg, 1987) Recently, Gloeckler et al (1990) have cloned genes of B sphaericus involved in conversion of pimelic acid to desthiobiotin and identified a gene (bioW) likely encoding pimeloyl-CoA synthetase E coli bioC and bioH auxotrophs thought to be defective in the synthesis of pimeloyl-CoA (Eisenberg, 1985) are not rescued by transformation with the B sphaericus bioW gene Additionally, no

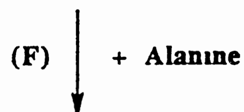
significant homology was found between the E coli bioC gene and any of the B sphaericus bio genes These results may suggest that bioC and bioH gene products are not involved in conversion of pimelic acid to pimeloyl-CoA and that pimeloyl-CoA may be synthesized from a precursor other than pimelic acid in E coli (Gloeckler et al , 1990) Because of these results and the inability to detect direct conversion of pimelic acid to pimeloyl-CoA in any microorganism, questions still remain concerning the exact role of pimelic acid in biotin synthesis

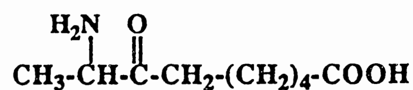
The biochemical steps of biotin synthesis common to E coli and other microorganisms are presented in the pathway outlined in Figure 6 This pathway begins with the condensation of pimeloyl-CoA and alanine to form KAP which is catalyzed by KAP synthetase encoded by the bioF gene in E coli This enzyme has been partially purified, shown to have a molecular weight of 45 kD, and requires pyridoxal 5'-phosphate for activity (Eisenberg, 1973) KAP is converted into DAP by the 85-kD enzyme DAP aminotransferase, which also requires pyridoxal 5'-phosphate as cofactor for activity and uses S-adenosyl-L-methionine as an amino donor (Eisenberg and Stoner, 1971, 1979, Pai, 1971, Stoner and Eisenberg, 1975a,b) This enzyme is encoded by the bioA gene (Eisenberg, 1973) Desthiobiotin synthetase, encoded by the bioD gene, converts DAP to desthiobiotin in a reaction that involves the incorporation of CO_2 into a ureido structure (Cheeseman and Pai, 1970, Eisenberg and Krell, 1969a,b, Pai, 1969a) This enzyme has also been purified and appears to be a dimer with a molecular weight of 23 kD per subunit (Eisenberg, 1985) The final step of biotin synthesis involves the incorporation of sulfur into desthiobiotin to form biotin This reaction has not been demonstrated in cell-free extracts but has been

Figure 6 Biosynthesis of biotin in *Escherichia coli* F, A, D, and B represent the corresponding *bio* genes of *E coli* Figure taken from Shellhammer and Meinke (submitted)

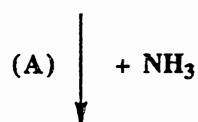


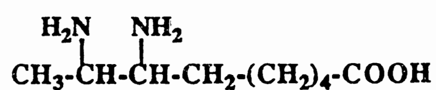
Pimeloyl - CoA



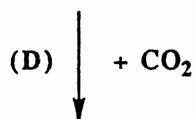


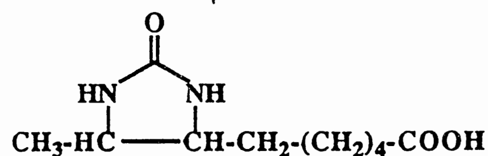
7-Keto-8-aminopelargonic Acid



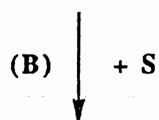


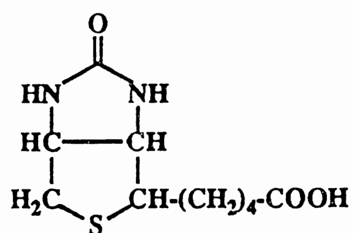
7,8-Diaminopelargonic Acid





Desthiobiotin





Biotin

observed using intact cells of E coli (Pai and Lichstein, 1966) E coli biotin auxotrophs defective in conversion of desthiobiotin to biotin belong to only one complementation group that defines the bioB locus (Eisenberg, 1985) Although the enzyme has not been purified the probable bioB gene product is referred to as biotin synthetase (Pai, 1972) Table III summarizes the characteristics of E coli biotin auxotrophs and the nature of their specific defects Bacteria and fungi that synthesize their own biotin have been shown to utilize the above pathway (Figure 6) Most other organisms except plants require biotin as a vitamin supplement

Four of the genes required for the biosynthesis of biotin in E coli (bioA, B, D, and F) code for enzymes with known functions (Table III) Additional biotin loci in E coli include a permeability gene (bioP) required for biotin utilization and a regulatory gene (bioR, bir) that codes for a repressor protein (holoenzyme synthetase) with a dual function (1) negative regulation of the biotin operon, and (2) attachment of biotin-AMP to the apoenzyme of acetyl-CoA carboxylase (Eisenberg, 1985) Regulatory and structural regions of the biotin operon in E coli have been sequenced and thoroughly analyzed at the molecular level (Barker et al , 1981, Eisenberg, 1985, Howard et al , 1985, Otsuka et al , 1988) Amino acid sequences of biotin biosynthetic enzymes have been determined based on the nucleotide sequence of open reading frames in the bio operon (Otsuka et al , 1988) The predicted sequence of the bioA gene product, DAP aminotransferase, revealed this enzyme to be similar to ornithine aminotransferase The bioF gene product, KAP synthetase, appears to be related to 5-aminolevulinic acid synthetase

TABLE III

OVERVIEW OF BIOTIN AUXOTROPHS OF *ESCHERICHIA COLI*

Gene Designation	Biotin Vitamers That Rescue Mutant	Precursors Excreted	Enzyme Disrupted
bioF	KAP DAP Desthiobiotin Biotin	None	KAP synthetase
bioA	DAP Desthiobiotin Biotin	KAP	DAP amino-transferase
bioD	Desthiobiotin Biotin	DAP	Desthiobiotin synthetase
bioB	Biotin	Desthiobiotin	Biotin synthetase

Numerous studies have been published on the biosynthesis and utilization of biotin and associated vitamers in fungi (Koser, 1968, Pearson et al , 1986) Some fungi synthesize biotin by the pathway described for *E coli* while others such as *Saccharomyces cerevisiae* and *Neurospora crassa* require the presence of biotin or related vitamers in the growth medium Many of these biotin-requiring microorganisms can still produce biotin when supplied with desthiobiotin or other biotin precursors (Iwahara et al , 1966, Naumova and Vorob'eva, 1984) The biotin requirement exhibited by these organisms is therefore probably caused by a defect in the initial stages of biotin synthesis Biotin auxotrophs have been described in *Aspergillus* (Pontecorvo, 1953), *Ophiostoma* (Fries, 1947), and *Penicillium* (Bonner, 1946), where wild-type strains do not require biotin, but these mutants have received relatively little attention in recent years The general conclusion from these studies is that fungi apparently utilize the bacterial pathway to produce biotin, but in many cases this pathway is not completely expressed

The importance of biotin for human health has been demonstrated in part through clinical studies of patients with biotin deficiencies and heritable defects in biotin-dependent carboxylases Heritable human disorders caused by a defect in pyruvate carboxylase, propionyl-CoA carboxylase, and methylmalonyl-CoA carboxylase have been examined in detail (Sweetman and Nyhan, 1986) These deficiencies of individual carboxylases cannot be treated with supplemental biotin Two types of multiple or combined carboxylase deficiencies have also been described in humans The first is caused by a defect in holocarboxylase synthetase, the enzyme that normally attaches biotin to carboxylase

apoenzymes The second is caused by a defect in biotinidase, the enzyme that normally removes biotin from associated proteins in the diet and generates free biotin for use in growth and development Patients with these disorders have been shown to respond well to biotin supplements

Until recently, little was known about the biosynthesis of biotin in plants (Thompson et al , 1986), mainly because of the lack of biotin auxotrophs The temperature-sensitive biotin-requiring ecotypes of Arabidopsis described by Langridge (1965) and Langridge and Griffing (1959) were the only apparent biotin auxotrophs known in plants for nearly 25 years Seedlings from two ecotypes (Pitztal and Blains) require supplemental biotin at 32°C These plants do not grow at this temperature when biotin is replaced with desthiobiotin and appear to be defective in the conversion of desthiobiotin to biotin (Langridge, 1965, Langridge and Griffing, 1959) Experiments to determine if these ecotypes were indeed defective in the conversion of desthiobiotin to biotin were not conducted, nor were analyses performed to determine if desthiobiotin accumulated when grown at 32°C with supplemental biotin My attempts to further characterize some of these ecotypes were unsuccessful because they did not exhibit the expected biotin auxotrophy at 32°C I did observe that Pitztal and Blains ecotypes grown for 3 to 4 weeks at high temperature (32°C) and then transferred to room temperature for 7 to 10 days died on basal medium while ones on biotin-supplemented medium continued to grow Further analysis of these plants was not attempted, and Pitztl and Blains ecotypes therefore did not contribute significantly to the elucidation of biotin synthesis in higher plants or to the analysis and characterization of biol

Plant Auxotrophs

Auxotrophic mutants with defined nutritional requirements have been used in a variety of microorganisms to study the genetic control of biosynthetic pathways (Fincham et al , 1979) Most of these defects have been in the biosynthesis or utilization of an amino acid, vitamin, or nucleoside The scarcity of plant auxotrophs has prevented the use of a similar approach to the analysis of biochemical pathways in plant systems A few auxotrophs have been described in lower plants such as the alga *Chlamydomonas* (Ebersold et al , 1962, Gowans, 1960), the moss *Physcomitrella* (Ashton and Cove, 1977, Engel, 1968), and the liverwort *Sphaerocarpos* (Schieder, 1976) Auxotrophs have been difficult to recover in higher plants, possibly due to lethality prior to germination, gene duplication, alternative biochemical pathways, coupling of biochemical and photosynthetic processes, or limited uptake or transport of the essential nutrient (Blonstein, 1986, Langridge, 1958, Last et al , 1991, Li et al , 1967, Nakamura et al , 1985). One of the most extensive collections of plant auxotrophs is the nitrate reductase-deficient mutants isolated in culture and at the seedling level (Wray, 1986) Several approaches have been used in an attempt to isolate plant auxotrophs The first approach involves screening at the seedling level and identifying abnormal plants that are rescued by the addition of a specific nutrient This approach has proven to be very successful in the isolation and characterization of numerous thiamine auxotrophs of *Arabidopsis* (Li and Rédei, 1969, Rédei, 1975c, Rédei and Acedo, 1976) It has also led to the discovery of a proline auxotroph of maize (Dierks-Ventling, 1982, Gavazzi et al , 1975, Racchi et al ,

1978), putative thiamine auxotrophs of tomato (Langridge and Brock, 1961) and Plantago (Murr and Spurr, 1973), and the temperature-sensitive vitamin-requiring ecotypes of Arabidopsis described earlier (Langridge, 1965, Langridge and Griffing, 1959) Additionally, a chlorotic mutant of tomato that can be rescued by the addition of nicotinamine has also been described by Procházka and Scholz (1984)

A second approach has been the isolation of auxotrophic cell lines in culture following either positive or negative selection As mentioned in recent reviews by Blonstein (1986) and King (1984) a variety of auxotrophic cell lines from Nicotiana, Datura, and Hyoscyamus have now been isolated in culture The specific nutrients required for growth include an extensive list of amino acids, vitamins, and purines The problem with this approach has been the failure of many regenerated plants to express the nutritional requirement and transmit this trait to subsequent generations The above results however are inconsistent with the hypothesis of gene duplication and alternative biochemical pathways as reasons for the scarcity of plant auxotrophs

A third approach has been to analyze the growth of arrested embryos from lethal mutants cultured on basal and enriched media Sheridan and Neuffer (1980) examined the response in culture of arrested embryos from over 100 defective-kernel mutants of maize in an attempt to identify auxotrophs Although several mutant lines displayed superior growth on an enriched medium, the only auxotroph identified among this group of mutants was later shown to be allelic to the pro-1 seedling-lethal described by Gavazzi et al (1975) The response of 17 embryo-lethal mutants of Arabidopsis was subsequently examined in culture (Table I) in an attempt to isolate auxotrophs (Baus et al , 1986) The biotin

auxotroph described in this dissertation was the only mutant that responded normally on enriched media but failed to grow on basal media (Table I)

Tryptophan auxotrophs of Arabidopsis have also been isolated by germinating M_2 seeds in the presence of 5-methylanthranilic acid and tryptophan (Last and Fink, 1988) Wild-type seeds convert the 5-methylanthranilate into toxic 5-methyltryptophan Mutant seeds defective in tryptophan biosynthesis do not produce the toxin and therefore survive on tryptophan included in the medium Three different complementation groups have been identified among these tryptophan auxotrophs One of these auxotrophic mutants, trp1-1, appears to lack the enzymatic activity corresponding to the second step in the tryptophan pathway, anthranilate phosphoribosyl (PR)-transferase (Last and Fink, 1988) This mutant was extensively characterized because it had an easily discernable fluorescent phenotype caused by accumulation of anthranilic acid Lethality apparently does not occur prior to germination because each mutant line was recovered from a viable M_2 seed Mutant embryos of heterozygous plants are apparently rescued by tryptophan that is either supplied by surrounding maternal tissue or produced by activation of different genes during embryo development

Another tryptophan auxotroph, trp2-1, has recently been described that appears to be defective in a gene coding for the β subunit of tryptophan synthase (Last et al , 1991) The enzyme tryptophan synthase catalyzes the synthesis of tryptophan from indole-3-glycerol-phosphate This enzyme consists of two subunits (1) the α subunit which removes glycerolphosphate from indole-3-glycerolphosphate yielding indole, and (2) the β subunit which catalyzes the conversion of indole to

tryptophan Analysis of the trp2-1 mutant has revealed the presence of a second tryptophan synthase gene, TSB2, that is unlinked to TSB1 TSB1 produces most of the tryptophan synthase β mRNA in leaf tissue The trp2-1 tryptophan-requiring mutant has about 10% of the wild-type tryptophan synthase β activity and appears to be defective in TSB1 (Last et al , 1991) Appearance of the trp2-1 mutant phenotype is dependent on the genetic background of the plant and the light intensity under which this auxotroph is grown Activity of the TSB2 gene is apparently too low to rescue the trp2-1 auxotroph when the mutation of TSB1 is present in "Columbia" strain plants grown under high light intensity (Last et al , 1991) In a different genetic background or under low light conditions, the trp2-1 mutation may be masked by expression of the second tryptophan synthase gene The discovery of two tryptophan synthase genes in *Arabidopsis* indicates that genetic redundancy may contribute to the low frequency of auxotrophs in higher plants Isolation of the trp2-1 mutant however demonstrates that such auxotrophs can be isolated under the appropriate experimental conditions

Developmental Mutants

Developmental mutants have been used in a variety of animal systems to study the genetic control of morphogenesis and cellular differentiation (Meinke, 1986, 1991, Sang, 1984, Wilkins, 1986) The experimental approach has been to first establish a model system, and then isolate mutants with abnormal patterns of development, analyze mutants with interesting phenotypes, induce additional mutations to recover several mutants of a specific locus or phenotype, characterize a number of hypomorphic and hypermorphic alleles, clone and sequence

mutant alleles in order to determine the molecular basis of abnormal development, and isolate extragenic suppressors to identify other genes involved in the same developmental pathway (Meinke, 1986, 1991) Lethal mutants have also been used to identify genes that perform essential functions during different stages in the life cycle (Suzuki et al , 1976) Mutations affecting morphogenesis and differentiation in plants have been known for many years and have been used to answer many basic questions concerning plant development (Marx, 1983, Scandalios, 1982, Thomas and Grierson, 1987) The field of plant developmental genetics has emerged several years after studies were initiated with Drosophila to gain a better understanding of animal development (Meinke, 1986, 1991) If the same questions concerning development applied to both plant and animal systems, there would be no need to analyze mutants defective in this process in plants, however, plant and animal development do differ in many interesting ways (Walbot, 1985) The ability of plants to regenerate from somatic cells in culture, the developmental plasticity of apical meristems, and the expression of many genes during both the haploid and diploid phases of the life cycle provide some examples of how plants do differ from animals These characteristics also demonstrate that plants exhibit many unique features amenable to developmental studies

The genetic control of plant embryo development has been approached partly through the isolation and characterization of embryo-lethal mutants (Meinke, 1986) The most extensive studies have dealt with defective kernel mutants of maize (Clark and Sheridan, 1986, Mangelsdorf, 1926, Neuffer and Sheridan, 1980, Sheridan and Clark, 1987, Sheridan and Neuffer, 1980, 1981, 1982, Sheridan and Thorstenson, 1986),

embryo-lethal mutants of Arabidopsis (Meinke, 1986, Muller, 1963), and variant cell lines of carrot that are unable to complete somatic embryogenesis in vitro (Breton and Sung, 1982, Giuliano, et al , 1984) The viviparous mutants of maize, which occasionally do not survive beyond the seedling stage, have also been extensively studied (Dooner, 1985, Robichaud, et al , 1980)

