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ACETYL-COENZYME A CARBOXYLASE AND THE
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Sarah Catherine Hunter

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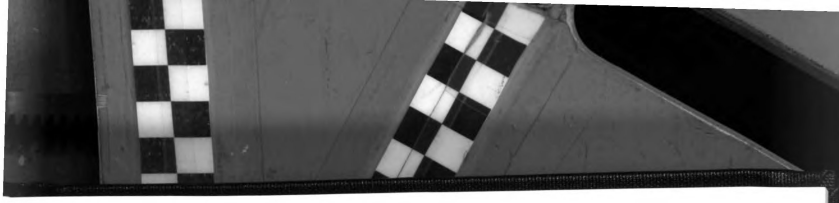

ACETYL-COENZYME A CARBOXYLASE AND THE REGULATION OF FATTY ACID SYNTHESIS

By

Sarah Catherine Hunter

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ABSTRACT

ACETYL-COENZYME A CARBOXYLASE AND THE REGULATION OF FATTY ACID SYNTHESIS

By

Sarah Catherine Hunter

While seeds often contain the bulk of a plant's store of fatty acids in the form of triacylglycerols, fatty acids are found throughout plants and fatty acid synthesis (FAS) is one of the primary metabolic pathways. Some plants make "unusual" fatty acids, stored almost exclusively in seeds, which have desirable industrial properties. Factors limiting the use of these oils for industry are 1) plants may not be suitable for crop use and 2) seed may have neither enough total oil, nor a high enough proportion of the unusual fatty acid in the oil, to be cost-effective for industrial use. Current efforts to manipulate both quality and quantity of oil have had mixed results. This is due, in part, to our incomplete knowledge about the components and regulation of FAS in plants. One goal of this thesis has been to extend our understanding of how plants regulate FAS. In plants *de novo* FAS occurs primarily in plastids and the rate of FAS is regulated in part by acetyl-CoA carboxylase (ACCase). The dicot ACCase investigated in this thesis contains four subunits, one of which, *AccD*, is encoded on the plastidial genome.

This work presents new data about the regulation of ACCase. In lysates of light-incubated chloroplasts, ACCase activity is at least two-fold higher than from

dark-incubated ones. Additionally, acetyl-CoA activates ACCase in a chloroplast lysate. This activation occurs at 10 μM acetyl-CoA, which is similar to the acetyl-CoA concentration found in chloroplasts. When ACCase is assayed *in vitro* under conditions which simulate the *in vivo* concentrations of metabolites, ACCase activity is five- to ten-fold lower than that required to sustain known *in vivo* rates of FAS. Thus, we still have an incomplete understanding of factors which regulate this enzyme.

This thesis also presents a model of the major initial reactions of FAS. This model was built using Stella II software, pool sizes of FAS metabolites, kinetic characterizations of FAS enzymes, and common sense. The simulation allows enzyme expression to be manipulated, either singly or jointly, between 0- and 25-fold. The model's predictions match several results reported from over- and underexpression experiments - even when those results have been non-intuitive. The model predicts that the set of FAS enzymes which limit or co-limit flux vary depending on illumination and products (18:1Δ9 or 16:0). The model also predicts a method involving concurrent overexpression of three enzymes by which total FA exported from plastids can be increased.

Finally, this work documents unsuccessful attempts to relocate *AccD* to the nuclear genome, reports the sequence of *Oenothera hookeri AccD*, and maps *AccD* on the plastidial genome of *O. hookeri*.

to the primary producers of the planet

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Many people have contributed to this research and my graduate education. John Ohlrogge, my advisor, accepted me into his lab, suggested a fascinating (albeit frustrating) research problem, and gave me guidance over the years. He has the ability to be patient at the appropriate time and push at the appropriate time. This work would not have been completed had he not had both those abilities. My committee members, Ken Keegstra, Ken Nadler, and Jon Walton also provided assistance, ideas, and feedback. The work on *Oenothera* was started in Barb Sears' lab when I needed a stable place to work my first summer. Without the support and co-operation of the above people, this work would not have been possible. The majority of my funding came from the Michigan State University Biotechnology Training program, however the Michigan Agriculture Experiment Station, the College of Natural Science, and the Graduate School also supported me at times throughout this process. The Cell and Molecular Biology Program, the Biotechnology Training Program, Paul Taylor funds from the Botany Department, the National Plant Lipid Cooperative, and the Conference Organizers at Iowa State all contributed travel funds for meetings. Additional research and travel support came from John and Mary Krimmel.

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LIST OF ABBREVIATIONS

ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
ACS	acetyl-CoA synthetase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BAP	benzyladenine
BC	biotin carboxylase
BCCP	biotin carboxyl carrier protein
chl	chlorophyll
CoA	Coenzyme A
CT	carboxyltransferase (transcarboxylase)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
FAS	fatty acid synthesis
Hepes	N-(Hydroxyethyl) piperazine-N'-2-ethanesulfonic acid)
KAS	3-ketoacyl-acyl carrier protein synthase
MCT	malonyl CoA:ACP transacylase
MCTE	medium chain thioesterase
MF	multifunctional
min	minutes
MS	multi-subunit
NAA	naphaleneacetic acid
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nd	not detected
ORF	open reading frame
PAGE	polyacrylimide gel electrophoresis
PFK	Phosphofructokinase
RuBisCO	ribulose-1,5-bisphophate carboxylase/oxygenase
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error of the mean
TAG	triacylglycerol
TLC	thin layer chromatography

COMMON NAMES OF FATTY ACIDS

laurate	12:0
myristic	14:0
palmitate	16:0
petroselinic	16:1Δ4
stearate	18:0
oleate	18:1Δ9, 18:1
ricinolenic	18:1Δ9, 12OH
cis-vaccenic	18:1Δ11
crepenynic	C18:1Δ9, 12-C≡C
linoleic	18:2
linolenic	18:3
erucic	22:1Δ13

Chapter 1

INTRODUCTION

Oilseeds produce around 80 million metric tons of oil per year and the total value of the vegetable oils produced is approximately 70 billion US dollars/year. About two-thirds of the oil produced is used for human consumption, while the rest is used by industry (Chrispeels and Sadava, 1994). While seeds often contain the bulk of a plant's store of fatty acids in the form of triacylglycerols (oils), fatty acids are found throughout plants: as major components of cell membranes, as signaling molecules, as membrane anchors for proteins, and as components of waxes in the cuticle. Thus, fatty acid synthesis is one of the primary metabolic pathways. Recent reviews of fatty acid synthesis can be found in Ohlrogge and Browse (1995), Harwood (1996), and Ohlrogge and Jaworski (1997).

Fatty acids consist of a hydrocarbon chain and a carboxyl group. Thus, they are extremely hydrophobic at one end and hydrophilic at the carboxy end. Free fatty acids are generally toxic to cells, and therefore are bound to other components: glycerol backbones in the case of triacylglycerols (TAG), or phospho-, sulfo-, or galacto-glycerols in the case of membranes. There are also acyl carrier proteins (ACP's) used during fatty acid synthesis. The common fatty acids in plants have 16 and 18 carbon acyl groups, with zero to three double bonds. These double bonds are almost always in the *cis* configuration. The double bonds are usually in the Δ9, Δ12, and Δ15 positions. While the common names are occasionally used,

more often fatty acids are designated by their chain length and the number of double bonds. Thus, 18:1Δ9, often just 18:1 (oleate), is a fatty acid with 18 carbons and one double bond. 16:0 (palmitate) has sixteen carbons and no double bonds. The other major fatty and "common" fatty acids found in all plants are 18:2 (linoleic) and 18:3 (linolenic).

Plants can also manufacture "unusual" fatty acids (reviewed in van de Loo et al., 1993). These fatty acids are generally found in seed oils and may be shorter or longer than the common fatty acids, may have double bonds in non-standard positions, or may have additional functional groups: hydroxy groups in the case of ricinoleic acids or acetylenic groups in the case of crepenynic acid. These unusual fatty acids often have desirable industrial properties. However, the use of fatty acids in industry is limited by practical considerations: the plant which makes a specific desirable fatty acid may not be adaptable to large-scale agriculture or may not make and store the unusual fatty acid in a high enough quantity to be cost-effectively purified.

Synthesis of Fatty Acids

In plants, almost all *de novo* fatty acid synthesis takes place in plastids. In contrast, in other eucaryotic systems FAS occurs in the cytosol. Furthermore, plants also have a different type of fatty acid synthetase than other eucaryotes. Animals and yeast have a type I fatty acid synthetase. In this case, the numerous

functions required to increase an acyl chain by two carbon units are encoded on the same gene and reside on the same protein for animals or on two genes/proteins for yeast. This is not true of the plant FAS system. The plant FAS system consists of a type II enzyme, which is very similar to bacterial fatty acid synthetase. Type II fatty acid synthetase consists of at least six separately encoded proteins that are easily separable but may be in a loose association. Thus, rather than being structurally related to other eucaryotic fatty acid synthetases, the plant fatty acid synthetase appears to be most closely related to bacterial fatty acid synthetase.

How is a fatty acid made in a plant? Figure 1.1 shows the major initial reactions of FAS. Malonyl-ACP is the building block of FAS. An initial condensation between malonyl-ACP and acetyl-CoA is catalyzed by 3-ketoacyl acyl carrier protein synthase III (KAS III). This yields a four carbon 3-ketoacyl-ACP, 3-ketobutyryl-ACP, which is then reduced, dehydrated, and reduced a second time to yield a saturated four carbon acyl chain attached to ACP via a thioester bond. Further elongation of the acyl chain occurs when a second, third, fourth, etc., malonyl-ACP is added to the reactive thioester end of the chain. Normally, this process continues until the acyl chain is 16 or 18 carbons long. Sixteen carbon acyl chains can be hydrolyzed via thioesters and exported from the plastid, or they can be retained in the plastid by the action of acyltransferases which incorporate them into membrane lipids. Almost all eighteen carbon fatty acids are desaturated at the Δ9 position before being exported or incorporated

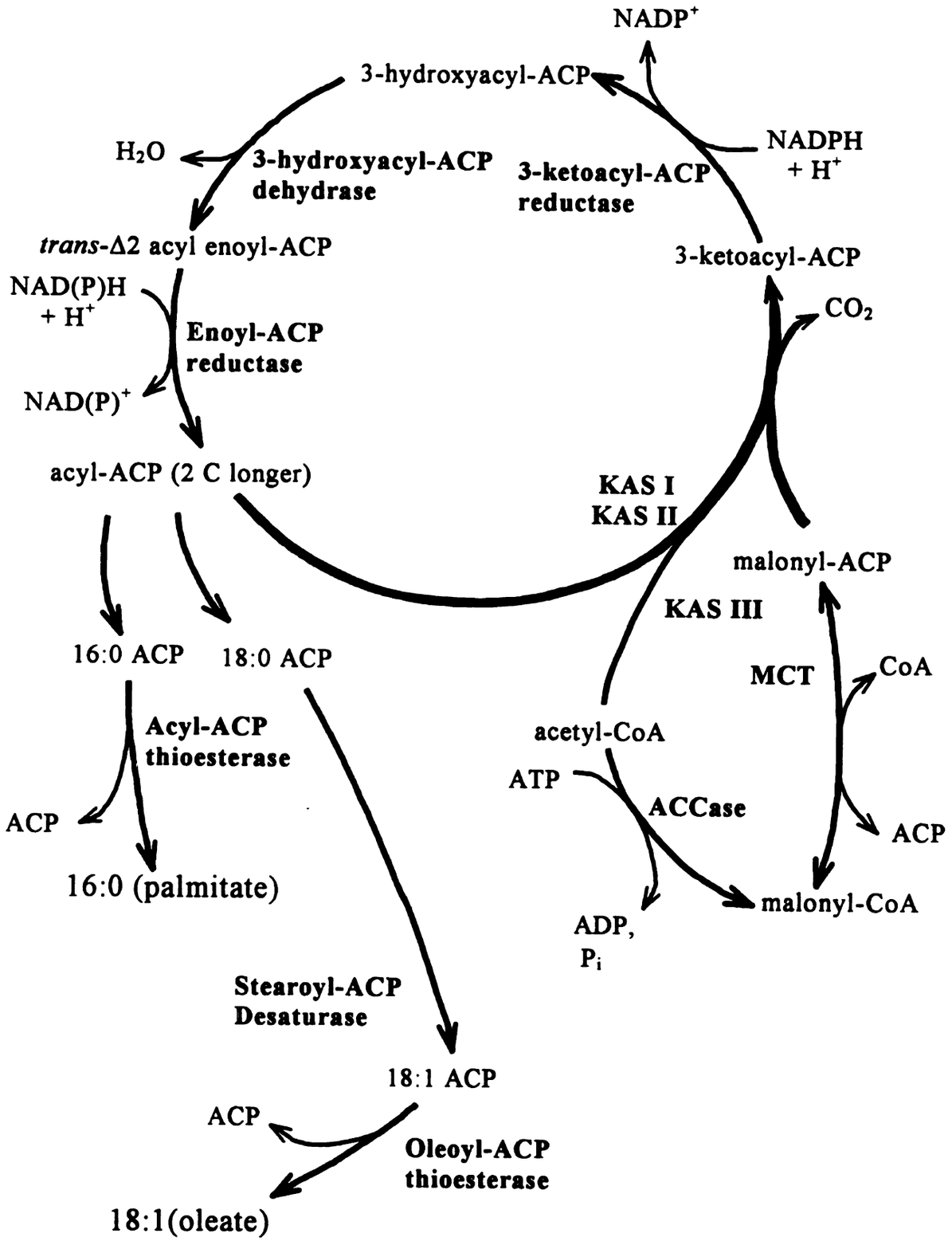

Figure 1.1. Major plastidial reactions of FAS.

Enzymatic steps are in boldface type. See text for further explanation. Figure after Ohlrogge and Browse (1995).

into membrane lipids in the plastid. Further desaturation occurs outside the plastid. Malonyl-ACP, the building block, is formed by the carboxylation of acetyl-CoA, an ubiquitous metabolite, and the subsequent transfer of the malonyl moiety from CoA to ACP. Acetyl-CoA carboxylase (ACCase, E.C. 6.4.1.2) catalyzes the first reaction. Malonyl-CoA:ACP transacylase catalyzes the second.

Regulation of FAS

Although FAS is a primary pathway, it is still unclear how a plant cell regulates the amount of fatty acids it produces (reviewed in Ohlrogge and Jaworski, 1997). There are numerous demands for fatty acids that vary according to cell type and developmental state. For example, plant cells undergoing rapid expansion require fatty acids for membrane synthesis, developing seeds require fatty acids in the form of TAG during oil deposition, and epidermal cells require fatty acids for wax and cuticular lipids. To make the regulation more complex, there is also a compartmentalization issue: most, if not all, of the *de novo* FAS occurs in plastids. Membrane phospholipids, triacylglycerols, waxes, etc. are formed in the cytosol. Therefore, there must be some mechanism of communication between the two compartments.

Light affects the rate of fatty acid synthesis both *in vitro* and *in vivo*. Under optimal conditions, which includes 200 μE * m^{-2} *sec^{-1}, *in vitro* rates of fatty acid synthesis are 1-2 nmoles 2 C units (acetate) * $hour^{-1}$ * mg chl^{-1} in intact

chloroplasts (Roughan, 1987). This value agrees with recent unpublished data on the *in vivo* rate of FAS (Bao et al., in preparation). Although isolated chloroplasts were unable to synthesize fatty acids without light, in leaf discs which were not exposed to light, the rate of FAS dropped six to ten-fold compared to leaf disks exposed to light (Browse et al., 1981). In the *in vivo* studies, essentially no FAS was seen without illumination (Bao et al., in preparation).

A number of studies have attempted to determine the mechanism of light activation of FAS. Light dependence has been attributed to changes in the stromal pH (Sauer and Heise, 1983). Many, but not all, of the FAS enzymes have pH optima between 8 and 8.5. Differences in ATP/ADP ratio, Mg^{2+}, and reductant concentrations have also been proposed as regulatory factors (Sauer and Heise, 1983). A number of these explanations have been questioned: ATP and reducing power are not sufficient to substitute for light activation (e. g. Stumpf et al., 1963; Nakamura and Yamada, 1975). In Chlamydomonas, electron flow through photosystem I is required for FAS, but the relationship is not linear (Picaud et al., 1991).

Proteins of fatty acid synthesis

The plant fatty acid synthesis system is made up of at least 11 enzymes as shown in Figure 1.1. The first committed step, carboxylation of acetyl-CoA, is performed by acetyl-CoA carboxylase (ACCase). The transfer of the malonyl

moiety to ACP is catalyzed by malonyl-CoA:ACP transacylase (MCT). There are three enzymes which perform the condensing reactions, 3-ketoacyl-acyl carrier protein synthase I, II, and III (KAS I, KAS II, and KAS III). The two reduction steps are performed by 3-ketoacyl-ACP reductase and enoyl-ACP reductase. The dehydration step is catalyzed by 3-hydroxyacyl-ACP dehydrase. There are two common thioesterases, oleoyl-ACP thioesterase and a less specific acyl-ACP thioesterase, and finally, a stearoyl-ACP desaturase. All of the above reactions, except ACCase, require ACP, a small (~9 kDa) protein, which is attached to the acyl chain during processing.

ACCase is a rate-determining enzyme of FAS

ACCase plays a major role in determining the rate of FAS. ACCase shares many *in vitro* properties with regulatory enzymes. Most importantly, however, *in vivo* data also suggest a regulatory role for ACCase. If flux through a pathway can be increased or decreased, concomitant changes in the pool sizes of intermediates in the pathway can indicate which enzyme(s) is (are) regulatory. When flux is decreased, metabolites prior to the regulatory step will increase, and those after the regulatory step will decrease. The converse is true if flux is increased: metabolite pools prior to a regulatory step will decrease and metabolite pools after the regulatory step will increase (Rolleston, 1972). Flux through FAS can be decreased by removing light and for isolated chloroplasts, it can be increased by Triton X-100. Through analysis of pool size data under states of high flux and low flux, Post-Beittenmiller et al. (1991, 1992) determined that ACCase is

regulatory for dicot systems. Analysis of control coefficients of barley and maize showed that ACCase is regulatory in gramineaceous systems (Page et al., 1994). Of course, other enzymes also play a role in rate-determination: KAS III, the enzyme that performs the initial condensation reaction, is likely also a player (Verwoert et al., 1995).

ACCase structure

ACCase is a biotin-containing enzyme with three functional subunits: a biotin carrier protein (BCCP), a biotin carboxylase (BC), and a carboxyltransferase (CT). Knowledge of the overall structure of ACCase has recently been revised (reviewed in Sasaki et al., 1995). It varies according to both cellular localization and plant species as shown in Figure 1.2. There is both a type I (also called homomeric or multifunctional) ACCase and a type II (also called heteromeric or multisubunit) ACCase. Dicots and most monocots have a type II ACCase in their plastids, but they also have a type I ACCase, presumably in the cytosol. Gramineaceous plants have only type I ACCases. However, there have been reports of homomeric ACCase in *Brassica napus* plastids (Schulte et al., 1997), so our picture of ACCase localization and structures may still be incomplete.

ACCase genes

The genes that encode three of the four subunits of ACCase are located on the nuclear genome. However, the gene that codes for the β-CT subunit, *AccD*, is retained in the plastidial genome in dicots and presumably most non-grass

Figure 1.2A. ACCase structure.

The functional units of ACCase can be on the same polypeptide or on different polypeptides. Multifunctional (MF) ACCase has the three functional subunits, a biotin carboxylase (BC), a biotin carrier protein (BCCP), and a carboxyltransferase (CT) on one peptide. Multisubunit (MS) ACCase has at least four separable subunits: BC, BCCP, α-CT and β-CT. Both α-CT and β-CT are necessary for the carboxyltransferase function. Figure after Ohlrogge and Browse (1995).

Figure 1.2B. ACCase localization.

Dicots and most monocots have both MS-ACCase and MF-ACCase. The MS-ACCase is located in the plastid and MF-ACCase is presumably in the cytosol. Grasses have only MF-ACCase. Figure after Ohlrogge and Browse (1995).

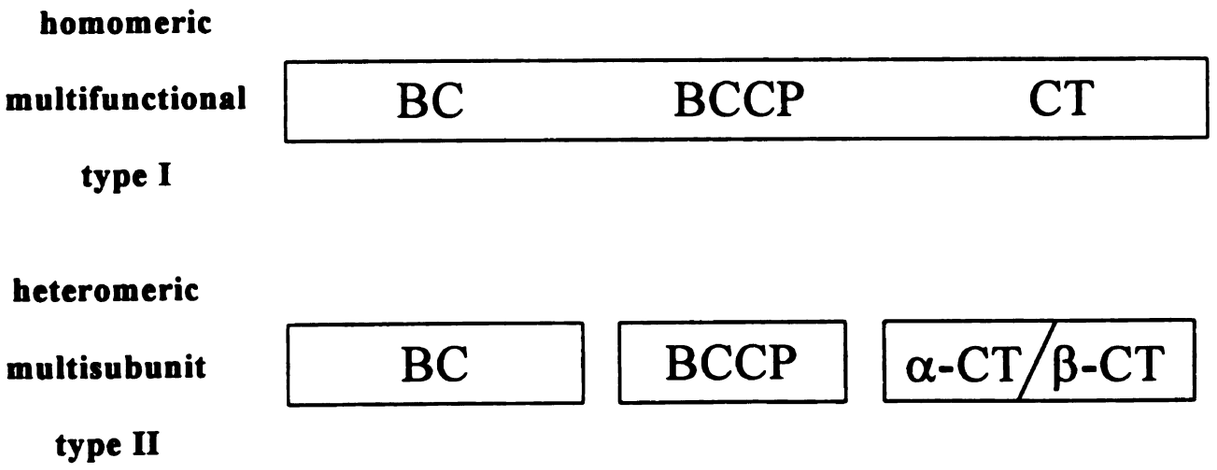

Figure 1.2A. ACCase structure

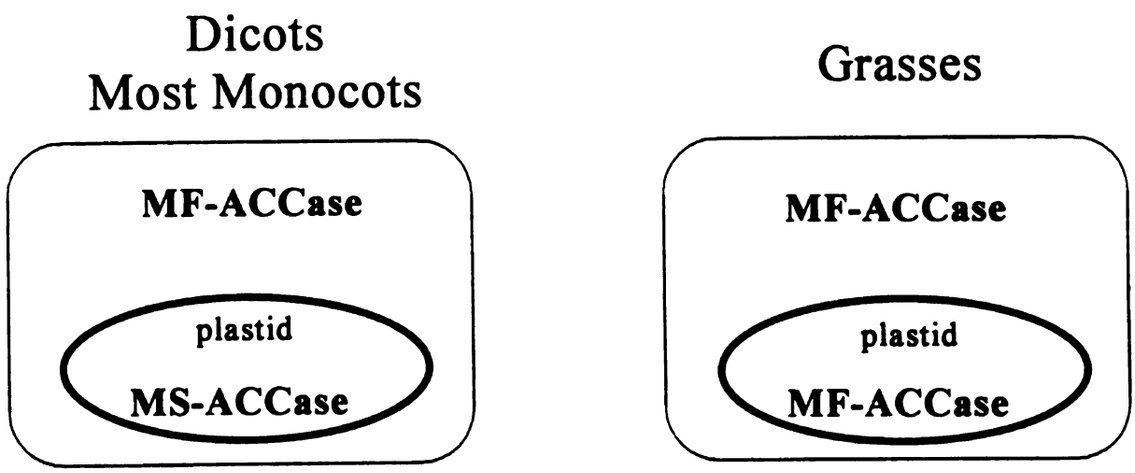

Figure 1.2B. ACCase localization.

monocots. In rice the plastidial genome has an ORF one-fifth the size of tobacco *AccD* with strong homology to the gene. However, it is clearly truncated and not a functional gene.

Oils Are an Important Commodity

Aside from curiosity, there are economic reasons to investigate the regulation of FAS in plants. Plants currently provide valuable seed oils, most of which are used for human consumption. While traditional breeding methods have been able to alter the amount of oil in seeds (e. g. high and low oil corn) and have been able to alter fatty acid composition (e. g. low- and high-erucic rapeseed lines), this process has been time-consuming. Plants make a wide variety of unusual fatty acids which could become important industrial feedstocks if the quantity and quality of the fatty acid in the seed oil of crop plants could be increased (reviewed in Ohlrogge, 1994; Töpfer et al., 1995). Thus, many efforts have focused on genetically engineering plants to alter and increase their oils (e. g., Voelker et al., 1992; Broun and Somerville, 1997). These efforts have met with limited success (e. g. Roesler et al., 1997; and reviewed in Ohlrogge and Jaworski, 1997), especially when the goal has been an increase in total oil content. What controls how much and what types of fatty acids a plant makes? It is vital to answer these questions if plant oils are to increase their agricultural value. Thus, understanding the regulation of *de novo* plant fatty acid synthesis is

useful. Furthermore, ACCase regulation, as a major determinant of the rate of FAS, needs to be understood.

The work described below adds to, confirms, and furthers the body of knowledge known about the regulation of ACCase and FAS. Chapter 2 presents data on the metabolic regulation of ACCase. A more precise, albeit time-consuming, ACCase assay was developed to investigate the differences between activity in light-incubated and dark-incubated lysed chloroplasts. Even when assayed in the same environment (e. g. pH, and ATP, acetyl-CoA, and Mg^{2+} concentrations), light-incubated chloroplasts had at least two-fold higher activity than dark-incubated ones. This difference decreased over time, until ACCase activity in dark-incubated lysed chloroplasts rose to the activity of light-incubated ones. Chapter 2 also presents the discovery of an additional activator for ACCase in chloroplast lysates: its substrate, acetyl-CoA. This activation occurs at low concentrations of acetyl-CoA that are likely to occur in chloroplasts. Chapter 2 also introduces a theme discussed in Chapter 3. Which of the observations made *in vitro* can be applied *in vivo*? Knowledge of the concentrations of chloroplastic metabolites are reasonably precise, yet ACCase activity in a simulated chloroplast environment is five- to ten-fold lower than that required to sustain known *in vivo* rates of FAS. Additionally, reductants have been shown to activate ACCase *in vitro*, but evidence in Chapter 2 questions whether or not the activation is physiologically relevant.

Chapter 3 takes a different approach. Although Chapter 2 clearly argues against indiscriminate application of *in vitro* knowledge to *in vivo* systems, the approach taken in Chapter 3 is a step further removed from *in vivo* systems than *in vitro* data. Chapter 3 presents a computer simulation of the initial reactions of FAS. This computer simulation was built using metabolite pool sizes in the light, characterizations of FAS enzymes, and common sense. As detailed above, FAS is a complex pathway and many of the details of its regulation may be non-intuitive. The computer simulation allows enzymes to be expressed, either singly or jointly, between 0 and 25-fold. The model's predictions match many results from transgenic over- and underexpression experiments, even when the results have been non-intuitive. KAS III overexpression is a case where the non-intuitive results from plants have been matched by the model. The model was then used to investigate limiting and co-limiting enzymes in the light and dark for production of both 18:1 and 16:0. The model predicts that different enzymes take over the major regulatory role(s) depending on both products and environment. Although there is precedent for this in carbohydrate metabolism, this is not something that has been seriously considered for FAS in the past. The model was also used to predict a method by which total FA exported from the plastids could be increased.

Chapter 4 illustrates that testing the ideas put forth in Chapter 3 will not be straightforward. Two attempts to relocate the plastidial gene of ACCase, *AccD*,

to the nucleus are documented. Initially, the goal was to alter *AccD* expression to test coordinate regulation of subunits and, hopefully, alter the rate of FAS. Both these attempts failed. Chapter 4 details the attempts, and provides ideas as to why the relocation failed and how it might be made to succeed. In addition, Chapter 4 presents the sequence of *Oenothera hookeri AccD*, and maps its location on the plastidial genome.

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Chapter 2

REGULATION OF SPINACH CHLOROPLAST ACETYL-COA CARBOXYLASE[1]

Abstract

We have investigated several factors which influence acetyl-CoA carboxylase (ACCase) activity in lysed spinach chloroplasts. 1) When assayed after rapid lysis of light-incubated chloroplasts, ACCase activity was two-fold higher than activity from dark-incubated chloroplasts. Within five minutes after lysis, activity from dark incubated chloroplasts increased, suggesting a transient inactivation or inhibition of ACCase in the dark. 2) When lysed chloroplast suspensions were incubated with 30 to 100 μM acetyl-CoA *before* starting assays, activity was four-fold higher than if suspensions were not pre-incubated with acetyl-CoA. CoA, malonyl-CoA, propionyl-CoA and butyryl-CoA also activated ACCase. Full acetyl-CoA activation required MgATP and was essentially complete after eight minutes. ACCase activity decreased upon removal of acetyl-CoA by gel-filtration and was partially restored by readdition of acetyl-CoA. Thus, ACCase activation by acetyl-CoA was reversible. 3) Dithiothreitol and thioredoxin stimulated ACCase activity, but only in

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preparations where ACCase activity was low. 4) ACCase was assayed in concentrations of ATP, ADP, NADPH, $NADP^+$, Mg^{2+}, and CO_2/HCO_3^- which are estimated to occur in the stroma of chloroplasts under illumination or darkness. ACCase activity from lysed chloroplast suspensions was ten-fold higher when illuminated conditions were used. However, this activity was still five-fold to ten-fold lower than the rates required to sustain known *in vivo* rates of fatty acid synthesis and *in vitro* rates achieved under optimum assay conditions with saturating substrates.

Introduction

In spite of the central role of fatty acid synthesis (FAS) in plants and numerous studies of FAS regulation, it is still unclear how a plant cell regulates the amount of fatty acids it produces (reviewed in Ohlrogge and Jaworski, 1997). FAS is six- to ten-fold higher in illuminated leaves than non-illuminated ones (Browse et al., 1981), and a number of studies have attempted to determine the factors involved in this light control. Some of the light-dependence of FAS can be attributed to changes in the stromal pH, Mg^{2+} concentration, ATP/ADP ratio, and reductants (Sauer and Heise, 1983). However, not all of the changes in FAS can be accounted for by these factors. Early studies showed ATP and reducing power are not able to substitute for light and thus imply the role of light in fatty acid synthesis is more than simply to provide those substrates (Sauer and Heise, 1983; Stumpf et al., 1963; Nakamura and Yamada, 1975). Studies in *Chlamydomonas* mutants lacking photosystem I show light activation of fatty acid synthesis is photosystem I dependent, but the relationship between photosystem I electron flow and FAS light activation is not linear (Picaud et al., 1991). Thus, light does more than provide metabolites for FAS. Furthermore, while it is clear light does play a regulatory role, the mechanisms by which this happens are not clear.