A large collection of recessive embryo-lethal mutants of Arabidopsis thaliana has been isolated following EMS seed mutagenesis in David Meinke's laboratory (Meinke, 1985, 1986, 1991, Meinke et al , 1985) These mutants become arrested at various stages of embryo development and exhibit a wide range of phenotypes (Meinke, 1985) Additional mutants have been identified in M_2 populations (Franzmann et al , 1989) and T-DNA-tagged lines (Errampalli et al , 1991) Analysis of these mutants has included determination of segregation ratios and patterns of abnormal development (Marsden and Meinke, 1985, Meinke, 1985, Meinke and Sussex, 1979a,b), gametophytic expression of mutant genes (Meinke, 1982), extent of cellular differentiation (Meinke et al , 1985, Patton and Meinke, 1990), accumulation of seed storage proteins (Heath et al , 1986), response of mutant embryos in culture (Baus et al , 1986, Franzmann et al , 1989), tests for temperature sensitivity, genetic complementation, and mapping of mutant genes relative to morphological and molecular markers (Patton et al , 1991) Arrested embryos from a wide range of mutants with relatively late lethal phases have produced plants in culture (Franzmann et al , 1989) Many of these plants have characteristic abnormalities ranging from the absence of roots to altered leaf morphology, trichomes, and pigmentation Other mutant plants are surprisingly normal Two mutant lines have been shown

to produce siliques containing 100% aborted seeds with the expected mutant phenotype following self-pollination and 100% phenotypically normal seeds when crossed with wild-type pollen These results provide further evidence that lethal mutations may disrupt genes critical for the completion of embryogenesis but not other stages of the life cycle The biol auxotroph described in the remaining chapters is defective in a gene that is probably constitutively expressed because supplemental biotin is required by this mutant throughout the life cycle (Schneider et al , 1989)

CHAPTER III

BIOTIN LEVELS IN MUTANT AND WILD-TYPE TISSUES[2]

Introduction

One approach to identifying the biochemical defect in biotin auxotrophs is to measure biotin levels in mutant cells Defects in biotin synthesis and degradation should reduce the amount of total biotin (free and protein-bound forms), whereas defects in the attachment and release of biotin from carboxylases should alter only the relative levels of free and protein-bound biotin Two experimental methods were used to assay for biotin The first involved coating microtiter plates with streptavidin, a bacterial protein that binds both free and protein-bound forms, and allowing the biotin present in tissue extracts to compete with biotinylated alkaline phosphatase for streptavidin binding sites (Bayer et al , 1986) This method therefore measures only total biotin, and differences in free and total biotin levels cannot be distinguished The second method involved measuring the growth of Lactobacillus plantarum which responds only to free biotin in aqueous extracts or total biotin in plant extracts hydrolyzed with sulfuric acid to release biotin bound to carboxylases (Scheiner, 1985) Differences in free and total biotin levels were therefore determined only with L plantarum microbiological assays In this chapter I demonstrate that

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[2]Most of the work described in this chapter has been published recently in Plant Physiology (Shellhammer and Meinke, 1990)

arrested embryos from the *biol* auxotroph of *Arabidopsis thaliana* contain virtually no detectable free or total biotin This lack of biotin appears to result from a defect in biotin synthesis that is specific to this mutant and not simply associated with developmental arrest in embryonic lethals

Materials and Methods

Plant Material

Arabidopsis thaliana (L) Heynh strain "Columbia" was grown in pots at 23 ± 3°C beneath 40-W fluorescent lights maintained on daily 16-h light/8-h dark cycles (Heath et al , 1986) Plants heterozygous for recessive lethal mutations isolated following EMS seed mutagenesis (Franzmann et al , 1989, Meinke, 1985) were identified by the presence of siliques containing 25% aborted seeds following self-pollination (Meinke and Sussex, 1979a) The mutants listed in Table IV were used as controls for biotin measurements Arrested *biol*/*biol* embryos were removed from heterozygous siliques and placed in culture as described previously (Baus et al , 1986) Homozygous *biol* plants were produced on regeneration medium containing the inorganic salts of Murashige and Skoog (1962), B5 vitamins (Gamborg et al , 1968), ≥ 10 nM biotin or desthiobiotin, 3 % (w/v) sucrose, 0 8% (w/v) Difco purified agar, 1 0 mg/l 6-benzylaminopurine, and 0 1 mg/l NAA Callus was produced on regeneration medium lacking 6-benzylaminopurine but containing 0 5 mg/l kinetin and 2 0 mg/l of either 2,4-D or NAA Plants from dry seeds were grown on basal medium containing inorganic salts (Murashige and Skoog, 1962) 3% (w/v) glucose 0 8 % (w/v) agar 0 55 mM inositol 5 µM thiamine hydrochloride, and biotin, added before autoclaving because its

TABLE IV

OVERVIEW OF MUTANTS CHOSEN FOR BIOTIN ASSAYS

Mutant	Lethal Phase	Colors[a]	
		Embryos	Seeds
bio1	Globular-mature cotyledon	1-2	2-3
emb20-2	Globular-heart	1	1
emb29	Globular-linear cotyledon	1	1
emb24	Globular-mature cotyledon	2-3	2-3
emb20-3[b]	Linear-mature cotyledon	1-2	1-2
111H-2B2	Mature cotyledon	1-2	1-2

[a]Arrested embryos and aborted seeds were either creamy white (1), very pale yellow-green (2), or pale green (3) Table taken from Shellhammer and Meinke (1990)

[b]Previously known as emb26 but found to be allelic to emb20-2 (Patton et al , 1991)

stability at high temperatures is well documented (Scheiner, 1985) All media were adjusted to pH 5 7 with NaOH, autoclaved for 15 min at 122°C and 18 psi, and poured into 60 X 20-mm sterile Petri plates Cultures were maintained at room temperature on 16-h light/8-h dark cycles Rescued biol/biol seeds were obtained from homozygous mutant plants initiated in culture from embryos, transplanted to soil, and supplemented daily with biotin or desthiobiotin (1 ml/plant of a 0 5 mM solution added directly to the soil) to promote continued growth and development (Schneider et al , 1989)

Isolation of Seeds and Embryos

Wild-type seeds at various stages of development were isolated from immature siliques under a dissecting microscope and transferred with fine-tipped forceps to 1 5-ml microfuge tubes placed on ice Developmental stages of embryos were determined by the size and color of seeds as well as the position of the silique along the stem Aborted seeds were collected from heterozygous siliques with green seeds that had completed morphogenesis but not the final stages of maturation and desiccation Aborted seeds used for biotin assays were generally inflated and lacked brown pigment in the seed coat. Microfuge tubes containing either 100 or 600 seeds were stored at -20°C until required for preparation of extracts

Isolated embryos and seed coats were obtained by dissecting normal and aborted seeds on cover slips located on the surface of agar plates lacking biotin Premature desiccation was prevented by transferring the isolated embryos and seed coats to a small piece of moistened filter paper adjacent to the cover slip Some of the liquid endosperm was

retained along with the seed coats but most was lost during dissection Filters containing 100 pieces of tissue were transferred to 1 5-ml microfuge tubes and stored at -20°C The presence of small amounts of filter paper in these samples did not interfere with biotin measurements

Preparation of Extracts

Extracts for microbiological assays of free biotin were prepared from groups of 600 seeds or 20 to 100 mg (FW) of tissue autoclaved in 0 5 ml of deionized water for 15 min at 122°C and 18 psi Extracts were further homogenized with a glass rod and centrifuged 1 5 min at 15,600g to remove cellular debris The supernatant was diluted to 10 ml with deionized water and was used immediately for assay of free biotin Extracts for assay of total biotin required acid hydrolysis to release biotin from associated proteins because L plantarum responds only to free biotin Extracts from seeds, embryos, and seed coats were prepared by autoclaving 100 pieces of tissue in 40 μl of 2 N H_2SO_4 Further homogenization of acid-hydrolyzed extracts was generally not required Extracts were neutralized with 16 μl of 5 N NaOH, diluted to 1 5 ml with water, and centrifuged 1 5 min at 15,600g to remove cellular debris The resulting supernatant was used immediately for total biotin assays Extracts from seedlings grown in culture were prepared by autoclaving leaf tissue (20-100 mg FW) in 200 μl of acid followed by neutralization with 80 μl of 5 N NaOH, dilution to 1 2 ml with water, and centrifugation The resulting supernatant was further diluted to 8 ml with water and then used for biotin assays

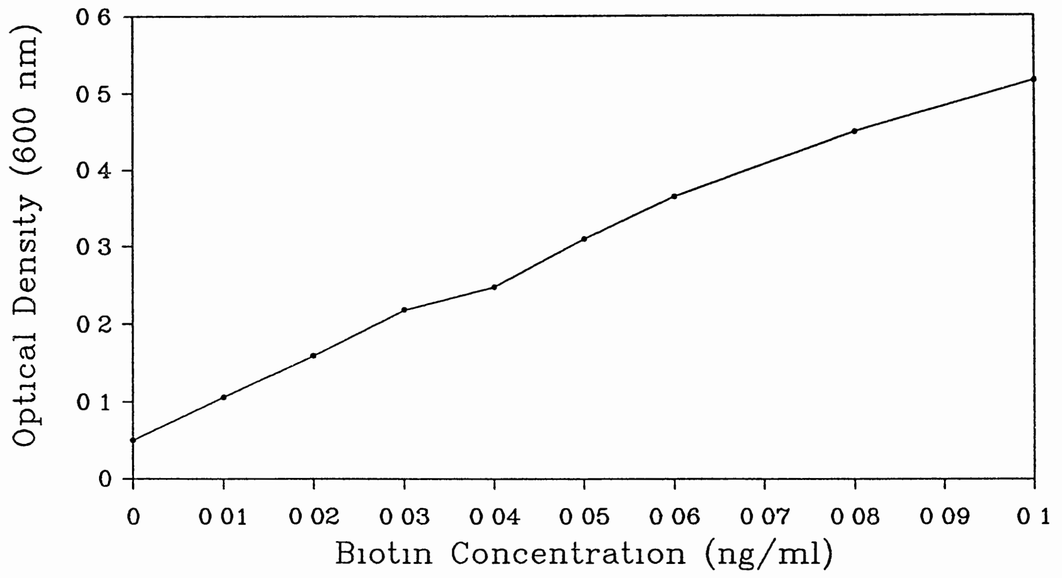
Extracts prepared for streptavidin assays of total biotin were not

hydrolyzed with acid because streptavidin binds both free and protein-bound forms of biotin Groups of 600 seeds were autoclaved in 0 5 ml of PBS for 15 min at 122°C and 18 psi to facilitate biotin extraction, homogenized briefly with a glass rod, and centrifuged for 1 5 min The resulting supernatant was used immediately for biotin assays Leaf, silique, and callus extracts were prepared by autoclaving 20 to 100 mg (FW) tissue in 1 ml of PBS

Lactobacillus Microbiological Assay

L plantarum 8014 was obtained from the American Type Culture Collection (Rockville, Maryland) Cultures of L plantarum were grown on agar slants containing Difco MRS medium (18 h at 37°C) and subsequently stored at 4°C Subcultures were made monthly and prior to use in microbiological assays Biotin was assayed by slight modification of the method of Scheiner (1985) Assays were reduced in volume from 10 ml to 5 ml for seedling extracts and seed extracts prepared from 600 seeds Volumes were further reduced to 1 ml for assay of seed, seed coat, and embryo extracts prepared from 100 pieces of tissue Standard curves were generated for each assay from control tubes containing 0 01 to 0 1 ng/ml biotin (Figure 7) The amount of bacterial growth in assay tubes was determined by measuring the optical density at 600 nm with a Shimadzu UV-160 spectrophotometer Dehydrated biotin assay medium was purchased from Difco All stock solutions, reagents, and sources of deionized water were sterilized to prevent microbial biotin contamination A detailed protocol for microbiological assay of biotin using L plantarum is described in the appendix

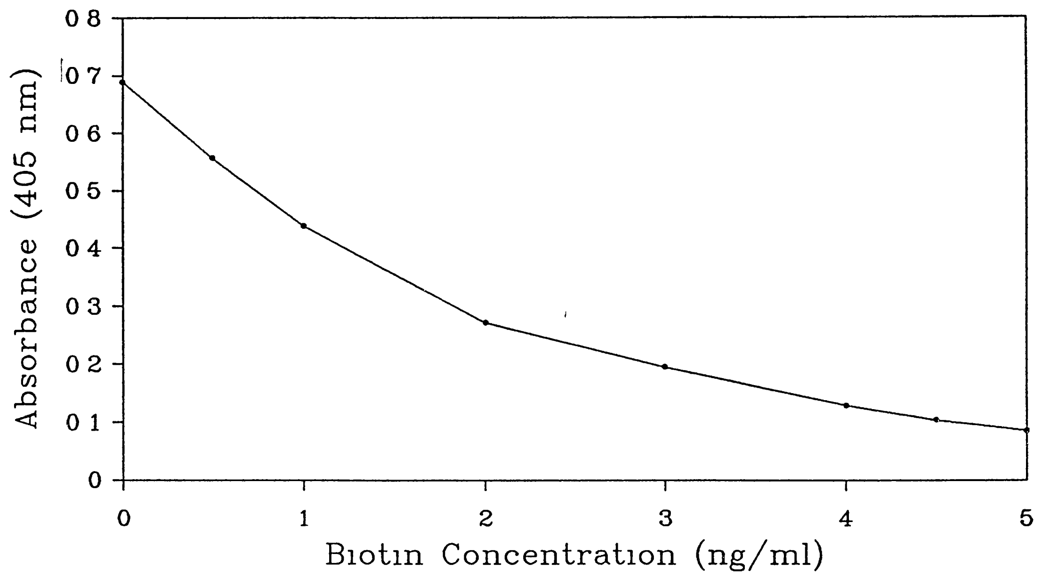
Figure 7 Example of standard curve for *Lactobacillus* microbiological assay of biotin


Optical Density (600 nm)
0 6
0 5
0 4
0 3
0 2
0 1
0
0
0 01
0 02
0 03
0 04
0 05
0 06
0 07
0 08
0 09
0 1
Biotin Concentration (ng/ml)

Streptavidin Binding Assay

Total biotin in PBS extracts was assayed by modification of the method of Bayer, et al (1986) Immulon #2 96-well microtiter plates (Dynatech Laboratories) were incubated 3 h at 37°C with 100 µl/well of a 10 µg/ml solution of streptavidin in 15 mM sodium carbonate buffer, pH 9 6 Plates were washed four times with PBS, incubated 1 h at 37°C with 100 µl/well of 0 3% (w/v) bovine serum albumin in 15 mM sodium carbonate buffer, and washed again with PBS Aliquots of 10 ng/ml biotin standard in PBS were added to a series of 7 wells in duplicate for generating standard curves (Figure 8) Standards ranged in concentration from 0 5 to 5 0 ng/ml biotin Two wells were not coated with biotin to serve as negative controls Aliquots of 25 to 100 µl of plant extract were added to the remaining wells in duplicate The final volume of liquid in each well was adjusted to 100 µl with PBS Plates were incubated 30 min at 25°C, washed with PBS, and incubated 30 min at 25°C with 100 µl/well of a 1 unit/ml solution of biotinylated alkaline phosphatase in PBS Wells were washed again with PBS, and 100 µl of 1 mg/ml p-nitrophenyl phosphate in substrate buffer (1 M diethanolamine, 100 mg/l $MgCl_2$, pH 9 8) was added to each well Plates were incubated at 37°C for 1 h or until a discernable yellow color appeared in control wells not coated with biotin standard Color reactions were stopped by adding 100 µl of 3 N NaOH to each well The contents of each well were transferred to a 1 5-ml semi-micro cuvette, brought to a volume of 1 ml with substrate buffer, and the absorbance was read at 405 nm All reagents for this assay were purchased from Sigma Chemical Company

Figure 8 Example of standard curve for streptavidin binding assay of biotin



0 8
0 7
0 6
0 5
0 4
0 3
0 2
0 1
0
Absorbance (405 nm)
0
1
2
3
4
5
Biotin Concentration (ng/ml)

Results

Biotin in Plant Tissues

Most cells require only trace amounts of biotin for general metabolism (Dakshinamurti and Bhagavan, 1985) A comparison of biotin levels in mutant and wild-type cells therefore requires sensitive methods of biotin detection Biotin assays with plant tissues are particularly challenging because so little is known about the biosynthesis and accumulation of biotin in plants (Thompson et al , 1986) Previous studies have been limited to measuring the biotin content of common feedstuffs (Scheiner and De Ritter, 1975) The values summarized in Tables V and VI were obtained to test the applicability of biotin assay methods to _Arabidopsis_ and to provide a framework for subsequent studies with mutant and wild-type seeds Results of free biotin assays did reveal some differences between the biotin content of mutant and wild-type tissues but these differences were not striking enough to draw any solid conclusions (Table V) Total biotin levels observed in wild-type tissues of _Arabidopsis_ (Table VI) however were within the range of values reported for other plants (Scheiner and De Ritter, 1975) and indicated that measuring total biotin levels may produce more significant results The reduced biotin content of wild-type plants grown in culture was probably caused by decreased vigor and higher water content of leaves in culture and by the absence of microorganisms that may contribute biotin to plants grown in pots

It has been demonstrated previously that rescued _bio1/bio1_ seeds germinated on a basal medium turn pale after 7 to 10 days in culture and fail to develop beyond the cotyledon stage (Schneider et al , 1989)

TABLE V

LACTOBACILLUS MICROBIOLOGICAL ASSAY OF FREE BIOTIN IN MUTANT AND WILD-TYPE TISSUES OF ARABIDOPSIS THALIANA

Plant Material Analyzed	Biotin Content[a]
Mature green siliques from wild-type plants grown in pots	54 ± 10
Cauline leaves from wild-type plants grown in pots	17 ± 8
Wild-type seedlings initiated from dry seeds on 5 μM biotin, transplanted to soil, and grown 14 d in pots without supplemental biotin	10 ± 3
bio1 seedlings initiated from rescued dry seeds on 5 μM biotin, transplanted to soil, and grown 14 d in pots without supplemental biotin	7 ± 2

[a]Each value represents the mean biotin content in pg free biotin per mg fresh weight ± SD Each mean was calculated from at least three independent assays

TABLE VI

STREPTAVIDIN AND MICROBIOLOGICAL ASSAYS OF TOTAL BIOTIN IN MUTANT AND WILD-TYPE TISSUES OF *ARABIDOPSIS THALIANA*

Plant Material Analyzed	Biotin Content[a]	Assay Method
Mature green siliques from wild-type plants grown in pots	140 ± 40	Streptavidin
Cauline leaves from wild-type plants grown in pots	120 ± 80	Streptavidin
Rosette leaves from wild-type plants grown on a basal medium	18 ± 4	Streptavidin
Wild-type seedlings grown for 7-10 d on a basal medium	18 ± 5	*Lactobacillus*
bio1 seedlings grown for 7-10 d on a basal medium	4 ± 2	*Lactobacillus*
bio1 seedlings initiated on 10 nM biotin, grown 14 d, and then grown for 7 d on a basal medium	40 ± 10	*Lactobacillus*
bio1 seedlings initiated on 5 μM biotin, grown 14 d, and then grown for 7 d on a basal medium	60 ± 28	*Lactobacillus*

[a]Each value represents the mean biotin content in pg total biotin per mg fresh weight ± SD Each mean was calculated from at least three independent assays Data taken from Shellhammer and Meinke (1990)

Results summarized in Table VI are consistent with the model that these homozygous mutant seedlings stop growing when they deplete residual biotin obtained from parental plants Senescence can be delayed and biotin levels increased by germinating rescued seeds on plates containing biotin and then transferring mutant seedlings to a basal medium The loss of biotin in mutant plants is therefore not simply an indirect consequence of senescence Assays of total biotin in bio1 callus tissues initiated on biotin and subcultured periodically to fresh media lacking biotin were conducted to determine if bio1 tissues eventually depleted the supplemental biotin Results of total biotin assays on callus tissues revealed that bio1 callus still contained biotin even after 180 d of growth on basal media (Table VII) Complete absence of biotin was observed only when callus tissue became chlorotic and ceased enlargement Whether this lack of biotin was a result of depletion of the residual biotin or the indirect consequence of callus death was unclear because gradual depletion of biotin was not observed, and the sudden absence of biotin appeared to coincide with callus death Biotin levels may have remained constant possibly because of activation of an alternative biosynthetic pathway for biotin during callus growth However, the slow rate of growth of callus tissue compared to that of developing embryos may better explain the levels of biotin observed The long period of callus viability and the presence of a relatively constant level of biotin in bio1 callus tissue subcultured to basal medium does support the hypothesis that reduced synthesis of biotin rather than increased biotin degradation causes lethality in bio1

TABLE VII

STREPTAVIDIN ASSAY OF TOTAL BIOTIN IN MUTANT AND WILD-TYPE CALLUS TISSUES OF ARABIDOPSIS THALIANA

Callus Tissue Analyzed	Biotin Content[a]
Wild-type callus initiated on 5 μM biotin and subcultured to basal media every 20 d for 180 d	32 ± 16
biol callus initiated on 5 μM biotin and subcultured to basal media every 20 d for 180 d	35 ± 12
Wild-type callus initiated on 10 nM biotin then subcultured every 20 d to fresh basal media for 40 d	32 ± 7
biol callus initiated on 10 nM biotin and grown 20 d	374 ± 0
biol callus initiated on 5 μM biotin then subcultured every 20 d to fresh basal media for 160 d	59 ± 16
Wild-type callus initiated on 5 μM biotin then subcultured every 20 d to fresh basal media for 160 d	53 ± 38
biol callus initiated on 10 nM biotin and then subcultured every 20 day to fresh basal media for 80 d	86 ± 13

[a]Values represent the mean biotin content in pg total biotin per mg fresh weight ± SD Each mean was calculated from at least three independent assays

Biotin in Intact Seeds

The remainder of this study dealt with biotin levels in seeds produced by plants grown in the absence of supplemental biotin Results of free biotin assays with mutant and wild-type seeds are summarized in Table VIII These results were somewhat more substantial than assays on other tissues and indicated that biol aborted seeds contained less free biotin than any other seeds analyzed I then decided to measure total biotin levels to determine if biol seeds did indeed contain less biotin or if most of the biotin was actually present in bound form and escaped detection in free biotin assays (Table IX) Biotin contents were expressed as pg/seed rather than pg/mg fresh weight because the precise weight of small groups of seeds could not be readily determined If one assumes an average weight of 30 μg/seed as reported previously (Heath et al , 1986), the amount of total biotin detected in wild-type seeds at the cotyledon stage of development (3-4 pg/seed or approximately 100 pg/mg FW) was equivalent to the value reported in Table VI for intact siliques Lower levels of biotin were found in wild-type seeds at earlier stages of development

One difference between these methods is that streptavidin binds to both desthiobiotin and biotin whereas Lactobacillus responds only to biotin (data not shown) Streptavidin assays therefore measure combined levels of biotin and desthiobiotin in tissue extracts Accumulation of desthiobiotin may have contributed to some of the strikingly higher levels of total biotin observed in some seeds (eg wild-type linear cotyledon, wild-type globular-heart, and emb20-2) when assayed by the streptavidin method (Table IX) However, other seeds (emb29, emb24, and

TABLE VIII

LACTOBACILLUS MICROBIOLOGICAL ASSAY OF FREE BIOTIN IN MUTANT AND WILD-TYPE SEEDS OF ARABIDOPSIS THALIANA

Type of Seed Analyzed	Biotin Content[a]
Wild-type mature cotyledon	0 80 ± 0 04
bio1 aborted	0 12 ± 0 06
emb20-2 aborted	1 30 ± 0 04
emb29 aborted	0 56 ± 0 06
emb24 aborted	1 05 ± 0 11
emb20-3 aborted	0 94 ± 0 02
111H-2B2 aborted	0 86 ± 0 06

[a]Each value represents the mean biotin content in pg free biotin per seed ± SD Each mean was calculated from at least three independent assays

TABLE IX

STREPTAVIDIN AND MICROBIOLOGICAL ASSAYS OF TOTAL BIOTIN IN MUTANT AND WILD-TYPE SEEDS OF *ARABIDOPSIS THALIANA*

Type of Seed Analyzed[a]	pg Total Biotin per Seed[b]	
	Streptavidin Method	*Lactobacillus* Method
Wild-type mature cotyledon	3 51 ± 0 88	3 14 ± 0 82
Wild-type linear cotyledon	4 26 ± 0 34	1 78 ± 0 28
Wild-type globular-heart	1 48 ± 1 11	0 70 ± 0 28
bio1 aborted	0 70 ± 0 52	0 74 ± 0 17
emb20-2 aborted	2 70[c]	1 38 ± 0 60
emb29 aborted	0 63 ± 0 35	1 65 ± 0 21
emb24 aborted	1 40 ± 0 26	2 03 ± 0 53
emb20-3 aborted	3 46 ± 1 70	3 57 ± 0 75
111H-2B2 aborted	1 17 ± 0 64	2 70 ± 1 50

[a]See Table IV for developmental stages of arrested embryos in aborted seeds

[b]Each mean ± SD was calculated from at least three independent assays Data taken from Shellhammer and Meinke (1990)

[c]Only one assay performed

111H-2B2) contained strikingly lower levels of total biotin compared to levels measured with the _Lactobacillus_ method (Table IX) Overall, results obtained with the two methods were relatively consistent considering the small size of the seeds analyzed

Biotin levels in aborted seeds from six mutant lines with characteristic lethal phases and patterns of abnormal development (Table IV) were determined using both the microbiological and streptavidin methods (Table IX) Aborted seeds from every mutant line tested contained at least some total biotin prior to desiccation Biotin deficiency is therefore not a common feature of developmental arrest in embryo-lethal mutants Aborted seeds produced by _bio1_ heterozygotes appeared to contain lower levels of biotin than wild-type seeds at an equivalent (linear) stage of development, but the difference was not particularly striking, and at least one other mutant (_emb29_) produced aborted seeds that appeared with streptavidin assays to contain reduced levels of biotin

Some of the observed variabilities in biotin content of _bio1_ seeds may have been caused by factors such as plant age, silique position, and embryo stage (Table X) Analysis of variance indicated that the position of heterozygous (_bio1_/_BIO1_) siliques along stems correlated with the biotin content of _bio1_ seeds and seed coats Plant age however correlated with the biotin content only in seed coats Observed differences in biotin content of _bio1_ seed coats may have also been caused by variable amounts of liquid endosperm lost during dissection of seeds Assays with intact seeds could therefore not provide a definitive answer to the question of whether _bio1_ tissues contained unusually low levels of biotin

TABLE X

CORRELATION OF PLANT AGE, SILIQUE POSITION, AND EMBRYO STAGE WITH BIOTIN CONTENT IN BIO1

Tissue	Biotin Content[a]	Silique Position[b]	Plant Age[c]	Embryo Stage[d]
Seeds	0 64 ± 0 01	Top	Old	2-3
	1 02 ± 0 13	Middle	Young	1-3
	0 82 ± 0 19	Bottom	Young	2-3
Seed coats	0 53 ± 0 14	Top	Old	2-3
	0 98 ± 0 06	Middle	Young	1-3
	0 86 ± 0 17	Bottom	Young	2-3
Embryos	0 02 ± 0 03	Top	Old	2-3
	0 00 ± 0 00	Middle	Young	1-3
	0 08 ± 0 01	Bottom	Young	2-3

[a]Values represent the mean total biotin content in pg per individual ± SD Each mean was calculated from at least three independent assays

[b]Siliques were taken from either top (near the shoot apex), middle (between the shoot apex and the four or five oldest siliques at the bottom of inflorescences), or the bottom (oldest siliques at the bottom of inflorescenes)

[c]Plant ages were classified as either old (7-8 weeks) or young (5-6 weeks)

[d]Embryo stages were classified as either 1 (globular), 2 (heart), 3 (linear to curled cotyledon)

Biotin in Isolated Embryos

Aborted seeds produced by phenotypically normal biol/BIO1 plants were expected to contain at least some biotin in the maternal seed coat This heterozygous tissue constitutes a significant portion of the fresh weight of immature seeds (Figure 9) and should not have been defective in either biotin synthesis or transport The question then became whether the biotin detected in biol aborted seeds was located exclusively in the seed coat (as indicated in Table X) or whether part of this biotin was present in the arrested embryo I chose to address this question by performing total biotin assays with groups of isolated embryos and seed coats obtained from the same collection of aborted seeds The results of these experiments as summarized in Table XI indicate that biol arrested embryos contained only trace amounts of biotin and that virtually all of the biotin detected in biol aborted seeds could be attributed to the maternal seed coat As was the case for biol seeds, analysis of variance indicated that silique position contributed to some of the observed variability in biotin content of biol embryos (Table X) Developmental stages also contributed somewhat to the variabilities in biotin content measured in arrested biol embryos but did not contribute to differences observed in seeds and seed coats (Table X)

The absence of biotin in biol arrested embryos could not be explained by either the small size or pale color of mutant embryos The five mutants chosen as controls for these experiments produced arrested embryos that were either slightly smaller (emb20-2, emb29), equivalent (emb24), or slightly larger (emb20-3, 111H-2B2) than biol arrested

Figure 9 Drawing of seeds of *Arabidopsis thaliana* at three different stages of development Pictured are seeds at (A) fertilization, (B) globular embryo stage, and (C) maturity Scale bar = 200 μm Drawing adapted from Muller (1963) and taken from Meinke (1979)

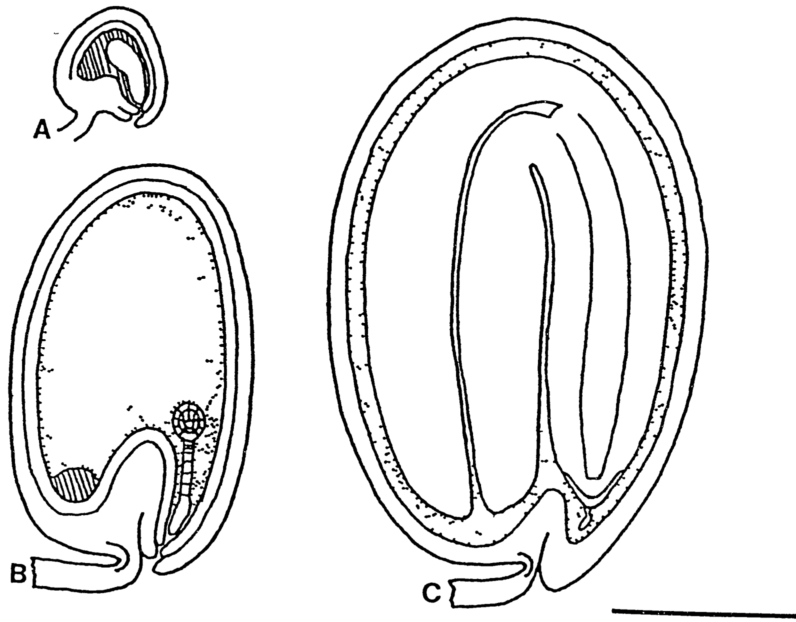

A
B
C

TABLE XI

<u>LACTOBACILLUS</u> MICROBIOLOGICAL ASSAY OF TOTAL BIOTIN IN EMBRYOS AND SEED COATS ISOLATED FROM MUTANT AND WILD-TYPE SEEDS OF <u>ARABIDOPSIS THALIANA</u>

Type of Seed Analyzed	pg Biotin per Individual[a]	
	Embryo	Seed Coat
Wild-type linear cotyledon	0.39 ± 0 06	0 59 ± 0 25
<u>bio1</u> aborted	0 03 ± 0 04[b]	0 72 ± 0 24
<u>emb20-2</u> aborted	0 26 ± 0 16[c]	0 67 ± 0 14
<u>emb29</u> aborted	0 22 ± 0 13[c]	0 96 ± 0 40
<u>emb24</u> aborted	0 31 ± 0 10[c]	0 93 ± 0 16
<u>emb20-3</u> aborted	1 65 ± 0 82[c]	1 04 ± 0 50

[a]Each value represents the mean of several independent assays ± SD Data taken from Shellhammer and Meinke (1990)

[b]Significantly lower than wild-type and mutant controls at P < 0 05 determined by t-test

[c]Not significantly different from wild-type control at P < 0 05 determined by t-test

embryos Arrested embryos from *emb20-2* and *emb29*, which were on the average less than half the size of *biol* arrested embryos, contained almost 10 times more biotin than *biol* arrested embryos These mutants also produced arrested embryos that were paler than *biol* arrested embryos The depletion of biotin in *biol* arrested embryos was therefore not an indirect consequence of reduced chlorophyll accumulation or chloroplast function Biotin levels in isolated embryos and seed coats (Table XI) even correlated reasonably well with values obtained for intact seeds (Table IX) The absence of biotin in *biol* arrested embryos was therefore not caused by the loss of liquid endosperm during dissection All of these results are consistent with the model that *biol* arrested embryos lack biotin because they are defective in biotin synthesis