The carboxylation of acetyl-CoA to produce malonyl-CoA by acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) is the first committed step of FAS and ACCase is the enzyme considered most responsible for the primary regulation of

FAS. Many of the properties of ACCase are consistent with it being a control enzyme. ACCase is the first committed step of the pathway. Metabolite pool size analyses under states of high flux (light-incubated leaves or chloroplasts) and low-flux (dark-incubated leaves or chloroplasts) are consistent with a regulatory role (Post-Beittenmiller et al., 1991 and 1992b). Flux control coefficients for ACCase from barley and maize leaves have been estimated to be 0.5 to 0.6 which also indicate that ACCase plays a major role in FAS regulation (Page et al., 1994). Furthermore, in animals and other non-plant systems, ACCase has been determined as a primary regulatory step (Salati and Goodridge, 1996).

Understanding of plant ACCase structure has been substantially redefined in the last five years. In most plants, such as spinach or pea, plastidial ACCase is an easily dissociated multisubunit enzyme (Kannangara and Stumpf, 1972; Sasaki et al., 1995; Alban et al., 1994). However, grasses have a large homodimeric multifunctional plastidial enzyme (Sasaki et al., 1995) and there is evidence that a multifunctional ACCase exists in *Brassica napus* chloroplasts (Schulte et al., 1997). In most cases, the multisubunit and multifunctional enzymes appear to be regulated in a similar manner. Both have pH optima near 8.0 to 8.5 (Alban et al., 1994; Nikolau and Hawke, 1984; Bettey et al., 1992; Sauer and Heise, 1984; Sasaki et al., 1997), both are competitively inhibited by ADP and to a lesser extent AMP (Nikolau and Hawke, 1984; Bettey et al., 1992; Sauer and Heise, 1984; Eastwell and Stumpf, 1983). Citrate and long chain acyl-CoA are considered key regulators of animal ACCase (Salati and Goodridge 1996; Allred

and Reilly, 1997). Long chain acyl-CoA inhibits plant ACCase (Shintani and Ohlrogge, 1995), but reports on citrate regulation of plant ACCase have been inconsistent (Alban et al., 1994; Nikolau and Hawke, 1984; Mohan and Kekwick, 1980).

The biochemical environment of the chloroplast stroma undergoes numerous changes upon illumination. Stromal H^+ concentration decreases ten-fold, ATP concentration increases almost three-fold, the ATP/ADP ratio goes up four-fold, the NADPH concentration doubles, and Mg^{2+} concentration increases from 1 mM to 3 mM (Heldt 1979; Heineke et al., 1991). It has been suggested that these differences can account for the stimulation of ACCase in the light, and therefore FAS (Nikolau and Hawke, 1984). However, other studies suggest that changing metabolite concentrations may not be the sole method for light modulation of ACCase activity (Sauer and Heise, 1984; Sasaki et al., 1997). In 1984 Sauer and Heise reported results on the activity of ACCase before, during and after intact spinach chloroplasts were illuminated. They observed that even when assayed at the same pH, Mg^{2+} and ATP concentrations, lysates of light-incubated chloroplasts had approximately three-fold higher ACCase activity than lysates of dark-incubated chloroplasts. More recently, Sasaki et al. reported that reduced thioredoxin activates ACCase and is the link between light activation and FAS (1997).

While much research has focused on the role of light and its direct or indirect effects, it would be surprising if there were not aspects of plant ACCase regulation independent of light. Fatty acids are a required precursor of membrane phospholipids, signaling pathways, carbon and energy storage. These precursors are necessary even in the absence of light. Additionally, in bacterial systems, fatty acid synthesis is tightly coupled to phospholipid synthesis (Cronan and Rock, 1996). Thus, non-light factors such as feedback regulation and source or sink control may also be involved in FAS regulation (Ohlrogge and Jaworski, 1997).

Although over the past several years increasingly strong evidence has accumulated that ACCase plays a major role in the rate determination of leaf FAS, the factors which determine ACCase activity are still unclear. There is now a more complete model of ACCase structure and more detailed knowledge of pool sizes for FAS metabolites, more detailed knowledge of the metabolite concentrations of the chloroplast stroma and more detailed knowledge of the changes which occur upon illumination. Because additional information on ACCase regulation should provide better understanding of how plant cells regulate their fatty acid production, the objectives of this study were to re-evaluate, verify and further study factors affecting spinach chloroplast ACCase activity.

Materials and Methods

Intact chloroplasts were isolated using a Percoll cushion according to the method of Roughan (1987) and suspended in incubation buffer (330 mM sorbitol, 25 mM Hepes/KOH pH 8.0, 2.0 mM Na_3EDTA, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 0.5 mM K_2HPO_4). Percoll gradient-purified chloroplasts for control experiments were isolated according to Perry et al. (1991) using homogenization buffer from Roughan (1987) instead of grinding buffer and incubation buffer for washes and suspension instead of import buffer.

All suspensions were adjusted to 0.4 mg chlorophyll/ml before use. Chlorophyll (chl) was assayed in methanol (Wintermans and De Mots, 1965). "Light" refers to an incubation with at least 150 $\mu E * m^{-2} * s^{-1}$ for 5 to 10 minutes. "Dark" incubations were done in foil-covered tubes. "Non-illuminated" chloroplasts were kept in room light, which does not stimulate FAS. All enzyme assays were done in room light.

ACCase activity assay A

ACCase activity was measured as the acetyl-CoA dependent fixation of $H^{14}CO_3^-$ into acid-stable, heat-stable products (Sauer and Heise, 1984). The final concentration of assay components was 100 mM Tricine/KOH (pH 8.5), 0.5 mM acetyl-CoA, 1 mM ATP, 2 mM $MgCl_2$, 30 mM KCl, 10 mM $NaHCO_3$ and 0.2% Triton X-100. Chl content, specific activity of $H^{14}CO_3^-$ and assay volume varied

and are described in figure legends. Assays were started by adding chloroplast suspension to assay components and stopped by bringing the reaction to 1N HCl. Samples were dried at 65°C, and the acid-stable radioactivity was counted by liquid scintillation. Control samples without acetyl-CoA were included in each series of assays and enzyme activity was calculated after subtracting these control results.

The Triton X-100 and low osmoticum in the assay buffer caused immediate lysis of chloroplasts after their addition to the assay mix. In some experiments chloroplasts were lysed separately from ACCase assay by dilution into lysis buffer (125 mM Tricine/KOH, pH 8.5, 0.33% Triton X-100). Usually additions to the lysis buffer were made. These additions are indicated in figure legends. Assays were then started by the addition of lysed chloroplast suspension to assay mix which contained the remaining components to bring the final assay to the standard assay conditions detailed above.

ACCase activity assay B

In a different set of experiments the products of $H^{14}CO_3^-$ fixation were separated via TLC. Final assay concentrations were the same as in Assay A. Reactions were started by the addition of chloroplast suspension and stopped by the addition of 1/6 volume of 15% trichloroacetic acid. Thioesters were hydrolyzed with the addition of 1/10 volume of 2N NaOH. Samples were spotted on a TLC plate (Whatman, K6 or K6F Silica Gel, 60 Å), then developed in 95% ethanol:

25% ammonium hydroxide: water (78: 12.5: 9.5, v/v/v) for 2 to 2.5 hours (Copius-Peereboom, 1969) in a saturated chamber. TLC plates were imaged and radioactivity determined by a Instant Imager (Packard Instrument Company, Meriden, CT). Relative ACCase activity was determined using the net counts of bands which co-migrated with a malonate standard.

Removal of acetyl-CoA from activated chloroplast lysates

Micro Bio-Spin® P-6 (Bio-Rad) columns were equilibrated with column buffer (125 mM Tricine/KOH, pH 8.5, 47 mM KCl, 1.6 mM ATP, 3 mM $MgCl_2$, 0.33% Triton X-100). A maximum of 75 µl of activated lysate was added to the column, columns were spun at approximately 1500 x *g*, eluant recovered and, if necessary, pooled.

In a control experiment, 0.5 mM radiolabeled acetyl-CoA (1 mCi/mmol) was combined with chloroplast lysate, added to the column, and the column spun as above. The column was washed thrice with 250 µl column buffer. Eluants and washes were counted for radioactivity via scintillation counting. Less than five percent of the radiolabeled acetyl-CoA was recovered in the eluant from the first spin. Thus, this method was able to remove almost all acetyl-CoA from chloroplast lysates to perform deactivation experiments.

ACCase assays in different metabolic environments

Non-illuminated chloroplasts were assayed using assay A for 10 minutes using the assay conditions detailed above, or substrate concentrations and pH were adjusted to mimic the light or dark stromal environment (Winter et al., 1994). ADP, NADPH, and $NADP^+$ were also added. Final concentrations of altered assay components are given in Table 2.1.

Results

We chose to investigate regulation of ACCase in chloroplast lysates rather than a partially purified ACCase preparation for several reasons. First, we and others (Shorrosh et al, 1996; Sasaki et al. 1993) have observed membrane association of ACCase which may influence regulation of the enzyme and would be lost during enzyme preparation. Second, some changes in ACCase activity occur rapidly (e.g. Sauer and Heise, 1984) and the time required to prepare partially purified ACCase preparations may obscure these changes. Third, if unknown regulatory factors or other enzymes are required for interactions with ACCase, partial purification may eliminate them. Thus, a crude chloroplast extract may more accurately mimic an *in vivo* system than a partially purified system, particularly for studying rapid changes in ACCase regulation.

Table 2.1. Composition of light and dark stromal environments and resultant ACCase activity.

	light-like environment	dark-like environment	light pH / other dark conditions	dark pH / other light conditions
pH	8.0	7.0	8.0	7.0
ATP	0.68 mM	0.24 mM	0.24 mM	0.68 mM
ADP	0.29 mM	0.41 mM	0.41 mM	0.29 mM
$NaHCO_3$ (CO_2)	0.74 mM (10 μM)	0.67 mM (84 μM)	0.74 mM (10 μM)	0.67 mM (84 μM)
NADPH	0.11 mM	0.05 mM	0.05 mM	0.11 mM
$NADP^+$	0.2 mM	0.2 mM	0.2 mM	0.2 mM
$MgCl_2$	3 mM	1 mM	1 mM	3 mM
activity	0.20 ± 4%	0.02 ± 16%	0.20 ± 12%	0.08 ± 7%

Twenty μl of non-illuminated chloroplast suspension was assayed with the concentrations of H^+, ATP, HCO_3^-, and Mg^{2+} altered from the standard assay. ADP, NADPH and $NADP^+$ were also added. Final assay volume was 100 μl, specific activity of $H^{14}CO_3^-$ was 20 mCi/mmol. The concentration of CO_2 and HCO_3^- was calculated based on pH and a total of 0.75 mM $H^{14}CO_3^-$ in the assay mix; the actual substrate for ACCase is HCO_3^- (Cronan and Rock, 1996). Activity was determined by the average of eight separate assays using two different chloroplast suspensions. ACCase activity in standard assay conditions was 0.8 ± 10% nmole CO_2 fixed * $hour^{-1}$ * μg chl^{-1}.

We used cushion-purified chloroplasts in order to minimize extraction time and chloroplast handling. Although unlikely, multifunctional ACCase could be present as a cytosolic contaminant or a plastidial isoform (Schulte et al., 1997). Multifunctional ACCase has the ability to carboxylate propionyl-CoA to create methyl malonyl-CoA, whereas the multisubunit ACCase does not (Dehaye et al., 1994). To check the purity of cushion-purified chloroplasts and confirm that any multifunctional ACCase present did not contribute to our results, we used ACCase assay B, substituting propionyl-CoA for acetyl-CoA to check for propionyl-CoA carboxylase activity. In this experiment, all products of the assay remained at the origin of the TLC plate. This strongly suggests that all ACCase activity described in the experiments below is due to the activity of the multisubunit ACCase.

Analysis of products of ACCase assay by TLC

The assay system most frequently used for ACCase measures the acetyl-CoA dependent fixation of $^{14}CO_2$ into acid stable materials (e.g. Alban et al., 1994; Sauer and Heise, 1984; Sasaki et al., 1997). For assays with crude chloroplast extracts lasting several minutes or with purified enzyme, fixation of $H^{14}CO_3^-$ and $^{14}CO_2$ by controls in the absence of acetyl-CoA was minor (less than 10-30%) compared to the fixation in the presence of acetyl-CoA. However, in order to study ACCase light activation, previous studies (Sauer and Heise, 1984) and those described here require assays of less than one minute duration. Under these short assay times, fixation of $H^{14}CO_3^-$ into products other than malonyl-CoA was

substantial, typically 50 to 90% of the total counts fixed. This background presumably reflects fixation of $^{14}CO_2$ by ribulose 1,5-bisphosphate carboxylase using endogenous pools of substrates.

To establish that the standard bicarbonate fixation assay provides a true measure of ACCase activity, we analyzed the acid stable products by silica gel TLC. After stopping the reaction, acyl-CoA's were hydrolyzed with NaOH, yielding radiolabeled malonate from the malonyl-CoA produced by ACCase. When the assay products were separated by silica gel TLC, three acid stable radiolabeled products were seen (Figure 2.1). A band with R_f = 0.34 co-migrated with a malonate standard. Its formation was acetyl-CoA, ATP, and time dependent. Occasionally, a minor upper band was seen which co-migrated with acetate. The remaining radioactive products remained at the origin. Their formation was acetyl-CoA independent but ATP dependent.

The TLC assay separated the malonate produced by the ACCase assay from other acid-stable $H^{14}CO_3$ fixation products and thereby ensured that products which did not co-migrate with the malonate band were not included in the assay results. Assay A, on the other hand, uses controls without acetyl-CoA to differentiate the products fixed by ACCase from those fixed by other enzymes and for short assay times background can be substantial as discussed above. Our results using assay B which are described below confirmed assay A results from (Sauer and Heise, 1984). Thus the results provided by the standard assay system, assay A, were

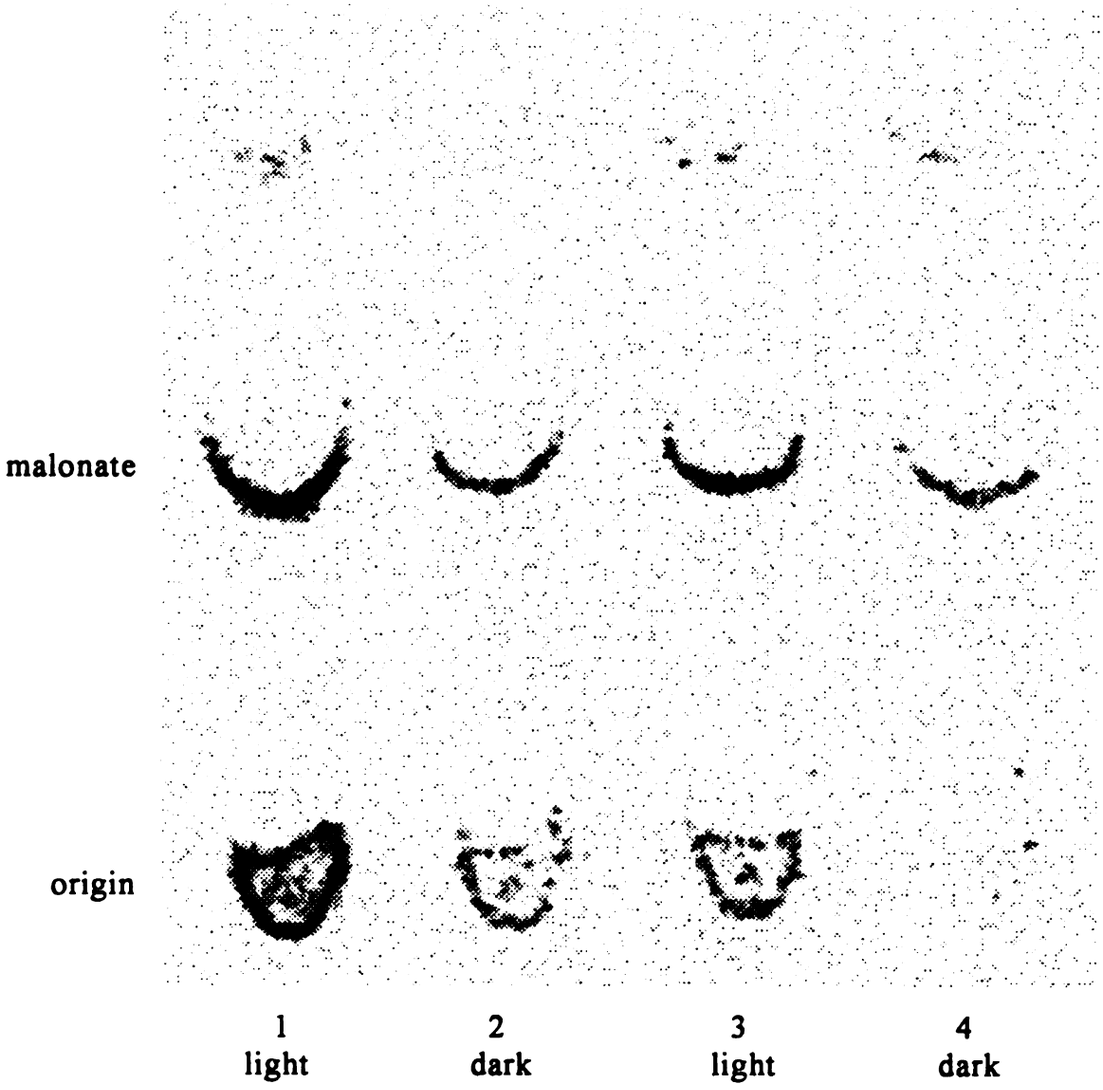

Figure 2.1. Representative autoradiogram of acid-stable products separated on silica gel.

After hydrolysis of the CoA's from the products by altering the pH to 12, samples were spotted and run in an ethanol/ammonium hydroxide solvent system. Two replications are shown on this plate, light-incubated (lanes 1, 3) or dark-incubated (lanes 2, 4) lysed chloroplasts were assayed immediately after lysis. Assay B was used. Final assay volume was 60 µl with 3.2 µg chl, specific activity of $H^{14}CO_3^-$ was 20 mCi/mmol.

reliable; the vast majority of product fixed in the presence of acetyl-CoA was malonyl-CoA and the high background could be adequately assessed by including samples without acetyl-CoA. Because analysis of the products by TLC and imaging the plates was slow, we preferentially used assay A for most experiments.

ACCase activity is two-fold higher in lysates of a light-incubated chloroplast suspension than lysates of a dark-incubated chloroplast suspension

ACCase activity was assayed by rapidly diluting an intact chloroplast suspension into Triton X-100 lysis/assay buffer. The TLC assay B was used in these experiments to distinguish products formed by ACCase from the high background ^{14}C fixation. When assayed for 30 seconds immediately after lysis, the ACCase activity in lysates was two-fold higher from light-incubated than dark-incubated chloroplasts. The average light/dark ratio was 1.9 (SE of the ratios = 0.1, SD of the ratios = 1.1, n = 32). Light- or dark-incubated chloroplasts which had been lysed for five minutes before measuring activity had no significant difference in activity (average light/dark ratio = 0.9, SE = 0.1, SD = 0.2, n = 8). Relative ACCase activity in lysed, dark-incubated chloroplasts increased over the five minute lysis period, eliminating the light/dark difference seen in lysed and immediately assayed chloroplasts (data not shown). Therefore, the influence on ACCase activity appears to be transient and easily reversible. These results confirm and extend those of Sauer and Heise (Sauer and Heise,

1984), where a three-fold difference between light and dark activity of ACCase was reported.

ACCase activity increases after pre-incubation of chloroplast lysates with CoA, acetyl-CoA and short-chain acyl-CoA's

In one minute assays of rapidly lysed chloroplasts ACCase activity was 0.4 nmole CO_2 fixed * $hour^{-1}$ *μg chl^{-1} (n=31). However, when assayed for 10 minutes under the same conditions, ACCase activity was 1.5 nmole CO_2 fixed * $hour^{-1}$ *μg chl^{-1} (n=22), or approximately four-fold higher. This result suggested a time dependent activation of ACCase may occur in the presence of assay components. Indeed, as shown in Figure 2.2A, ACCase activity of spinach chloroplast lysates increased over time, perhaps in response to components in the assay. In order to examine this activation, chloroplasts were lysed and pre-incubated in the presence of selected substrates. Figure 2.2B graphs the effect of adding acetyl-CoA, MgATP, or HCO_3^- to the lysis buffer before assaying ACCase activity for one minute using assay A. When chloroplasts were lysed in the presence of 30 to 833 μM acetyl-CoA *before* starting the ACCase assay, activity was increased by four-fold within eight minutes. Maximal acetyl-CoA activation required MgATP. Other substrates of the reaction, MgATP and HCO_3^-, did not significantly activate spinach ACCase upon pre-incubation with the enzyme. Acetyl-CoA activation occurred in suspensions regardless if they were kept in room light, foil-wrapped, or illuminated. There is considerable variability in these assays. Hence, we believe that higher concentrations of acetyl-CoA

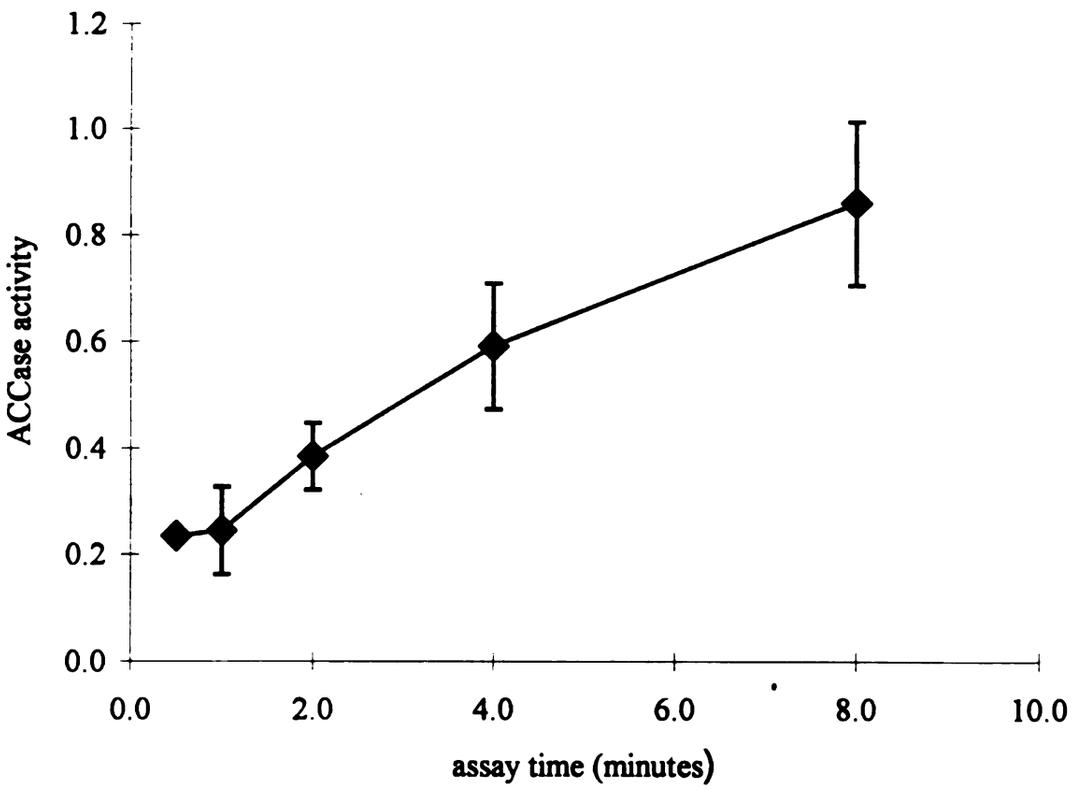

Figure 2.2A. Specific activity of ACCase vs. total assay time.

ACCase was assayed for varying times by adding 275 µl non-illuminated chloroplast suspension to 1100 µl assay buffer, then stopping 250 µl aliquots after 0.5, 1, 2, 4, and 8 minutes with 1 vol 2N HCl. Data are the average of three separate assays using the same chloroplast suspension, activity is given in nmole CO_2 fixed * $hour^{-1}$ * µg chl^{-1}. Specific activity of $H^{14}CO_3^-$ was 0.1 mCi/mmol.

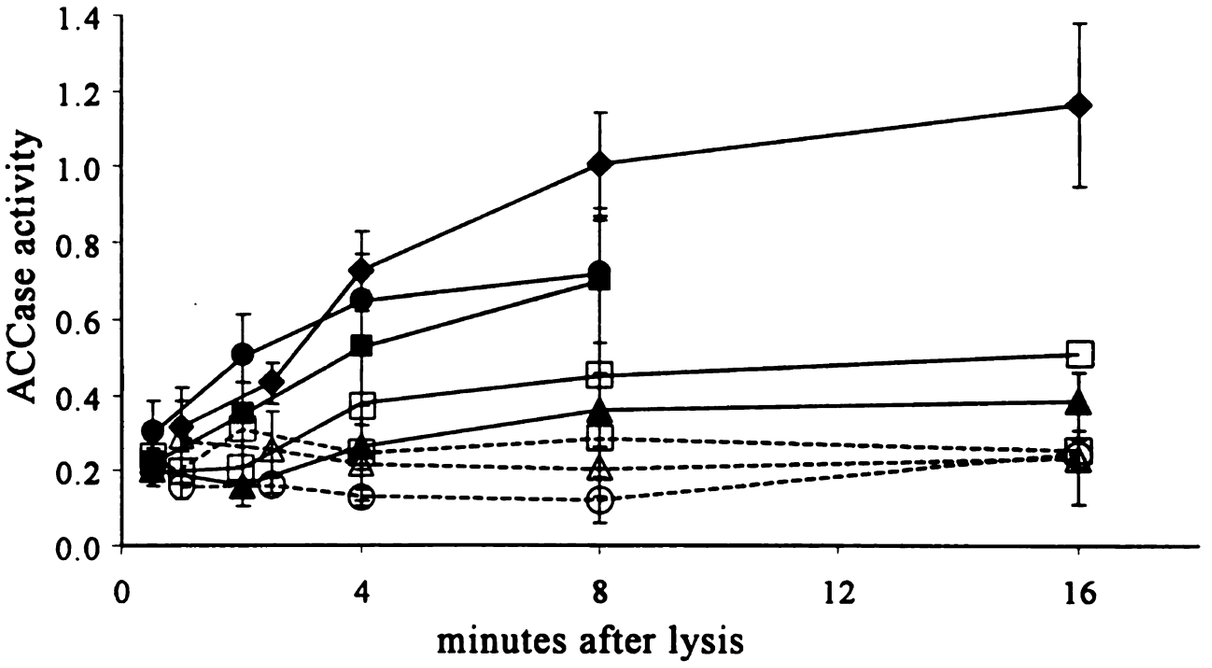

Figure 2.2B. ACCase assay of chloroplast suspension lysed for various times.

Chloroplast suspension was lysed by five-fold dilution, then lysates were incubated for various times before ACCase assay. After the specified time, a 112.5 µl aliquot of lysed suspension containing 9 µg chl was added to assay mix. All assays were one minute. Lysis buffer (-⊖-) had additions of 3.33 mM $MgCl_2$, 1.67 mM ATP, and 50 mM KCl (-⊟-); 10 mM $NaHCO_3$ and 50 mM KCl (-△-); 100 µM acetyl-CoA and 50 mM KCl (⊟); 833 µM acetyl-CoA, 3.33 mM $MgCl_2$, 1.67 mM ATP and 50 mM KCl (◆); 100 µM acetyl-CoA, 3.33 mM $MgCl_2$, 1.67 mM ATP and 50 mM KCl (■); 30 µM acetyl-CoA, 3.33 mM $MgCl_2$, 1.67 mM ATP and 50 mM KCl (●); 10 µM acetyl-CoA, 3.33 mM $MgCl_2$, 1.67 mM ATP and 50 mM KCl (▲). Data are averages of at least 3 replications. Final volume was 150 µl, specific activity of $H^{14}CO_3^-$ was 10-15 mCi/mmol. Activity is given in nmoles CO_2 fixed * $hour^{-1}$ * µg chl^{-1}.

(e. g., 833 μM) most likely do not give significantly higher levels of activation. To determine if acetyl-CoA activation was reversible, acetyl-CoA was removed from the activated lysate by spinning through a gel filtration column. At least 95% of the acetyl-CoA was removed by this procedure. Immediately after the spin-column, ACCase activity was similar, or perhaps slightly higher, than the ACCase activity just prior to the spin column. However, 16 minutes after removal of acetyl-CoA, activity was reduced to about half of the maximal activation. Readdition of 200 to 400 μM acetyl-CoA to the same lysate caused ACCase activity to increase to about two-thirds of the maximal activation (Figure 2.3). Thus, the influence of acetyl-CoA is at least partially reversible.

The activation described above was not specific to acetyl-CoA; other short chain acyl-CoA's and CoA also activated ACCase. Table 2.2 shows that over a period of 8 minutes, CoA, malonyl-CoA, propionyl-CoA and butyryl-CoA also activated ACCase three- to five-fold above *initial* activity. These activation experiments were done in a lysed chloroplast system, thus stromal enzymes were present. Since acetyl-CoA synthetase (ACS) is highly active in chloroplast lysates (Roughan and Ohlrogge, 1994; Golz and Lichtenthaler, 1992), CoA added to the lysate could be converted to acetyl-CoA by ACS. To test if the activation of ACCase in presence of CoA was occurring due to CoA conversion to acetyl-CoA, ethyl adenylate, an ACS inhibitor (Golz and Lichtenthaler, 1992), was added to the lysis buffer. Table 2.2 shows that both acetyl-CoA and CoA

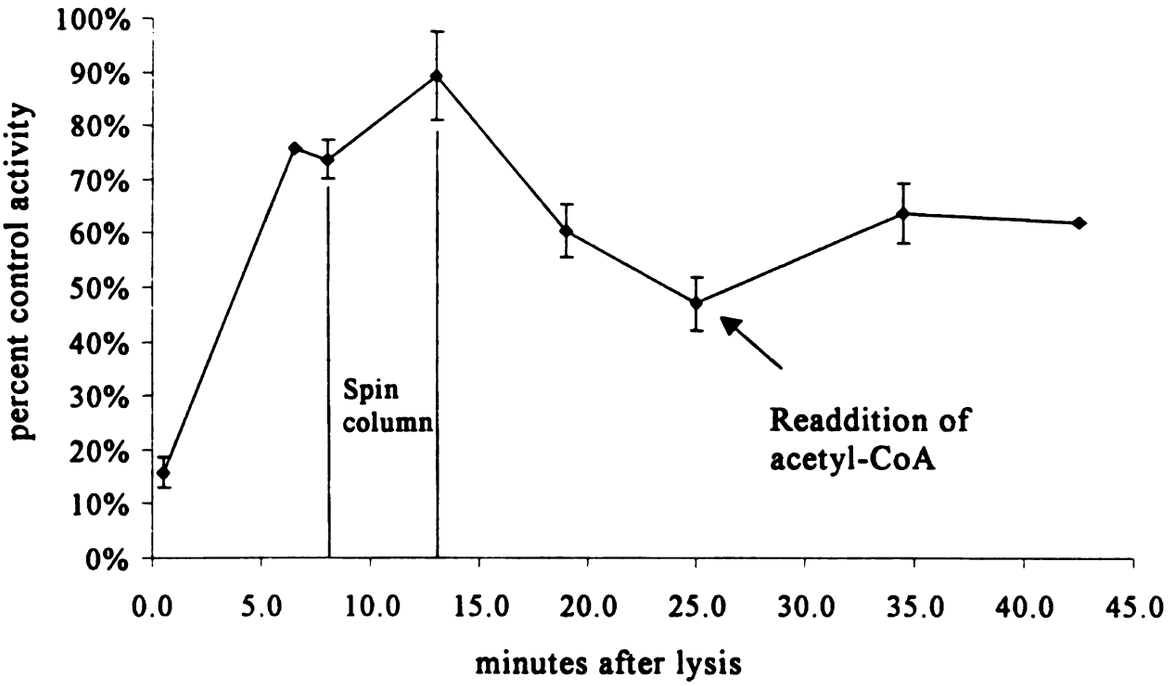

Figure 2.3. Activity of ACCase after removal of acetyl-CoA.

Non-illuminated chloroplast suspension was lysed by five-fold dilution. Lysis buffer additions were 100 μM acetyl-CoA, 50 mM KCl, 3.33 mM $MgCl_2$, and 1.67 mM ATP. 40 μl aliquots of lysate were assayed for 1 minute at the times indicated. After 8 minutes acetyl-CoA was removed via a spin column prepared as described in Materials and Methods. After spinning, aliquots of elutant were assayed for 1 minute at the times indicated. After 25 minutes, acetyl-CoA was added to the elutant and aliquots were assayed. ACCase activity was normalized using the activity of a 10 minute assay done concurrently as 100% activity. N = 3 for times 0.5, 8, 13, 19, 25, and 34.5 minutes. N=1 for times 6.5 and 42.5 minutes. Assay A was used. Final assay volume was 50 μl (with 3.2 μg chl), specific activity of $H^{14}CO_3^-$ was 10 mCi/mmol.

Table 2.2. Effect of ethyl adenylate, CoA, malonly-CoA, and short-chain acyl-CoA's on ACCase activation and activity.

Additions to Lysis Buffer	One minute assay			Standard assay
	0.5 minute lysis	8 minute lysis	fold activation	
100 μM acetyl-CoA	0.10 ± 0.02	0.42 ± 0.11	4	1.26 ± 0.04
100 μM acetyl-CoA, 50 μM ethyl adenylate	0.17 ± 0.15	0.33 ± 0.22	2	1.17 ± 0.05
100 μM CoA	0.07 ± 0.02	0.38 ± 0.11	5	not determined
100 μM CoA, 50 μM ethyl adenylate	0.06 ± 0.02	0.26 ± 0.15	4	1.14 ± 0.04
100 μM malonyl-CoA	0.05 ± 0.04	0.15 ± 0.03	3	0.61 ± 0.07
100 μM propionyl-CoA	0.06 ± 0.05	0.22 ± 0.03	4	0.96 ± 0.05
100 μM butyryl-CoA	0.09 ± 0.03	0.36 ± 0.02	4	1.15 ± 0.07

One minute assays: Non-illuminated chloroplast suspension was lysed by five-fold dilution and incubated for 30 seconds or 8 minutes before assay. Lysis buffer contained 1.67 mM ATP, 3.33 mM $MgCl_2$, and 50 mM KCl as well as additions listed above. After the indicated time, 112.5 μl of lysed chloroplast suspension (equivalent to 9 μg chl) was added to 37.5 μl assay mix. Specific activity of $H^{14}CO_3^-$ was 10 mCi/mmol. Assays were analyzed as in assay A. **Standard assays:** 22.5 μl of non-illuminated chloroplast suspension was added to 127.5 μl assay mix and assayed for 8 or 16 minutes. Specific activity of $H^{14}CO_3^-$ was 2 mCi/mmol, assays were analyzed as in assay A. Activity is given in nmole CO_2 fixed * $hour^{-1}$ *μg chl^{-1}.

activated ACCase in the presence of ethyl adenylate, thus any effect of CoA was not due to its conversion to acetyl-CoA by ACS from the chloroplast lysate.

Table 2.2 also shows the effects of short-chain acyl-CoA's on 8 and 16 minute ACCase activity. The presence of 100 μM malonyl-CoA depressed ACCase activity approximately 50%. 100 μM propionyl-CoA depressed activity about 25%. Ethyl adenylate with acetyl-CoA, ethyl-adenylate with CoA, or butyryl-CoA did not significantly affect 8 and 16 minute ACCase activity.

ACCase activity of chloroplast lysates in the presence of DTT and thioredoxin

There have been reports that reducing agents such as DTT and thioredoxin stimulate ACCase activity both in spinach chloroplasts (Sauer and Heise, 1984) and partially purified pea ACCase (Sasaki et al., 1997). Sauer and Heise observed a 50% increase in ACCase activity when chloroplasts were pre-incubated with 5 mM DTT before chloroplast lysis and ACCase assay. Additionally, in a partially purified ACCase preparation from pea, Sasaki et al. (1997) report almost a six-fold increase in ACCase activity when the preparation includes 2 mM DTT or is pre-incubated with a combination of 50 μM DTT and 16 μM *E. coli* thioredoxin. In agreement with these results, we have observed stimulation by DTT and DTT plus *E. coli* thioredoxin on a clarified pea lysate as well as a resuspended and desalted ammonium sulfate precipitate of the pea lysate (data not shown). However, with lysed spinach chloroplasts, DTT stimulated ACCase only under conditions when activity was low (Figure 2.4).

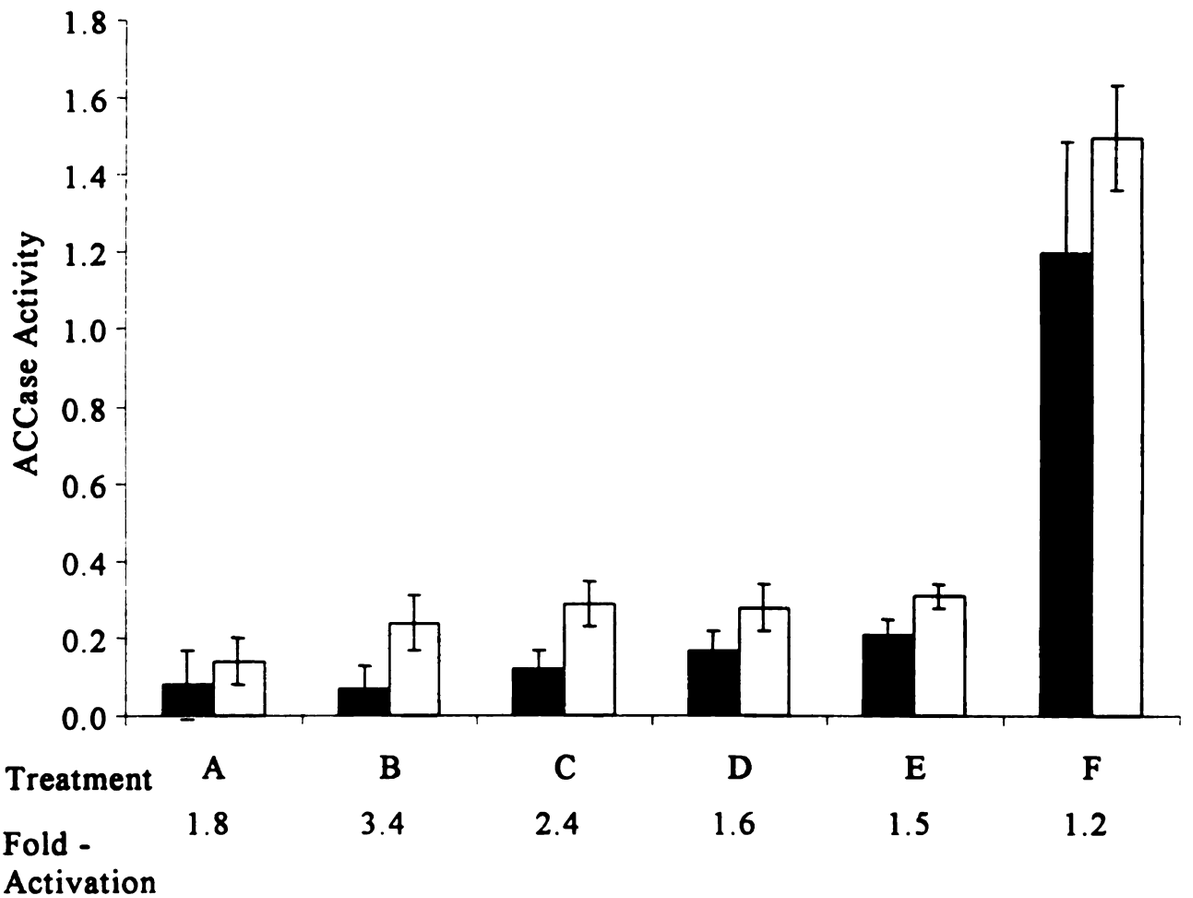

Figure 2.4. Effect of DTT on ACCase activity (nmole CO_2 fixed * $hour^{-1}$ * μg chl^{-1}).

Treatments A through E: Chloroplast suspension was lysed by five-fold dilution into lysis buffer (with additions of 1.67 mM ATP, 3.33 mM $MgCl_2$, 50 mM KCl) containing no (■) or 2.67 - 5 mM DTT (□). After 1 (A), 2.5 (B), 4 (C), 8 (D) or 16 (E) minute(s) ACCase activity was assayed for 1 minute. Assay A was used, the final volume was 100 to 150 μl (5 - 9 μg chl), specific activity of $H^{14}CO_3^-$ was 10 mCi/mmol. Data are average of n=6 trials. **Treatment F**: Chloroplast suspension was assayed using standard assay conditions in the absence (■) or presence (□) of 0.05 or 1.0 mM DTT. Assay A was used, final assay volume was 100 to 150 μl (5 - 9 μg chl), specific activity of $H^{14}CO_3^-$ was 0.5 mCi/mmol. Data are average of n=4 trials.

With 1 minute assays immediately after lysis using assay A, we saw a stimulation by DTT of about two-fold, however, with 15 minute assays, where ACCase activity is higher, DTT had no significant effect. DTT at 50 μM and reduced *Spirulina* thioredoxin (50 to 151 μM) had no significant effect on the ACCase activity of lysed spinach or pea chloroplasts (data not shown).

ACCase activity in different biochemical environments

The effects of pH, Mg^{2+}, ATP and ADP on the activity of chloroplast ACCase have been described (Sauer and Heise, 1984; Sasaki et al., 1997; Eastwell and Stumpf, 1983). ADP is a competitive inhibitor of ATP (Eastwell and Stumpf, 1983) and stromal ADP levels rise in the dark (Heineke et al., 1991). Although the effects of pH and Mg^{2+} together have been detailed (Sauer and Heise, 1984; Sasaki et al., 1997), ACCase activity in the expected environment of the chloroplast, either in the light or dark, with respect to ATP, ADP, pH, NADPH, $NADP^+$, Mg^{2+} and CO_2/HCO_3^- simultaneously, has not been described. Table 2.1 shows the effects that a light-like or a dark-like environment had upon ACCase activity. When assayed under the conditions described, ACCase activity was about ten-fold higher in a light-like environment (0.2 nmole CO_2 fixed * $hour^{-1}$ *μg chl^{-1}) than a dark-like one (0.02 nmole CO_2 fixed * $hour^{-1}$ * μg chl^{-1}). When ACCase activity was assayed at pH 7 with concentrations of metabolites expected in the light, activity was four-fold greater than activity in a wholly dark-like environment. But, when assayed at the pH of illuminated chloroplast stroma (pH 8), ACCase activity was the same, 0.2 nmole CO_2 fixed * $hour^{-1}$ *μg

chl^{-1}, regardless of other metabolite concentrations. Thus, most of the influence of light-like and dark-like environments can be attributed to pH.

Discussion

Light activation (or dark deactivation) of ACCase in a lysed chloroplast suspension

When lysed and assayed in the same buffer, the ACCase activity of a chloroplast extract is two-fold higher if chloroplasts were pre-incubated in light. Based on a value of 15 μg chl/μl stroma (Winter et al., 1994) and the final concentration of chl in the assay of 0.08 μg chl/μl, lysis into assay buffer dilutes the stromal contents more than 100-fold, therefore, any metabolite differences of the illuminated vs. dark chloroplast suspensions would be overshadowed by the conditions in the assay buffer. Therefore, this difference in ACCase activity is likely to be either an enzyme activation connected to light, or a deactivation of the enzyme in the absence of light. Interestingly, when the chloroplasts are lysed for five minutes prior to assay, the ACCase activity of the dark-incubated chloroplasts increases relative to that of a short lysis period. Thus the regulation is most likely a reversible dark inactivation rather than a light activation. These data indicate that light regulation of ACCase is not solely a response to changes in ATP, pH, Mg^{2+}, etc., but that other factors are likely involved in establishing a lower activity of ACCase in dark chloroplasts. Because fatty acid synthesis must respond to a wide variety of cellular demands, we expect that metabolic feedback

controls on the enzyme are crucial to coordinating the production of fatty acids in the plastid with their use at the endoplasmic reticulum or other locations. Indeed, we have observed that exogenous fatty acids feedback on the rate of fatty acid synthesis in tobacco suspension cultures and that the site of this control is ACCase (Shintani and Ohlrogge, 1995). Thus, one possible explanation for the lower rate of ACCase in dark chloroplasts is that ACCase is inhibited by some feedback mechanism related to the lower demand for fatty acids in the dark.

ACCase activity after pre-incubation of chloroplast lysates with substrates

We observed an increasing activity of ACCase over assay time (Figure 2.2A) which could be attributed to the activating presence of acetyl-CoA in the assay buffer. As shown in Figure 2.2B, pre-incubation with 30 to 833 μM acetyl-CoA increases ACCase activity by about four-fold. At 10 μM acetyl-CoA, two-fold activation is seen. The other substrates of the reaction, MgATP and HCO_3^- do not appear to activate the spinach chloroplast enzyme. Laing and Roughan (1982) reported activation of ACCase by CoA, but did not investigate acetyl-CoA activation. We saw no significant difference between CoA, acetyl-CoA and other short chain acyl-CoA's when used for activation, and therefore these activators most likely use the same mechanism. The addition of ethyl adenylate at 12.5-fold its k_i effectively blocked ACS activity, hence, any activation by CoA is due to the effect of CoA, not the effect of acetyl-CoA.

Removal of acetyl-CoA by gel filtration decreased activity, and furthermore, ACCase was partially reactivated by the readdition of acetyl-CoA. Since the column buffer contained all ACCase assay components except acetyl-CoA and HCO_3^-, only acetyl-CoA, HCO_3^- and small molecules from the diluted stroma were removed by the spin column. If there were small molecules from the stroma which were removed from the lysate by the spin column, and these molecules were essential for acetyl-CoA activation of ACCase, readdition of acetyl-CoA would not have reactivated ACCase. Since readdition of acetyl-CoA at least partially reactivated ACCase, any small stromal molecules which may have remained on the column were not required for reactivation. Therefore, acetyl-CoA activation appears to be easily reversible, although full activation may require additional components.

As shown in Figure 2.2B, ACCase is activated at low concentrations of acetyl-CoA, but does this occur *in vivo*? The concentration of total CoA's in chloroplasts is 10-20 μM, and the vast majority occurs as acetyl-CoA (Post-Beittenmiller et al., 1992b; Roughan 1997). Therefore, at the concentrations where we observe activation, it is likely that acetyl-CoA, rather than CoASH, or other acyl-CoA, is the form of CoA available to activate ACCase *in vivo*. Concentrations where we observe activation *in vitro* are similar to those that occur *in vivo* and thus acetyl-CoA activation of ACCase may be physiologically important. In order for this activation to be used *in vivo*, acetyl-CoA concentrations must change, at least locally to ACCase. Chloroplast acetyl-CoA

concentrations are very similar in light or dark leaves or chloroplasts. However, a transient decrease in acetyl-CoA occurs for two to three minutes after transfer of spinach chloroplasts from light to dark, but then stabilizes to light levels (Post-Beittenmiller et al., 1992b). The effect of acetyl-CoA upon ACCase is rapid: changes in the activity are apparent 30 seconds after lysis, which is in line with the rapid light-dependent increase in FAS (Browse et al., 1981).

The calculated concentration of stromal acetyl-CoA (10-20 μM) is well below the K_m of ACCase for acetyl-CoA (60-100 μM, Sauer and Heise, 1984; Mohan and Kekwick, 1980). Furthermore, at this concentration of acetyl-CoA and other stromal metabolites, calculated ACCase activity is several-fold lower than that needed to sustain *in vivo* rates of FAS (Table 2.1, Roughan 1997). The K_m of spinach plastid ACCase for acetyl-CoA is about five times the calculated stromal concentration of acetyl-CoA. Based on this and other information, Roughan (1997) proposed that compartmentalization of FAS enzymes and substrates, including acetyl-CoA, may be occurring. Thus, the acetyl-CoA concentration actually presented to the ACCase active sites may be substantially higher than 10-20 μM. Although acetyl-CoA levels in seed plastids are not known, acetyl-ACP and malonyl-ACP levels are higher in seeds than in leaves (Post-Beittenmiller et al., 1991, Post-Beittenmiller et al., 1992a). If acetyl-CoA levels are likewise higher in seeds than leaves, this may suggest that acetyl-CoA activation of seed ACCase is associated with high rates of FAS during triacylglycerol accumulation in developing seeds.

Activation of ACCase by reductants

Our results show that when ACCase activity is low, DTT has a stimulatory effect, increasing activity approximately two-fold. However, in cases where ACCase activity is higher, DTT by itself or in combination with *Spirulina* thioredoxin has no significant stimulatory effect either in spinach or pea chloroplast lysates. Although it has been reported that partially purified pea ACCase and spinach lysates are stimulated several fold by DTT and *E. coli* thioredoxin (Sauer and Heise, 1984; Sasaki et al., 1997) the activity of ACCase in those preparations was one-third to one-half (for pea, Sasaki et al., 1997) and one-tenth (for spinach, Sauer and Heise, 1984) the amount required to support observed *in vivo* rates of FAS (Browse et al., 1981; Roughan, 1987). From our results, this is in the range where added DTT does have an effect. Possibly the preparation method of the partially purified pea ACCase altered its state to make the enzyme more sensitive to reductants than we observe in chloroplast lysates. Activation by reducing agents could be concurrent with acetyl-CoA activation - both operate when ACCase activity is relatively low.

ACCase activity in different biochemical environments

To a first approximation, the biochemical environment of the stroma is an important determinant of ACCase activity from lysed spinach chloroplasts, contributing a ten-fold difference in *in vitro* activity (Table 2.1). Furthermore, most of this effect appears to be due to the difference between light and dark stromal pH. But even in this model environment, *in vitro* ACCase activity is at

least five-fold lower than that required to sustain known rates of acetate incorporation by intact chloroplasts (1-2 μmole * $hour^{-1}$ * mg chl^{-1}, Roughan, 1987) or the *in vitro* rate of ACCase under optimized assay conditions. Thus, in the standard ACCase assay, where substrates are present well above their K_m's and the reaction is optimized for ACCase, *in vitro* activity is similar to *in vivo*. This is not true for the model stromal environments. The expected concentration of HCO_3^- in the stroma is approximately one quarter of the spinach chloroplast K_m for HCO_3^- (3.0 mM, Mohan and Kekwick, 1980). Thus, if ACCase activity in optimized assays is at v_{max} and ACCase follows Michaelis-Menten kinetics, this lack of HCO_3^- would reduce ACCase activity in the light-like assay to about one fifth of v_{max} activity. The difference in pH between standard assay conditions and illuminated stromal conditions further reduces ACCase activity by one half (Sauer and Heise, 1984). These changes alone can explain the difference in activity between a standard (optimized) ACCase assay and the model stromal environment. However, the low activity of ACCase under estimated stromal conditions suggests either the model environment is inaccurate or/and the ACCase enzyme *in vitro* is less active than *in vivo*. Roughan (1997) argues that the enzymes, substrates and cofactors for fatty acid synthesis must be compartmentalized to account for the types of differences discussed above. Our data support this conclusion, although at this time there is no clear picture of the nature of a FAS metabolon, nor can we rule out that ACCase must be in some "native" environment (membrane association?) to achieve maximum activity. Finally, in non-photosynthetic plastids those factors which are best understood to

influence FAS, such as light-dependence and pH changes, are unlikely to control ACCase, and other controls, such as substrate activation or feedback inhibition may play a dominant role in the regulation of this enzyme.

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Chapter 3

COMPUTER SIMULATIONS OF THE INITIAL REACTIONS OF FAS

Abstract

Biosynthesis of the major fatty acids produced by plastids, palmitic and oleic (16:0 and 18:1Δ9), requires 43 and 50 enzymatic steps respectively; thus *de novo* fatty acid synthesis (FAS) is a complex process. Through genetic engineering alterations of lipid composition have been achieved (e.g. increased laurate in rapeseed, addition of epoxy fatty acids in Arabidopsis) and oil quantity has been increased in Arabidopsis and *Brassica*. However, these accomplishments are balanced by numerous failures to achieve expected results and thus techniques for metabolically engineering a plant to produce a predetermined quantity and/or quality of fatty acids is still largely "hit-or-miss." In addition to the conventional biochemical and genetic methods which are currently applied in metabolic engineering this chapter presents an additional approach: the use of modeling and computer simulations to control conceptual complexity and focus experiments in promising areas.

A modeling program, Stella II, was used to create a computer simulation of the initial reactions of FAS. This simulation 1) integrates and combines the large amount of biochemical data available from studies of both enzyme kinetics and

metabolite pool sizes; 2) allows enzyme activities to be easily altered before or during a simulation; 3) mimics the behavior of metabolite pools under both high and low rates of FAS; 4) mimics the behavior of several transgenic over- and underexpression studies; and 5) qualitatively predicts most of the changes in metabolic pools seen in a light/dark transition, which consists of a six- to ten-fold drop in the rate of FAS.

While the model is still primitive, it provides a proof-of-concept that models of the complex FAS pathway can be built, can mimic (or predict) changes in pool size and can mimic (or predict) the consequences of over- and underexpression of genes. This model predicts a series of results, a number of which correspond to known properties. In addition, it predicts that the set of enzymes which limit and co-limit the production of fatty acids are different depending upon 1) whether 18:1Δ9 or 16:0 fatty acid is considered and 2) if the system is in a state of high flux or low flux. The model also predicts that in order to substantially increase the amount of FA produced, ACCase, KAS III, and KAS I will all have to be concurrently overexpressed. Any one enzyme or pair alone will not suffice. The model also predicts that increasing expression of genes in the latter part of the pathway will have very little effect on the overall rate of FAS.

Introduction

How can the amount and type of fatty acids synthesized by a plant be altered? Almost forty years after the first papers on plant fatty acid synthesis were published, key aspects of plant fatty acid synthesis regulation are not understood. The majority of fatty acids are used in membranes and as storage products, although small amounts of specific fatty acids such as jasmonate and myristate play important cellular roles in signaling and anchoring proteins to membranes. Eighty to 85% of the FA in a non-seed plant cell is derived from 18:1Δ9, whereas about 15-20% is derived from 16:0 or its derivatives. Even in plants such as castor (*Ricinus communis)* or California Bay (*Umbellularia californica)* which are known for synthesizing large amounts of unusual fatty acids such as ricinolenic (18:1Δ9, 12-OH) or laurate (12:0) in seeds, 16:0 and 18:1 fatty acids are still the major components of membranes. Ideally, we would like to be able to genetically engineer specialty crop plants to create more or less oil and/or change the composition of that oil.

To engineer fatty acid synthesis we need a clear understanding of the limits and regulators of fatty acid and triacylglycerol production. *De novo* FAS occurs in plastids and many factors affect the rate. In leaves one major regulator is light; FAS is six- to ten-fold higher in light-incubated chloroplasts than in dark-incubated chloroplasts (Browse et al., 1981). Light affects FAS in numerous ways: the pH of the stroma increases one unit, thus enzyme activity is affected,

and the concentrations of substrates and cofactors change. Light also indirectly generates the NAD(P)H used in the two reduction steps in each cycle of FAS. In chloroplasts the first committed and a rate-determining step of FAS is performed by acetyl-CoA carboxylase (ACCase). ACCase activity is at least two-fold higher in extracts of light-incubated chloroplasts than in extracts of dark-incubated chloroplasts, even when the assay conditions are identical (Chapter 2). Light also indirectly generates ATP which is required for the initial carboxylation of acetyl-CoA. The concentration of a cofactor, Mg^{2+} also increases over two-fold in the light from 1 mM to 3 mM (Heldt, 1979). Reduced thioredoxin can also stimulate ACCase activity, and Sasaki et al. have proposed this as the connection between light and FAS regulation (1997). The "on/off" mechanism of FAS is still not completely understood, although ACCase has been identified as a probable control point and some factors which influence its activity are known.

Figure 3.1 shows the metabolic pathway of the plastidial reactions of FAS. Briefly, acetyl-CoA is carboxylated. Then the three carbon moiety of the resultant malonyl-CoA is transferred to acyl carrier protein (ACP) and this malonyl-ACP is used as a building block for fatty acid synthesis. The initial elongation reaction, catalyzed by KAS III, condenses malonyl-ACP and acetyl-CoA to form 3-ketobutyryl-ACP. The 3-ketobutyryl-ACP is reduced to 3-hydroxybutyryl-ACP, dehydrated to *trans*-Δ2-butenoyl-ACP, then reduced again to butyryl-ACP. This butyryl-ACP is then elongated by another 2 carbons by condensation with another malonyl-ACP, and the cycle of reduce, dehydrate, reduce repeats. This cycle is

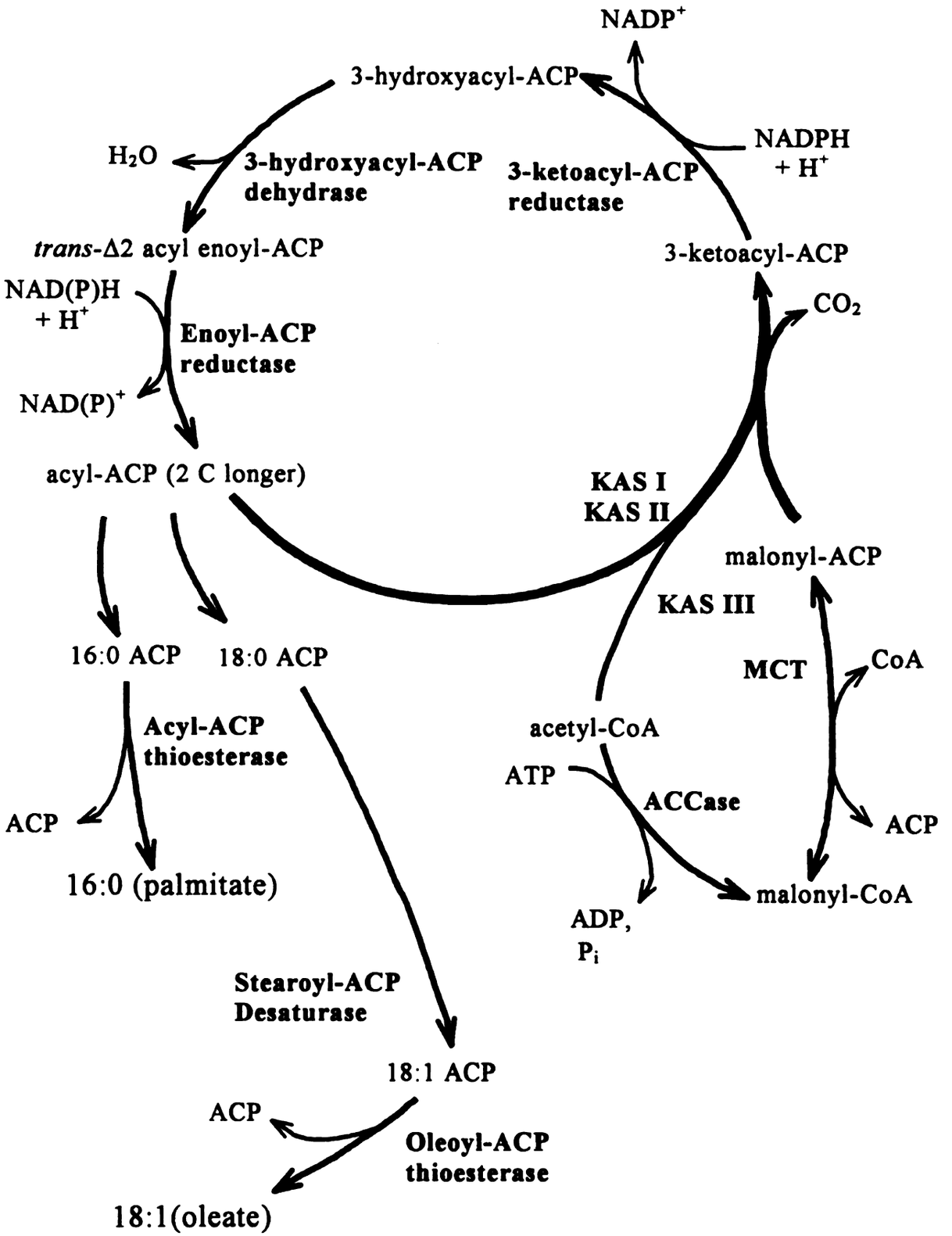


Figure 3.1. Major plastidial reactions of FAS.