Discussion

The biotin auxotroph of *Arabidopsis* described in this study represents the first example of an embryonic lethal with a defined biochemical defect early in embryogenesis that can be rescued by the addition of a specific nutrient Until recently, very little was known about the biosynthesis and utilization of biotin in plants (Thompson et al , 1986) The site of biotin synthesis, the identity of early biochemical intermediates, and the nature of biotin transport remain to be determined Plant cell lines with elevated levels of biotin have been recovered following selection for resistance to pimelic acid (Watanabe et al , 1982), but these variants were not regenerated into plants or examined in detail Most of the information on biotin synthesis and transport in higher plants has come from analysis of the

biotin auxotroph described in this report It has been shown previously that biotin can be transported from the soil to immature seeds where it can rescue bio1/bio1 embryos produced by heterozygous and homozygous mutant plants (Schneider et al , 1989) Maternal sources of biotin are insufficient to rescue mutant embryos produced by heterozygous plants grown in the absence of supplemental biotin

The results presented in this chapter suggest that bio1 arrested embryos are defective in either the biosynthesis or degradation of biotin Several lines of evidence are potentially inconsistent with increased degradation Mutant embryos are rescued by extremely low levels of biotin in culture and continue to grow for several months following subculture to fresh media lacking biotin (Schneider et al , 1989) Mutants with high rates of biotin turnover would be expected to deplete this supplemental biotin more rapidly Increased degradation also represents a gain of function that should be inherited as a dominant trait, in contrast to a biosynthetic defect, which represents a loss of function more likely to be inherited as a recessive trait It therefore appears that bio1 embryos are defective in biotin synthesis Further analysis of this biotin auxotroph is described in following chapters, and results of those studies continue to support this hypothesis

CHAPTER IV

ANALYSIS OF BIOCHEMICAL DEFECT IN BIO1[3]

Introduction

As described in the previous chapter, microbiological assays with the biotin-requiring bacterium Lactobacillus plantarum demonstrated that bio1 arrested embryos contain virtually no detectable biotin The absence of free and protein-bound biotin (Table XI) and the ability of relatively low concentrations of biotin or desthiobiotin to rescue mutant tissues (Schneider et al , 1989) suggest that bio1 is defective in biotin synthesis Further analysis of this mutant has been hindered by the absence of biotin auxotrophs in well-characterized eucaryotic systems (e g Saccharomyces cerevisiae), the limited availability of known bacterial precursors of biotin, and the inability of standard microbiological assays to detect these precursors in plant tissues Recent access to chemically-synthesized biotin intermediates (DAP and KAP) has allowed further biochemical analysis of bio1 and has provided additional information on the nature of biotin synthesis in plants

In this chapter I demonstrate that bio1 mutant embryos are rescued by the biotin intermediate DAP but are not rescued by KAP, the immediate precursor of DAP in bacteria (Figure 6) The absence of detectable KAP in both mutant and wild-type tissues, as measured by thin-layer

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[3]Sections of this chapter have been recently submitted to Plant Physiology

chromatography and microbiological assays, suggests that this compound is either metabolized into other products in plant cells or is not a precursor of biotin in plants The final steps of biotin synthesis in bacteria must be conserved in plants because rescued biol/biol tissues convert both DAP and desthiobiotin into biotin I believe that the initial steps of biotin synthesis are also likely to be conserved in plants I attempted to confirm this model by growing wild-type plants in the presence of [^{14}C]pimelic acid but failed to detect incorporation of label into biotin with either intact plants or cell-free extracts These results are difficult to interpret because the specific activity of the [^{14}C]pimelic acid was low, the pathway produces only trace amounts of biotin in plants, and questions remain concerning the role of pimelic acid in biotin synthesis in bacteria (Eisenberg, 1987, Gloeckler et al , 1990) My current model is that plants and bacteria produce biotin through the same pathway, that biol is defective in conversion of KAP into DAP, and that KAP fails to accumulate to detectable levels in biol tissues

Materials and Methods

Bacterial Strains and Biotin Vitamers

Biotin auxotrophs of *Escherichia coli* used in microbiological assays for biotin precursors were obtained from the *E coli* Genetic Stock Center (Yale University, New Haven, Connecticut) Cultures of *E coli* were grown on agar slants containing Difco nutrient agar (18 h at 37°C) and subsequently stored at 4°C Subcultures were made monthly and prior to use in microbiological assays

Desthiobiotin was purchased from United States Biochemical

Corporation and, as shown in Table XII, contained no contaminating biotin as determined by microbiological assay with L plantarum (Scheiner, 1985) Biocytin (biotin bound to the ϵ-amino group of lysine) was purchased from Sigma Chemical Company and contained less than 0 04% biotin as determined by assay with L plantarum (Table XII) The sulfate salt of DAP was synthesized from desthiobiotin (du Vigneaud et al , 1942c) and was provided independently by Bob Baxter (University of Edinburgh, Scotland) and Nicholas Shaw (Biotechnology Research Group, Lonza, Switzerland) This precursor contained no contaminating biotin as determined by assay with L plantarum (Table XII) DAP was also shown to contain less than 0 01% desthiobiotin as determined by assay with the E coli bioD auxotroph which responds only to desthiobiotin and biotin (Eisenberg, 1973) Chemically-synthesized KAP was obtained in the final months of this project from Dr Max Eisenberg (Columbia University, New York) and contained no contaminating biotin vitamers Activity of biotin precursors was demonstrated with the appropriate E coli auxotrophs

Partial Purification of KAP

Biochemical analysis of biol was delayed for several years by failure to identify either a source of purified KAP or a practical method of direct chemical synthesis Cultures of Penicillium chrysogenum utilized in the initial purification of this biotin precursor (Eisenberg and Maseda, 1970) were also not maintained I therefore attempted a large-scale purification of KAP from the E coli bioA auxotroph, which is defective in the conversion of KAP to DAP and excretes KAP when grown in the presence of biotin (Eisenberg, 1973)

TABLE XII

LACTOBACILLUS AND ESCHERICHIA COLI MICROBIOLOGICAL ASSAYS OF SYNTHETIC BIOTIN VITAMERS[a]

Synthetic Vitamer Analyzed	Vitamer Assayed for		
	Biotin	Desthiobiotin	DAP
Biocytin	0 032%[b]		
Desthiobiotin	ND[c]		
DAP	ND	0 0066%[b]	
KAP	ND	ND	ND

[a]Assays were conducted with either L plantarum to detect biotin, E coli bioD to detect desthiobiotin, or E coli bioA to detect DAP

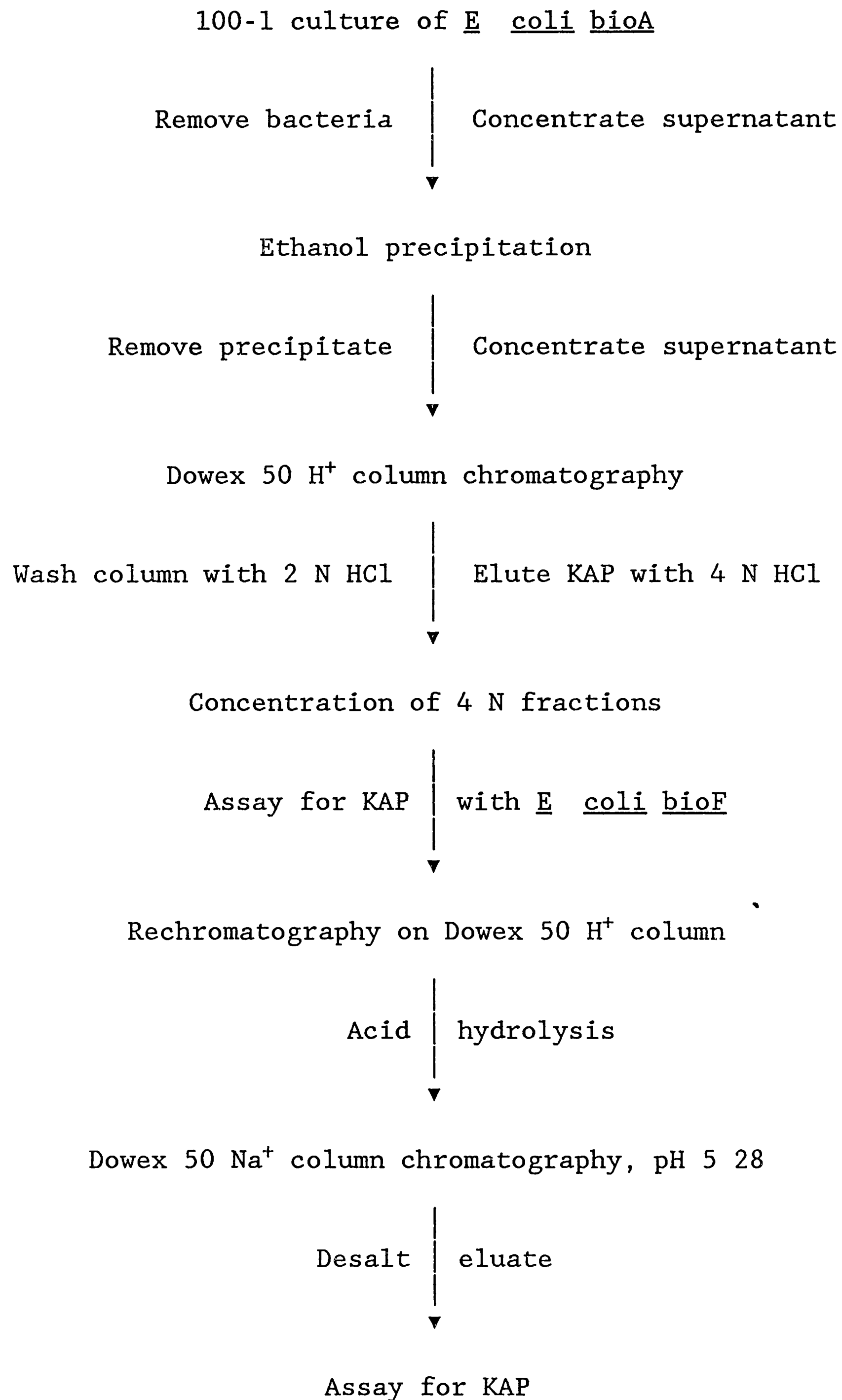
[b]Mole %

[c]Not detected

Although this purification scheme was not completed to homogeneity, the fraction obtained was greatly enriched for KAP and provided a valuable standard for subsequent studies

A 100-l culture of E coli bioA was produced with the assistance of Michael McInerney and David Nagle (Oklahoma University, Norman) E coli cells were suspended in 1 ml of glucose-salts medium containing 1 5 g/l K_2HPO_4, 0 8 g/l KH_2PO_4, 4 g/l $(NH_4)_2SO_4$, 0 1 g/l $MgSO_4$ $7H_2O$, 5 g/l glucose, and 4 g/l Difco vitamin assay casamino acids (Pai, 1969a) The resulting suspension was transferred to 200 ml of glucose-salts medium supplemented with 0 1 ng/ml biotin and incubated with shaking for 24 h at 37°C This culture was transferred to 2 l of glucose-salts medium lacking biotin to derepress the bio operon (Pai and Lichstein, 1966) Following incubation for 3 h at 37°C with shaking, the resulting culture was used as the inoculum for 100 l of glucose-salts medium supplemented with 0 2 ng/ml biotin This large-scale culture was grown in a Braun 100-l fermentor at 37°C for 3 h with stirring at 200 rev/min and aeration of 3 l/min Cells were removed by filtration with a 0 45 μm Millipore membrane, and the effluent was poured into 30 X 55 X 5-cm pans and heated to reduce the volume This material was further concentrated under aspirator vacuum to 2 5 l by rotary evaporation at 55°C KAP was partially purified by modification of the method of Eisenberg and Maseda (1970) as outlined in Figure 10 Portions (400-ml) of concentrated supernatant were brought to 1 l with deionized water and mixed with 3 l of 95% ethanol This mixture was stored at 4°C for 1 h, and the precipitate was removed by centrifugation at 2,500g for 5 min at 2 °C The supernatant was concentrated by rotary evaporation, and the residue was dissolved in 2 l of deionized water The resulting solution was

Figure 10 Scheme for partial purification of KAP


100-l culture of E coli bioA
Remove bacteria
Concentrate supernatant
Ethanol precipitation
Remove precipitate
Concentrate supernatant
Dowex 50 H+ column chromatography
Wash column with 2 N HCl
Elute KAP with 4 N HCl
Concentration of 4 N fractions
Assay for KAP
with E coli bioF
Rechromatography on Dowex 50 H+ column
Acid
hydrolysis
Dowex 50 Na+ column chromatography, pH 5 28
Desalt
eluate
Assay for KAP

adjusted to pH 2 2 with concentrated HCl and chromatographed on a column (14 X 43 cm) filled to a height of 32 cm with 4 Kg of Dowex 50-X8 (100-200 mesh) H^+ form resin (Sigma) The column was washed with 18 l of deionized water followed by 6 l of 2 N HCl This was followed by elution with 20 l of 4 N HCl and collection of 2-l fractions which were concentrated by rotary evaporation The resulting concentrate was dissolved in 20 ml of deionized water, neutralized with 1 N NaOH, and assayed for KAP by E coli bioF Assays were also conducted using E coli bioA to confirm that the biological activity detected by growth of the bioF strain was due to KAP and not biotin or other precursors

The above procedure was repeated on three additional 400-ml portions of supernatant Pooled KAP fractions were then concentrated by rotary evaporation and the residue was dried over NaOH pellets under vacuum (0 1 mm Hg) to remove residual HCl This dried material was dissolved in 500 ml of 6 N HCl and refluxed for 6 h to hydrolyze material that interfered with further purification The hydrolysate was concentrated by rotary evaporation, dissolved in 1 l of deionized water, adjusted to pH 2 2 with concentrated HCl, and filtered through acid-washed diatomaceous earth (Sigma) The filtrate was chromatographed again on the Dowex 50-X8 H^+ column as described above

Pooled KAP fractions from the previous chromatographic procedure were concentrated and the residue was dissolved in 60 ml of deionized water Half of the resulting solution was adjusted to pH 2 2 and chromatographed on Dowex AG 50W-X8 (200-400 mesh) Na^+ form resin (Bio-Rad) in a jacketed column (4 X 55 cm) filled to a height of 45 cm Elution was carried out at 50°C by pumping 6 l of 0 38 N sodium citrate buffer, pH 5 28, at a rate of 8 0 ml/min through the column Fractions (500-ml) were collected and assayed for KAP with E coli bioF KAP

fractions were desalted by chromatography on Dowex AG 50W-X8 (100-200 mesh) H^+ form resin in a column (3 5 X 20 cm) filled to a height of 12 cm The column was washed with 1 l of 0 5 N HCl and KAP was eluted with 2 l of 4 N HCl HCl was removed by rotary evaporation and drying over NaOH The resulting material was dissolved in 5 ml of deionized water and used as a source of partially purified KAP in experiments where pure KAP was not required Further purification was not attempted because a small amount of synthetic KAP had by that time been obtained The partially purified KAP was nevertheless useful as a standard in microbiological assays and allowed further experiments to be conducted without exhausting the small supply of synthetic KAP

Paper Chromatography of Biotin Vitamers

Standard solutions of synthetic biotin and desthiobiotin were initially analyzed by paper chromatography (Williams and Kirby, 1948) in an attempt to identify these biotin vitamers in plant tissues Fresh stock solutions of biotin and desthiobiotin standards were prepared in 50% ethanol prior to each experiment to minimize oxidation of biotin (Wright et al , 1954) and possible decomposition of precursors Ten μl of standard were spotted on Whatman 3MM chromatography paper (20 X 20 cm) The spotted paper was then rolled into a cylinder approximately 8 cm in diameter and stapled together so that the samples applied to the paper were located in a row along one end of the cylinder The paper cylinder was placed upright, with the dried samples near the bottom end, in a developing tank containing solvent [1-butanol glacial acetic acid water, 60 15 25 (v v v)] This mixture was prepared by combining the components in a bottle and mixing them by vigorous shaking (Eisenberg and Krell, 1969a,b) The organic (upper) phase was used as

the developing solvent, and the aqueous (lower) phase was placed in a beaker in the developing tank but did not contact the chromatogram The chromatogram was developed for 4 h in a tank lined with clean chromatography paper to provide a saturated system Following development, the chromatogram was dried thoroughly and then visualized by exposure to I_2 vapors (Gadsden et al , 1960) produced by placing approximately 2 g of I_2 crystals in a clean developing chamber containing the chromatogram Biotin and desthiobiotin appeared as dark reddish-brown spots