Enzymatic steps are in boldface type. See text for further explanation. Figure after Ohlrogge and Browse (1995).

repeated six times until the chain is 16 carbons in length. These elongations are performed by KAS I. The final steps of FAS are either hydrolysis of the 16:0 moiety from ACP to yield 16:0 fatty acids or a further elongation by KAS II to 18:0-ACP, then desaturation to 18:1Δ9-ACP followed by hydrolysis to yield 18:1Δ9 fatty acids. Acyl transferases can also move 16:0 and 18:1Δ9 from ACP onto glycerol for use in the chloroplast glycerolipids (Roughan and Slack, 1982; Somerville and Browse, 1991).

The biochemistry of plant FAS is anything but simple. Of the 15 or so proteins involved in common fatty acid production, 10 have been partially purified or purified in some plants. Sixteen of the 18 or so genes have been cloned; of these, 10 have been used for under- or overexpression studies. However, the pathway is actually more complex than that shown in Figure 3.1. Enzymes specific to unusual fatty acid synthesis are still being discovered. A few examples: desaturase for petroselinic acid production (Cahoon et al., 1992), thioesterase for 12:0 production (Pollard et al., 1991), and KAS IV in *Cuphea* endosperm (Dehesh et al., 1998). Furthermore, there are specific co-factors which interact with these enzymes: ACP isoforms in *Thunbergia alata* and coriander which yield higher activity when used in *in vitro* desaturation assays (Suh et al., 1999) and likely a specific ferredoxin for the *T. alata* desaturase (Schultz and Ohlrogge, 1998).

Where do the myriad of factors which regulate FAS act? Through analysis of metabolite pools in spinach leaf chloroplasts, Post-Beittenmiller et al. have argued

that ACCase must be rate limiting (1991, 1992). This work relies on the theory that it is possible to compare the pool sizes of metabolites in a pathway under states of high or normal flux and low flux. Under a state of low flux, the rate-limiting enzyme will be saturated and a backlog of some metabolite will be waiting to be processed, increasing the pool size just *before* the rate-limiting enzyme. Pool sizes downstream from the rate-limiting enzyme will be smaller than in the normal or high flux state (Rolleston, 1972). The studies of Post-Beittenmiller et al. used the difference in rates of FAS between the light and dark to alter flux (1991, 1992). Since Triton X-100 stimulates FAS in isolated chloroplasts, this method was also used to alter the rate of flux (Post-Beittenmiller et al., 1992). The studies showed that 1) in light-incubated chloroplasts, malonyl-CoA and malonyl-ACP were detectable, but were not detected in dark-incubated chloroplasts, 2) although the concentration of acetyl-CoA, the substrate for ACCase, was constant, the concentration of acetyl-ACP increased five-fold in dark-incubated chloroplasts. Likewise, when FAS is stimulated by Triton X-100, the acetyl-CoA pool size decreases slightly, but the malonyl-CoA pool size increases several-fold (Post-Beittenmiller et al., 1992). Taken together, these data point to ACCase as a major component of FAS rate-determination. Other enzymes in the pathway may also be regulatory. From results of an overexpression experiment of the *E. coli* KAS III homolog, *fabH*, in *Brassica napus* and *E. coli*, Verwoert et al. (1995) suggested that regulation of FAS is shared by a number of enzymes in the pathway.

In any case, a logical next step to alter oil yield in plants was to overexpress ACCase. Roesler et al. (1997) targeted the homomeric cytosolic ACCase to the plastid using a napin promoter. They saw a two-fold increase in ACCase activity in the seed, but only a 5% oil increase in plants during a growth chamber experiment (Ohlrogge, personal communication; Roesler et al., 1997). This pattern has held in field trials (Ohlrogge, personal communication). Why did total oil yield not increase more than 5%?

Although there may be many reasons, one possible explanation is that some regulatory and/or controlling components remain unidentified. Antisense experiments in other primary pathways have also yielded unexpected results. Phosphofructokinase (PFK) is often described as a rate-limiting enzyme in glycolysis. PFK was overexpressed with no final change in respiration and only a slight increase in the intermediate metabolite, pyruvate (Burrell et al., 1994). Thus, if more than one enzyme is operating close to its maximal activity in a pathway, increasing just that one enzyme may not significantly increase flux and the final product. In tryptophan synthesis in yeast, genes were singly overexpressed ten- to fifty-fold (Niederberger et al., 1992). In most cases, overexpression had little effect on tryptophan accumulation or flux. The largest single-gene increase in flux was 30%, which was achieved when the enzyme involved had twenty-fold higher activity. Overexpression of four genes increased flux up to 2.5-fold. An eight-fold increase in flux was finally obtained: it took fifteen- to twenty-five-fold overexpression of the five genes in the pathway.

Non-intuitive regulation is also apparent in cyclic pathways. Reactions that are reversible *in vivo* are generally not candidates for pathway control points (Fell, 1997). However, aldolase, an enzyme in the Calvin Cycle that catalyzes a reversible reaction, inhibits photosynthesis when antisensed (Haake et al., 1999). The activity of fructose-1,6-bisphosphatase is decreased in these plants under both high and low irradiance growth conditions (Haake et al., 1999). Underexpression of enzymes that are postulated to play a controlling role, e.g., RuBisCO, shows the "rate-limiting" enzymes to have 40-60% excess capacity under non-stress conditions. (Haake et al., 1999; Stitt, 1999) The above examples from both linear and cyclic pathways, as well as experience specific to fatty acid synthesis, argue that altering final product levels may require manipulation of many genes.

In a short pathway, it may be possible to test single and multi-gene overexpression in a few experiments. This is not as feasible in a long pathway. With the advent of powerful and relatively easy-to-use computer simulation programs there is an additional approach that may be used. In this chapter, a computer simulation is described which incorporates the pool sizes of FAS metabolites, enzyme kinetic data (K_m's) and, when available, the relative activity of enzymes. This model was tested to see if it behaved in a "plant-like" manner under states of high and low flux. It can easily run multiple over- and underexpression studies. Ideally, the model will suggest a limited number of bench experiments for increasing oil production in plants. The computer simulation allows control of the conceptual complexity of FAS, and enforces consistent logic to scenarios tested. This chapter

describes 1) the simulation, 2) the test cases used to validate the model, 3) the model's predictions, and 4) the implications of those predictions for both increasing oil production in plants and conceptualizing the regulation of plant fatty acid synthesis.

Materials and Methods

Materials

Computer Program - Stella II

Stella II (version 5.1, High Performance Systems, Inc., Hanover, NH) is a general-purpose modeling program available for both Windows and Macintosh platforms. Models in Stella consist of "stocks," "flows," "reservoirs," "connectors" and "converters." For the FAS model, "stocks" represent metabolite pools and are represented by rectangular boxes. Stocks move from one pool to another by means of a "flow." The enzymes of FAS are the flows, and are represented by uni- or bi-directional pipes with faucets. Flows have user-defined rate equations which are based on Michaelis-Menten equations for this model . A "reservoir" is an infinite stock; these represent metabolite pools that are in constant, infinite supply in the model. The only true reservoir used in this model of FAS is the source of carbon, assumed to be acetate. A "converter" modifies the flow rate; these are gene expression factors in this implementation. A "connector" is a link between two other objects, either stocks or flows. Connectors may be

used to define the value of the flow in a model. Connections from stocks (metabolite pools) to flows (enzymes) allow pool size data to be used in the rate equations.

Kinetic Data and Metabolite Pool Sizes

The simulation was built using metabolite pool sizes in the light and kinetic data from enzymes. The pool sizes of FAS metabolites are shown in Table 3.1. This table also contains changes that occur during a light to dark transition when that data was available. The pool sizes have been determined in a variety of ways and general agreement of the values has been achieved over the last few years. Table 3.2 contains kinetic values for the enzymes of FAS gleaned from the literature.

While kinetic data and pool size data were used as a base, they alone were not enough to create a realistic model of FAS. Therefore, other common-sense criteria were applied and are listed in Table 3.3. These criteria were used to tweak the system in order to get the system to behave in a biologically reasonable manner.

Numerical Methods

Stella II generates a system of differential equations, one for each flow into and out of a stock. This system of equations can be solved by standard mathematical methods which provide excellent approximations. Stella II provides three numerical methods for solving the system of rate equations generated: Runge-

Table 3.1. Pool sizes of FAS intermediate metabolites from spinach and pea chloroplasts.

Values from references which were given in nmol per mg chl were adjusted according to Winter et al., 1994. Additional calculations have been performed on values in italics. Details of those calculations are given in the notes below the table. Nd = not detected.

Metabolite	μM		References and Notes
	Light	Dark	
Acetyl-CoA	10-20	same as light	Post-Beittenmiller et al., 1992 [1, 2]
CoA	nd, < 0.8	nd	Post-Beittenmiller et al., 1992 [2, 3]
Acetyl-ACP	0.5 – 1.0 *0.6*	*2.7*	Post-Beittenmiller et al., 1991, 1992 [4]
Total ACP	8	same as light	Post-Beittenmiller et al., 1991, 1992;
	10-20		Kuo and Ohlrogge, 1984
free ACP	1.7 – 1.9 *4.6*	decreases	Post-Beitenmiller et al., 1991, 1992 [4]
Malonyl-CoA	0.2 – 1.2	nd	Post-Beittenmiller et al., 1992
Malonyl-ACP	nd, *0.5–2.8*	decreases stays same	Post-Beittenmiller et al., 1991, 1992 [4, 5]
	0.4		Williamson and Wakil, 1966 [6]
4:0-ACP	*0.3*		Post-Beittenmiller et al., 1991 [4]
6:0-ACP	*0.5*		Post-Beittenmiller et al., 1991 [4]
	0.17 – 0.13		Roughan, 1998
8:0-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	0.33 – 0.40		Roughan, 1998
10:0-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	0.24 – 0.31		Roughan, 1998
12:0-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	0.26 – 0.26		Roughan, 1998
14:0-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	0.24 – 0.23		Roughan, 1998
16:0-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	1.23 - 0.73		Roughan, 1998
18:0-ACP	*0.5*		Post-Beittenmiller et al., 1991 [4]
	0.64 – 0.54		Roughan, 1998
18:1-ACP	*0.2*		Post-Beittenmiller et al., 1991 [4]
	1.06 – 1.11		Roughan, 1998
Total esterified ACP	5		Kopka, et al., 1995

Table 3.1, continued.

3-ketoacyl-ACP	nd < 0.005		Roughan, 1998
trans Δ2 enoyl-ACP	nd < 0.005		Roughan, 1998
3-hydroxyacyl-ACP, 8 C	0.023 – 0.016		Roughan, 1998
3-hydroxyacyl-ACP, 10 C	0.049 – 0.040		Roughan, 1998
3-hydroxyacyl-ACP, 12 C	0.070 – 0.081		Roughan, 1998
3-hydroxyacyl-ACP, 14 C	0.103 – 0.137		Roughan, 1998
3-hydroxyacyl-ACP, 16 C	0.089 – 0.109		Roughan, 1998
3-hydroxyacyl-ACP, 18 C	0.094 – 0.091		Roughan, 1998

Notes:

1) Acetyl-CoA shows a transient decrease in the dark over 2-3 minutes after light/dark transition, but it returns to its original value after 6-8 minutes.

2) Essentially all of the CoA pool is found in acetyl-CoA. The pool size is slightly lower in light-incubated chloroplasts under optimal conditions for FAS than in freshly isolated chloroplasts

3) The detection limit was 0.8 μM

4) The reported values include the total acyl-ACP concentration and the proportion of specific acyl-ACP's of this total determined by western blot analysis. These data were combined to yield the values in this table.

5) Detection methods differed.

6) Value calculated from the k_{eq} for the reaction and malonyl-CoA concentration.

Table 3.2. Kinetic data for FAS enzymes used in the Stella Model.

Enzyme	K_m (μM)		References
ACCase	acetyl-CoA	60-300	Bettey et al., 1992; Alban et al., 1994; Mohan and Kekwick, 1980, Sauer and Heise, 1984
ACT	acetyl-CoA	8	Shimakata and Stumpf, 1983
MCT	ACP	34	Post-Beitenmiller et al., 1989; Shimakata and Stumpf, 1983; Guerra and Ohlrogge, 1986
	malonyl-CoA	9-16	
KAS III	acetyl-CoA	5-45	Gulliver and Slabas, 1994; Verwoert et al., 1995; Clough et al., 1992
	malonyl-ACP	14	
KAS I	6:0-ACP	7	Shimakata and Stumpf, 1982
	10:0-ACP	5-10	
	14:0-ACP	9	
KAS II	10:0-ACP	13	Shimakata and Stumpf, 1982
	14:0-ACP	14	
	16:0-ACP	3	
OTE	16:0-ACP	4	Dörmann, et al., 1993
	18:0-ACP	7	
	18:1Δ9-ACP	1	
Desaturase	18:0-ACP	0.38	McKeon and Stumpf, 1982
Fatty Acid Synthase	ACP	5	Post-Beittenmiller et al., 1989

Table 3.3. Additional criteria used to build the model.

Metabolite Pools and Enzyme Kinetics

The model must reach a steady state in the light.

The model must reach a steady state in the dark.

Total CoA pool must remain constant.

Total ACP pool must remain constant.

Under wild type simulations, 15 to 20% of the FA produced should be 16:0.

Steady state metabolite concentrations achieved in the model under wild-type (light) conditions should be approximately the same as measured *in vivo* concentrations.

Steady state metabolite concentrations achieved in the model under decreased ACCase activity (dark conditions) should be approximately the same as measured *in vivo* concentrations.

About 90% of the CoA should be found in free CoA under both light and dark conditions.

About 60% of the ACP should be non-esterified in the light.

The MCT reaction should be close to equilibrium.

Light to Dark Transition

The rate of FAS in the dark should be $1/10^{th}$ to $1/6^{th}$ of the rate in the light.

The acetyl-ACP concentration should climb four- to five-fold in the dark.

Non-esterified ACP should decrease after transition and most of the ACP in the dark should be in acetyl-ACP.

Malonyl-ACP levels should decrease in the dark.

Malonyl-CoA levels should increase in the dark.

Short and medium chain (C_4 to C_{14}) acyl-ACP's should remain constant.

Palmitoyl-ACP, stearoyl-ACP, and oleoyl-ACP concentrations should be lower after the light to dark transition.

Kutta 2, Runge-Kutta 4, and Euler's Method. In theory, the simulation should predict the same behavior, independent of the numerical method used to solve the problem. Due to computational issues, this is not always the case. Detailed explanations of the numerical methods are in appendix A.

Stella II provides both tabular and graphical views of stocks and flow rates as a function of time. Tabular data may be easily exported to other programs, e.g. Excel 97 (Microsoft Corporation, Redmond, WA) for data analysis.

Methods

Scope of the Model

An all-inclusive model of FAS would be extremely difficult to build. Furthermore, FAS is complex. ACP isoforms, probable differences in enzyme presence and specificity for unusual fatty acids would need to be incorporated. Given the limited knowledge of the enzyme kinetics and metabolic pools involved, it is likely that too many assumptions would be required to build a meaningful all-inclusive model at this time. Furthermore, a model that is too simplistic would likely not predict anything useful. This model is limited to the reactions of FAS that occur in the plastid and also to the production of 16:0 and 18:1Δ9 fatty acids. These two fatty acids or their derivatives comprise about 95% of the fatty acids found in plants, of which 15 to 20 % of the fatty acids produced are 16:0, the remainder is 18:1Δ9.

Implementation

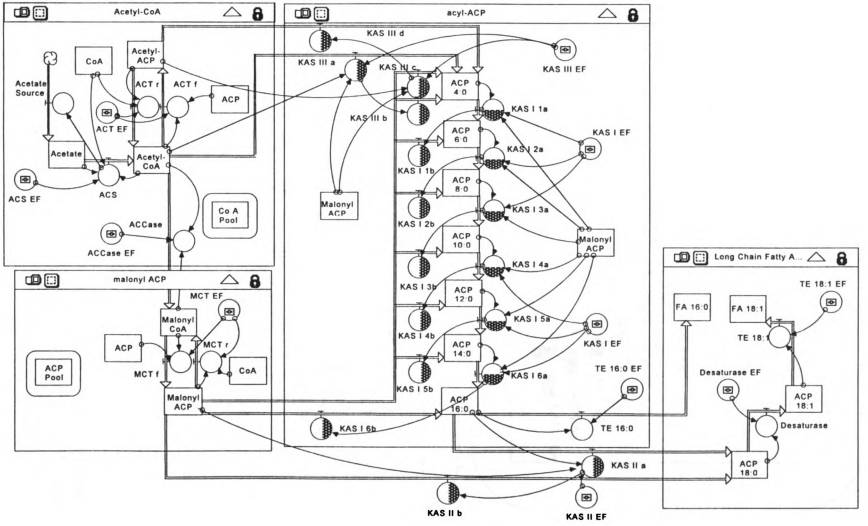
The biochemical detail of the model is shown in Figures 3.2A and 3.2B. In this model, all stocks (metabolite pools) are non-negative. In addition, altered gene expression and thus activity are represented by converters that can be manipulated by sliders, this model detail is shown in Figure 3.2C. All Stella objects are required to have unique names beginning with a letter, thus when an enzyme uses more than one metabolite pool, numbers and letters have been appended to form a unique name. For example, KAS III normally uses acetyl-CoA and malonyl-ACP to form 3-ketobutyryl-ACP, however, acetyl-ACP can also be used in place of acetyl-CoA with lower activity. Thus, the activities of KAS III are represented by `KAS III a` and `KAS III b` for the malonyl-ACP and acetyl-CoA condensation. Additionally, `KAS III c` and `KAS III d` represent the malonyl-ACP and acetyl-ACP condensation. Table 3.4 shows the components of the model and separates them by Stella object type. Table 3.5 shows the kinetic information and pool size data actually used in the model.

Assumptions

In order to create a workable model, simplifying assumptions almost always must be made. In the best possible case, these assumptions are not only correct, but have no bearing on the output and predictions of the model. In fact, often the most difficult part of creating a model is deciding which simplifications are valid (Bender, 1978). Modeling is an iterative process. As data become available and the model matures additional complexity is added. Many of the assumptions made

Figure 3.2A. Stella diagram of the model of the initial reactions of FAS.

The reactions of FAS have been separated by function into four large classes: 1) reactions involving acetyl-CoA, 2) reactions involving the building block malonyl-CoA, 3) the elongation reactions which create acyl-ACP's, and 4) the final reactions of the pathway. The details of flow into and out of CoA and ACP pools are shown in Figure 3.2B. Flow of the metabolites (square boxes) through the pathway is shown by double-lined arrows. Single lined arrows denote connections between components of the model, thus flow through ACCase is modulated by the concentration of acetyl-CoA, its substrate, and malonyl-CoA, its product. Flow rates are user-specifiedand in this model are all based on Michaelis-Menten kinetics. Expression factors (EF's) exist for all enzymes and are used to modulate wild-type activity before or during simulations. Some icons appear in multiple places, such as Malonyl-ACP or KAS III EF. These are the exact same Stella component, but have been duplicated to increase diagram simplicity. More details of the icons, their use in the model, and their meaning are presented in Table 3.4.


Acetyl-CoA
CoA
Acetyl-ACP
Acetate Source
ACT r
ACT f
ACP
ACT EF
Acetate
Acetyl-CoA
ACS EF
ACS
ACCase
Co A Pool
ACCase EF
malonyl ACP
MCT EF
Malonyl CoA
ACP
MCT r
MCT f
CoA
ACP Pool
Malonyl ACP
acyl-ACP
KAS III d
KAS III a
KAS III c
KAS III EF
ACP 4:0
KAS I 1a
KAS III b
ACP 6:0
KAS I EF
KAS I 2a
KAS I 1b
ACP 8:0
KAS I 3a
Malonyl ACP
KAS I 2b
ACP 10:0
Malonyl ACP
KAS I 4a
KAS I 3b
ACP 12:0
KAS I 5a
KAS I 4b
ACP 14:0
KAS I EF
KAS I 6a
TE 16:0 EF
KAS I 5b
ACP 16:0
KAS I 6b
TE 16:0
KAS II a
KAS II b
KAS II EF
Long Chain Fatty A...
TE 18:1 EF
FA 16:0
FA 18:1
TE 18:1
Desaturase EF
ACP 18:1
Desaturase
ACP 18:0

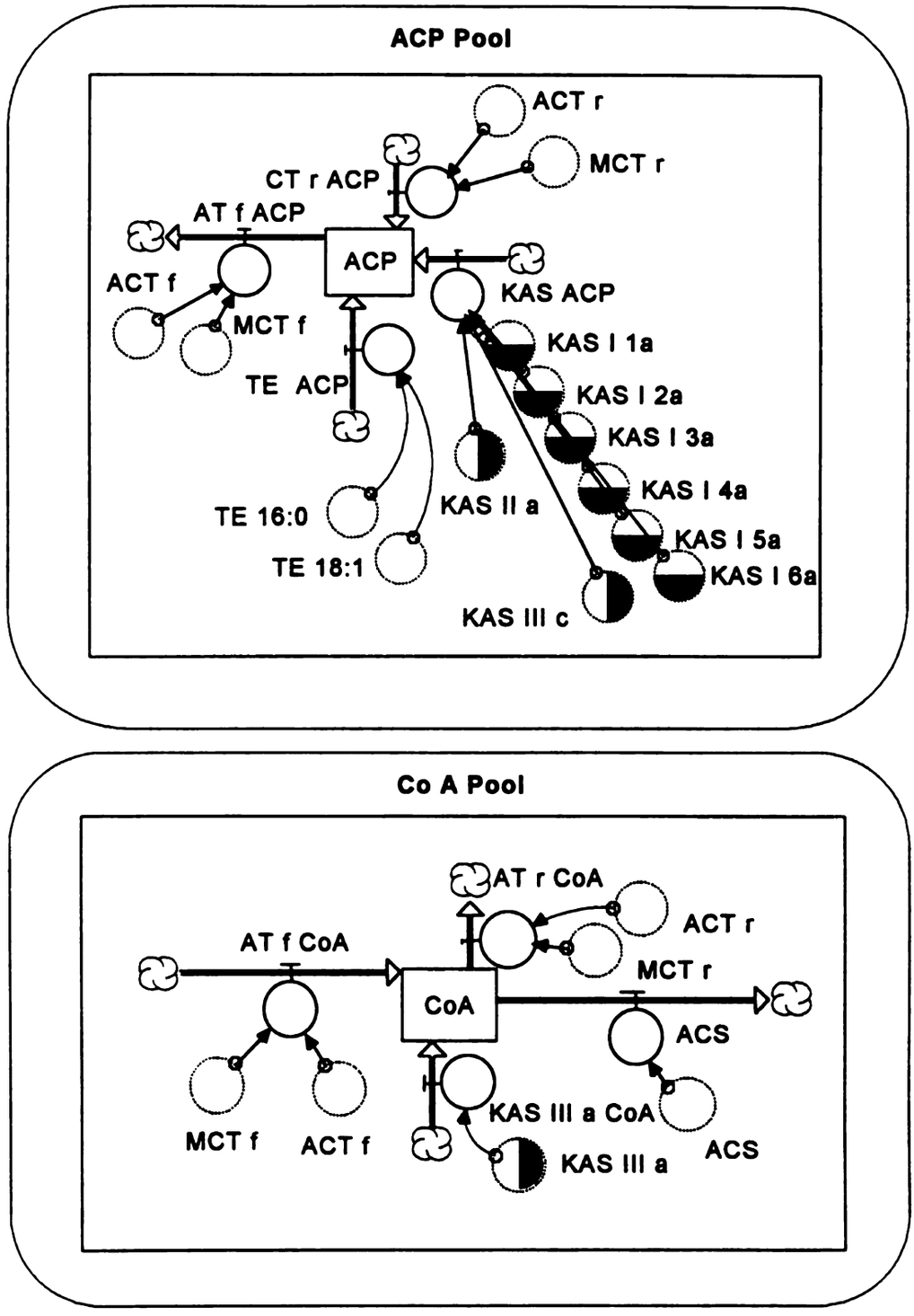


Figure 3.2B. Stella diagrams of total CoA and ACP pools.

These sub-models are used to force CoA and ACP pools to have constant values. The stocks and flows in dotted outline are repeat images from the upper layer shown in Figure 3.2A. Icons and their meaning are explained in Table 3.4 and the text.

Figure 3.2C. Stella diagram of the simulation layer of the model.

The top layer of the Stella model contains the four major classes of reactions used in the model: 1) reactions which create and use acetyl-CoA, 2) reactions involving malonyl-ACP, 3) the acyl-ACP elongation reactions, and 4) the final steps of fatty acid synthesis. Metabolite flow between the classes is indicated by thick arrows, while connections between the classes is indicated by thin arrows. The sliders on the right side of the diagram correspond to "expression factors" which are used to alter enzyme activity during a simulation. Sliders can have values ranging from 0.0 to 25.0, a wild-type value corresponds to 1.0. The value used in the simulation is shown in the slider's center box. In this case, ACCase EF was modified to be $1/8^{th}$ its initial (and wild-type value). The new value and the reset button, [U], reflect this change. Triangles allow navigation between layers.

The graph shows the metabolite levels of acetyl-CoA, malonyl-CoA, acetyl-ACP, malonyl-ACP, and free CoA. Initially the simulation was run with all expression factors set to 1.0. After the pools attained steady state values, the simulation was paused, and ACCase EF set to $1/8^{th}$ its initial value. This mimics a light to dark transition. The simulation was continued with this new value. The graph shows that the pool sizes of malonyl-ACP, malonyl-CoA, and CoA drop, the pool size of acetyl-ACP climbs, and the pool size of acetyl-CoA increases slightly. These changes are qualitatively consistent with *in vivo* pool size changes. Pool size concentrations are given in nmoles. The scale varies with the pool and is detailed on the y-axis.

ACS EF
ACCase EF
ACT EF
MCT EF
KAS III EF
KAS I EF
KAS II EF
TE 16:0 EF
Desaturase EF
TE 18:1 EF
0.00
1.00
0.13
25.0
Acetyl-CoA
Acyl-ACP
Malonyl-ACP
Long Chain Fatty ...
1: CoA
2: AcetylACP
3: AcetylCoA
4: MalonylACP
5: MalonylCoA
2500.0
20000.0
1250.0
10000.0
0.0
5.0
10.0
15.0
20.0
Graph 1: p1 (Early Metabolites)
Time
8:32 AM
Wed, Jun 07, 1999

Table 3.4. Components used in the Stella model of FAS.

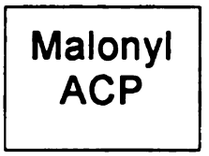
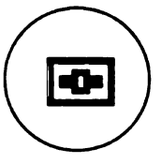
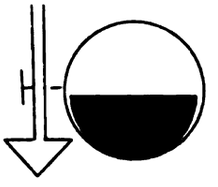
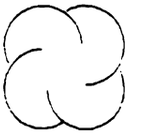
Stella Object	Icon	FAS model component
Stock metabolic pool		ACP acetyl-CoA acetyl-ACP acetate CoA malonyl-CoA malonyl-ACP 4:0 - 18:0 acyl-ACP's 18:1 ACP FA 16:0 FA 18:1
Converter expression factors (EF)		ACS EF ACT EF ACCase EF MCT EF KAS I EF KAS II EF KAS III EF Desaturase EF TE 16:0 EF TE 18:1 EF
Connector		n/a
Flow Enzymes		acetate source ACS ACT f, ACT r ACCase MCT f, MCT r KAS I 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b, 6a, 6b KAS II a, b KAS III a, b, c, d Desaturase TE 16:0 TE 18:1
Reservoir metabolites external to the model		n/a

Table 3.5A. Kinetic data used to implement the Stella Model.

Enzyme	Affinity (nM)		V_{max}
ACS	K_m: Acetate	10,000	1,2500
	K_m: CoA	500	
	K_i: Acetyl-CoA	2,500	
ACCase	K_m: Acetyl-CoA	2,000	5,000
	Ki: Malonyl-CoA	3,500	
ACT reverse*	K_m Acetyl-ACP	2750	420
	K_m CoA	2000	
ACT forward*	K_m acetyl-CoA	75000	840
	K_m ACP	20000	
MCT forward+	K_m Malonyl-CoA	2,000	100,000
	K_m ACP	2,500	
MCT reverse+	K_m CoA	2,000	42,000
	K_m Malonyl-ACP	5,000	
KAS III a/b	K_m Acetyl-CoA	2,500	1,250
(acetyl-CoA + malonyl-ACP)	K_m Malonyl-ACP	1,000	
KAS III c/d**	K_m Malonyl-ACP	1,000	50
(acetyl-ACP + malonyl-ACP)	K_m acetyl-ACP	10,000	
KAS I	K_m Malonyl-ACP	1,000	3,500
	K_m ACP_4:0 to 14:0	500	
KAS II	K_m Malonyl-ACP	1,000	8,000
	K_m ACP_16:0	1,000	
TE 16:0	K_m ACP_16:0	5,000	8,000
Desaturase	K_m ACP_18:0	380	4,000
TE 18:0	K_m ACP_18:1	5,000	8,000

* V_{max}'s chosen to give an equilibrium constant of 2.0 (Williamson and Wakil, 1966).

+ V_{max}'s chosen to give an equilibrium constant of 2.4 (Williamson and Wakil, 1966).

** V_{max} chosen to be $1/25^{th}$ V_{max} of acetyl-CoA + malonyl-ACP reaction based on data from Jaworski et al. (1989) and Clough, et al. (1992).

Table 3.5B. Initial pool size of metabolites used in the Stella model of FAS and values of model K_m's.

Metabolite	Initial Pool Size (μM)	Range of K_m's
Acetate	500	10.0
Acetyl-CoA Acetyl-CoA, ACT reaction	15	2.0 - 2.5 75.0
Malonyl-CoA	0.7	2.0 -3.5
Acetyl-ACP Acetyl-ACP, KAS III reaction	0.6	2.75 10.0
Malonyl-ACP	1.65	1.0 - 5.0
4:0-ACP	0.3	0.5
6:0-ACP	0.5	0.5
8:0-ACP to 16:0-ACP	0.2	0.5 - 5.0
18:0-ACP	0.5	.38
CoA	0.8	0.5 - 2.0
ACP-SH ACP-SH, ACT reaction	4.6	2.5 20.0
18:1-ACP	0.2	5.0
16:0-FA, 18:1-FA	0	n/a

while building the model can be removed when (if) more precise biochemical data become available, but, until then, there must be some estimates. Thus, the model presented here assumes that enzyme kinetic data measured *in vitro* can be meaningfully extrapolated to *in vivo* conditions. All of the enzymatic rates are based on Michaelis-Menten kinetics. Data for maximum enzyme rates are difficult to find, compare, interpret, and may not accurately reflect *in vivo* rates. Therefore, relative rates for the enzymes in the simulation have been used. For example, based on *in vitro* assays, the malonyl-CoA:ACP acyl transferase is an active enzyme: it has a high v_{max} and the rate at which it operates in the simulation under normal conditions is less than 10% of its v_{max}. ACCase is thought to be a rate-determining enzyme, its rate during simulation is 75% to 80% of its v_{max}. The model also assumes that the K_m for each acyl-ACP in the KAS I reaction is the same.