TLC and Bioautography of Biotin Vitamers

Plant extracts and standards were also analyzed by TLC to determine if biotin precursors accumulated in biol tissues Extracts were prepared by freezing 20 to 200 mg FW of seedling tissue in 1 5-ml microfuge tubes on dry-ice, homogenizing in 10 μl of 95% ethanol with a glass rod, and centrifuging 15 min at 15,600g to remove cellular debris The resulting supernatant was used immediately for TLC analysis Five μl of extracts and standards were spotted on silica gel chromatogram sheets (Kodak 6060) and developed for 2 h in a tank lined with Whatman 3MM chromatography paper saturated with solvent [benzene methanol glacial acetic acid acetone (70 20 5 5)] After the solvent front had traveled approximately 16 cm from the origin, chromatograms were dried and visualized by either I_2 visualization, as described above for paper chromatography, or bioautography For bioautography, dried chromatograms were cut into longitudinal strips (2 X 16 cm) that included the origin and developed portion of the sample Strips were then cut transversely into segments 1 X 2 cm beginning 0 5 cm below the origin Each segment was placed into a test tube (16 X 125 mm),

autoclaved 5 min at 122°C and 18 psi, and immersed in 2 ml of sterile glucose-salts medium

Biotin vitamers in chromatogram segments were identified using bioautography (Wright et al , 1954) with the bioF auxotroph of E coli which responds to biotin, desthiobiotin, DAP, or KAP (Eisenberg, 1973) A 10-ml culture of bioF in Difco nutrient broth was incubated with shaking at 37°C for 18 to 22 h in a New Brunswick gyratory shaking water bath Bacterial cells were harvested from 1 ml of the resulting culture by centrifugation for 1 min at 15,600g and resuspended in 1 ml of 0 9% (w/v) sterile saline This procedure was repeated with harvested cells three more times to completely remove residual biotin Washed cells were resuspended in 1 ml of 0 9% sterile saline and further diluted 1 500 with saline Each tube containing a single chromatogram segment in glucose-salts medium was inoculated with 1 drop (approximately 50 μl) of bacterial suspension delivered from a sterile 1-ml pipet Inoculated tubes were incubated 18 to 22 h at 37°C with shaking, and the amount of bacterial growth was determined by measuring the optical density at 600 nm with a Shimadzu UV-160 spectrophotometer Optical density was plotted against distance from the origin to determine R_F values for each biotin vitamer

Microbiological Assays of KAP in Plant Tissues

Extracts from mutant and wild-type seedlings grown in culture were prepared with a mortar and pestle by homogenizing 4 g FW of leaf tissue in 4 ml of 0 15 M sodium phosphate buffer, pH 7 0 Cellular debris was removed by centrifugation at 27,000g for 30 min, and the supernatant was stirred at 25°C for 3 h with 40 mg of avidin-acrylic beads (Sigma) to remove residual biotin Avidin-acrylic beads were removed by

centrifugation for 1 5 min at 15,600g, and the resulting supernatant was used immediately for assay of KAP. Silique extracts were prepared by homogenizing 4 g FW in a Waring blender at high speed with 200 ml of deionized water This method produced extracts that were more homogenous than could be obtained by homogenization with a mortar and pestle, possibly due the presence of stem material that was isolated with siliques Cellular debris was removed by centrifugation at 10,000g for 30 min After adding 600 ml of 95% ethanol with stirring, the resulting mixture was stored at 4°C for 1 h to facilitate removal of material that precipitated and interfered with accurate measurement of bacterial growth The precipitate was removed by centrifugation at 2,500g for 10 min, and the residue produced following rotary evaporation at 55°C was dissolved in 4 ml of sodium phosphate buffer, pH 7 0 The resulting solution was stirred with 80 mg of avidin-acrylic beads at 25°C for 3 h The beads were removed by centrifugation, and the supernatant was used for assay of KAP

Extracts were assayed for KAP by modification of the method used for biotin assays described previously (Scheiner, 1985, Shellhammer and Meinke, 1990) except that E coli bioF was used as the assay organism Assays were conducted in a final volume of 1-ml using glucose-salts medium Standard curves were generated from controls containing 0 08 to 0 4 ng/ml biotin Inoculum was prepared as described earlier for bioautography of biotin vitamers except that washed cells were diluted 1 1000 with 0 9% sterile saline Tubes were incubated at 37°C with shaking for 18 to 22 h The amount of bacterial growth was determined by measuring optical density at 600 nm

Labeling with [^{3}H]Biotin and [^{14}C]Pimelic Acid

Wild-type seedlings used for labeling studies were cultured on basal medium At the time of labeling, seedlings were 7 to 10-d old and contained two cotyledons, one or two pairs of leaves, and several roots extending 5 to 10 mm into the medium Seedlings were labeled with 0 1 μCi/plant of either [8,9-^{3}H(N)]biotin (specific activity, 45 Ci/mmole, New England Nuclear) or [1,7-^{14}C]pimelic acid (specific activity, 8 mCi/mmole, ICN Biomedicals) by pipetting 5 μl of a 0 02 μCi/μl solution of label in liquid basal medium near the roots beneath the agar surface Cultures were maintained at room temperature under continuous light Labeled seedlings were harvested after 1 to 3 d by cutting the hypocotyl above the surface of the medium Harvested seedlings were placed in 1 5-ml microfuge tubes on ice, transferred to a Nalgene 30-ml, 0 45-μm sterile filter unit, rinsed 3 times with wash medium [2% sucrose (w/v) and 0 5% (w/v) casein hydrolysate in deionized water], and blotted dry with filter paper Washed seedlings were weighed and frozen in 1 5-ml microfuge tubes on dry-ice, homogenized with a glass rod in 2 ml/g FW of 95% ethanol, and centrifuged 15 min at 15,600g to remove cellular debris Supernatant (100 μl) was transferred to 10 ml of Ecolume liquid scintillation cocktail (New England Nuclear) and counted 10 min on a Beckmann LS 7500 liquid scintillation counter

To determine uptake by seeds and embryos, immature siliques from wild-type plants were harvested by cutting with a razor blade at the stem-petiole junction Cut ends of 50 siliques were immediately placed in a 1 X 3 5-cm vial containing 5 μCi of label in 400 μl of liquid basal medium Vials were sealed with parafilm and kept at room temperature under continuous light for 1 to 3 d Labeled siliques were rinsed three

times with wash medium, homogenized with a mortar and pestle in 4 ml/g FW of 0 15 M sodium phosphate buffer, pH 7 0, and centrifuged 15 min at 15,600g Supernatant (100 μl) was transferred to scintillation cocktail for detection of radioactivity

To test for incorporation of pimelic acid into biotin, labeled tissues were homogenized with either 2 ml/g (seedlings) or 4 ml/g (siliques) of 0 15 M sodium phosphate buffer, pH 7 0 Cellular debris was removed by centrifugation at 15,600g for 15 min Supernatant was stirred 2 h at 25°C with 40 mg avidin beads to recover any radioactive biotin Beads were removed by centrifugation at 15,600g for 1 5 min and resuspended in 1 ml of fresh buffer Washing was repeated until radioactivity was no longer detected in the supernatant After the final wash, beads were resuspended in 100 μl of buffer and transferred to 10 ml of liquid scintillation cocktail

Incorporation studies also utilized cell-free extracts prepared by homogenizing unlabeled plant tissue with a mortar and pestle in 1 ml/g FW of 0 15 M sodium phosphate buffer, pH 7 0 at 4°C Cellular debris was removed by centrifugation at 27,000g for 30 min at 4°C [1,7-^{14}C]pimelic acid (0 1 μCi) was added to 0 5 ml of cell-free extract and incubated at 30°C for 2 to 18 h Labeled cell-free extracts were treated with avidin-acrylic beads as described above to detect incorporation of labeled pimelic acid into biotin

Activity of the biotin pathway in these cell-free extracts was determined by measuring the conversion of unlabeled DAP into desthiobiotin (Pai, 1969a) Cell-free extracts were first stirred with 40 to 80 mg of avidin-acrylic beads to remove residual biotin Beads were then removed by centrifugation DAP (250 nmoles in 25 μl of

deionized water) was added to 0 5 ml of cell-free extract in a microfuge tube This solution was incubated at 37°C for 2 h, placed 5 min in a boiling water bath, and centrifuged 1 5 min at 15,600g The supernatant was assayed for desthiobiotin with the bioD auxotroph of E coli, which responds to biotin or desthiobiotin but not DAP Controls lacking either DAP or cell-free extract were assayed to confirm that the desthiobiotin detected was not due to biotin or desthiobiotin contamination Extracts were also assayed with the bioB auxotroph of E coli, which responds only to biotin, to demonstrate that the activity detected with the bioD strain resulted from the presence of desthiobiotin rather than biotin

Results

Response of Mutant Embryos in Culture

Arrested embryos supplemented with either biotin, desthiobiotin, or DAP enlarged and turned green during the first 7 to 10 d in culture and subsequently produced rosettes with normal leaves and trichomes (Table XIII) Microbiological assays of mutant tissues grown in the presence of DAP or desthiobiotin demonstrated that these precursors were converted to biotin (Table XIV) Arrested embryos remained white and enlarged only slightly on a basal medium lacking biotin or supplemented with KAP or a mixture of pimelic acid, alanine, and CoA (Table XIII) Rescued seeds from homozygous biol plants watered with biotin (Schneider et al , 1989) also produced normal plants when germinated on media supplemented with either biotin, desthiobiotin, or DAP (Figure 11) Mutant seedlings initiated on basal media in the presence or absence of KAP failed to produce any leaves and died after 2 weeks The response

TABLE XIII

RESPONSE OF <u>BIO1</u> EMBRYOS ON BASAL MEDIA SUPPLEMENTED WITH INTERMEDIATES OF BACTERIAL BIOTIN SYNTHESIS[a]

Medium Supplement	Concentration (μM)	Response in Culture
Biotin	≥ 0 01	Normal plants
Desthiobiotin	≥ 0 01	Normal plants
DAP	0 1-100	Normal plants
KAP[b]	0 1-1 0	No growth
Pimelic acid	250	No growth
Pimelic acid/alanine	250/250	No growth
Pimelic acid/alanine/CoA	100/100/100	No growth
Biocytin	0 1-1 0	Normal plants

[a]Arrested embryos were removed from aborted seeds and cultured on a basal medium supplemented with specific nutrients Data taken from Shellhammer and Meinke (submitted)

[b]Synthetic KAP from Max Eisenberg

TABLE XIV

LACTOBACILLUS MICROBIOLOGICAL ASSAY OF TOTAL BIOTIN IN TISSUES OF ARABIDOPSIS THALIANA GROWN IN THE PRESENCE OF BIOTIN PRECURSORS

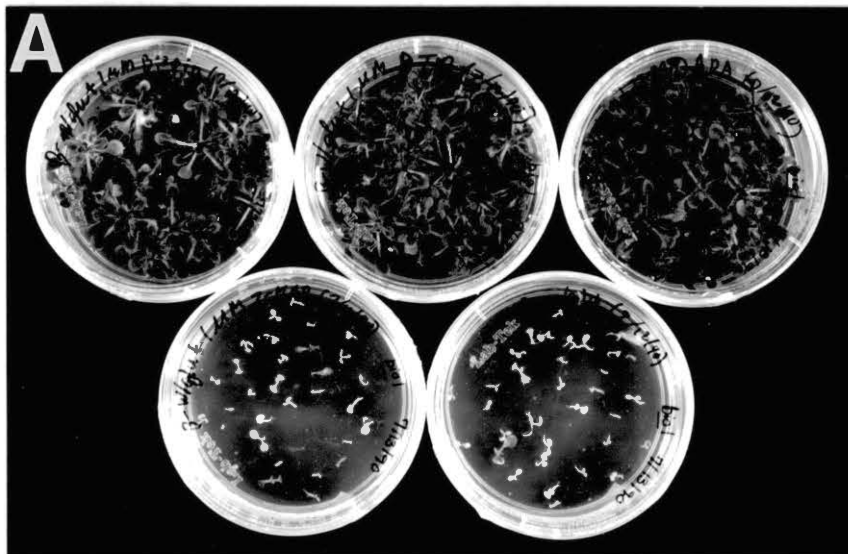
Type of Tissue Analyzed	Biotin Content[a]
Wild-type leaf tissue from seedlings grown 7-10 d on a basal medium	34 ± 14
Wild-type leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 5 μM desthiobiotin	35 ± 2
Wild-type leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 100 nM DAP	31 ± 2
bio1 cotyledon tissue from seeds germinated on basal medium and grown 7-10 d[b]	ND[c]
bio1 leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 5 μM desthiobiotin	26 ± 1
bio1 leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 100 nM DAP	4 ± 1
bio1 leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 10 nM biotin	5 ± 1
bio1 leaf tissue from seedlings grown 7-10 d on a basal medium supplemented with 5 μM biotin	376 ± 0

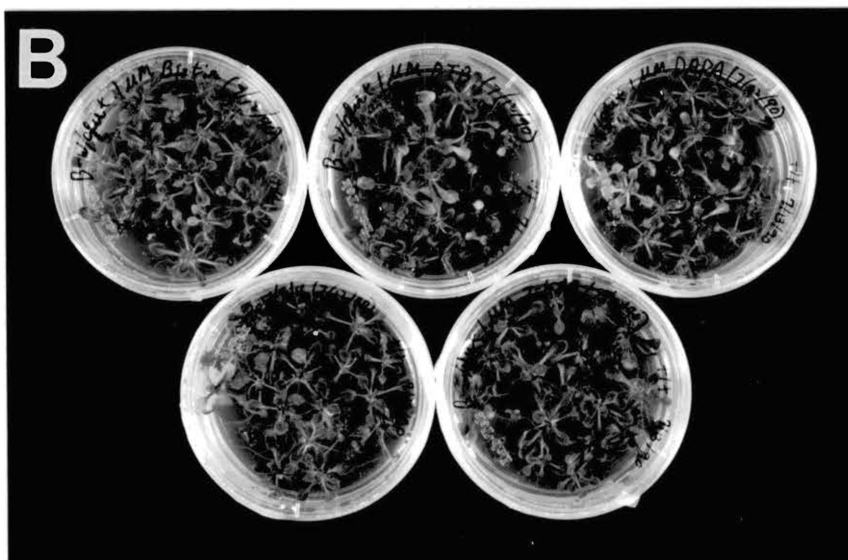
[a]Each value represents the mean biotin content in pg total biotin per mg FW of tissue ± SD

[b]Cotyledons had become chlorotic after 7-10 d probably due to depletion of maternal biotin

[c]Not detected

Figure 11 Response of _bio1_ in culture Seedlings of _bio1_ (A) and wild-type (B) were grown for 2 weeks on basal media supplemented with biotin vitamers Plates contained 1 μM of either biotin (top left), desthiobiotin (center), 7,8-diaminopelargonic acid (top right), or 7-keto-8-aminopelargonic acid (bottom left) The bottom right plate lacked biotin vitamers Figure from Shellhammer and Meinke (submitted)





of mutant embryos to pimeloyl-CoA was not tested because this biotin vitamer is not commercially available. Chemical synthesis of pimeloyl-CoA was not attempted because problems were anticipated with both uptake and hydrolysis of this compound by plant cells. The ability of rescued seeds to produce normal plants on media supplemented with biocytin (biotin bound to lysine) demonstrated that <u>bio1</u> can utilize a bound form of biotin (Table XIII).

<u>Partial Purification of KAP</u>

Attempts to supplement tissue culture media with crude <u>E. coli bioA</u> supernatant, which contained KAP excreted by this auxotroph, were unsuccessful due to inhibition of seed germination by an unknown component. Partial purification of KAP from 800 ml of concentrated supernatant yielded a solution consisting of approximately 20 μg of this vitamer and a substantial amount of contaminating amino acids (Eisenberg and Maseda, 1970). Seeds cultured on media supplemented with 100 nM partially purified KAP still failed to germinate, possibly due to co-purification of the component responsible for the toxicity of crude supernatant.