The model was implemented to ensure that the total CoA and ACP pools remained constant, allowing only the length of the acyl group attached to the cofactor to change. Flow into and out of these pools is detailed in Figure 3.2B. The model does not include ATP and NAD(P)H availability. The model combines the condensation, reduction, and dehydration steps that make up each cycle of fatty acid elongation. Because the major acyl-ACP pools detected *in vivo* are saturated acyl-ACP's, it is unlikely the dehydration and two reduction steps play an important role in the regulation of fatty acid synthesis. Although feedback has been reported on both ACCase (Shintani and Ohlrogge, 1995) and the *E. coli*

enoyl-ACP reductase (Heath and Rock, 1995), the model currently has no feedback loops. The feedback regulator for ACCase has not been identified (Ohlrogge and Jaworski, 1997).

One objective of the model is to test the consequences of altered gene expression. Therefore, in the model all of the enzymes can be under- or overexpressed between 0- to 25-fold, either singly or multiply. Gene expression is assumed to be linearly proportional to enzyme activity. This assumption is probably not true *in vivo*, especially at high levels of gene expression. The model assumes the source of acetyl-CoA is acetyl-CoA synthetase (ACS). While ACS may not be the source *in vivo*, acetate feeding is the most effective way to synthesize fatty acids in isolated chloroplasts (Roughan et al., 1979). Most of the data describing the regulation of FAS has used isolated chloroplasts, so this assumption is valid for the experimental system, if not the plant.

Model validation

Any model must behave in a physically and biologically realistic manner. Table 3.3 lists the criteria used to confirm that the model does so. Some of these criteria were invoked while building the model, thus they are a list of desirable model traits and cannot strictly be used to test the model. However, these criteria are

important to consider. The model arrives at a steady state after simulation begins. In addition, it maintains constant total CoA and total ACP pools.[1]

Since metabolite pool sizes change after the light to dark transition, and since ACCase activity decreases in the dark, a light to dark transition is mimicked during simulation by decreasing ACCase activity to $1/8^{th}$ its normal activity. Under these conditions, the major metabolites in the model reach new steady state conditions: acetyl-CoA remains about constant, malonyl-CoA and malonyl-ACP pools decrease. Although short and medium-chain acyl-ACP pools remain constant *in vivo*, they do not in this simulation. Overall, the rate of FAS declines to one quarter of normal. The acetyl-ACP concentration increases and the free ACP concentration decreases. Since these changes in the simulation qualitatively and occasionally quantitatively match the actual changes observed in a light to dark transition, this indicates the model behaves in a plant-like manner.

Comparison of model predictions to transgenic plant results

In some cases, overexpression experiments have already been performed. In these experiments, the foci have been on seed oil, several steps removed from plastidial fatty acid synthesis. Thus, interpreting the data from seed experiments may be problematic. However, since 16:0 and 18:1Δ9 are precursors to most seed acyl

[1] The default action Stella-II takes when combining two molecules is to create two molecules. Unless forced to do otherwise, Stella II creates two 3-ketobutyryl-ACP molecules from one acetyl-CoA and one malonyl-ACP. The half shaded circles used in all the condensation reactions correct for this.

groups, these overexpression experiments can be used to help validate the model and computer simulations. Table 3.6 lists overexpression and antisense experiments, their results and whether the model predicted a similar result. Overexpression of either KAS I or KAS II singly has not increased seed oil content (Kinney and Hitz, 1995), nor is it predicted to in the simulation. Overexpression of ACP does not increase seed oil (Suh and Ohlrogge, unpublished data; Kinney and Hitz, 1995).

In many cases the results of overexpression experiments have been intuitive, but not in all cases. KAS III is such a case. At least two different KAS III overexpression experiments have been performed. Jaworski and Hinneburg-Wolf (1998) expressed spinach KAS III in *Nicotiana*. KAS III activity increased 40- to 50-fold, yet the total fatty content acid remained constant. The 18:3 to 16:0 ratio decreased from 3.1 to 2.0, indicating an increase in 16:0 fatty acids. Neither the total composition of all fatty acids nor the growth rate of transgenic plants was described. In a second set of overexpression experiments, Verwoert et al. expressed the *E. coli* homolog of KAS III, *fabH*, in both *E. coli* and *Brassica napus* (1995). In *E. coli*, 14:0 levels increased, 18:1Δ9 levels decreased and cell growth was arrested. *Brassica* plants behaved differently. In *Brassica*, the mole percent of 18:1Δ9 decreased, and the mole percent of 18:2 and 18:3 increased concomitantly (Verwoert et al., 1995). The amount of 14:0 and 16:0 fatty acids remained constant. Due to variation in the data, no significant differences in total lipid content were reported. These results are puzzling. Why does overexpression

Table 3.6. Expression experiments and model predictions.

In vivo experiments are reported on the right, if the model approximates the same behavior, a + is in the left hand column, if not, a - is in the column. Model predictions, when different, are detailed in the right column.

+	ACCase overexpression increases total FA at least 5% (Roesler et al., 1997).
+	ACP overexpression by two- to three-fold has no effect on total FA (Suh and Ohlrogge, unpublished; Post-Beittenmiller et al., 1989).
+	Desaturase antisense decreases 18:1Δ9 by half (Kinney and Hitz, 1995).
+	Desaturase overexpression increases 18:1Δ9 slightly, may decrease saturated FA (Kinney and Hitz, 1995).
-	KAS I and KAS II dual overexpression has little effect on 16:0/18:1Δ9 ratio (Kinney and Hitz, 1995). The model predicts that more 18:1Δ9 will be produced and less 16:0, decreasing the 16:0/18:1Δ9 ratio.
-	KAS I antisense increases 16:0 and 18:0 almost two-fold (Kinney and Hitz, 1995). The model predicts a linear decrease in both 16:0 and 18:1 when KAS I is antisensed (Figure 3.4).
+	KAS I antisense decreases 18:1Δ9 about 30% (Kinney and Hitz, 1995).
+	KAS I overexpression has little effect on 16:0/18:1Δ9 ratio (Kinney and Hitz, 1995).
+	KAS II antisense increases 16:0 approximately 3-fold (Kinney and Hitz, 1995).
-	KAS II antisense causes no change or a slight decrease in 18:1Δ9 (Kinney and Hitz, 1995). The model predicts that KAS II antisense will co-limit 18:1Δ9 production, which would cause a large decrease in 18:1Δ9.
-	KAS II overexpression has little effect on 16:0/18:1Δ9 ratio (Kinney and Hitz, 1995). The models predicts an increase in 18:1Δ9 and a decrease in 16:0 when KAS II is overexpressed, thus the 16:0/18:1Δ9 ratio would decrease.
+	KAS III overexpression increases levels of 16:0, it may or may not decrease total FA (Jaworski and Hinneburg-Wolf, 1998; Verwort et al., 1995).
+	Oleoyl thioesterase antisense may increase saturated FA slightly, decrease 18:1Δ9 slightly (Kinney and Hitz, 1995).
+	Oleoyl thioesterase overexpression has little effect on ratios, perhaps a slight decrease in saturated FA, and a slight increase in 18:1Δ9 (Kinney and Hitz, 1995).

Table 3.6, continued

+	Acyl thioesterase antisense increases 18:1Δ9 by 5-fold, decreases 16:0 by 7-fold. 18:0 also may decrease slightly (Kinney and Hitz, 1995).
+	Acyl thioesterase overexpression causes a two-fold increase in 16:0 (Kinney and Hitz, 1995).
-	Acyl thioesterase overexpression may cause a slight increase in 18:0 and 18:1Δ9 (Kinney and Hitz, 1995). The model predicts that TE 16:0 overexpression will cause a decrease in the amount of 18:1Δ9.

of KAS III often result in increased shorter-chain fatty acids? When the computer simulation is run, the model predicts a similar outcome. In this case, the ratio of 18:1Δ9 to 16:0 changes from 2.4 to between 1.2 and 1.4 when KAS III is overexpressed from two- to four-fold. At this expression level, total carbon in fatty acids is depressed by 10% to 60%. At high levels (15- to 20-fold) of KAS III overexpression, the simulation predicts free ACP will drop to zero and the bulk of ACP will be found in short-chain acyl-ACP's. The malonyl-CoA pool also increases in this simulation. This is due to the conversion of acetyl-CoA to malonyl-CoA, but the lack of ACP hinders the malonyl-CoA:ACP transacylase reaction. When spinach KAS III is overexpressed in tobacco, ACP levels increase approximately two-fold (Jaworski and Hinneburg-Wolf, 1998). This could be a response to the limited supply of free ACP which would occur in the plant. Although many of the fine details are not consistent between the simulation and *in vivo* data, the computer simulations of the model qualitatively predict several of the unexpected effects of KAS III overexpression and furthermore provides an explanation. This is surprising: the model does not incorporate feedback regulation on the enzymes and it has no mechanism to sense and respond to fatty acid demand or the 18:1Δ9/16:0 ratio. Given the numerous cases sketched above, where the predictions of the model mimic the actual behavior of plants, it is likely that simulations run with the model can yield useful information which may be applied to manipulate oil synthesis in seeds.

Results and Predictions

Since the model behaves similarly to a plant, the questions become 1) can the model teach us anything useful about the regulation of FAS? 2) can the model pinpoint areas where our assumptions may be misleading us? and finally, 3) does the model concur with other predictions of metabolic regulation? These issues are discussed below.

ACCase, KAS I, and KAS III limit or co-limit flux through FAS in the light

A classic method for determining limiting and co-limiting enzymes in a pathway is to graph flux through the pathway as a function of enzyme activity (Furbank and Taylor, 1995; Haake et al., 1999). This approach is shown in Figure 3.3. In this approach, the graph of a limiting enzyme is a straight line with a positive slope, a graph of a co-limiting enzyme is slightly bowed above a straight line. Enzymes which are not limiting until their activity is severely impaired are horizontal lines until their activity is close to zero, then the flux takes a precipitous drop. Enzymes can also be detrimental to a pathway, in this case, the flux through the pathway increases as enzyme activity is decreased. The simulation was run with each enzyme underexpressed in turn. Graphs of total carbon incorporated vs. enzyme activity were generated for all enzymes, these graphs are shown in Figure 3.4. All enzymes were tested for both under- and overexpression. As shown in Figure 3.4, when total carbon in fatty acids or 18:1Δ9 free fatty acid is considered, ACCase is limiting, KAS I and KAS III are co-limiting at most

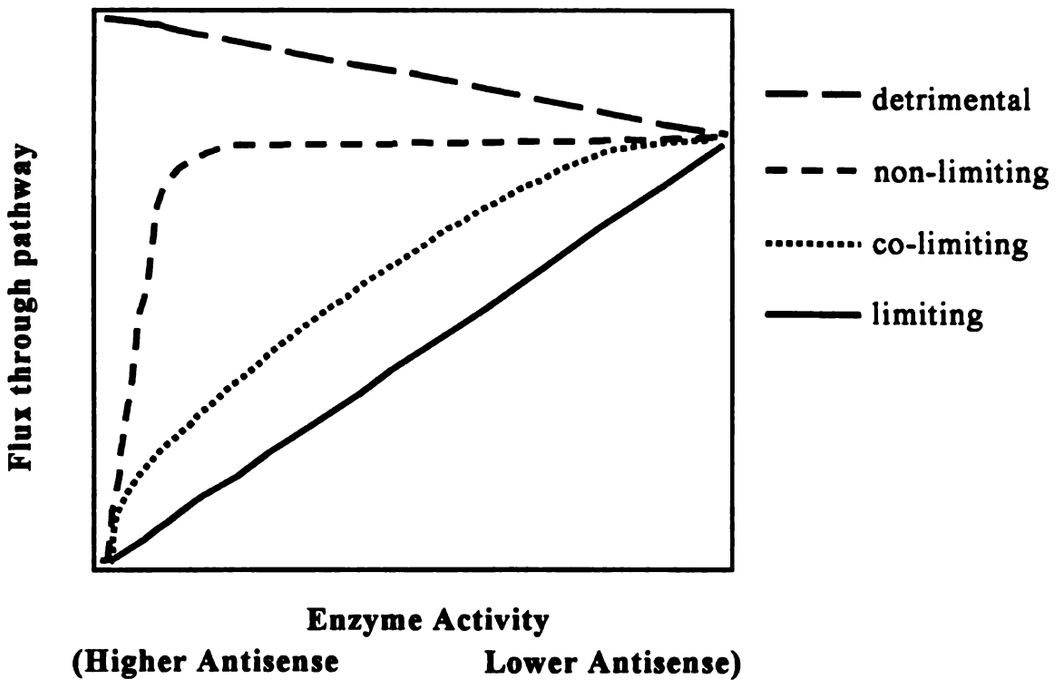


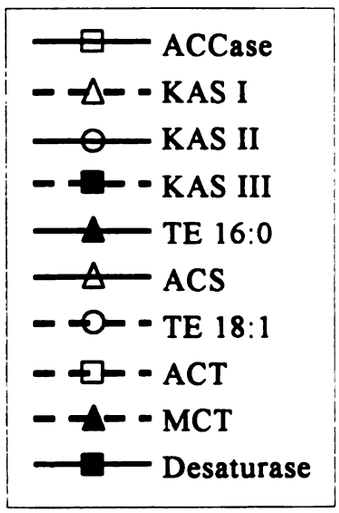
Figure 3.3. Theoretical consequences of altering enzyme activity and its effect on pathway flux.

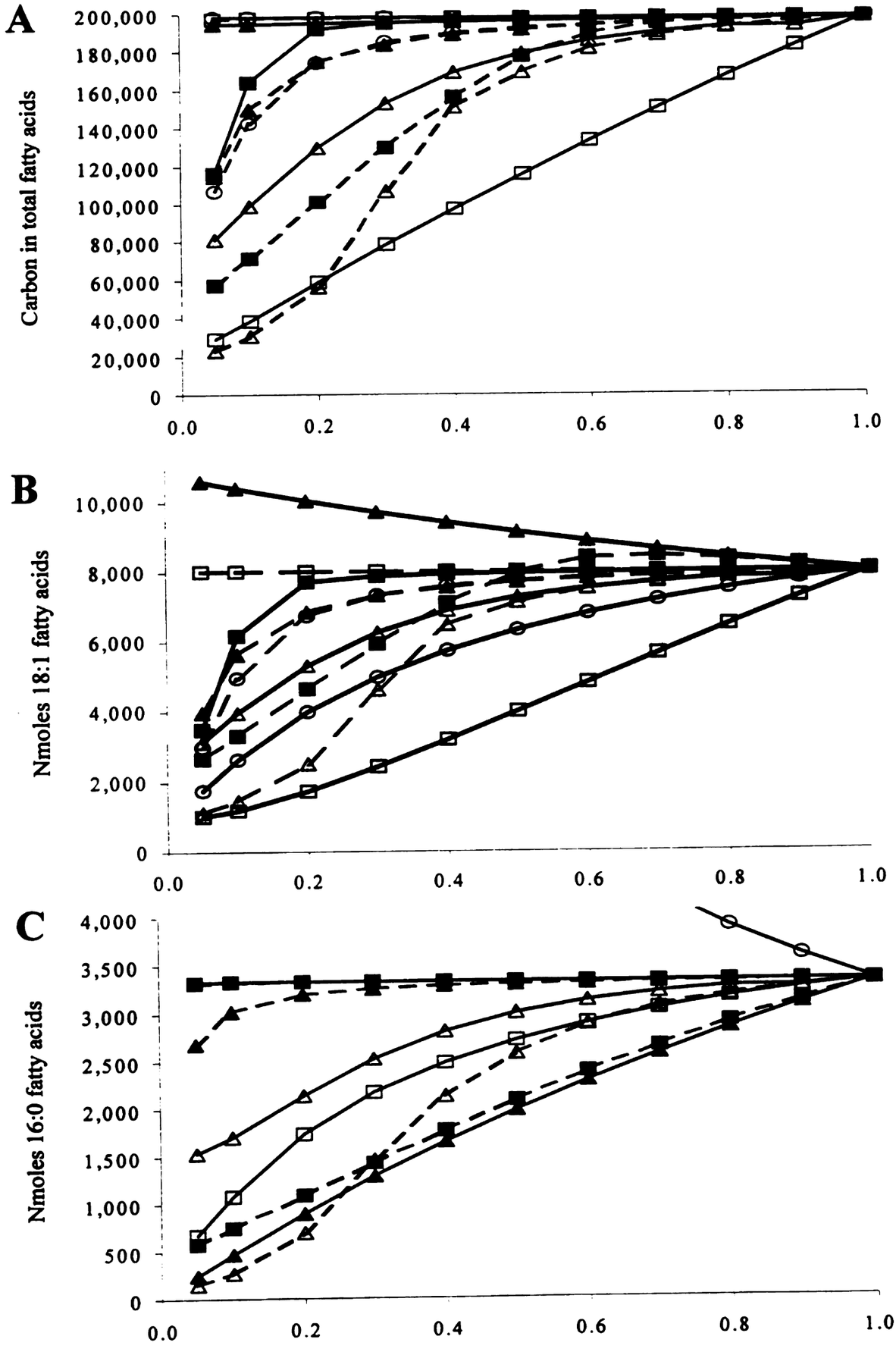
Altering the activity of an enzyme which limits flux causes a linear change in the pathway flux. Altering the activity of a co-limiting enzyme causes the line to bow slightly. Altering the activity of a non-limiting enzyme has no or little effect until the activity of the non-limiting enzyme becomes very low. Lines characterizing the relationship between enzyme activity and flux for enzymes which are detrimental to flux have negative slopes.

Figure 3.4. Flux through the FAS pathway vs. enzyme activity under states of high flux.

Computer simulations were run with the expression factor of each enzyme set to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 0.9 and 1.0. The final pool sizes of FAS products, 16:0 and 18:1Δ9 are shown in **theoretical** nmoles. **A)** Total carbon theoretically incorporated into FAS products. TE 16:0, KAS II, and ACT have essentially no effect on total carbon incorporated. **B)** Flux into 18:1Δ9 products only. **C)** Flux into 16:0 products only. The expression of KAS II is highly detrimental to the production of 16:0 and the graph has been cropped to show finer detail. The KAS II line is seen at the far right edge of the graph. ACT, Desaturase and TE 18:1 have no effect on the flux into 16:0.

Legend




A
Carbon in total fatty acids
200,000
180,000
160,000
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
0.0
0.2
0.4
0.6
0.8
1.0
B
Nmoles 18:1 fatty acids
10,000
8,000
6,000
4,000
2,000
0
0.0
0.2
0.4
0.6
0.8
1.0
C
Nmoles 16:0 fatty acids
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
0.0
0.2
0.4
0.6
0.8
1.0

expression levels. ACS is also co-limiting to some extent but this may be artifactual since the *in vivo* source of acetyl-CoA for FAS is unknown. The remainder of the enzymes, ACT, MCT, KAS II, the desaturase, and the thioesterases are not limiting.

Counter-intuitively, the limiting enzymes are different when the product of FAS is 16:0 free fatty acid rather than 18:1Δ9. In this case, TE 16:0, KAS I and KAS III underexpression have the most effect on limiting flux toward 16:0. ACCase and ACS are co-limiting. ACT, MCT, the desaturase, and TE 18:1 are non-limiting. KAS II underexpression increases flux through the 16:0 free fatty acid pathway.

ACCase, KAS I, and KAS II limit or co-limit flux through FAS in the dark

FAS is six to ten-fold higher in light-incubated leaf discs than in dark-incubated leaf-discs (Browse et al., 1981), in addition ACCase activity is at least two-fold higher in lysed, light-incubated chloroplasts than in dark-incubated ones (Chapter 2). Incubation of chloroplasts in the dark is mimicked by lowering ACCase activity in the model to one-eighth its wild-type value. That is, we set the expression factor, `ACCase EF`, to 0.125; in essence, this turns off the lights in the model. The same set of simulations as those shown in Figure 3.4 were run. The results, where the lights are "off," are shown in Figure 3.5.

Figure 3.5 shows that when total carbon in fatty acid or 18:1Δ9 fatty acids are considered, the limiting enzyme is ACCase, and KAS I is co-limiting. However,

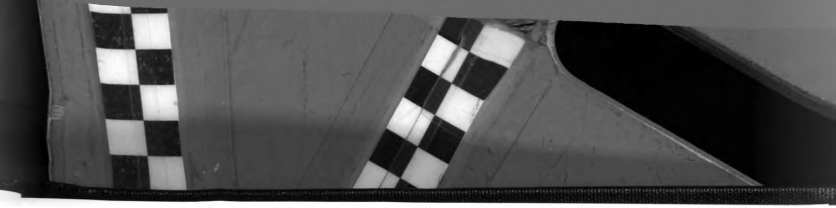
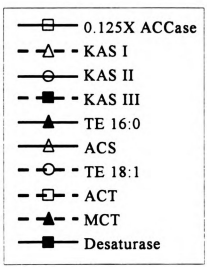
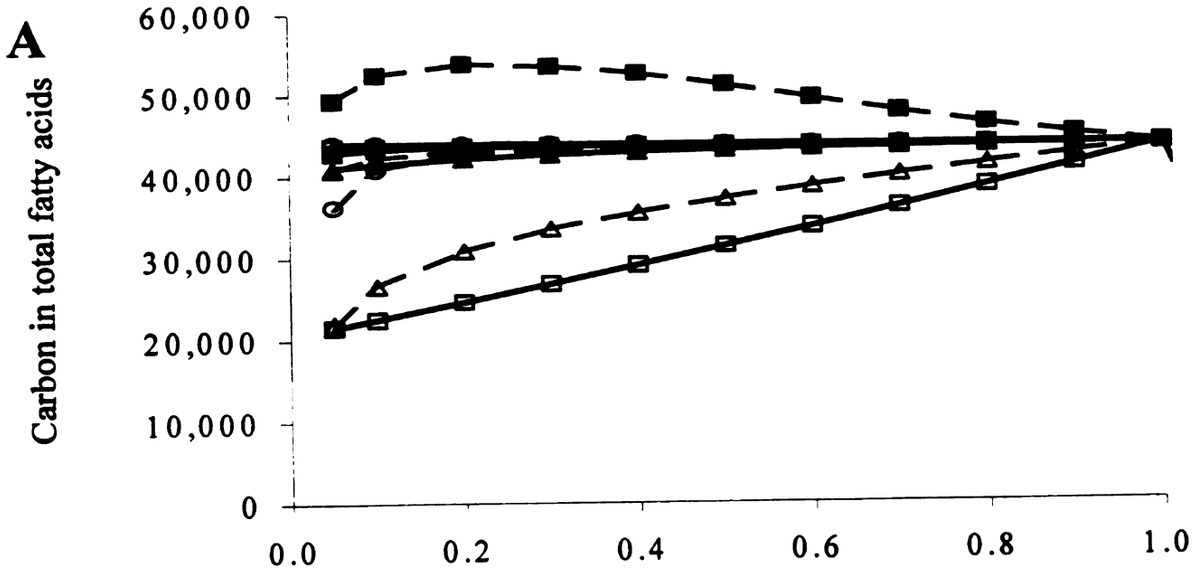


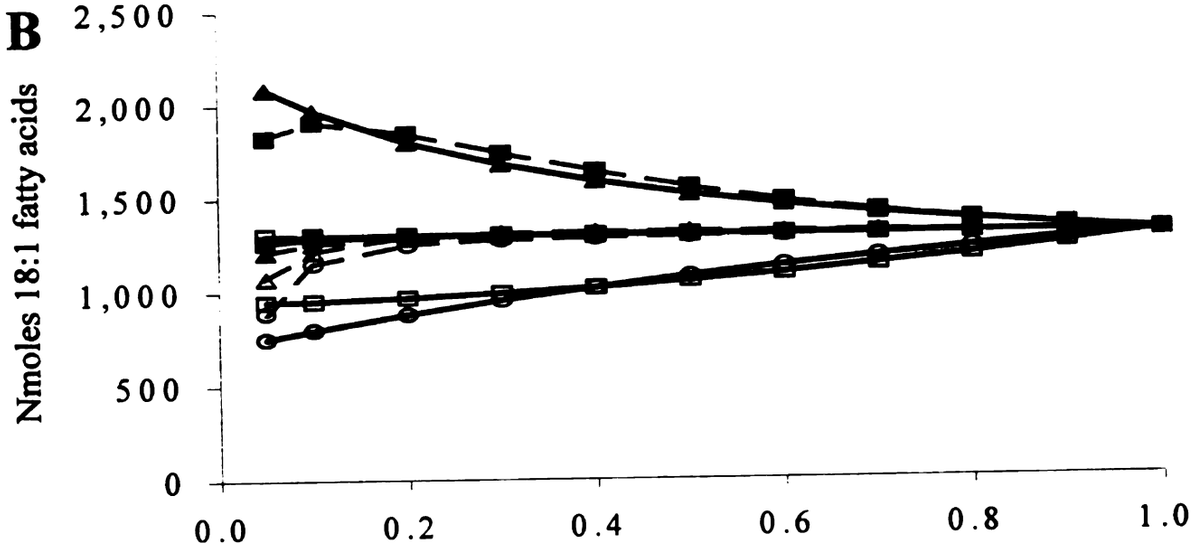
Figure 3.5 Flux through the FAS pathway vs. enzyme activity under states of low flux.

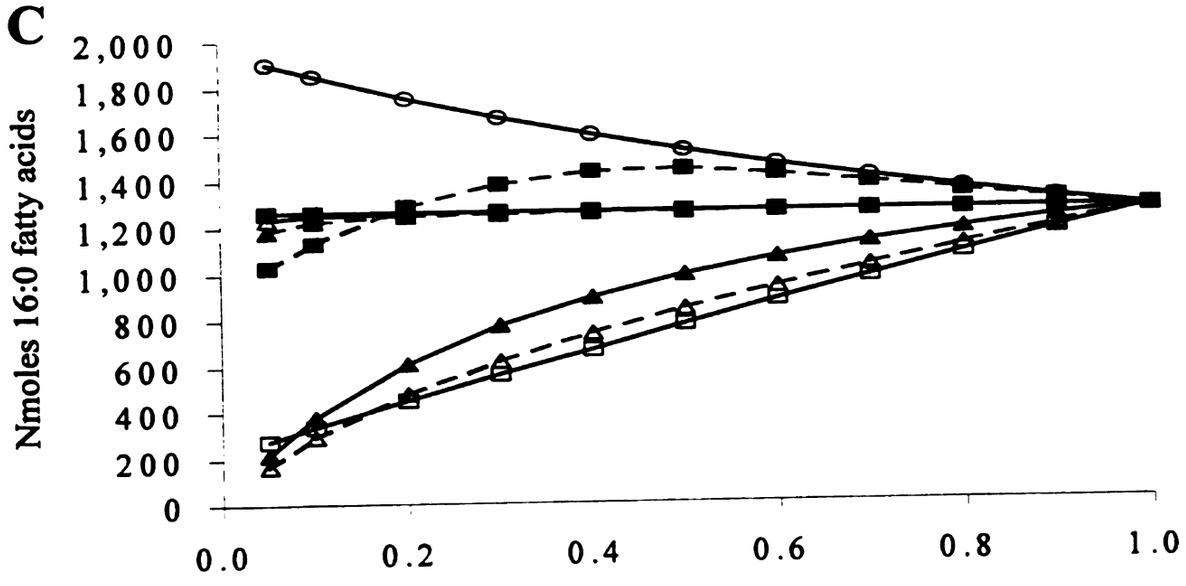
Computer simulations were run with ACCase EF set to 0.125. Then each expression factor of each enzyme was set to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 0.9 and 1.0. The final pool sizes of FAS products, 16:0 and 18:1Δ9 are shown in **theoretical** nmoles. **A)** Total carbon theoretically incorporated into FAS products. KAS II, TE 16:0, ACS, TE 18:1, ACT, MCT, and the Desaturase have essentially no effect on carbon incorporated into total fatty acids. **B)** Flux into 18:1Δ9 products only. KAS I, ACS, TE 18:1, ACT, MCT, and the Desaturase have essentially no effect on carbon incorporated into 18:1Δ9 fatty acids.
C) Flux into 16:0 products. ACS, ACT, MCT, and the Desaturase have essentially no effect on carbon incorporated into 16:0 fatty acids.

Legend




A
Carbon in total fatty acids
60,000
50,000
40,000
30,000
20,000
10,000
0
0.0
0.2
0.4
0.6
0.8
1.0


B
Nmoles 18:1 fatty acids
2,500
2,000
1,500
1,000
500
0
0.0
0.2
0.4
0.6
0.8
1.0


C
Nmoles 16:0 fatty acids
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
0.0
0.2
0.4
0.6
0.8
1.0

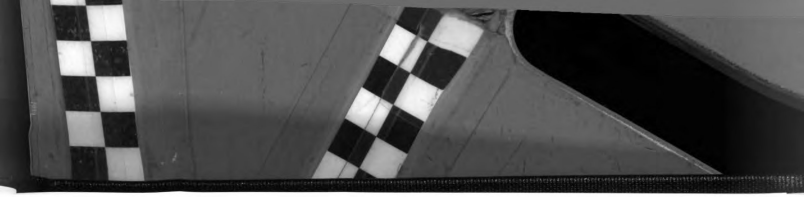
unlike the predictions in the light, KAS III becomes detrimental to total fatty acid production, whereas in the light KAS III is co-limiting. These different roles are upheld when total fatty acid synthesis is broken down into 18:1Δ9 and 16:0 components. In the dark, ACCase and KAS II are limiting, and KAS III and the 16:0 thioesterase become detrimental. In the case of 16:0 production in the dark, ACCase and KAS I are limiting, the 16:0 thioesterase is co-limiting. KAS II is detrimental and KAS III borders on detrimental. These relationships are summarized in Table 3.7. Similar to FAS simulation in the light, a different set of enzymes limit and co-limit 18:1Δ9 and 16:0 production. However, these sets are different in the light and dark. That is, KAS II and, to some extent, ACCase, limit 18:1Δ9 production in the dark, but in the light, 18:1Δ9 production is limited by ACCase alone while KAS II is co-limiting. So, not only does the model question the assumption that 18:1Δ9 and 16:0 production are regulated similarly but it also predicts that regulation may be controlled by different sets of enzymes under states of high flux (light) or low flux (dark). There is precedent for this: the regulatory role of RuBisCO varies with irradiance (reviewed in Stitt and Schultze, 1994). These possible complexities in regulation will need to be explored further if we want to engineer plants to produce fatty acids on demand. The model may not be correct, but the predictions are worth exploring further.

Increase of Fatty Acid Synthesis by Enzyme Overexpression

One major purpose in creating this computer simulation was to attempt to predict which enzymes, when overexpressed, would allow the most effective increase in

Table 3.7. Summary of limiting and co-limiting enzymes in the light and dark.

Fatty Acid	Enzyme Role	Light	Dark
Total	Limiting	ACCase	ACCase
	Co-limiting	KAS I KAS III (ACS)	KAS I
	Detrimental	none	KAS III
18:1Δ9	Limiting	ACCase	KAS II ACCase
	Co-limiting	KAS I KAS II KAS III (ACS)	none
	Detrimental	TE 16:0	TE 16:0 KAS III
16:0	Limiting	TE 16:0 KAS III	ACCase KAS I
	Co-limiting	KAS I ACCase (ACS)	TE 16:0
	Detrimental	KAS II	KAS II (KAS III)



fatty acid synthesis. Since overexpression of medium chain thioesterases and desaturases have already been well-explored and shown to change the composition of seed oils (Knutzon et al., 1992; Voelker et al., 1992; and reviewed in Kinney, 1994), increased production of 18:1Δ9 was the focus of this study. The simulation's predictions of overexpression of any one of the four enzymes which limit or co-limit 18:1Δ9 production are shown in Figure 3.6. It is clear from the graphs that the model does not predict a continual increase in yield when any single enzyme is overexpressed. After two-fold overexpression of ACCase, 18:1Δ9 production is only predicted to increase 50%. Flux does not increase substantially further when ACCase is overexpressed up to five-fold. Predictions of double-enzyme overexpression experiments are shown in Figure 3.7. Once again the model makes some sobering predictions: 18:1Δ9 can only be increased two-fold, despite several-fold simultaneous overexpression of two enzymes. The model predicts the highest 18:1Δ9 yield when KAS II/ACCase dual expression is used. KAS I/ACCase overexpression is only slightly lower. An increase in 16:0 is perhaps not as limited. KAS I/KAS III overexpression is predicted to yield a 2.5-fold increase in 16:0, the largest in the simulation.

Since single and double overexpression experiments do not appear to significantly increase FAS, will triple-enzyme overexpression make a difference? The results of simulated overexpression of three of the four major limiting enzymes are shown in Figure 3.8. In this case, The largest increase in pathway flux to 18:1Δ9 is predicted if ACCase, KAS I and either KAS II or KAS III are overexpressed

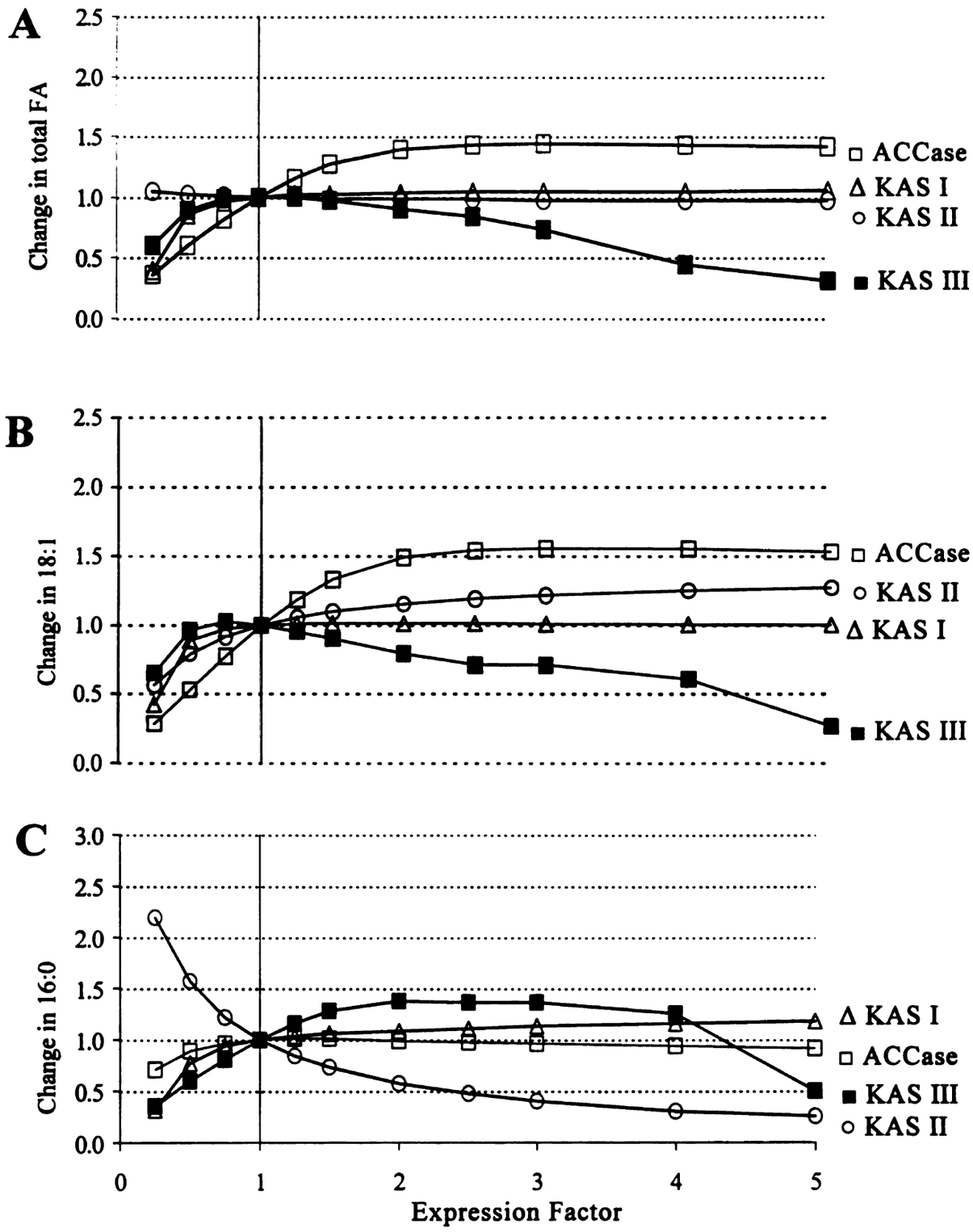


Figure 3.6. The effect of altered expression levels of single enzymes in the model.

The simulation was run with each enzyme expressed 0.05 to five-fold. The ratio of altered-expression production/wild-type expression production vs. EF is plotted. **A)** total carbon in fatty acids, **B)** carbon in 18:1Δ9, **C)** carbon in 16:0.

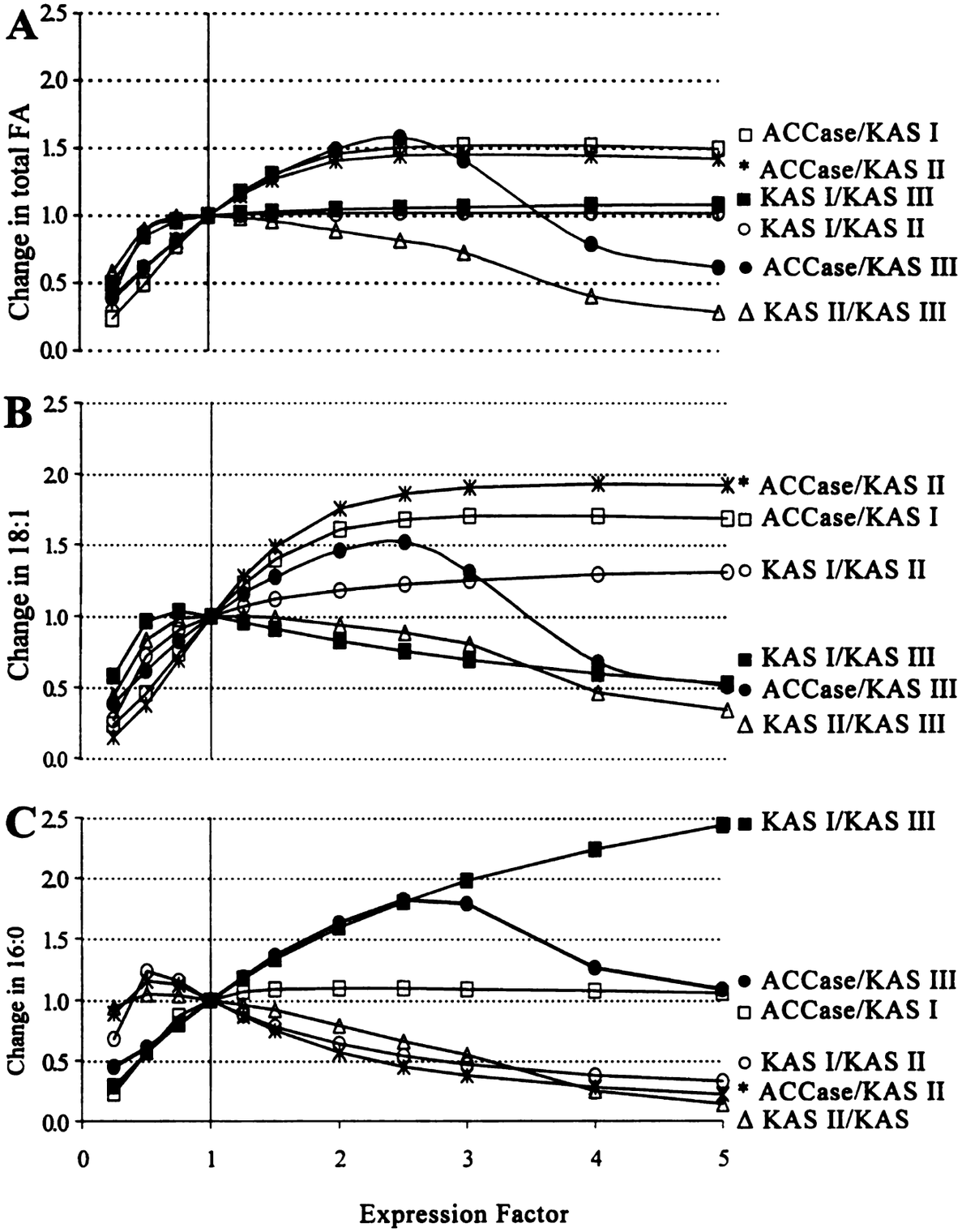


Figure 3.7 .Model predictions of the effect of double enzyme expression.

The simulation was run with each pair of enzymes expressed by 0.05 to five-fold. The ratio of altered-expression production/wild-type production vs. EF is plotted. **A)** total carbon in fatty acids, **B)** carbon in 18:1Δ9, **C)** carbon in 16:0.

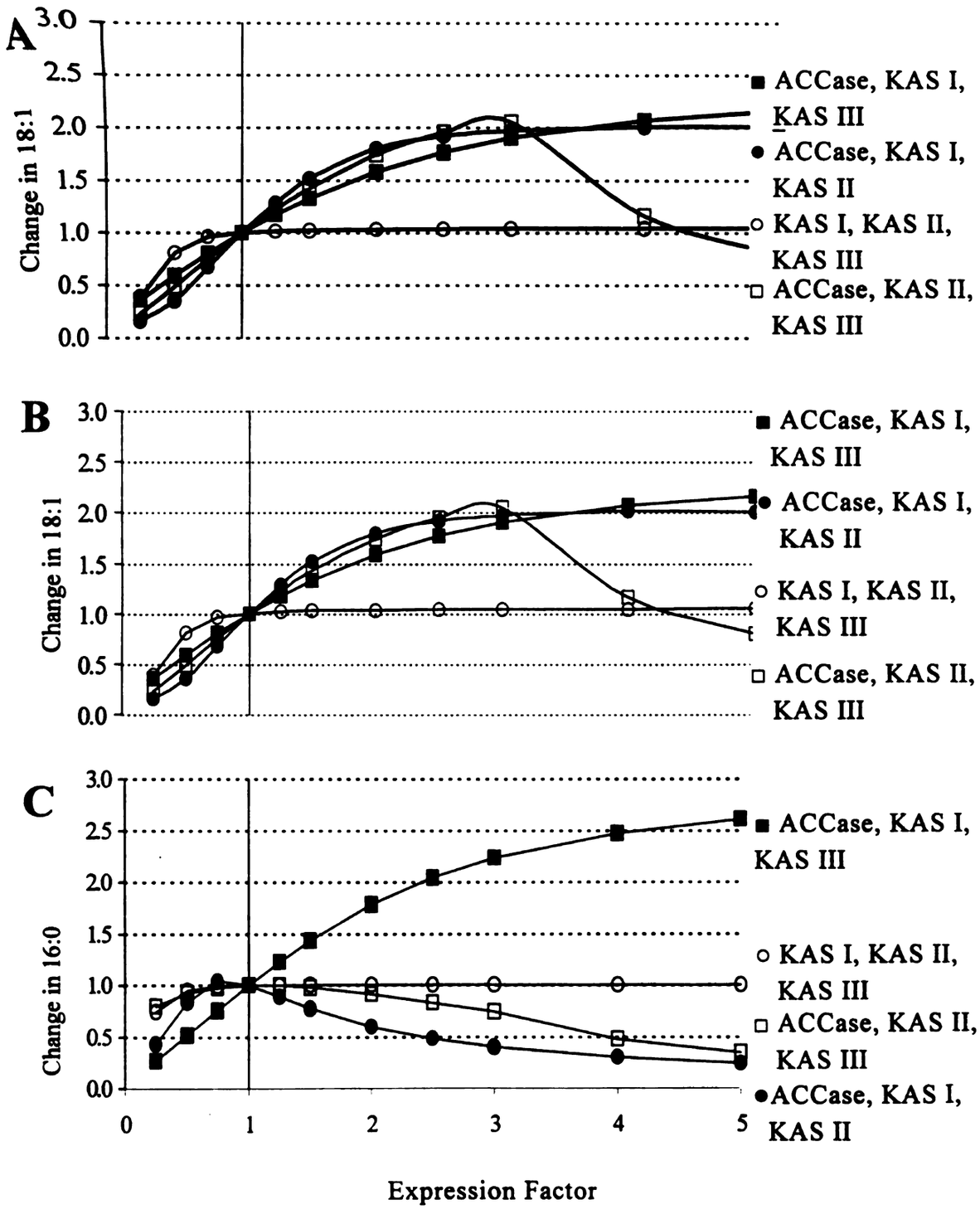


Figure 3.8. Predictions of the effect of the altered expression of three enzymes.

The simulation was run with each triplet of enzymes expressed by the same amount, from 0.05- to five-fold. The ratio of altered-expression production/wild-type production vs. EF is plotted. **A)** total carbon in fatty acids, **B)** carbon in 18:1Δ9 , **C)** carbon in 16:0.

Figure 2.8. Predictions of the effect of the altered expression of three enzymes.

The simulation was run with each triplet of enzymes expressed by the same amount, from 0.05- to five-fold. The ratio of altered-expression production/wild-type production vs. EF is plotted. A) total carbon in fatty acids. B) carbon in 18:1Δ9, C) carbon in 16:0.

simultaneously. Figure 3.9 shows the effect of overexpressing all four of the enzymes simultaneously. In this case, the graph is almost linear indicating these enzymes in consort are limiting according to the scheme in Figure 3.3. Additionally, concurrent five-fold overexpression of ACCase, KAS I, KAS II, and KAS III yields almost a three-fold increase in flux to 18:1Δ9. If this were actually true *in vivo*, it is likely that transport or downstream processes would start to limit TAG production.

Overexpression of the enzymes at the end of the pathway does not increase FAS

One universal method proposed to increase metabolic methods is to overexpress all enzymes of the pathway (Kacser and Acerzena, 1993). Clearly at this time, this is not feasible for FAS as it would require at least 14 enzymes to be overexpressed. However, this highly theoretical treatment also predicts that enzymes near the end of a pathway have more control over the output of the pathway than those at the start. This is due to end products relieving feedback inhibition at the start of the pathway (Kacser and Acerzena, 1993). Therefore, enzymes at the end of the pathway were overexpressed in the Stella FAS model. Equal and concurrent overexpression of the two thioesterases, the desaturase, and KAS II are shown in Figure 3.10. It is clear that overexpression of these terminal enzymes does not significantly alter total carbon in fatty acid or the 18:1Δ9/16:0 ratio.

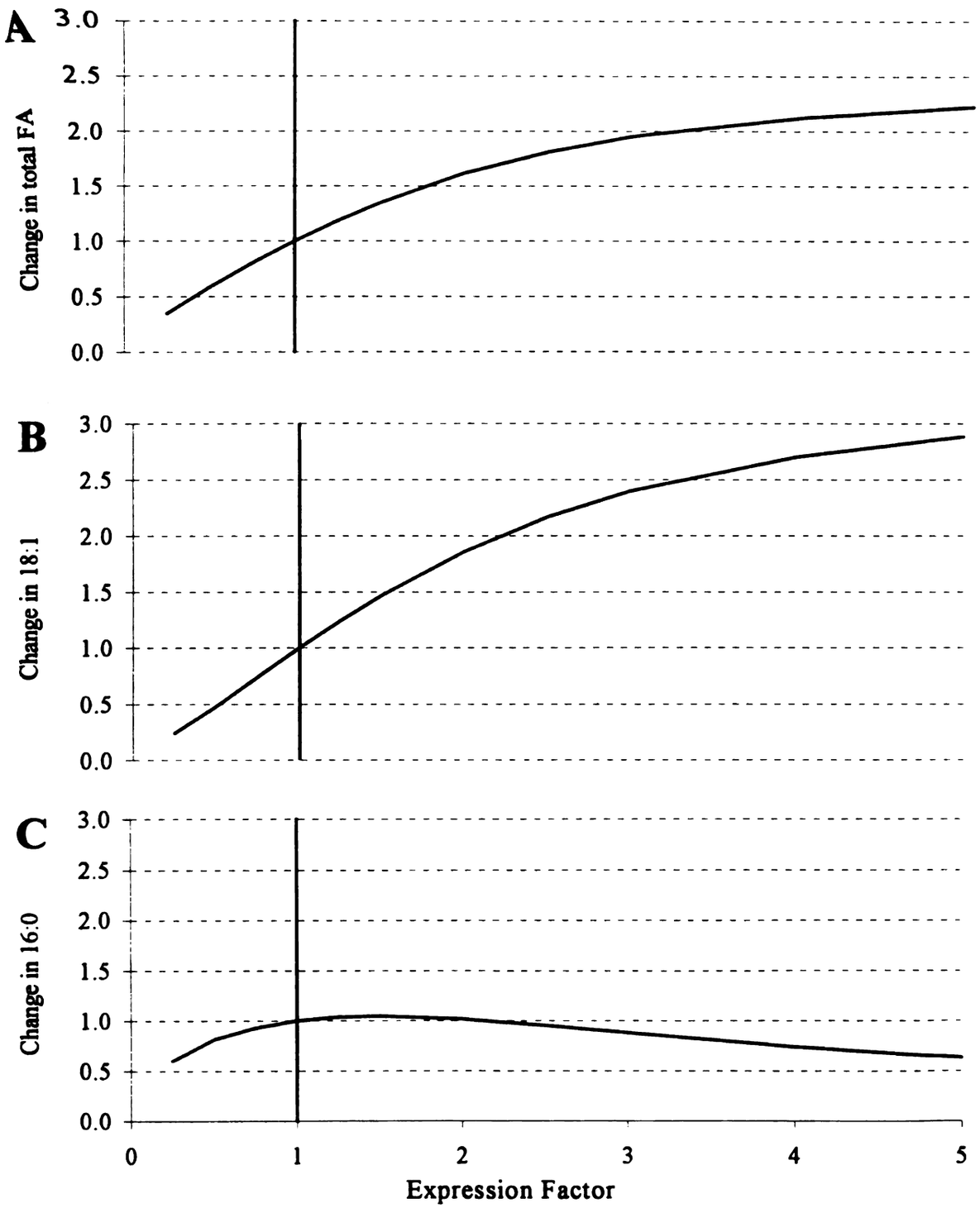


Figure 3.9. Concurrent altered expression of ACCase, KAS I, KAS II, and KAS III.

The simulation was run with the enzymes expressed the same amount, from 0.05- to five-fold. The ratio of altered-expression production/wild-type expression production vs. EF is plotted. **A)** total carbon in fatty acids, **B)** 18:1Δ9 , **C)** 16:0.

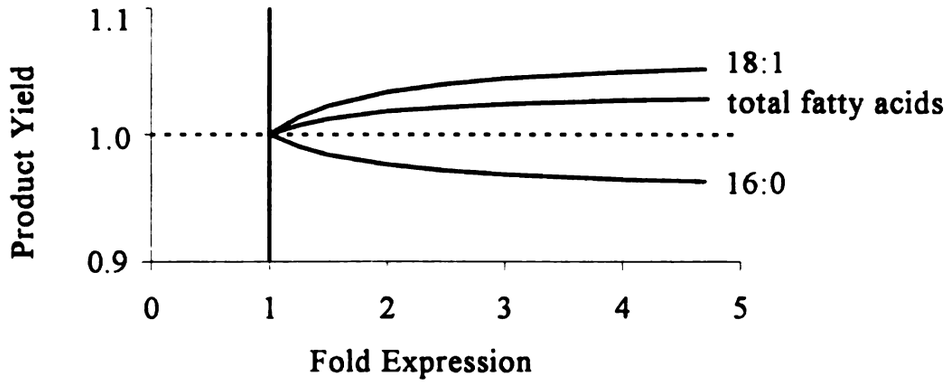


Figure 3.10. Overexpression of the enzymes at the end of FAS pathway.

TE 16:0, TE 18:1, KAS II, and desaturase were all simultaneously overexpressed one- to five-fold. The amount of total, 16:0, and 18:1 fatty acids produced during overexpression were compared to the amount of total 16:0, and 18:1 fatty acids produced during wild-type expression. The ratio of overexpression production/wild-type production vs. level of expression is plotted. Only a slight increase in total fatty acids is seen.

Discussion

In many modeling situations it is easy to forget that the model is not the *in vivo* system. One purpose of any model is to allow us to understand the system under study more completely and to point out areas where further work is needed. This model of FAS and the computer simulations are no exception. The model presented above is a vast simplification of a living plant system, and therefore the model's predictions may not hold *in vivo*. However, the model raises some interesting questions. These questions are 1) is the accumulation of 16:0 regulated differently than the accumulation of 18:1Δ9? and 2) is FAS regulated in the same manner under states of high flux (light) and low flux (dark)? While these questions may not be novel questions, investigating them becomes more important because the model raises them. A short diversion into science philosophy will explain this.

Progress in science depends on making worthwhile observations that either help confirm or (even better) invalidate conceptual models (Popper, 1953; Platt, 1964). So, when testing a conceptual model there are criteria which make a test, usually a bench experiment, worth doing. One criterion of a "good" test is that any prediction made from the conceptual model must be logically deducible (Giere, 1984). This is sometimes difficult to ensure with a simple model; however, with a complex model system, such as the FAS model presented here, it would be essentially impossible to integrate and check every assumption and connection without some sort of external control. The computer simulation provides this

consistent and complete check on deducibility. Therefore, the issues raised in the computer simulations are logical and deducible consequences of the model and not simply well-reasoned, though perhaps humanly flawed, opinion.

Model validation relies heavily on in vivo KAS III overexpression experiments

The KAS III overexpression data are critical for model validation. This is due to the non-intuitive nature of the results - both in the model and *in vivo*. Certainly the model's predictions do not quantitatively match reality, however they do qualitatively. The *E. coli* KAS III homolog, *fabH*, was under and overexpressed in *E. coli* by Tsay et al. (1992) as well as Verwoert et al. (1995). Membranes were analyzed for 14:0, 16:0, 18:0 and 18:1 (*cis*-vaccenic) fatty acids. In the case of KAS III underexpression, 16:0 in *E. coli* membranes decreased by slightly over 12% while 18:1 (*cis*-vaccenic) increased by 18%. With overexpression of KAS III, membrane 14:0 increased 10-fold, 16:0 increased 24%; conversely 18:0 decreased 60% and 18:1 (*cis*-vaccenic) decreased 86% (Tsay et al., 1992). The authors did not comment on growth rate. Verwoert et al. (1995) observed the same pattern and also reported significantly slower growth of *E. coli* overexpressing *fabH*. They proposed the accumulation of 14:0 may be a stress response of *E. coli* and not a direct consequence of KAS III overexpression (Verwoert et al., 1995). However, if KAS III overexpression decreases fatty acid availability for cell membranes, signaling, and growth, then this would probably be stressful *in vivo*.

The KAS III overexpression simulations used to validate the model suggest some additional experiments for existing overexpressing plants. In a wild-type plant and in the normal model simulation over half the ACP is non-esterified (ACP-SH). KAS III overexpression decreases the amount of ACP-SH to very low levels in the Stella model. KAS III overexpression in tobacco plants results in a two-fold increase in total ACP (Jaworski and Hinneburg-Wolf, 1998). Is the *in vivo* increase enough to maintain wild-type ratios of acyl-ACP pools? Or is ACP expression at its maximal level.? Given the model predictions of very low ACP-SH, it is more probable that ACP expression is as high as possible.

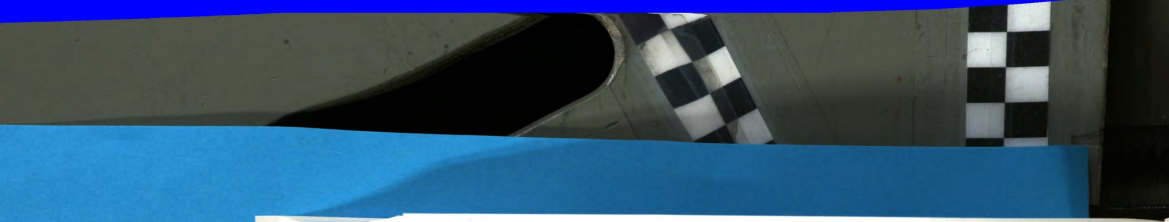
Limiting and co-limiting enzymes in the light

Most of the predictions the model makes in Figures 3.4 and 3.5 are intuitive. Post-Beittenmiller et al. (1991, 1992) determined that ACCase is a limiting factor in total fatty acid synthesis in the light, and the simulation reflects this as well. The model predicts that decreasing the activity of the 16:0 thioesterase (TE) will increase the amount of 18:1Δ9 produced. This makes sense, since 16:0-ACP is a substrate for both the 16:0 thioesterase and KAS II reactions. Decreasing the 16:0 thioesterase activity increases the amount of 16:0-ACP available for KAS II elongation. Likewise, the predictions the simulation makes for the limiting and co-limiting steps of 16:0 production are logical. The final step catalyzed by the 16:0 TE is limiting. In the production of 16:0, the initial condensation, performed by KAS III, is also limiting. Here the bottleneck seems to be the initiation of fatty acid synthesis rather than the co-limiting reactions of malonyl-CoA synthesis and

intermediate elongations catalyzed by ACCase and KAS I respectively. In a manner similar to the production of 18:1Δ9, decreasing the activity of the competing KAS II reaction in the final branch point of 16:0 production increases the production of 16:0.

One surprising prediction the model makes is apparent when the simulation's predictions for the regulation of 18:1Δ9 and 16:0 production are compared. The production of 18:1Δ9 is limited by ACCase, whereas the production of 16:0 is limited by the thioesterase and KAS III. Since ACCase becomes co-limiting when the production of 16:0 is considered and KAS III becomes co-limiting when the production of 18:1Δ9 is considered, it appears that ACCase and KAS III may switch regulatory roles when different products are examined. KAS I is co-limiting in the production of both 18:1Δ9 and 16:0. Thus, FAS may be regulated differently depending on whether the production of 16:0 or 18:1Δ9 is considered. Until now, 18:1Δ9 and 16:0 fatty acids have been combined when considering the regulation of FAS. The pathway of fatty acid synthesis is taught and studied as one pathway, in spite of different exit points. This assumption needs to be more fully examined.

In many cases, oil composition has been altered by changing the expression level of the thioesterases and desaturases (reviewed in Kinney, 1994). For example, Knutzon et al. (1992) antisensed the 18:0 desaturase and increased the 18:0 level in seeds. Overexpression of an acyl-ACP thioesterase increased the amount of



16:0 and 18:0 (Kinney, 1994). In many of the cases where enzyme overexpression studies have been done, results for both 16:0 and 18:1Δ9 have been reported. Often, the model predicts only part of the results correctly (Table 3.6). For example, when KAS II is antisensed, 16:0 increases approximately three-fold and there is essentially no significant change in the amount of 18:1Δ9 (Kinney and Hitz, 1995). The model predicts the increase in 16:0, but predicts a 50% decrease in 18:1Δ9. Likewise, thioesterase overexpression causes a two-fold increase in 16:0 and no significant change in 18:1Δ9 (Kinney and Hitz, 1995). The model correctly predicts the increase in 16:0, but predicts a 25% decrease in 18:1Δ9. In both cases, the model predicts reduced levels of 18:1Δ9 which are not seen *in vivo*. This may be due to strict requirements for 18:1Δ9 which control its production *in vivo*.

It is clear from the above discussion that there may be a very fine balance between FAS enzyme activities, not only for ACCase, KAS I, KAS II, and KAS III, but also for the set of terminal enzymes: the thioesterases and the desaturase. In addition, slight differences in enzyme substrate affinities may change the composition of the fatty acids produced and exported by the plastids. Alternatively, the enzymes of fatty acid synthesis may be flexible and subject to fine regulation. Both scenarios are speculative at this point in time. If varying substrate affinities are important for regulation, there are both positive and negative consequences for manipulation of seed oils. On the positive side, if small changes are needed, then perhaps the enzymes involved can be engineered for

specificity. For example, Shankin and co-workers (Cahoon et al., 1997) have changed a Δ6 desaturase to a Δ9 desaturase and vice versa with five or fewer amino acid changes. On the negative side, a consequence of fine and precise control is that precise biochemical data for the enzymes will be required to model FAS on a quantitative level. Furthermore, the enzyme data must be valid *in vivo*. Given this requirement, it is perhaps surprising that the model mimics reality in any way.