<u>Paper Chromatography of Biotin Vitamers</u>

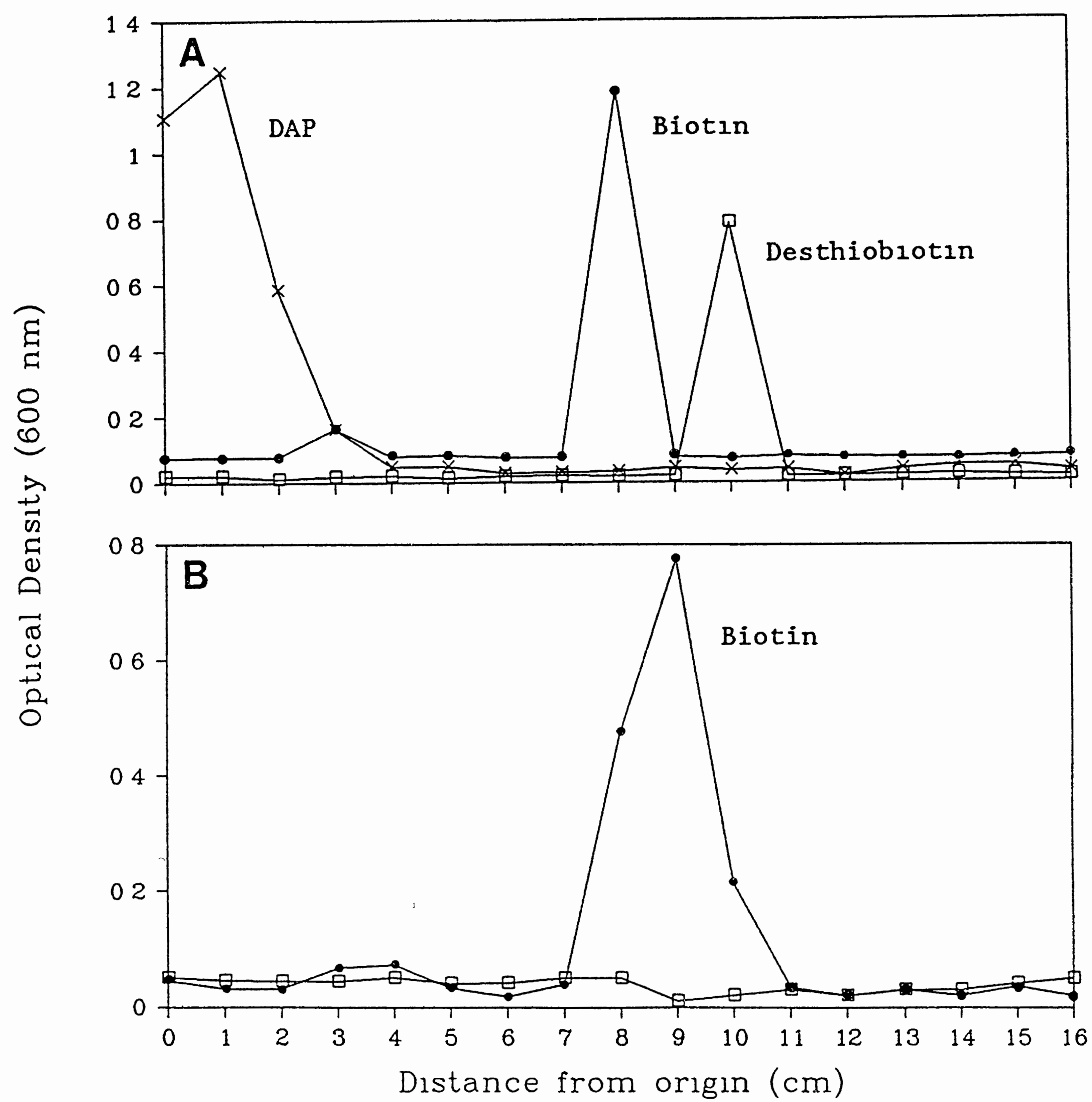
Three methods were used to assay for the accumulation of biotin precursors in mutant tissues. The first two involved analysis of vitamers separated by paper chromatography or TLC. These approaches were used extensively in the initial analysis of bacterial auxotrophs defective in biotin synthesis (Cheeseman and Pai, 1970). Paper chromatography was used primarily to resolve biotin and desthiobiotin which are structurally similar and do not separate well under most

chromatographic conditions Results that I obtained with this technique however were difficult to interpret because biotin and desthiobiotin usually migrated together When good resolution was finally achieved by modification of the solvent mixture, the compounds appeared as 3 to 4-cm long streaks rather than small, distinct spots Subsequent analysis of vitamer accumulation in biol was therefore attempted by TLC and microbiological assays rather than paper chromatography

TLC and Bioautography of Biotin Vitamers

The second technique, TLC analysis, did not resolve biotin and desthiobiotin very well, but the compounds when visualized by I_2 vapors appeared as small distinct spots Iodine visualization however was not sensitive enough to detect biotin in wild-type tissues Further analysis of biotin vitamers in plant tissues therefore required the more sensitive visualization technique of bioautography which involved microbiological assay of vitamers eluted from chromatogram segments A broad peak corresponding to biotin was observed when wild-type Arabidopsis tissues were examined by this method (Figure 12B) The absence of peaks corresponding to desthiobiotin and DAP standards (Figure 12A) in extracts from mutant tissues (Figure 12B) was consistent with the model that biol is defective in conversion of KAP to DAP The absence of a KAP peak was more difficult to interpret because I was unable to elute KAP standards from chromatograms Subsequent analysis of biotin precursors in mutant and wild-type tissues therefore utilized more sensitive microbiological assays

Figure 12 Bioautography of biotin vitamers Standards (A) included biotin (•), desthiobiotin (□), and DAP (X) Bioautography of wild-type seedlings (B, •) revealed a broad peak corresponding to biotin No biotin vitamers were detected in extracts of bio1/bio1 seedlings grown on a basal medium supplemented with 10 nM biotin (B, □)


A
B
DAP
Biotin
Desthiobiotin
Biotin
Optical Density (600 nm)
Distance from origin (cm)
1 4
1 2
1
0 8
0 6
0 4
0 2
0
0 8
0 6
0 4
0 2
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Microbiological Assays of KAP

The third technique utilized avidin-acrylic beads which provided a simple method for removing residual biotin from plant tissues and allowed direct microbiological assay of KAP using *E coli bioF* (Table XV) Extracts supplemented with partially purified KAP were used to demonstrate that plant tissues did not interfere with detection of this biotin precursor The absence of KAP in wild-type tissues was expected because of the low level of biotin synthesis in plants The absence of KAP in leaf tissues obtained from *bio1/bio1* seedlings grown in the presence of biotin at first appeared inconsistent with the model that *bio1* is defective in conversion of KAP to DAP Mutant seedlings also failed to accumulate KAP when grown in the presence of high concentrations of pimelic acid and alanine, the apparent precursors of biotin in bacteria I then assayed for KAP in silique tissue because biotin synthesis in developing seeds may occur at a higher rate than in leaf tissue The results summarized in Table XV demonstrated that KAP failed to accumulate even in siliques from *bio1/bio1* plants watered with biotin, pimelic acid, and alanine

Labeling with [^{3}H]Biotin and [^{14}C]Pimelic Acid

I became interested in the role of pimelic acid in biotin synthesis in plants when *bio1* embryos failed to grow in the presence of this compound (Schneider et al , 1989) I first wanted to determine whether the failure of *bio1* to respond to pimelic acid was caused by a lack of uptake Labeled biotin was used as a control because its uptake was already demonstrated by the observation that biotin rescues *bio1*

TABLE XV

MICROBIOLOGICAL ASSAY OF KAP

Plant Material Analyzed	KAP Added[a]	KAP Detected[b]
Wild-type seedlings grown for 4 weeks on a basal medium	-	-
Wild-type seedlings grown for 4 weeks on a basal medium	+	+
biol seedlings grown for 4 weeks on 100 nM biotin, 10 μM pimelic acid, and 10 μM alanine	-	-
Siliques from wild-type plants grown in pots	-	-
Siliques from wild-type plants grown in pots	+	+
Siliques from biol plant in pots watered with 5 mM biotin, 1 mM pimelic acid, and 1 mM alanine	-	-

[a]Some extracts contained supplemental KAP to demonstrate that microbiological assays could detect KAP in plant tissues

[b]Assays were capable of detecting ≥ 100 pg KAP per mg plant tissue

Labeled pimelic acid was used to trace movement of this compound in wild-type plants Results summarized in Table XVI demonstrated that pimelic acid was indeed translocated from the culture medium to the leaves of young seedlings Label was most readily detected in whole seedlings, which were in contact with the culture medium, but even young leaves that never touched the medium contained detectable amounts of label The failure of pimelic acid to rescue biol is therefore not caused by a lack of uptake This experimental approach was not possible with KAP because labeled samples were not available

I also tested wild-type plants for their ability to convert labeled pimelic acid into biotin because the initial steps of biotin synthesis in plants have not been documented I reasoned that conversion of labeled pimelic acid into biotin would indirectly provide evidence for the role of both pimeloyl-CoA and KAP in biotin synthesis in plants Failure to recover labeled biotin from seedlings was probably caused by the low specific activity of the pimelic acid employed and the low level of biotin synthesis in plants Immature siliques were then examined because they were considered likely to be more active in biotin synthesis Wild-type siliques took up labeled biotin and pimelic acid but did not incorporate detectable levels of labeled pimelic acid into biotin I also looked for incorporation of pimelic acid into biotin in cell-free extracts of wild-type plants No incorporation of label into biotin was detected Activity of the biotin pathway in extracts was demonstrated by assaying for conversion of unlabeled DAP into desthiobiotin (Table XVII) Results obtained suggested that biotin synthesis in plants occurs at such a low rate that incorporation of [^{14}C]pimelic acid into biotin may be difficult to detect

TABLE XVI

UPTAKE OF LABELED PIMELIC ACID BY WILD-TYPE SEEDLINGS OF *ARABIDOPSIS THALIANA*

Plant Material	Labeling Period (h)	DPM per mg FW[a]
Whole seedlings	16	2522 ± 282
	24	2206 ± 100
Cotyledons and young leaves	8	111 ± 92
	16	232 ± 145
	24	297 ± 102
Young leaves	8	47 ± 19
	16	59 ± 25

[a]Each value represents the mean number of DPM above background ± SD Means were calculated from at least three independent experiments

TABLE XVII

CONVERSION OF DAP TO DESTHIOBIOTIN IN CELL-FREE EXTRACTS OF *ARABIDOPSIS THALIANA*

Sample Analyzed	Desthiobiotin Detected[a]
Wild-type extract + 250 nmoles DAP	63 0 ± 25 0
Wild-type extract	0 6 ± 0 7
250 nmoles DAP	1 2 ± 2 1

[a]Values represent the mean number of pmoles desthiobiotin per ml of sample ± SD Means were calculated from at least three independent experiments

Discussion

The results presented in this chapter suggest that <u>bio1</u> is defective in conversion of KAP to DAP This model is based on the failure of synthetic KAP to rescue mutant embryos in culture Even 1 μM KAP was insufficient to promote growth of <u>bio1</u> embryos This concentration represents 10 times the quantity of DAP and 100 times the amount of biotin required to rescue mutant embryos Higher levels of KAP were not tested because only a small amount of the purified synthetic compound was available Attempts to repeat the direct chemical synthesis of KAP and provide additional material for this project were unsuccessful Studies with bacterial mutants have nevertheless shown that KAP is much more effective than DAP at rescuing the <u>bioF</u> auxotroph of <u>E coli</u> (Eisenberg, 1973) The failure of <u>bio1</u> embryos to respond in culture was not caused by KAP decomposition because biological activity was demonstrated with appropriate bacterial auxotrophs Samples of KAP obtained from <u>E coli bioA</u> supernatant were not used for culture studies because even the most purified fractions were toxic to wild-type embryos and seedlings

The failure of KAP to rescue <u>bio1</u> embryos might also be caused by limited uptake of this biotin vitamer I attempted to test for KAP uptake by growing both mutant and wild-type seedlings on media supplemented with KAP (and biotin for <u>bio1</u> seedlings), using avidin beads to remove residual biotin from plant extracts, and testing for the presence of KAP translocated to young shoots by assaying with <u>E coli bioF</u> Although no evidence of KAP uptake was found, relatively small amounts of KAP were added to the medium, and existing methods of KAP

detection probably lacked the sensitivity required to detect transport of low levels of this biotin vitamer to young leaves

An alternate model consistent with the failure of KAP to rescue biol embryos in culture might be that KAP is not a biotin precursor in plants and that DAP is synthesized instead from an unknown intermediate Although the entire pathway from pimeloyl-CoA to biotin is conserved among bacteria and fungi, some biochemical pathways have been described in which early steps differ while final steps are conserved among distantly related species One example of such a pathway is heme biosynthesis which begins with the condensation of glycine and succinyl-CoA which yields δ-aminolevulinate in animals, fungi, and some bacteria (Kikuchi et al , 1958) In plants and many bacteria, however, glutamate is converted into γ,δ-diketovalerate which is then transaminated to form δ-aminolevulinate (Avissar et al , 1989, Beale and Castelfranco, 1974, Foley and Beale, 1982) Remaining steps of heme biosynthesis are conserved among bacteria, fungi, animals, and plants (Umbarger and Zubay, 1988)

Another question that remains to be addressed is why biol tissues do not accumulate high levels of KAP as might be expected for an auxotroph defective in the synthesis of DAP KAP is known to form inactive dimers at high pH (Eisenberg and Maseda, 1970) but appears to be relatively stable when added to plant extracts The most likely explanation is that KAP is metabolized into other products by intact plant cells or accumulates to such low levels that it escapes detection In microbial systems, biotin auxotrophs excrete KAP into the growth medium where conversion into other products may be limited The conversion of KAP into DAP is therefore the most likely defect in biol,

but with limited supplies of purified KAP and assay methods that lack the required sensitivity, definitive identification of the biochemical defect in this plant auxotroph will require either radioactively labeled KAP or molecular isolation of the mutant gene

Recent advances in molecular characterization of bacterial genes involved in biotin synthesis may facilitate both the analysis of biol and elucidation of early steps of biotin synthesis in plants The bioA gene which codes for DAP aminotransferase has been cloned and sequenced from both E coli (Otsuka et al , 1988) and Bacillus sphaericus (Gloeckler et al , 1990) Partial sequences are also available for bioA genes from Citrobacter freundii and Salmonella typhimurium (Shiuan and Campbell, 1988) These bacterial genes exhibit a high degree of sequence similarity with ornithine aminotransferases (Otsuka et al , 1988) that function in general metabolism and some similarity with glutamate 1-semialdehyde aminotransferases (Elliott et al , 1990) that play an important role in plant tetrapyrrole synthesis (Grimm, 1990).

The recent elucidation of bioA sequences from different bacteria raises the possibility of identifying the corresponding plant gene by screening genomic libraries with heterologous probes constructed from conserved regions Alternatively, it might be possible to complement a bacterial bioA mutant with cloned cDNAs from wild-type Arabidopsis plants This approach has already been used to isolate housekeeping genes from higher plants (Delauney and Verma, 1990) but might, in the case of biotin synthesis, be limited by low rates of transcription Chromosome walking could also be used to isolate the biol gene, particularly since the biol locus has been mapped to within 0 5 cM of an existing RFLP (Patton et al , 1991), but this method is tedious even in

model systems with small genomes A final approach would be to determine whether the bacterial bioA gene can rescue bio1 tissue following Agrobacterium-mediated plant transformation This approach may be worth pursuing now that the probable defect in bio1 has been identified One potential problem associated with this approach is the question of the intracellular location of biotin synthesis in plants Information of this type is important because biotin synthesis may occur in chloroplasts In the following chapter I will address the question of where biotin synthesis is located and whether other steps of biotin synthesis can be characterized in plants by using isolated chloroplasts

CHAPTER V

ANALYSIS OF BIOTIN SYNTHESIS IN SUBCELLULAR FRACTIONS OF ARABIDOPSIS THALIANA

Introduction

Until recently very little was known about the biosynthesis and utilization of biotin in plants Much of the work described in previous chapters provided valuable information regarding these aspects and the nature of the biochemical defect in the biol auxotroph of Arabidopsis One aspect that remains to be addressed is the subcellular localization of biotin synthesis in plants Information regarding the subcellular localization of biotin synthesis in plants may be important in future studies conducted with biol One approach to the analysis of the genetic and biochemical defect in this biotin auxotroph is to attempt genetic complementation of biol with the cloned E coli bioA gene introduced by Agrobacterium-mediated transformation of homozygous (biol/biol) mutant tissue This approach may require addition of a transit sequence to the cloned gene if biotin synthesis in plants is located in chloroplasts

Additional information on the subcellular localization of biotin synthesis may also be helpful in studying the specific enzymatic steps involved in biotin synthesis in plants and aid in the biochemical analysis of the defect in biol If biotin synthesis is localized in chloroplasts isolation of these organelles will enrich for enzymes of

biotin synthesis and possibly aid in assaying for biotin synthetase and DAP aminotransferase activities which cannot be detected in crude cell-free extracts of wild-type plants I have therefore reasoned that if this approach resulted in detection of DAP aminotransferase activity in wild-type chloroplasts, the same method could be used to determine if bio1 is indeed defective in conversion of KAP to DAP, catalyzed by DAP aminotransferase

As discussed in previous chapters, biotin is a cofactor of carboxylases One of the most well-characterized carboxylases in higher plants is acetyl-CoA carboxylase This enzyme performs an essential function in the initiation of fatty acid biosynthesis by catalyzing the formation of malonyl-CoA from acetyl-CoA and CO_2 (Lane et al , 1974) Acetyl-CoA carboxylase has been purified from higher plants and found to be localized primarily in chloroplasts (Nikolau et al , 1984) Because biotin is required by this chloroplast-specific enzyme as the carrier of transferred CO_2 groups it seems reasonable that biotin synthesis may be localized in chloroplasts as well

In this chapter I demonstrate that biotin synthesis does not appear to be localized in chloroplasts because desthiobiotin synthetase activity is present in cytosolic fractions rather than in chloroplast fractions Two enzymes chosen as controls were amylase, which is located primarily in the cytosol, and transglycosylase, which is located in chloroplasts (Lin et al , 1988) Assays for these marker enzymes indicated that subcellular fractions were enzymatically active and relatively pure Other enzymes of biotin synthesis, biotin synthetase and DAP aminotransferase, could not be detected in either cell-free extracts or subcellular fractions Failure to detect these two enzymes

may indicate that the pathway for biotin synthesis in plants operates at a very low rate This hypothesis is supported by the presence of only trace amounts of biotin in plant tissues, the absence of biotin precursors in bio1 and wild-type tissues, and the low level of conversion of DAP to desthiobiotin in cell-free extracts and cytosolic fractions

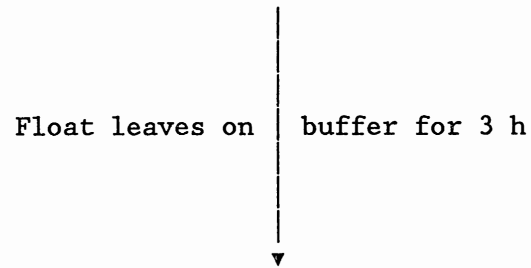
Materials and Methods

Chloroplast Isolation

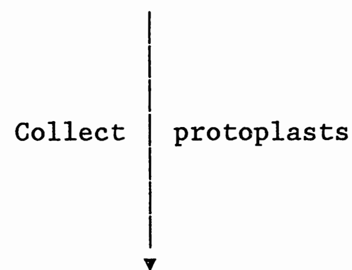
Chloroplasts were isolated by modification of procedures described previously (Lin et al , 1988, Somerville et al , 1981) Figure 13 outlines the procedure used for chloroplast isolation Protoplasts were isolated from 4-week-old wild-type plants by removing the lower epidermis from rosette leaves using fine-tipped forceps and floating the stripped leaves on 15 ml of protoplast isolation buffer containing 0 5 M sorbitol, 10 mM MES (pH 5 5), 1 mM $CaCl_2$, 1 6% (w/v) each of Macerase and Cellulysin (Calbiochem) in a glass petri dish Digestion of cell wall components was conducted at 37°C after the surface of the buffer was completely covered with leaves After 3 h of digestion, protoplasts had settled to the bottom of the dish while the undigested upper epidermis remained on the surface of the buffer Digests were then stored at 4°C until a total of approximately 5 g FW of tissue had been collected and digested Subsequent procedures were conducted at 4°C unless otherwise noted Undigested debris was removed by filtration through one layer of Miracloth (Calbiochem) and protoplasts were collected by centrifugation for 4 min at 164g and resuspended in 5 ml of

Figure 13 Outline of chloroplast isolation procedure

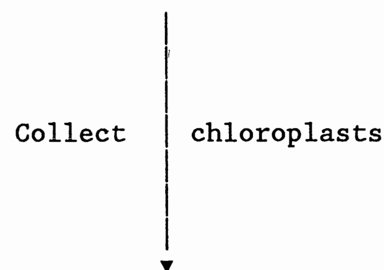
Remove lower epidermis from leaves



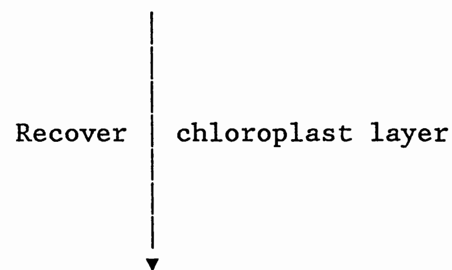
Filter through Miracloth



Wash and disrupt protoplasts



Centrifuge chloroplasts on Percoll gradients



Collect chloroplasts

protoplast isolation buffer lacking Macerase and Cellulysin Protoplasts were collected by centrifugation again for 5 min at 164g and resuspended in 5 ml of protoplast disruption buffer containing 0 3 M sorbitol, 20 mM Tris-HCl (pH 7 5), and 0 1% (w/v) bovine serum albumin The suspension was transferred to a 10-ml syringe and was gently expelled through a 15-μm nylon filter which was attached to the syringe Chloroplasts were collected by centrifugation at 365g for 5 min and resuspended in 0 5 ml of protoplast disruption buffer The supernatant was saved as the cytosolic fraction and resuspended chloroplasts were layered on 24 ml of a gradient of 10 to 80% (v/v) Percoll, containing 1% (w/v) Ficoll 400, 3% (w/v) polyethylene glycol 8,000, 0 33 M sorbitol, 50 mM Tris-HCl (pH 7 5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbic acid, and 2 mM dithiothreitol The gradients were centrifuged for 8 min at 10,000g Intact chloroplasts were collected and diluted in 1 ml of chloroplast isolation buffer containing 375 mM sorbitol, 35 mM Tris-HCl (pH 7 5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM dithiothreitol The chloroplasts were collected by centrifugation until rotor speed reached 4,000g Chloroplasts were either used intact or disrupted in 1 ml of 10 mM Tris-HCl, pH 7 5 buffer and stored at 4°C until used Protein concentrations were determined with Bio-Rad dye reagent by the method of Bradford (1976) using bovine serum albumin as standard

Enzyme Assays

Amylase To assess the activity and purity of cytosolic and chloroplast preparations, amylase assays were used because this activity is localized primarily in the cytosol rather than the chloroplast (Lin et al , 1988) Amylase activity was typically measured in 1 5-ml

reactions containing 0 375 mg starch (0 5 ml Sigma starch solution), 40 μmole sodium acetate buffer (pH 6 0), 0 5 ml of 0 002 N iodine in 2% (w/v) KI solution (Sigma Chemical Company) and 0 2 to 1 0 mg of protein in either cell-free extracts or subcellular fractions Mixtures were incubated until the dark blue starch-iodine color turned pale green indicating that all the starch had been degraded Reaction volumes were reduced to 75 μl for assaying activity in small amounts of cytosol and chloroplast fractions using equal volumes of substrate, fraction, and iodine solution

Transglycosylase This enzyme was chosen as a marker because it is localized mainly in chloroplasts (Lin et al , 1988) Transglycosylase activity was measured in 250-μl reactions containing 10 μmole sodium acetate buffer (pH 6 0), 2 48 μmoles maltotriose (Peat et al , 1956) and 0 2 to 1 0 mg of protein in cell-free extracts or subcellular fractions Mixtures were incubated at 37°C for 30 min and then immersed in boiling water for 30 s to terminate reactions The resulting suspensions were centrifuged 1 5 min at 15,000g to remove precipitates that formed during heating of tubes in boiling water Released glucose was measured by adding 10 μl of the terminated reaction mixture to 1 5 ml of glucose (HK) diagnostic kit (Sigma Chemical Company) and then following the reduction of NAD to NADH + H^+ in the presence of hexokinase and glucose-6-phosphate dehydrogenase provided in the diagnostic kit Reduction of NAD was measured at 340 nm on a Shimadzu UV-160 spectrophotometer Increase in absorbance was directly proportional to the amount of glucose added to the kit

Desthiobiotin Synthetase To determine the subcellular location of biotin synthesis, desthiobiotin synthetase activity was measured in subcellular fractions by modification of procedures described by Pai (1969a) and Cheeseman and Pai (1970) Standard assay mixtures contained 50 μmoles Tris-HCl (pH 7 5), 250 nmoles DAP, 250 nmoles ATP (disodium salt), 2 5 μmoles $MgCl_2$, 5 μmoles freshly prepared $NaHCO_3$, and 0 2 to 1 0 mg of protein in cell-free extracts or subcellular fractions in a final volume of 0 5 ml Reactions were incubated at 37°C for 2 h and then terminated by heating for 5 min in a boiling water bath Enzyme activity was determined by measuring the amount of desthiobiotin formed as assayed microbiologically with E coli bioD

Biotin Synthetase In an attempt to measure activity of other biotin synthetic enzymes biotin synthetase activity was measured by modification of the methods of Pai and Lichstein (1966) and Pai (1972) by assaying for biotin produced from desthiobiotin The standard reaction mixture contained 5 μmoles Tris-HCl buffer (pH 7 5), 1 5 μmoles $MgSO_4$, 22 μmoles glucose, 0 5 nmole desthiobiotin, and 0 2 to 1 0 mg protein in cell-free extracts or subcellular fractions in a final volume of 0 5 ml Mixtures were incubated at 37°C for 2 h and stopped by heating for 5 min in a boiling water bath Precipitates were removed by centrifugation, and the supernatant was assayed for biotin with E coli bioB

DAP Aminotransferase To determine if bioI is defective in conversion of KAP to DAP, DAP aminotransferase activity was measured by modification of the procedure of Eisenberg and Stoner (1971) Standard reaction mixtures contained 75 μmoles Tris-HCl (pH 7 5), 5 nmoles KAP,

1 25 µmoles S-adenosyl-L-methionine, 0 1 µmole pyridoxal 5'-phosphate, 2 5 µmole dithiothreitol, and 0 2 to 1 0 mg protein from cell-free extracts or subcellular fractions in a final volume of 0 5 ml Enzyme (extract), buffer, and dithiothreitol were mixed and incubated at 37°C for 10 min A mixture of the remaining components in buffer were then added to start the reaction After 2 h at 37°C, reactions were stopped by heating for 5 min in a boiling water bath, and precipitates were removed by centrifugation Supernatants were assayed for DAP with E coli bioA strain 109

Enzymes in extracts and fractions containing less than 3 mg of protein/ml were concentrated by addition of ammonium sulfate to 50% saturation, collection of precipitated protein by centrifugation at 15,600g for 15 min at 4°C, and reconstitution in 100 µl of 50 mM Tris-HCl buffer, pH 7 5 (Eisenberg and Stoner, 1979) Controls lacking either substrate or enzyme (extract) were conducted in all enzyme assays to demonstrate that the product detected was not introduced with any of the reagents or attributed to the presence of background levels in extracts

Results

Activity and Purity of Subcellular Fractions

Amylase Activity and Localization As summarized in Table XVIII, cytosolic fractions contained most of the amylase activity while disrupted chloroplast fractions contained relatively little Activity was readily detected in cell-free extracts and cytosolic fractions Chloroplast fractions did contain detectable amylase activity indicating

TABLE XVIII

RESULTS OF ENZYME ASSAYS IN SUBCELLULAR FRACTIONS OF *ARABIDOPSIS THALIANA*

Enzyme	Fraction	Activity[a]
Amylase	Cell-free extract	8 44 ± 3 50[b]
	Cytosolic fraction	15.00 ± 7 69[b]
	Chloroplast fraction	2 63 ± 0 59[b]
Transglycosylase	Cell-free extract	16 50 ± 0 71[c]
	Cytosolic fraction	ND[d]
	Chloroplast fraction	20 16 ± 4 50[c]
Desthiobiotin synthetase	Cell-free extract	26 80 ± 2 99[e]
	Cytosolic fraction	37 76 ± 1 02[e]
	Chloroplast fraction	ND
Biotin synthetase	Cell-free extract	ND
	Cytosolic fraction	ND
	Chloroplast fraction	ND
DAP aminotransferase	Cell-free extract	ND
	Cytosolic fraction	ND
	Chloroplast fraction	ND

[a]Values represent the mean specific activity of each enzyme ± SD Means were calculated from at least three independent experiments

[b]μg starch degraded/mg protein/min

[c]nmole glucose released/mg protein/min

[d]Not detected

[e]pmoles desthiobiotin produced/mg protein/h

that some contamination of chloroplast fractions with cytosolic enzymes did occur Additionally, some chloroplast-specific amylase isozymes do exist and may have contributed to the low level of activity detected in chloroplast fractions (Lin et al , 1988) These results therefore provided evidence that cytosolic fractions were enzymatically active but may have contained some contaminating chloroplast-specific enzymes Amylase assays nevertheless served as useful controls for assessing the enzymatic activity and relative purity of subcellular fractions

Transglycosylase Activity and Localization The data presented in Table XVIII show that transglycosylase activity was detected in cell-free extracts and chloroplast fractions but not in cytosolic fractions Although one would expect some contamination of cytosolic fractions with chloroplast-specific enzymes, no transglycosylase activity was detected in disrupted chloroplast fractions Failure to detect transglycosylase activity in chloroplast extracts may reflect the inability of the assay method to detect small amounts of this enzyme present as a contaminant in cytosolic fractions Transglycosylase assays did however provide useful data for assessing the enzymatic activity and purity of disrupted chloroplast fractions

Desthiobiotin Synthetase Activity and Localization

As shown in Table XVIII, desthiobiotin synthetase activity was detected only in cell-free extracts and cytosolic fractions No desthiobiotin synthetase activity was detected in disrupted chloroplast fractions indicating that desthiobiotin synthesis is not located in chloroplasts Failure to detect any contaminating activity in

chloroplast fractions may indicate the lack of sensitivity required to detect low levels of desthiobiotin synthetase that may be present in chloroplast fractions as contaminants Desthiobiotin synthetase activity provided a useful marker enzyme for determining the subcellular localization of biotin synthesis in plants and indicated that this pathway is not localized in chloroplasts Biotin synthesis may however be localized in some other subcellular compartment such as mitochondria because organelles of this sizes may be present in cytosolic fractions

Assays for Other Enzymes of Biotin Synthesis

Biotin Synthetase As shown in Table XVIII, biotin synthetase could not be detected in cell-free extracts In *E coli*, conversion of DAP to desthiobiotin could be detected in crude cell-free extracts (Pai, 1969a), however, conversion of desthiobiotin to biotin was demonstrated only in intact bacterial cells (Pai and Lichstein, 1966) This observation led me to assay for biotin synthetase activity in intact protoplasts and intact chloroplasts As summarized in Table XIX, no conversion of desthiobiotin to biotin could be detected in intact protoplasts or chloroplasts Enzyme activities in intact protoplasts and chloroplasts were either lower compared to activities measured in cytosolic fractions and disrupted chloroplasts (amylase and desthio-biotin synthetase) or completely absent (transglycosylase) possibly because of limited uptake of substrate by intact fractions Biotin synthetase assays did not provide information on plant biotin synthesis

DAP Aminotransferase Synthesis of DAP from KAP in *E coli* has been demonstrated in cell-free extracts when S-adenosyl-L-methionine and

TABLE XIX

RESULTS OF ENZYME ASSAYS IN INTACT PROTOPLASTS AND CHLOROPLASTS OF *ARABIDOPSIS THALIANA*

Enzyme	Fraction	Activity[a]
Amylase	Protoplasts	0 08 ± 0 02
	Chloroplasts	ND[b]
Transglycosylase	Protoplasts	ND
	Chloroplasts	ND
Desthiobiotin synthetase	Protoplasts	0 02 ± 0 01
	Chloroplasts	ND
Biotin synthetase	Protoplasts	ND
	Chloroplasts	ND
DAP aminotransferase	Protoplasts	ND
	Chloroplasts	ND

[a]Values represent the mean specific activity (see Table XVIII for definition) ± SD Means were calculated from at least three independent experiments

[b]Not detected

pyridoxal 5'-phosphate are added (Eisenberg and Stoner, 1971) Detection of DAP synthesized from KAP by this system requires an E coli bioA strain (109) that responds more readily to DAP than any other bioA strain (Eisenberg and Stoner, 1971) This bioA mutant requires 25 to 50 times less DAP for growth than do other bioA strains (Eisenberg and Stoner, 1979) Even with bioA-109, conversion of KAP to DAP was not detected in plant cell-free extracts (Table XVIII), intact protoplasts, or intact chloroplasts (Table XIX) Even though controls using E coli cell-free extracts demonstrated that DAP aminotransferase could be detected, assays using plant cell-free extracts, subcellular fractions, or intact protoplasts and chloroplasts were not useful in determining if bio1 is indeed defective in conversion of KAP to DAP Failure to detect DAP aminotransferase activity even in cell-free extracts (Table XVIII) indicates that other methods may be needed to definitively identify the biochemical defect in bio1

Discussion

Many biochemical pathways have been elucidated and analyzed in detail using experimental microorganisms such as *E coli* and *Saccharomyces cerevisiae* Plants provide a useful eucaryotic system for studying many aspects of these processes in multicellular organisms because they are capable of synthesizing most of their own biologically important compounds such as lipids, polysaccharides, vitamins, amino acids, and nucleosides *de novo* One aspect of biochemical pathways commonly analyzed in plant systems is the subcellular localization of such metabolic processes For example, many papers have been published on the details of fatty acid biosynthesis in model plant systems such as