Modeling, as a technique to understand systems, is widely practiced. Any abstraction or simplification of a problem is a model. Formalized mathematical models are also widely used (for examples, see Bender, 1978). The model of fatty acid synthesis presented in this chapter synthesizes data from three sources: biochemical data, successful and not-so-successful transgenic expression experiments, and a computer simulation package. The data presented by the model highlight areas where further work is indicated in order to engineer fatty acids, namely, more precise biochemistry. The model points out one area where reconceptualization of the FAS pathway may be in order: are there separate signals and mechanisms for 18:1Δ9 and 16:0 production? Finally, the model yields a sobering prediction if increased total flux is the goal: overexpress at least three enzymes. While the predictions of the model may be incorrect, modeling is an iterative process. This model, with its predictions and questions, is a starting point for further investigations.

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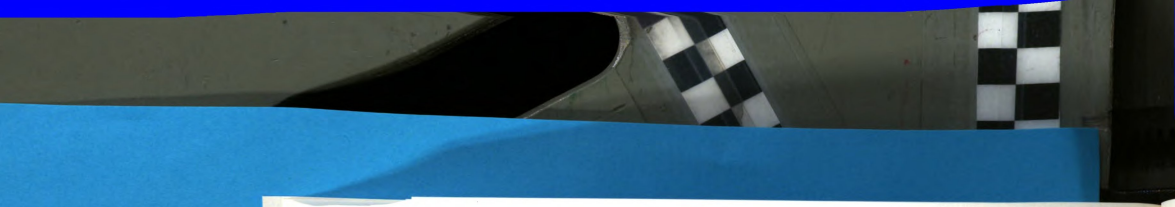


Chapter 4

EXPRESSION OF β-CT AND THE STRUCTURE OF *ACCD*

Abstract

Plastidial acetyl-CoA carboxylase (ACCase), one of the rate determining enzymes of fatty acid synthesis (FAS), is encoded by four different genes in dicot species. Although three of these genes are nuclear-encoded, the fourth, *AccD*, is encoded on the plastid genome. Originally the goal of the work presented in this chapter was to create transgenic tobacco plants with variable levels of the *AccD* protein, β-carboxylase transferase (β-CT). These plants would have allowed investigation of 1) coordinate regulation of ACCase subunits, 2) mutations in *AccD* structure, and 3) the effects of low rates of FAS. This chapter presents two unsuccessful attempts to relocate *AccD* to the nuclear genome. The first attempt used tobacco *AccD* behind the promoter and transit peptide from the small subunit of RuBisCO, while the second attempt used the pea *AccD* behind a 35S promoter and the transit peptide from the small subunit of RuBisCO. In neither set of transformed plants was any expressed β-CT protein detected, nor was message from the constructs detected.

Sequence analysis shows the pea and tobacco *AccD* sequences to be AT-rich with non-plant codon usage. In addition, many motifs which may cause non-desired transcript processing such as polyadenylation or degradation are found in the

coding regions. While none of these motifs is known to be sufficient for processing, the abundance of possible motifs may be the cause of non-expression of the protein.

In addition, this chapter presents preliminary sequence data for the *AccD* gene of *Oenothera hookeri*. The 5' end of the *Oenothera* sequence has essentially no conservation with the 5' end of the other *AccD* genes and contains many repeated motifs from 9 to 45 bp in length. The *AccD* gene was also mapped on the *Oenothera* plastidial genome. Rather than being between the *rbcL* and *petA* genes as in the tobacco, pea, pine, and liverwort plastidial genomes, it is located between the *petA* and *atpA* genes at the opposite end of the large single copy region of the plastome.

Introduction

Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) is one of the rate limiting enzymes of fatty acid synthesis (FAS). ACCase is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the building block of FAS. ACCase contains three functional subunits: a biotin carrier protein (BCCP), a biotin carboxylase (BC), and a carboxyl-transferase (CT). There are two different types of acetyl-CoA carboxylase in plants: a multi-subunit form and a multi-functional form. In dicots and most monocots both forms exist: the multi-subunit form is located in the plastids, while the multi-functional form is presumably located in the cytosol. In grasses, only the multi-functional form of ACCase exists. In this case, there are at least two isoforms of this greater-than 250 kDa protein, one of which is imported into plastids and used in *de novo* FAS and which therefore replaces the multi-subunit form found in most plant plastids.

ACCase structure and localization have been recently reviewed by Sasaki et al. (1995). The BCCP and BC subunits of the multi-subunit ACCase are each encoded by nuclear genes. In the multi-subunit ACCase, the carboxyl-transferase function is encoded by two genes: *AccA* encodes the α-CT subunit and *AccD*, the subject of this chapter, encodes the β-CT subunit. While α-CT is a nuclear-encoded gene, the β-CT gene is located on the plastidial genome. It is the only gene of plant lipid metabolism (of more than 100) which is plastome-encoded

and perhaps the only plastidial gene of known function which is not involved in photosynthesis or protein synthesis. Of the four genes which encode the multi-subunit form, only one gene has been identified for each of the two CT and the BC subunits, while at least two BCCP-encoding genes have been identified (Mekhedov et al., in preparation).

Proteins with multiple subunits may have coordinate expression of the genes encoding the subunits. For example, the gene which encodes the small subunit of RuBisCO is a nuclear gene, whereas the gene which encodes the large subunit is a plastidial gene. When the large subunit of RuBisCO is not available, the small subunit is imported into the chloroplast but rapidly degraded (Schmidt and Mishkind, 1983). Thus, RuBisCO subunits are kept in strict stoichiometric amounts. While the genes which encode the ACCase subunits are transcribed at the same time and thus expressed coordinately (Nikolau and Wurtele, 1998), underexpression of BC does not affect BCCP accumulation until very little BC is present (Shintani et al., 1995). However, since the *AccA* and *AccD* genes code for different subunits of the same functional component, over- or underexpression of these genes may still affect gene expression or protein stability of the other component.

Numerous genes from the ancestral plastidial genome have been lost or transferred to the nuclear genome during the evolution of plants (for review see Gray, 1992). Generally, the transfer of a single gene to the nucleus has occurred

in parallel in independent lineages (Martin et al., 1998). That is, even though there have been independent endosymbioses of chloroplasts, the same genes tend to be lost. Although *AccD* is present in dicot and most monocot plastidial genomes, it has been removed multiple times from the genomes of Cyanophora, Odontella, Euglena, and Porphyra (Martin et al., 1998). Other genes which have been removed with the same frequency are the *ndh* genes and the *cysA* and *cysB* genes (Martin et al., 1998). In the monocots, both *Zea mays* and *Orzya sativa* have vestiges of the *AccD* gene in their plastid genomes. However, in these species and other grasses, the present-day functional plastidial ACCase is a multi-functional, not a multi-subunit, enzyme.

The original purpose of the work of described in this chapter was two-fold 1) determine if β-CT and α-CT subunits accumulate in strict stoichiometric amounts similar to the large and small subunits of RuBisCO, and 2) alter the expression of β-CT using a light-regulated promoter, remove the *AccD* gene from the plastidial genome, and use those plants to study the effects of altered β-CT and presumably ACCase availability. This chapter describes unsuccessful attempts to move the β-CT gene from the plastidial genome to the nuclear genome. In addition, the chapter 1) presents evidence that the pea and tobacco *AccD* genes must be re-engineered to be functional in a nuclear background, 2) presents the sequence of the *Oenothera hookeri AccD* gene, 3) analyzes the tobacco and pea *AccD* sequences for possible transcript instability motifs and

polyadenylation signals, and 4) maps the *AccD* gene on the *O. hookeri* plastidial genome.

Materials and Methods

Materials

Restriction enzymes used in these studies were from Boheringer Mannheim. *E. coli* strains used were DH5α and DH5α'. *A. tumefaciens* strains used were LBA4404 and ABI (Monsanto). The *N. tabacum* variety was SR-1, and the pea variety was Little Marvel. Accession numbers for the genes involved are *N. tabacum*: Z00044, bases 59793 to 61331; *P. sativum*, X54750; *P. thunbergii* D17510; *B. napus*, Z50868; and *M. polymorpha* (liverwort) X04465.

Surface Sterilization of Seeds

Seeds were agitated in 10% Clorox bleach with 0.001% Tween-20 for 10-20 min, washed three times for 5-10 min each with sterile H_2O.

Construction of β-CT transformation vectors

The construction of plasmids p0921-3.3 and p0921-3.5 is shown in Figure 4.1. Standard protocols were used (Sambrook et al., 1989; Magic Minipreps, Promega; GeneClean II, Bio101, La Jolla, CA). One ng of genomic *N. tabacum* DNA (gift of Keith Roesler) was amplified with *Taq* polymerase (Boheringer Mannheim) using primers JO360 (5'- CAU CAU CAU CAU ATG CAT GCA

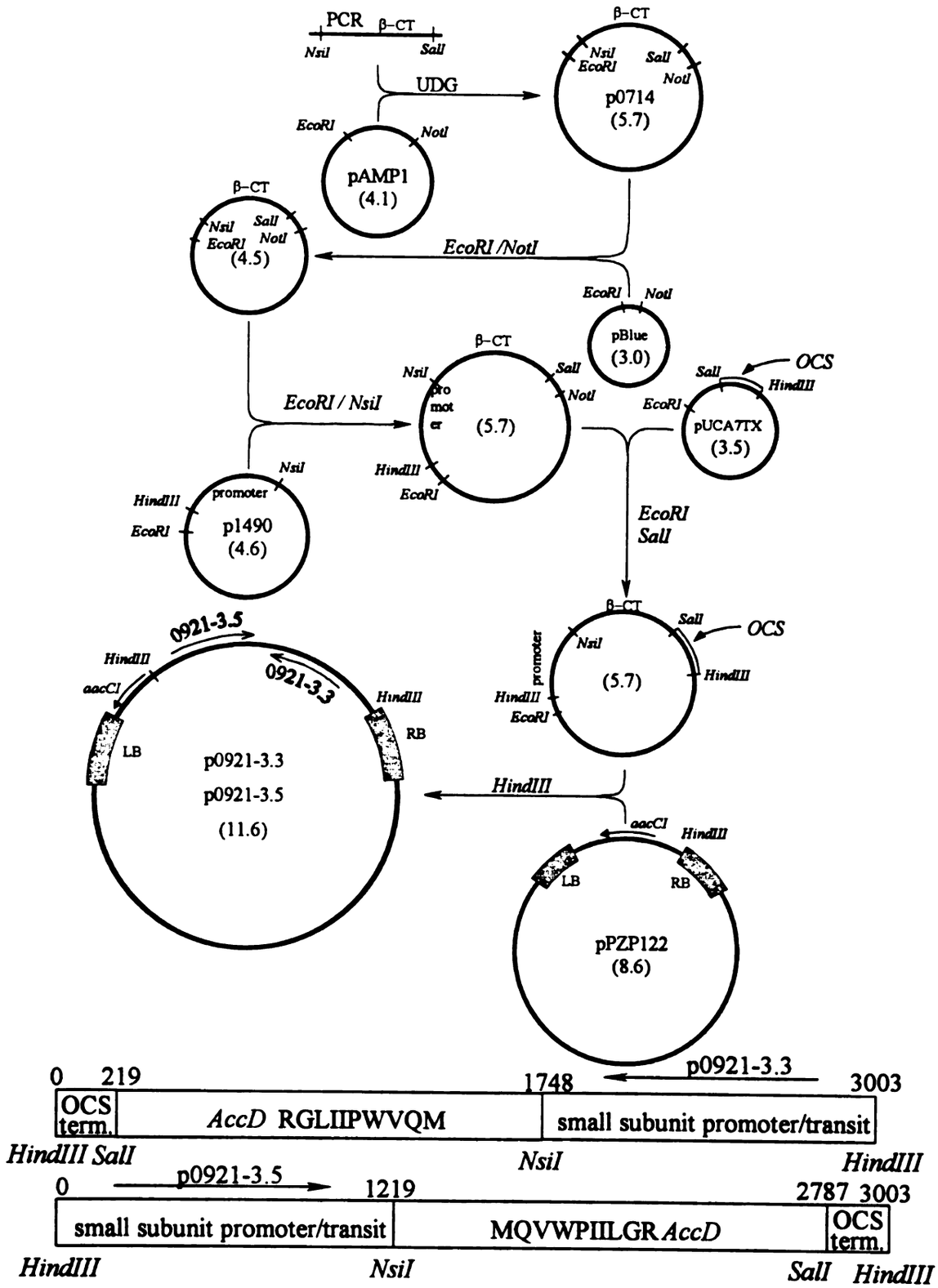


Figure 4.1. Construction of plasmids p0921-3.3 and p0921-3.5.

GGT GTG GCC AAT AAT TTT AGA GCG AAT GAC TA) and JO329 (CUA CUA CUA CUA GTC GAC TAC TTG ATT TTG CTT GA) for 30 cycles. Primer JO360 contains the sequence encoding the first 10 amino acids of the mature small subunit of RuBisCO in tobacco. Both primers were from the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility (East Lansing, MI) and contained deoxyUMP (U), used in conjunction with uracil DNA glycosylase to insert the PCR product into the pAMP1 vector (Gibco/BRL). Each PCR cycle consisted of a denaturation step (94°C, 1 min), a primer annealing step (45°C, 2 min) and an elongation step (72°C, 1.5 min). The thermocycling was preceded by an initial denaturation step of 94°C for 3 min and followed by a final elongation of 10 min at 72°C. The PCR product was inserted into the pAMP1 vector (Gibco/BRL) according to the manufacturers protocol. This plasmid was designated p0714. The *EcoRI/NotI* fragment from this vector was inserted into pBluescript II KS+ (Stratagene) The *EcoRI/NsiI* fragment from p1490 (Post-Beittenmiller et al., 1989) was added to the pBluescript II KS+ plasmid with the β-CT gene. This *EcoRI/NsiI* fragment contains the RuBisCO small subunit promoter and transit peptide. The termination signal was provided by insertion of the 3-kb *EcoRI/SalI* fragment from the pBluescript II KS+ construct into pUCA7-TX (provided by C. Gatz, University of Gottingen, Germany). The *HindIII* fragment containing the promoter, transit peptide, β-CT gene, and OCS terminator was inserted into the plant transformation vector pPZP122 (Hajdukiewicz et al., 1994). The plasmid with the β-CT gene construct coding in the same direction of the *AacC1* coding region was denoted p0931-3.3,

the plasmid with the β-CT construct coding in the opposite direction was denoted p0931-3.5. *AacC1* codes for gentamycin resistance. The constructs were sequenced by the Michigan State University DNA Sequencing Facility (East Lansing, MI) to verify that the junction between the transit peptide, additional amino acids, and the start of the *AccD* gene remained in frame.

A second construct was designed and built by Oskar Martínez de la Ílarduya (University of California, Riverside) and is depicted in Figure 4.2. Pea (Little Marvel) genomic DNA was amplified using custom primers from GibCO/BRL, AccM.DO (5'-NN GAA TTC TC ATG AAA AGA AAG TGG) and AccM.UP (5'-CTT CAT TTA TCA TTT GTC GTT T). The sense primer was designed to amplify 130 bases prior to the published start site of the pea genomic sequence. The *EcoRI* and *SacI* sites added during PCR were used to insert the PCR product, pea β-CT*, into pUC19. The *BspHI/SacI* fragment which contained the ATG start codon was transferred to pMON25661 to add the small subunit transit peptide and E93' termination signal. The *BglII/NotI* fragment from this vector was then ligated into pMON10098 to provide the 35S promoter and plant transformation sequences. The construct was sequenced to verify junctions.

Plant Transformation

Standard protocols were used for plant transformation. Surface sterilized leaf discs or strips were incubated in *A. tumefaciens* culture containing the desired plasmid, briefly blotted, then transferred to MS104 plates (4.31 g/l MS Salts

Figure 4.2. Pea β-CT* construct.

This construct has a 35S promoter with an E93' termination signal. In addition to the published coding region of the pea *AccD* gene, this construct carries 130 bases 5' of the coding region. The construct is in the pMON10009 vector (Monsanto) which contains sequence for plant transformation and kanamycin resistance, also behind a 35S promoter.

(Gibco/BRL); 30 g/l sucrose; 100 mg/l *myo*-inositol; 10 mg/l thiamine-HCl; 1 mg/l nicotinic acid; 1 mg/l pyridoxine-HCl; 8 g/l phytoagar (Gibco/BRL)) with 1.0 μg/ml benzyladenine (BAP) and 0.1 μg/ml naphaleneacetic acid (NAA). After two to four days, leaf pieces were transferred to MS104 plates with antibiotics (100 mg/l carbenicillin and either 150 mg/l kanamycin or 100 mg/l gentamycin depending on vector), and the hormones BAP and NAA. Shoots from these leaf pieces were transferred to root-induction media (MS104 with 100 mg/l carbenicillin and either 150 mg/l kanamycin or 100 mg/l gentamycin depending on vector), allowed to root, then transferred to soil.

Seed Germination Assays

Surface sterilized seeds from p0921-3.3- and p0921-3.5-transformed plants were germinated on seed germination medium (Fisher and Guiltinan, 1995) in the presence of gentamycin. Surface sterilized seed from β-CT*-transformed plants were germinated on filter paper wetted with 0.1X MS-Salts in the presence of 100 mg/l kanamycin (Knutzon et al., 1992). Seeds were scored resistant if they were green after two weeks.

Neomycin phosphostranferase assays

Neomycin phosphotransferase (NPT) assays were performed using a kit from Agdia, Inc. (Elkhart, IN) according to manufacturer's protocol.

Western Blotting

Protein was extracted from approximately 20 mg (fresh weight) tissue from transformed plants by freezing, grinding in 1X SDS sample buffer (0.25 M Tris, pH 6.8, 8% SDS, 20% glycerol, 0.04% bromphenol blue, 5 mM DTT), then boiling for 10 min. Clarified extract was separated on 10% or 7.5% SDS-PAGE gels and transferred to membranes and detected according to standard protocols (Hoefer Scientific Instruments, 1994). All antibodies were used at 1:1000 and were all gifts from Linda Savage. The MSU-1 and UMC-1 antibodies were prepared from *E. coli*-expressed tobacco β-CT protein. The MSU6(E) antibody was prepared from *E.-coli*-expressed pea β-CT protein and could detect as little as 10 ng protein. Neither tobacco nor pea antibodies cross-reacted with each other.

RNA Extraction 1

One hundred to 250 mg (fresh weight) tissue from young leaves was frozen in liquid N_2 and ground to a powder. Total RNA was extracted similarly to the method described in Shintani (1996). One ml of 80°C homogenization buffer (200 mM sodium borate, 30 mM EGTA, 1% SDS, 1% deoxycholate, 2% PVP 40,000, 10 mM DTT, pH 8.0) was added to the ground powder, followed by 1 ml of phenol: chloroform (1:1). The mixture was vortexed for several seconds, then centrifuged at 10,000 x *g* for 15 min. The aqueous layer was removed and saved. The organic phase was back-extracted with 1 ml H_2O, spun for 15 min at 10,000 x *g*, and the aqueous phase combined with the previously-saved aqueous phase.

The combined aqueous phases were extracted with one vol of chloroform and spun for 5 min at 2700 rpm to separate phases. The aqueous layer was adjusted to 2M LiCl and incubated overnight on ice. The precipitate was collected by centrifugation at 10,000 x *g* at 4°C. RNA pellets were dried under vacuum. Pellets were resuspended and reprecipitated by the addition of 1/10 vol of 2M potassium acetate and two vol of ice cold ethanol. The precipitate was recovered by centrifugation at 10,000 x *g* for 20 min, the pellet dried under vacuum, and resuspended in 200 µl H_2O. Total RNA was stored at -20°C for less than one week.

RNA Extraction 2

Fifty to 100 mg tissue from young leaves was frozen in liquid N_2 and ground to a powder. Ground powder was kept in liquid N_2 until all samples were ground. One ml TRIZOL® reagent (Gibco/BRL) was added and tubes were incubated 5 min, then spun 10 min at 12,000 x *g*, 4°C to pellet debris. The supernatant was transferred to a fresh tube and total RNA isolated using the manufacturer's protocol.

Northern Probes Construction

Engineered β-CT: Plasmid p0714 (from Figure 4.1) was digested with *NsiI/SalI*. This 1.2-kb fragment contains the first 10 codons from the mature small subunit of RuBisCO and the tobacco *AccD* coding region. This fragment was isolated using a Geneclean II kit (Bio 101 Inc., La Jolla, CA), then labeled with random

hexamers using ^{32}P-CTP. Unincorporated nucleotides were removed with a G-50 spin column (Sambrook et al., 1989).

Small Subunit Riboprobe: A linearized plasmid containing the tobacco RuBisCO small subunit coding region (gift of David Shintani) was transcribed in the antisense direction using α^{32}P-UTP and a MAXIscript™ In Vitro Transcription kit according to the manufacturer's protocol (Ambion). This yielded a 250-bp transcript, of which 180 bp matched the small subunit coding region; the remainder matched the multiple cloning site from pBluescript II KS+. Unincorporated nucleotides were removed with a G-50 spin column (Sambrook et al., 1989).

35S/β-CT probe: The 35S/β-CT* plasmid from Figure 4.2 was digested with *BglII/SacI*. This fragment contains the SSU transit peptide and the pea β-CT coding region. The fragment was inserted into pBluescript II KS+ cut with *BamHI/SacI*. This plasmid was transformed into *E. coli* to amplify it, then digested with *SacI/SmaI*. The resultant 2.1-kb fragment was isolated using a JETSORB kit (Genomed, Research Triangle Park, NC) and labeled with [α-^{32}P]dATP using Ambion's Strip-EZ™ DNA kit (Ambion, Austin, TX). Unincorporated nucleotides were removed with a Micro Bio-Spin® P-6 column (Bio-Rad Laboratories, Hercules, CA) using the manufacturer's protocol.

Kanamycin probe: Vector DNA from pMON28860 (Monsanto) was digested with *XhoI*. This yielded a 1.4-kb fragment containing the kanamycin-resistance coding region and the 3' nopaline synthase terminator region (NOS). This fragment was inserted into pBluescript II KS+ cut with *XhoI*. The plasmid was then transformed into *E. coli* to amplify it. Plasmid DNA was digested with *XhoI*. The 1.4-kb fragment was isolated with a JETSORB kit (Genomed, Research Triangle Park, NC), and the resultant fragment labeled with [α-^{32}P]dATP using Ambion's Strip-EZ™ kit. Unincorporated nucleotides were removed with a Micro Bio-Spin® P-6 column (Bio-Rad Laboratories, Hercules, CA) using the manufacturer's protocol.

Sequencing and Sequence Analysis

Plasmid pOjSalE (from B. Sears, Michigan State University) was used for sequencing the *O. hookeri AccD* gene. This plasmid contains 7 kb of the plastidial genome in pRL498 (Elhai and Wolk, 1988). Sequencing was performed by the Michigan State University DNA Sequencing Facility (East Lansing, MI) using primers made by the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility (East Lansing, MI). The gene was sequenced in one direction, from one to five times, depending on sequence data. *Oenothera* sequence data were assembled using a combination of tools: BLAST (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov), GCG Sequence Analysis Software Package 7.3-Unix (Genetics Computer Group, Madison, WI), and SeqWeb version 1.1 (Genetics Computer Group, Madison, WI). Unpublished *Oenothera*

sequence for alignment and comparison was also provided by R. G. Herrmann (Ludwig Maximilians University) in collaboration with Barbara Sears (Michigan State University).

Mapping of AccD on the O. hookeri plastome

A DNA blot was prepared from a clone library from *Oenothera hookeri* using standard protocols (Sambrook et al., 1998). Plasmid pET11aNtTC (gift from Linda Savage), a pET11a (Novagen) expression vector containing the complete tobacco *AccD* gene, was labeled with ^{32}P and hybridized to the DNA on the membrane. The blot was exposed to film for 4 h at -70°C.

Results

Transformation of N. tabacum with β-CT constructs

Leaf discs were incubated in *Agrobacterium* culture containing one of the three β-CT constructs detailed above and in Figures 4.1 and 4.2. Each construct contained an *AccD* gene fused to a chloroplast transit peptide from the small subunit of RuBisCO. Two constructs, p0921-3.3 and p0921-3.5 used a light-regulated promoter and conferred resistance to gentamycin and they differed in the orientation of the *AccD* gene in the plasmid. These constructs contained the *AccD* gene from tobacco. The third construct, β-CT*, used a 35S promoter and contained the *AccD* gene from pea.

Ten plants which went through the plant transformation protocol using plasmid p0921-3.5 and which grew roots on root-induction plates containing 100 μg/ml gentamycin were transferred to soil. Of these ten plants, four produced seed which germinated in the presence of 100 μg/ml gentamycin. Of the six plants transformed with p0921-3.3 two survived and produced seed capable of germinating on gentamycin. None of the plants transformed with the pPZP122 vector alone produced seed which germinated on gentamycin.

Thirteen plants transformed with the 35S/β-CT* construct were transferred to soil. Nine of these expressed the neomycin phosphotransferase (NPT) gene at various levels. These NPT expressing plants were the only ones able to germinate on media containing 150 μg/ml kanamycin. Six plants transformed with pMON10009 were transferred to soil. Of these, four expressed NPT and were able to germinate on media containing 150 μg/ml kanamycin. Most of the antibiotic-resistant lines which produced germinating seed had germination:non-germination ratios of 75%, although in some lines all seeds tested were resistant.

Immunoblots of transformed plants

Most of the plants transformed with the pPZP-derived vectors containing the tobacco *AccD* gene were checked for expression of the β-CT protein. Plants putatively transformed with p0931-3.3 and p0931-3.5 were probed with two antibodies raised against tobacco β-CT (gifts of Linda Savage). Neither antibody detected any β-CT protein in transgenic plants. Plants transformed with the

pMON-derived vector containing the pea *AccD* gene were also checked for expression of the β-CT protein. Although the MSU-6E antibody could easily detect 10 ng protein, no pea β-CT protein was detected in the transgenic plants.

RNA blots

As all attempts to detect β-CT protein in transgenic plants led to negative results, an indication was sought that the message for the β-CT protein was present. Figure 4.3 shows that mRNA corresponding to the plasmids from the plants transformed with p0921-3.3 and p0921-3.5 cannot be detected. The blot contains total RNA from nine lines, five of which are transformed according to seed germination tests on kanamycin. Figure 4.3A shows the blot after hybridization with the engineered β-CT probe. This probe contains 30 bases of the coding region from the small subunit of RuBisCO and 1536 bases which encode the *AccD* gene. It is clear that there is no correlation between transformation and hybridization. In order to confirm that the small subunit promoter was functional and the blot had detectable mRNA, a second hybridization was done to the same membrane. Figure 4.3B, shows the same blot probed with an RNA probe containing the antisense message to the small subunit transit peptide coding region. Since Figure 4.3B shows that small subunit message RNA was present, the blot did contain detectable mRNA, and the light-regulated small subunit promoter functional, at least for the native gene.

Figure 4.3. RNA blots from plants transformed with plasmids p0921-3.3 and p0921-3.5.

RNA was extracted from young leaves using extraction method 1. Twenty μg of total RNA was fractionated on a 1.2% agarose RNA gel (1X MOPS, 2% formaldehyde, 10 μg/ml ethidium bromide). RNA was transferred to Zeta-Probe® membrane (Bio-Rad Laboratories, Hercules, CA) using the manufacturer's Northern Blotting protocol. Membranes were prehybridized in 5X SSC, 10X Denhardt's, 0.1% SDS, 100 mM potassium phosphate, pH 6.8, 100 μg/ml salmon sperm DNA at 42°C overnight using a hybridization oven (Hybaid, Hybaid Instruments, Holbrook, NY) at the oven speed 6 setting. Lanes left to right: 1003- (pPZP122), 1302, 2301+, 2302+, 1503-, 2504+, 2505+, 2506+, 2507-. Lines which are denoted with + germinated on gentamycin containing media. **A)** Engineered β-CT probe (5 x 10^6 CPM/ml buffer) was added to hybridization buffer (5X SSC, 10X Denhardt's, 250 mM potassium phosphate, pH 6.8, 30% formamide, 100 μg/ml salmon sperm DNA) and the membrane was hybridized overnight at 42°C at the oven setting of speed 6. The membrane was washed four times. The first wash was at 50°C in 2X SSC, 0.5% SDS for 5 min; the second at 50°C in 2X SSC, 0.5% SDS for 20 min; the third at 65°C in 2X SSC, 0.5% SDS for 20 min; and the last at 65°C in 0.2X SSC, 0.5% SDS for 30 min. The membrane was phosphoimaged overnight. **B)** After imaging the blot was stripped by washing twice in 0.1X SSC, 0.5% SDS at 80°C for 20 min, the blot was prehybridized as above, then labeled small subunit riboprobe (4.9 x 10^5 CPM/ml buffer) added and hybridized overnight. The membrane was washed as follows. 1) and 2) 50°C for 20 min in 2X SSC, 0.5% SDS at speed 10; 3) 50°C for 20 min in 0.2X SSC, 0.5% SDS at oven speed setting 10; 4) 50°C for 20 min in 0.2X SSC, 0.5% SDS at speed 5; 6) 60°C for 30 min in 0.1X SSC, 0.5% SDS at oven speed setting 5. 7) The final washes were at 65°C and 70°C, each for 30 min in 0.1X SSC, 0.5% SDS at the oven's setting of speed 5.