Arabidopsis (Browse et al , 1985, 1986) This process as well as starch degradation and synthesis and amino acid biosynthesis has been shown to occur primarily in chloroplasts (Last et al , 1991, Liedvogel and Bauerle, 1986, Lin et al , 1988, Miflin and Lea, 1977, Ohlrogge et al , 1979) Other biochemical processes such as photosynthesis and certain steps of nitrogen assimilation also occur in chloroplasts (Lin et al , 1988, Wallsgrove et al , 1979) Activity of enzymes such as ribulose bisphosphate carboxylase, ADP-glucose pyrophosphorylase, starch synthetase, and transglycosylase have been shown to be predominantly localized in chloroplasts rather than cytosolic fractions (Lin et al , 1988) In Arabidopsis, tryptophan synthase-β activity is located in chloroplasts, and both genes coding for tryptophan synthase-β isozymes contain sequences indicative of a chloroplast-specific signal peptide (Last et al , 1991) Enzymes required for the synthesis of other amino acids such as methionine, cysteine, glycine, and serine are localized in chloroplasts as well (Bryan et al , 1977, Hess and Tolbert, 1966, Mazelis et al , 1976, Schmidt and Trebst, 1969, Shah and Cossins, 1970) Wallsgrove et al (1979) have determined the distribution of enzymes of nitrogen assimilation and found nitrite reductase and glutamate synthase activities localized mainly in chloroplasts

Although the entire pathway for biotin synthesis in plants has not been elucidated, at least one step appears to occur in the cytosol rather than in the chloroplast The results presented in this chapter indicate that desthiobiotin synthetase activity is located in the cytosol but not in disrupted chloroplast fractions Desthiobiotin synthetase activity was also detected at a very low level in intact protoplasts but not in intact chloroplasts Whether biotin synthesis

occurs in mitochondria or another organelle in plants remains to be determined In eucaryotic microorganisms, the specific intracellular location of biotin synthesis is also unknown

The data presented here suggest that a cloned _bioA_ gene will not require addition of a chloroplast-specific transit sequence for targeting the gene product into chloroplasts Unfortunately, _bio_ genes have not been cloned from eucaryotic microorganisms such as _Penicillium chrysogenum_ and _S_ _cerevisiae_, therefore, attempts to rescue _bio1_ by genetic complementation will require the cloned _E_ _coli_ gene Even though biotin synthetase and DAP aminotransferase were not detected in cytosolic or chloroplast fractions, it seems likely that the entire pathway may be located in the cytosol, based on the results of desthiobiotin synthetase assays, indicating that genetic complementation may be feasible without modification of the _E_ _coli_ _bioA_ gene

Little appears to be known about the subcellular localization of other vitamin biosynthetic pathways in plants while much is known about the biochemistry of the enzymatic steps For example, thiamine synthesis in plants proceeds through the same pathway used by _E_ _coli_ (Komeda et al , 1988) but the subcellular site of thiamine biosynthesis is unknown In addition, the _th-1_ thiamine auxotroph of _Arabidopsis_ has been shown to be defective in thiamine monophosphate pyrophosphorylase activity, but the subcellular localization of this enzyme and other enzymes of thiamine biosynthesis in plants remains to be determined

CHAPTER VI

CONCLUSION

The biosynthetic pathway for biotin in plants appears to be the same as the pathway used by bacteria and certain fungi The biol auxotroph of Arabidopsis is an embryo-lethal that appears to be defective in biotin synthesis Arrested biol embryos contain virtually no detectable biotin and can be rescued in culture by biotin, desthiobiotin, or DAP This mutant is not rescued by KAP or pimelic acid and does not produce detectable levels of KAP The current model for describing biotin synthesis in plants and the nature of the specific defect in biol is therefore as follows (a) the pathway for biotin synthesis is conserved in plants and microorganisms, (b) the lesion in biol disrupts the conversion of KAP to DAP in this pathway, and (c) biol fails to accumulate detectable levels of KAP Because biochemical approaches have not led to identification of the specific defect in biol, further research may require cloning of the BIO1 gene through various molecular techniques such as screening genomic libraries with heterologous probes, complementation of microbial bioA mutants with cloned cDNAs from wild-type Arabidopsis plants, or chromosome walking from a closely linked RFLP marker (Chang et al , 1988) Recently the biol mutation has been mapped and found to be located within 0 5 cM of an RFLP marker on chromosome 5 (Patton et al , 1991) This work will facilitate the cloning of BIO1 through chromosome walking and will eventually result in a better understanding of biotin synthesis in

higher plants and of the specific defect in biol Biochemical analysis of this auxotroph has helped to establish the relationship between a specific metabolic defect and a critical developmental pathway in higher plants

One point that remains to be addressed concerns the potential significance of studying biotin synthesis in higher plants Apart from the value of elucidating a biochemical pathway that remained poorly documented for many years, further analysis of biol and the initial steps of biotin synthesis in plants may have important applications to industrial biotin production, control of plant growth by inhibitors of biotin synthesis, studies on the role of biotin in root nodulation (Al-Mallah et al , 1990), and utilization of biotin transport systems to deliver macromolecules into plant cells (Horn et al , 1990) In addition, biol is a useful tool for studying the biological activity of biotin conjugates and analogs in plants Unfortunately, biotin analogs such as biotin sulfoxide, biotin sulfone, homobiotin, norbiotin, oxybiotin, and biotinol are not commercially available However, the analysis of biol has demonstrated that a bound form of biotin such as biocytin can be used as a source of biotin in plants

Currently many scientists are studying various aspects of biotin synthesis and utilization in plants and bacteria Jacques Joyard (Centre d'Études Nucléaires, Grenoble-Cedex, France) had attempted to determine the subcellular localization of biotin synthesis in plants but is currently interested in biotin-containing enzymes, especially acetyl-CoA carboxylase Nicholas Shaw (Biotechnology Research Group, Lonza, Switzerland) is interested in the enzymatic reactions of biotin synthesis in microorganisms and has prepared antibodies to E coli

desthiobiotin synthetase Bob Baxter (University of Edınburgh, Scotland, UK) studies the mechanism of each reaction involved in _E colı_ bıotin synthesis Finally, one company (Sumitomo Chemıcal Company, Japan) has interest in genetically engineering plants to produce increased amount of biotin These studies, in addition to the work described in this dissertation, have provided information that may one day result in commercial exploitation of the biotın pathway in controlling plant growth and in producing biotin though means other than chemical synthesis

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APPENDIX

PROTOCOL FOR MICROBIOLOGICAL ASSAY OF BIOTIN

USING *LACTOBACILLUS PLANTARUM*

Reagents

MRS Media

(1) Dissolve 55 g of MRS dehydrated medium (Fisher DF-0881-02) in 1 l of deionized water, heat to completely dissolve

(2) Divide resulting solution in half, add 7 5 g of Difco purified agar to one portion to make slants, and autoclave both halves for 15 min at 122°C and 18 psi

(3) Distribute 10-ml aliquots of warm (45°C) medium into sterile culture tubes (16 X 125 mm)

(4) Immediately place tubes containing agar in tilted rack and allow to cool at room temperature so that agar slants are formed Store MRS broth and slants at 4°C no longer than one year

0 9% (w/v) Sterile Saline

Dissolve 2 25 g NaCl in 250 ml of sterile deionized water, autoclave 15 min at 122°C and 18 psi, distribute 10-ml aliquots into sterile 16 X 125 mm culture tubes, and store at room temperature

50% Ethanol

Add 475 ml of deionized water to 525 ml of 95 % ethanol and mix well

2 N H_2SO_4

(1) Add 55 56 ml of concentrated H_2SO_4 slowly and with stirring to 750 ml of sterile deionized water

(2) Adjust volume to 1 l with sterile deionized water and store at 4°C

5 N NaOH

(1) Dissolve 40 g of NaOH in 100 ml of sterile deionized water and adjust volume to 200 ml with sterile deionized water

(2) Store in plastic bottle at 4°C Do not store in glass bottle because NaOH solutions will etch the glass and produce a cloudy precipitate

10 ng/ml Biotin Standard Stock Solution

(1) Dissolve 10 mg of biotin (Sigma) in 10 ml of 50% ethanol in disposable, plastic beaker

(2) Dilute the resulting 1 mg/ml solution to 10 ng/ml with 50% ethanol

(3) Sterilize by filtration through a Gelman 0 2 μm filter and store at 4°C no longer than 2 months

(4) Dispose of plastic beaker to prevent biotin contamination of future stocks

0 2 ng/ml Working Biotin Standard

Dilute 0 5 ml of 10 ng/ml biotin standard stock solution to 25 ml with sterile deionized water in disposable plastic beaker Prepare fresh working standard for each assay

2X Biotin Assay Medium

Dissolve 7 5 g of Difco dehydrated biotin assay medium (DF-0419-15-8 Fisher) in 100 ml of sterile deionized water, heat to dissolve completely, and autoclave 15 min at 122°C and 18 psi Store at 4°C

Procedures

Maintenance of Stock Cultures and Preparation of Inoculum

(1) Maintain *L plantarum* (American Type Culture Collection 8014) on MRS slants by growing for 18 h at 37°C and then storing at 4°C for no longer than one month

(2) Prepare inoculum from culture less that one month old by transferring one loop of cells to 10 ml of MRS liquid medium and incubating for 18 h at 37°C

(3) Harvest cells from resulting overnight culture by centrifugation of

1 ml at 15,600g for 1 5 mın

(4) Resuspend bacterial cells ın 1 ml of sterile 0 9% (w/v) saline and centrifuge again Repeat this procedure three addıtional tımes to remove residual bıotin and dilute washed cells 1 1000 with sterile 0 9% (w/v) saline

Preparation of Assay Tubes

(1) Prepare standard tubes by addıng appropriate volumes of water and 0 2 ng/ml biotin working standard to 16 X 125-mm culture tubes as indicated in Table XX

(2) Prepare unknown tubes by addıng water and alıquots of sample to be assayed to culture tubes as also ındıcated in Table XX

(3) Autoclave tubes 15 mın at 122°C and 18 psi, allow to cool to room temperature, and add 0 5 ml of sterile 2X biotin assay medium to each tube

(4) Inoculate each tube ın sterile hood wıth one drop of inoculum delivered from a sterile 1-ml disposable plastic pipette

(5) Vortex each tube to mix contents and ıncubate (wıthout shaking) for 18 to 24 h at 37°C in water bath or incubator

(6) After incubation, transfer the contents of each tube to a 1 5-ml semi-micro disposable cuvette and measure optical density at 600 nm on spectrophotometer

TABLE XX

CONTENTS OF TUBES FOR MICROBIOLOGICAL ASSAY OF BIOTIN USING *LACTOBACILLUS PLANTARUM*

Tube	Biotin Content[a]	Water (ml)	Biotin Standard (ml)
Blank	0 00	0 50	0 00
Standard 1	0 00	0 50	0 00
Standard 2	0 01	0 45	0 05
Standard 3	0 02	0 40	0 10
Standard 4	0 03	0 35	0 15
Standard 5	0 04	0 30	0 20
Standard 6	0 05	0 25	0 25
Standard 7	0 06	0 20	0 30
Standard 8	0 08	0 10	0 40
Standard 9	0 10	0 00	0 50
			Sample to be Analyzed (ml)[b]
Unknown 1A		0 30	0 20
1B		0 20	0 30
1C		0 10	0 40
1D		0 00	0 50

[a]Values represent the amount of biotin added to each standard tube in ng biotin per ml

[b]Aliquots of the sample to be analyzed rather than biotin standard are added to a series of unknown tubes

Operation of Shimadzu UV-160 Spectrophotometer

(1) Turn on power and wait for spectrophotometer to complete initialization procedure

(2) When initialization procedure is complete main menu will be displayed on monitor as shown below

MODE No = "1" ?

---

1 PHOTOMETRIC
2 SPECTRUM
3 TIME SCAN
4 KINETIC
5 QUANTITATIVE
6 MULTI-COMPONENT
7 MEMORY OUT
8 CONDITION SET
9 ATTACHMENT

(3) Type "8" (CONDITION SET) then "ENTER" Monitor will appear as shown below

PARAMETER No = "1" ?

---

1 BASELINE
2 CLOCK SET
3 CLOCK DISPLAY
4 LAMP CHANGE λ
5 LAMP SELECT
6 FILE DELETE

(4) Type "5" (LAMP SELECT) then "ENTER" to turn off UV lamp

(5) To get back to main menu, press "MODE"

(6) To setup parameters for generating a standard curve and analyzing samples type "5" (QUANTITATIVE) then "ENTER" The quantitative mode menu will be displayed as shown below

QUANT PARAMETER CHANGE Y/N ?

---

1 METHOD 1 λ
λ 1 = 800 0
2 STANDARD N = 5
3 SAMPLE No = 1
4 DATA PRINT NO
5 NON-LINEAR W CURVE NO
6 FILE

(7) Press "YES, 1, ENTER" to change wavelength (λ)

(8) Set wavelength to 600 nm by typing "1, ENTER, 600, ENTER"

(9) Set number of standards to 9 by pressing "YES, 2, ENTER, 9, ENTER"

(10) Set DATA PRINT to YES by pressing "YES, 4, ENTER"

(11) Change NON-LINEAR W CURVE to YES by pressing "YES, 5, ENTER" Menu will be displayed as follows

QUANT PARAMETER CHANGE Y/N ?

1 METHOD 1 λ
λ 1 = 600 0
2 STANDARD N = 9
3 SAMPLE No = 1
4 DATA PRINT YES
5 NON-LINEAR W CURVE YES
6 FILE

(12) Change parameters for calibration of standard (working) curve by pressing "NO" and then entering values from Biotin Content column of Table XX Press "NO" twice Monitor display will appear as follows

CALIBRATION SET SAMPLE, PRESS START KEY

STD No	CONC	ABS
1	0 0000	
2	0 0100	
3	0 0200	
4	0 0300	
5	0 0400	
6	0 0500	
7	0 0600	
8	0 0800	
9	0 1000	

(13) Place blank in reference chamber (near back of compartment) and cuvette containing standard 1 in sample chamber

(14) Press "START/STOP" to measure optical density of sample

(15) Remove cuvette and measure optical density of remaining standards

(16) Display WORKING CURVE by entering "YES"

(17) To measure optical density of unknowns, place cuvette in sample chamber and press "NO, START/STOP" Sample number (No), optical density (ABS), and biotin concentration (CONC) in ng biotin/ml assay solution will be printed for each sample as shown below

** QUANTITATIVE ANALYSIS **

No	ABS	CONC

(18) When finished, parameters can be saved by pressing "RETURN, YES, 6, ENTER" The most commonly used parameters for this assay are saved as "LP 1-MLA *" This program can be directly accessed by typing at the main menu "FILE, 19, ENTER, RETURN" A new standard curve must be generated for each assay To do this type "YES, ENTER, NO" and complete steps (8) through (16) above

(19) Measure optical density of unknowns as before in step (17)

(20) Turn off power

NOTE At any time the information displayed on the monitor can be printed by pressing "COPY"

Calculation of Biotin Content

Calculate biotin content of sample analyzed by the following equation

$$\frac{\text{ng biotin}}{\text{ml assay solution}} \times \frac{\text{ml assay solution}}{\text{ml sample assayed}} \times \frac{\text{total volume of sample}}{\text{amount of tissue}}$$

$$= \text{ng biotin/amount of tissue}$$

EXAMPLE

An extract of 100 seeds is prepared, and the total volume of the sample is adjusted to 1 5 ml before assay Aliquots (0 2, 0 3, 0 4, and 0 5 ml) of this sample are each assayed in a final volume of 1 ml of assay solution The following data are obtained

** QUANTITATIVE ANALYSIS **

No	ABS	CONC
1	0 159	0 0198
2	0 200	0 0270
3	0 250	0 0416
4	0 300	0 0492

CALCULATION FOR UNKNOWN SAMPLE 1A

No 1 = Unknown 1A (0 2 ml)

ABS = Optical density of sample (0 159)

CONC = Biotin content of sample (0 0198 ng biotin/ml of assay solution)

$$\frac{0\ 0198 \text{ ng biotin}}{\text{ml assay solution}} \times \frac{1 \text{ ml assay solution}}{0\ 2 \text{ ml of sample}} \times \frac{1\ 5 \text{ ml total sample}}{100 \text{ seeds}}$$

$$= 0\ 00148 \text{ ng biotin/seed}$$

Repeat calculation for remaining samples and average values to determine mean biotin content of tissue

Cleaning of Glassware used for Biotin Assays

(1) Glassware that has been in contact with ≤ 100 ng/ml biotin should be rinsed thoroughly with 95% ethanol followed by reverse osmosis water

(2) Glassware that has been in contact with ≥ 100 ng/ml biotin should be rinsed thoroughly with 5 N NaOH solution followed by reverse osmosis water

(3) Wash all glassware used for biotin assays separately from any other glassware

(4) Wash in hot, soapy, tap water

(5) Rinse thoroughly with reverse osmosis water followed by deionized water

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis ANALYSIS OF A BIOTIN AUXOTROPH OF ARABIDOPSIS THALIANA

Major Field Botany

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