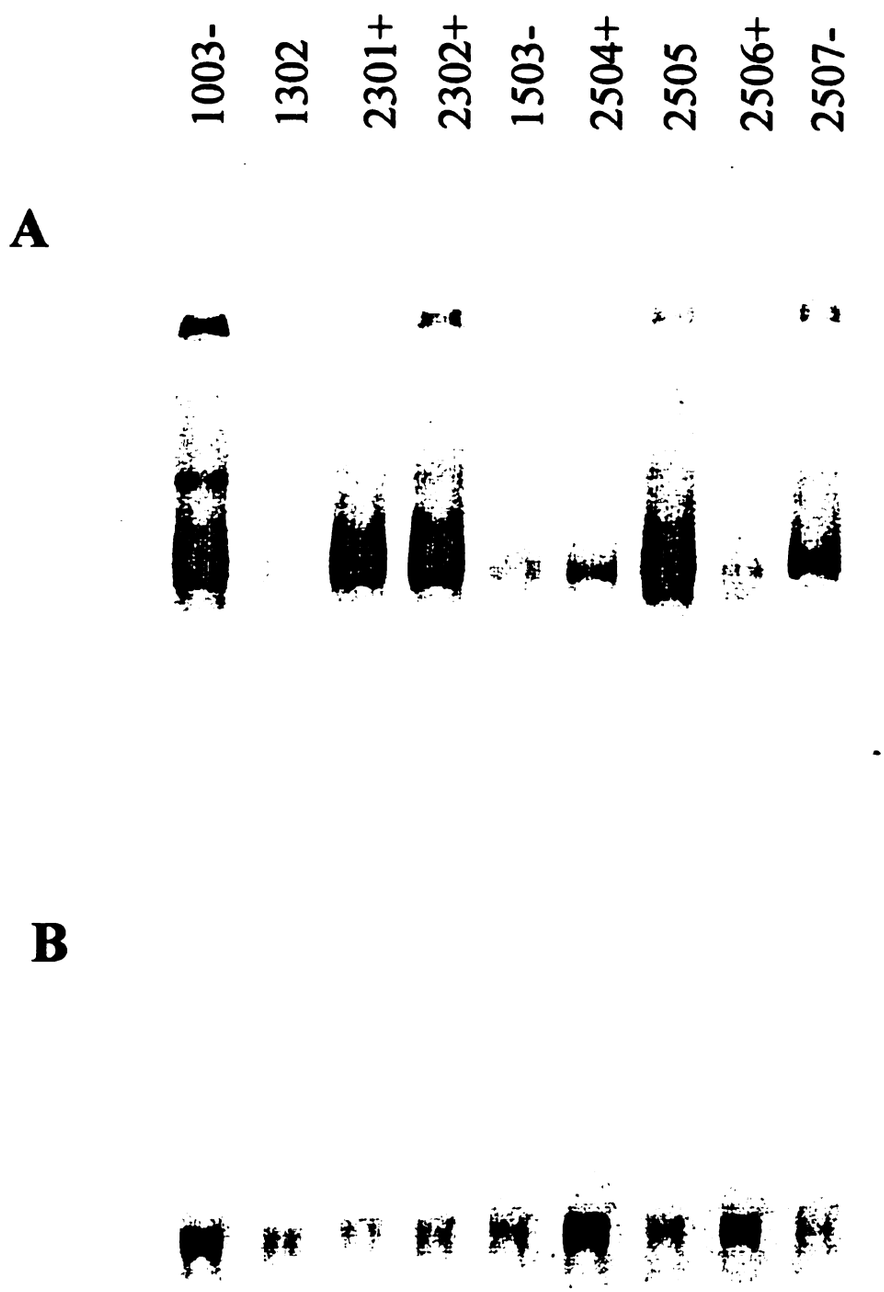

1003-
1302
2301+
2302+
1503-
2504+
2505
2506+
2507-
A
B

Figure 4.4 shows the Northern data from a second set of transformed plants. This set of plants was transformed with the plasmid 35S/β-CT*. Figures 4.4A and 4.4B show the blots probed with 35S/β-CT. Once again, no mRNA hybridizing to the pea β-CT gene was detected. Figures 4.4C and 4.4D show the same blots hybridized with a kanamycin gene probe. These panels confirm that the 35S promoter is expressed and the plants are transformed.

Sequence of Oenothera hookeri AccD

At the time this work was initiated, *AccD* sequences of higher dicot plants were available only for tobacco and pea. These two sequences diverged greatly at the N-terminal region of the protein. Therefore, to help obtain a more complete picture of the primary structure of this protein in plants and to take advantage of available materials, the genomic sequence of *O. hookeri AccD* was determined. The sequence was only determined in one direction, therefore, only preliminary sequence data are available. This data is shown in Figure 4.5. Possible start codons are at 319 and 556. There are five different direct repeat motifs in the 5' coding region of the gene, three of which are 20-21 bp in length. First, a 21-bp repeat with low C content is located at 644 and 689. Two imperfect repeats of this region are at 583 and 614. Second, adjacent to these repeats is another shorter 9-bp repeat. This repeat is located after the first three repeats, but before the fourth repeat. A third 45-bp repeat is located at 416 and 488. A fourth repeat of 21-bp with low TC content is located at 394 and 466 with imperfect copies at 728 and 761. The fifth repeat is a 20 bp repeat with no T's and one C. These

Figure 4.4. RNA blots from plants transformed with plasmid β-CT*.

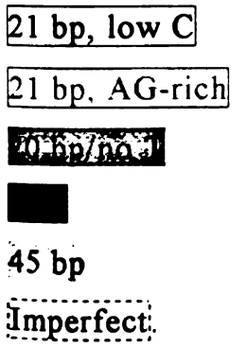
RNA was extracted from pooled apical meristems and small leaves of transformed lines using extraction method 2. Unless otherwise noted, 30 μg of total RNA was fractionated on 1.2% agarose RNA gels. RNA was transferred to Zeta-Probe membrane as in Figure 4.3. Membranes were prehybridized at least 2 hours. **A)** and **B)** β-CT probe (2.8 x 10^6 CPM/ml buffer) was added to hybridization buffer and membranes were hybridized overnight at 42°C at oven speed setting 6. Membranes were washed four times at 42°C. The first wash was in 2X SSC, 0.5% SDS for 10 minutes at oven speed setting 10; the second in 2X SSC, 0.5% SDS for 15 minutes at oven speed setting 7. The third and fourth washes both used 1X SSC, 0.5% SDS at oven speed setting 7, and each lasted 20 minutes. Membranes were phosphoimaged overnight. Blots were stripped using Ambion's Strip-EZ kit according to the manufacturer's protocol. **C)** and **D)** The same blots were prehybridized as above, then labeled kanamycin probe (2.1 x 10^6 DPM/ml buffer) was added to hybridization buffer and membranes were hybridized overnight and washed as in **A)** and **B)**. **A)** and **C)** Lanes left to right: 0.5 ng unlabeled β-CT probe, 0.5 ng unlabeled kanamycin probe, nothing, V-3+, V-13+, β-1+, β-2+, β–8, β–11+, 15 μg β–12, nothing, 2.5 ng unlabeled kanamycin probe, 0.1 ng unlabeled β-CT probe. **B)** and **D)** Lanes left to right: 2.5 ng unlabeled β-CT probe, 0.1 ng unlabeled kanamycin probe, nothing, β-13, β-10+, β-9+, nothing, V-15+, V-11+, V-1+, wild type, 0.5 ng unlabeled kanamycin probe, 0.5 ng unlabeled β-CT probe. Lines which are denoted with + expressed NPT and germinated on kanamycin containing media.

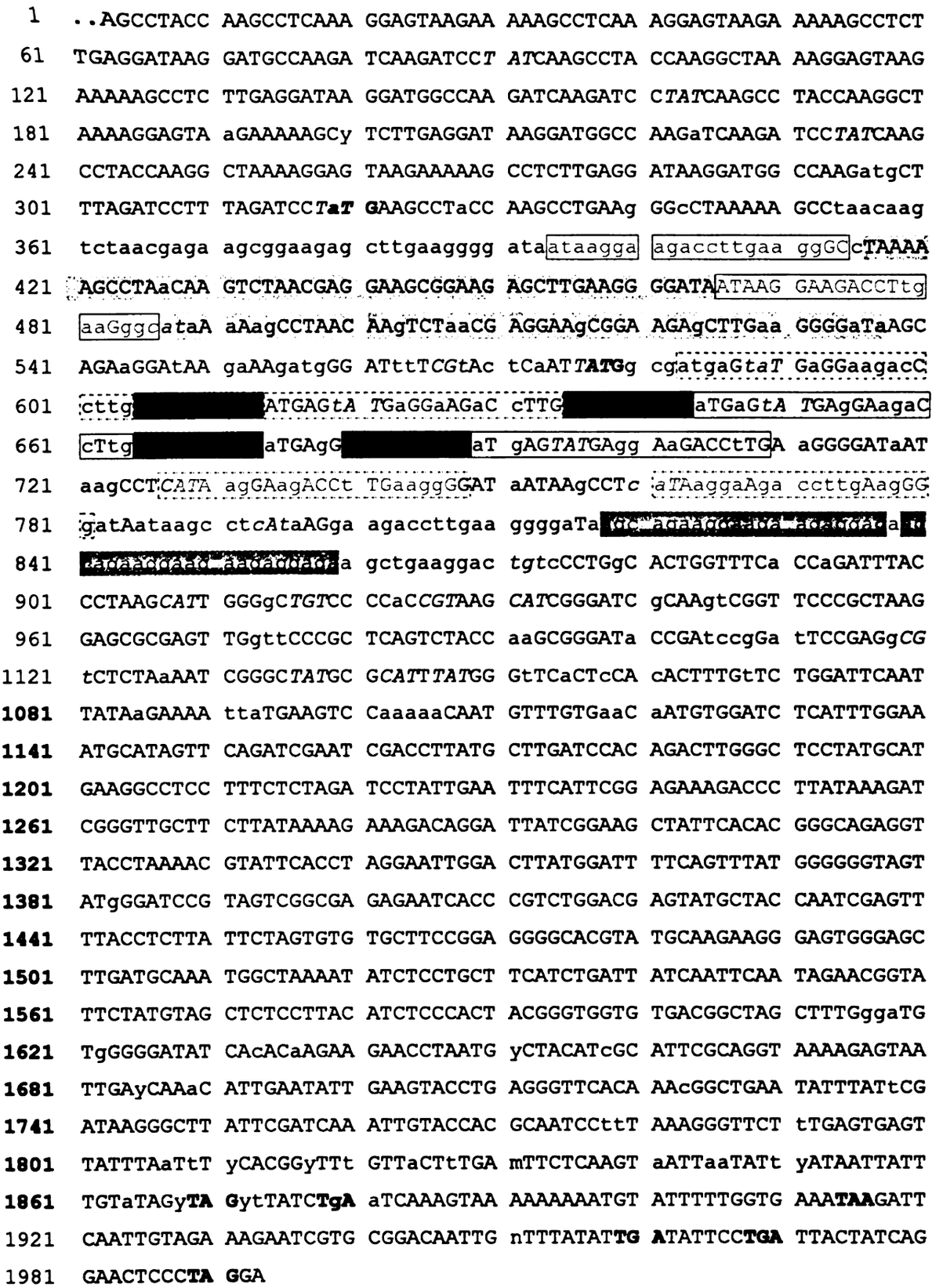
β-CT +
kan +
V-3+
V-13+
β-1+
β-2 +
β-8 –
β-11 +
β-12 –
kan +
β-CT +
A
B
C
D

Figure 4.5. Preliminary *O. hookeri AccD* sequence.

Since the sequence data is preliminary, upper case letters indicate a consensus between the different sequencing runs. Lower case letters indicate that different runs produced different results, with the most likely base given. Codes used when data did not yield a consensus are Y = C or T; M = A or C; and N = any base.

Potential start codons and the stop codon are in boldface. Boldfaced numbers on the left side indicate the sequence of the region where the protein is conserved (see Figure 4.6). The promoter used to transcribe *AccD* is a nuclear-encoded RNA polymerase. The YRT motifs required for this type of promoter are italicized. The only *AccD* promoter sequence published is for *N. tabacum* (-129 ATATAAAGGGGGTTCCAACATATTAATATATAGTGTAAGT-) (Leire and Maliga, 1999). Repeats described in the text are indicated as below.





..AGCCTACC AAGCCTCAAA GGAGTAAGAA AAAGCCTCAA AGGAGTAAGA AAAAGCCTCT
TGAGGATAAG GATGCCAAGA TCAAGATCCT ATCAAGCCTA CCAAGGCTAA AAGGAGTAAG
AAAAAGCCTC TTGAGGATAA GGATGGCCAA GATCAAGATC CTATCAAGCC TACCAAGGCT
AAAAGGAGTA aGAAAAAGCy TCTTGAGGAT AAGGATGGCC AAGaTCAAGA TCCTATCAAG
CCTACCAAGG CTAAAAGGAG TAAGAAAAAG CCTCTTGAGG ATAAGGATGG CCAAGatgCT
TTAGATCCTT TAGATCCTaT GAAGCCTaCC AAGCCTGAAg GGcCTAAAAA GCCtaacaag
tctaacgaga agcggaagag cttgaagggg ataataagga agaccttgaa ggGCcTAAAA
AGCCTAaCAA GTCTAACGAG GAAGCGGAAG AGCTTGAAGG GGATAATAAG GAAGACCTTg
aaGggcataA aAagCCTAAC AAgTCTaaCG AGGAAgCGGA AGAgCTTGaa GGGGaTaAGC
AGAaGGAtAA gaAAgatgGG ATttTCGtAc tCaATTATGg cgatgaGtaT GaGGaagacC
cttg[redacted]ATGAGtA TGaGGaAGaC cTTG[redacted]aTGaGtA TGAgGAagaC
cTtg[redacted]aTGAgG[redacted]aT gAGTATGAgg AaGACCtTGA aGGGGATaAT
aagCCTCATA agGAagACCt TGaaggGGAT aATAAgCCTc aTAaggaAga ccttgAagGG
gatAataagc ctcAtaAGga agaccttgaa ggggaTa[redacted]a
[redacted]a gctgaaggac tgtcCCTGgC ACTGGTTTCa CCaGATTTAC
CCTAAGCATT GGGgCTGTCC CCaCCGTAAG CATCGGGATC gCAAgtCGGT TCCCGCTAAG
GAGCGCGAGT TGgttCCCGC TCAGTCTACC aaGCGGGATa CCGAtccgGa tTCCGAGgCG
tCTCTAaAAT CGGGCTATGC GCATTTATGG GtTCaCTcCA cACTTTGtTC TGGATTCAAT
TATAaGAAAA ttaTGAAGTC CaaaaaCAAT GTTTGTGaaC aATGTGGATC TCATTTGGAA
ATGCATAGTT CAGATCGAAT CGACCTTATG CTTGATCCAC AGACTTGGGC TCCTATGCAT
GAAGGCCTCC TTTCTCTAGA TCCTATTGAA TTTCATTCGG AGAAAGACCC TTATAAAGAT
CGGGTTGCTT CTTATAAAAG AAAGACAGGA TTATCGGAAG CTATTCACAC GGGCAGAGGT
TACCTAAAAC GTATTCACCT AGGAATTGGA CTTATGGATT TTCAGTTTAT GGGGGGTAGT
ATgGGATCCG TAGTCGGCGA GAGAATCACC CGTCTGGACG AGTATGCTAC CAATCGAGTT
TTACCTCTTA TTCTAGTGTG TGCTTCCGGA GGGGCACGTA TGCAAGAAGG GAGTGGGAGC
TTGATGCAAA TGGCTAAAAT ATCTCCTGCT TCATCTGATT ATCAATTCAA TAGAACGGTA
TTCTATGTAG CTCTCCTTAC ATCTCCCACT ACGGGTGGTG TGACGGCTAG CTTTGggaTG
TgGGGGATAT CAcACaAGAA GAACCTAATG yCTACATcGC ATTCGCAGGT AAAAGAGTAA
TTGAyCAAaC ATTGAATATT GAAGTACCTG AGGGTTCACA AAcGGCTGAA TATTTATtCG
ATAAGGGCTT ATTCGATCAA ATTGTACCAC GCAATCCttT AAAGGGTTCT tTGAGTGAGT
TATTTAaTtT yCACGGyTTt GTTaCTtTGA mTTCTCAAGT aATTaaTATt yATAATTATT
TGTaTAGyTA GytTATCTgA aTCAAAGTAA AAAAAAATGT ATTTTTGGTG AAATAAGATT
CAATTGTAGA AAGAATCGTG CGGACAATTG nTTTATATTG ATATTCCTGA TTACTATCAG
GAACTCCCTA GGA

repeats are located at 818 and 839. The significance, if any of these repeats is unknown. The promoter sequence for the tobacco *AccD* transcript has been described (Leire and Maliga, 1999). This sequence has conserved motifs which consist of a YRT core in an AT-rich region. (Y = C or T). Due to the high GC content of the repeated motifs, there are few truly AT-rich regions in this area, however, YRT motifs are also shown in Figure 4.5.

When the putative *O. hookeri* protein is aligned with other β-CT proteins, the protein is more conserved at the carboxy end of the protein. Furthermore, there is no significant protein alignment of the amino-terminal end of the protein until the coding region is out of the highly repeated area of the gene (Figure 4.6).

Mapping of Oenothera hookeri AccD on the plastidial genome

Early attempts at obtaining the *Oenothera AccD* gene via PCR were unsuccessful. Due to the unconserved 5' regions of the tobacco and pea genes, these attempts used primers based on the highly-conserved 3' end of the gene that precedes it in both the tobacco and pea plastidial genomes - the large subunit of RuBisCO. Since these attempts were unsuccessful and the *Oenothera* plastidial genome has some unusual qualities, the location of *AccD* on the plastome was investigated. The map of the clones in the library and results of the hybridization are shown in Figure 4.7. An expression vector containing the tobacco *AccD* gene hybridized with the pOjSalE, Bam3a and Bam3b clones. This places the *AccD* gene between the *petA* and *atpA* genes in *O. hookeri*.

```
          1                                                  50
  tobacco} MTIHLLYFHA NRGQENSMER WWFNSMLFKK EFERRCGLNK SMGSLGPIEN
 B. napus} ~~~~~~~~~~ ~~~~~~~MEK SWFNLMFSKG ELEYRGELSK AMDSFAPIEK
      pea}  ~~~~~~~MI NEDPSSLTDM DNNIDSWKNN SENSSYSHAD SLADVSNIDN
Oenothera} ~~~~~~~~~~ ~~MKPTKPEG PKKPNKSNEK RKSLKGIIRK TLKGLKSLTS
Consensus} ---------- N------ME- -WFN-M--KK E-E-RG-L-K S--SL-PIEN

          51                                                 100
  tobacco} TNEDPNRKVK NIHS...W.. ....RNRDNS SCSNVDYLFG VKDIRNFISD
 B. napus} TTISKDRFIY DMDKNFYG.. ....WGERSS YYNNVDLLVN SKDIRNFISD
      pea} LLSDKIFSIR DSNSN..... ..........  ........IY DIYYAYDTND
Oenothera} LTRKRKSLKG IIRKTLKGIK SLTS.LTRKR KSLKGISRRI RKMGFSYSIM
Consensus} -T-DK-R-I- DI--N--G-- -------R-S ---NVD-L-- -KDIRNFISD

          101                                                150
  tobacco} DTFLVSDRNG DSYSIYFDIE NHIFE.IDND HSFLSELESS FYSY....RN
 B. napus} DTFFVRDSNK NSYSIYFDIE KKKFE.INND ...LSDLEIF FYSY....CS
      pea} TNITKYKWTN NINR.CIESY LRSQICEDID FNSDICDKVQ RTIIILIRST
Oenothera} AMSMRKTLEY DDEYEEDLE. .YDDEYEEDL EYDDEEYDDE YEEDLEGDNK
Consensus} DTF-V-D-N- -SYSIYFDIE ---FE--DND ----SELE-- FYSY------

          151                                                200
  tobacco} SNYRNNGFRG EDPYYN...S YMYDTQYSWN NHINSCIDSY LQSQICIDTS
 B. napus} SSYLNNRSKG DNDLHYD..P YIKDTKYNCN NHINSCIDSY FRSHICINSH
      pea} NDTNDISDTN DISDTNDTND TNAIYDPFDI SDTNDTNEIY DPFFILDIND
Oenothera} PHKEDLEGDN KPHKEDLEGD NKPHKEDLEG DKQKEEEEKQ KEEEEKLKDC
Consensus} S-Y--N---- D----ND--D Y--DT-Y--N NHINSCI-SY --S-ICI---

          201                                                250
  tobacco} IISGSENYGD SYIYRAVCGG ESRNSSENEG SSRRTRTKGS DLTIRESSND
 B. napus} FLSDSNNSNE SYIYNFICS. ESGSGKIRES KNDKIRTN.S NRNNLMSSKD
      pea} TNDTNDIYG. .IYDPDDIYE TNIKDICERY SEIYPRNREK STFVPIDYSD
Oenothera} PWHWFHQIYP KHWGCPHRKH RDRKSVPAKE RELVP..AQS TKRDTDPDSE
Consensus} --S-S-NYG- SYIY---C-- ESRKS---E- SE--PRT--S ------SSSD

          251                                                300
  tobacco} LEVTQKYRHL WVQCENCYGL NYKKFLKSKM NICEQCGYHL KMSSSDRIEL
 B. napus} FDITKNYNQL WIQCDNCYGL KYK...KVEM NVCEECGHYL KMTSSERIEL
      pea} PNCMEKLARL WVQCETCYGL NFKQFFRPKM NICEHCGEHL KMSSSDRIDL
Oenothera} ASLKSGYAHL WVHSTLCSGF NYKKIMKSKN NVCEQCGSHL EMHSSDRIDL
Consensus} ---T-KYAHL WVQCENCYGL NYKKF-KSKM N-CEQCG-HL KMSSSDRI-L
```

Figure 4.6. Protein alignment and consensus of selected *AccD* genes.

The *Oenothera AccD* genomic sequence was translated using GCG and aligned with predicted proteins from tobacco, *B. napus*, and pea. The protein alignment was based on one done by L. Savage as well as the GCG program, pileup. The repeated region of *Oenothera* corresponds to amino acids 1 to 181.

```
          301                                                        350
 tobacco} LIDPGTWDPM DEDMVSLDPI EFHSEEEP.. .......... ..........
 B. napus} SIDPGSWNGM DEDMVSADPI KFHSREEP.. .......... ..........
      pea} SIDRDTWNPM DEDMVSVDPI KFDSIKELGS EEESSKDRLD EDMLSPDPIE
Oenothera} MLDPQTWAPM HEGLLSLDPI EFHSEKDP.. .......... ..........
Consensus} SIDPGTWNPM DEDMVSLDPI -FHSE-EP-- ---------- ----------

          351                                                        400

 tobacco} .......... .......... .......... .......... ..........
 B. napus} .......... .......... .......... .......... ..........
      pea} LDSEEEESSKD RVDSEEEKDQ SYIDRLDSYQ EKTGLPETVQ TGTDQREEIH
Oenothera} .......... .......... .......... .......... ..........
Consensus} ---------- ---------- ---------- ---------- ----------

          401                                                        450
 tobacco} .......... .......... .......... YKDRIDSYQR KTGLTEAVQT
 B. napus} .......... .......... .......... YKKRIASAQK KTGLTDAIQT
      pea} PLFEDIMNQL DLYLQTAKNR VDSEEEKDQS YIDRLDSYQE KTGLPEAVQT
Oenothera} .......... ..YKDRVASY KRKTGLSEAI HTGRGYLKRI HLGIGLMDFQ
Consensus} ---------- --Y------- ---------- YKDRIDSYQ- KTGLTEAVQT

          451                                                        500
 tobacco} GIGQLNGIPV AIGVMDFQFM GGSMGSVVGE KITRLIEYAA NQILPLIIVC
 B. napus} GTGQLNGIPV ALGVMDFQFM GGSMGSVVGD KITRLIEYAT NQCLPLILVC
      pea} GTGQLNGIPL ALAVMDSEFI AGSMGCVVGE KITRLIEYAT NLLLPLIIVC
Oenothera} FMGGSMGSVV GERITRLDEY ATNRVLPLIL VCASGGARMQ EGSGSLMQMA
Consensus} GTGQLNGIPV ALGVMDFQFM -GSMGSVVGE KITRLIEYAT NQ-LPLIIVC

          501                                                        550
 tobacco} ASGGARMQEG SLSLMQMAKI SSALYDYQLN KKLFYVSILT SPTTGGVTAS
 B. napus} SSGGARMQEG SLSLMQMAKI SSVLCDYQSS KKLFYISILT SPTTGGVTAS
      pea} ASGGARMQEG SLSLMQMAKI SSALYNYQIN QKLFYVAILT SPTTGGVTAS
Oenothera} KISPASSDYQ FNRTVFYVAL LTSPTTGGVT ASFGMWGISH KKNLMXTSHS
Consensus} ASGGARMQEG SLSLMQMAKI SSALYDYQ-N KKLFYVSILT SPTTGGVTAS

          551                                                        600
 tobacco} FGMLGDIIIA EPNAYIAFAG KRVIEQTLNK TVPEGSQAAE YLFQKGLFDL
 B. napus} LGMLGDIIIA EPYAYIAFAG KRVIEQTLKK AVPEGSQAAE SLLRKGLLDA
      pea} FGMLGDIIIA EPNATIAFAG KRVIEQLLNK EVPEGSQSAD LLFDRGLLDA
Oenothera} QVKE*~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Consensus} FGMLGDIIIA EPNAYIAFAG KRVIEQTLNK -VPEGSQAAE -LF-KGLLDA

          601                                                   643
 tobacco} IVPRNLLKSV LSELFKLHAF F......... ..PLNQKSSK IK*
 B. napus} IVPRNPLKGV VSELFQLHAF F......... ..PLNKNEIK *~~
      pea} VVPRHLLKEF LTELFQFHGF V......... ..PLT*~~~~ ~~~
Oenothera} ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
Consensus} IVPRNLLK-V LSELFQLHAF F--------- --PLN----K -K-
```

Figure 4.6, continued.

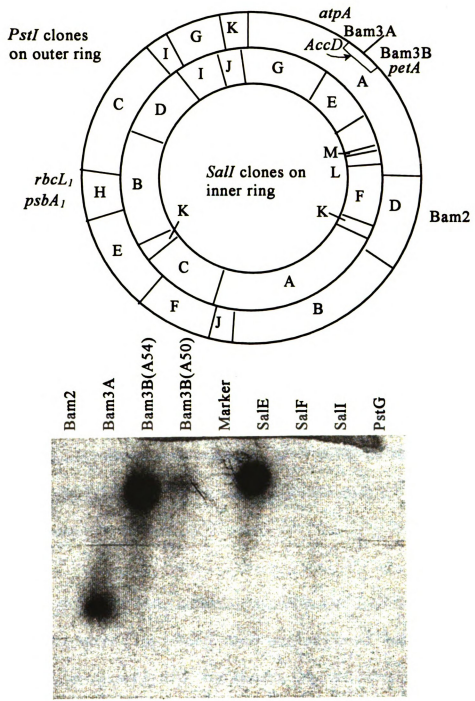


Figure 4.7 *O. hookeri* plastome maps and the location of the *AccD* gene.

Clones from the plastidial genome of *O. hookeri* were obtained from B. Sears, DNA prepared and blotted to membrane. The membrane was probed with an expression vector (from Linda Savage) containing the tobacco *AccD* coding region.

Discussion

Plant transformation and extra-plastidial expression of AccD

It is clear from the immunoblots and northern blots that moving the plastidial β-CT gene to the nuclear genome is not a straight-forward project. Although the efficiency of transformation was disappointing, especially with the pPZP-derived vectors, it is clear from the seed germination and NPT assays that the plants were able to incorporate single and multiple copies of foreign genes into their nuclear genomes. Thus, the plants were transformed. Both plant transformation vectors had the drug resistance genes adjacent to the left border which is transferred to the plant after the right border, therefore, the constructed *AccD* genes were transferred to the plant genome. In addition, the use of two different constructs, one based on the tobacco β-CT gene, the other based on the pea β-CT gene, decreases the probability that some non-conserved region is responsible for the lack of expression.

Why did this project not yield detectable expression of *AccD* when it was located in the nuclear genome? The tobacco *AccD* gene has an AT content of 63% while the pea *AccD* AT content is 65%. The average dicot AT content is 55% (Sinibaldi 1992, reviewed in Diehn et al., 1996). Thus the *AccD* transcript may contain instability motifs and polyadenylation signals which are AT-rich and which prevent its successful translation in a nuclear background. Problems of this sort have been well documented for the *Bacillus thuringiensis* toxin genes (for review

see Diehn et al., 1998). Figure 4.8 highlights potential problem areas in the *AccD* sequence. They are numerous in both tobacco and pea. While none of the regions indicated are known to be sufficient to cause incorrect processing and translation by themselves, the sheer number of problem areas suggests that relocation of *AccD* to the nuclear genome may require substantial modification of the gene. In addition to these instability motifs and polyadenylation signals, the codon usage of the pea and tobacco *AccD* genes are not plant-like. Plant genes have a G or a C in the third position about 40% of the time (Wada, 1992), while the pea *AccD* gene has an G or a C in the third position 28% of the time and tobacco 32% of the time.

Although the most likely explanation of the lack of β-CT protein is due to incorrect transcript processing or stability, there is also a small possibility that the β-CT DNA from the vectors was not stably incorporated into the plant genome. The techniques used to confirm this are generally Southern blotting or PCR (Register, 1997). This technique was not used on the tobacco plants because the gene is still present in numerous copies in the plastidial genome. While it is possible for occasional gene rearrangements or incomplete transfer of vector DNA to the plant genome, it is highly unlikely that every construct with both vectors chanced upon this problem, so if β-CT was rearranged out, it is likely to be due to a conserved region of the sequence.

Figure 4.8. Areas where the *AccD* transcript may be improperly processed.

The promoter (Leire and Maliga, 1999) is italicized. Start and Stop codons are in boldface. Sequence prior to the ATG start codon was not included in the construct, thus no potential problem areas in that region are marked. ATTTA instability motifs which may cause premature degradation (Ohme-Takagi et al., 1993) are in boldface and underlined. Near upstream elements (NUE's) are used as polyadenylation signals 40% of the time they are present. One mismatch with the consensus sequence has little effect on polyadenlyation efficiency (Rothnie, 1996). Although NUE's are difficult to find via sequence inspection, potential NUE's and NUE's with one mismatch are shaded and light gray respectively. The large box indicated by the dashed line indicates the region where the protein is conserved.

CATATTAATA TATAGTGAAG TGTTCCCCCA GATTCAGAAC TTTTTTTCAA
TACTCACAAT CCTTATTAGT TAATAATCCT AGTGATTGGA TTTTATGCTT
AGTCTGATAG GAAATAAGAT ATTCAAATAA ATAATTTTAT AGCGAATGAC
TATTCATCTA TTGTATTTTC ATGCAAATAG GGGGCAAGAA AACTCTATGG
AAAGATGGTG GTTTAATTCG ATGTTGTTTA AGAAGGAGTT CGAACGCAGG
TGTGGGCTA[redacted]TCAAT GGGCAGTCTT GGTCCTATTG AAAATACC[redacted]
[redacted]GATCCA AATCGAAAAG TGAAAAACAT TCATAGTTGG AGGAATCGTG
ACAATTCTAG TTGCAGTAAT GTTGATTATT TATTCGGCGT TAAGACATTC
GGAATTTCAT CTCTGATGAC ACTTTTTTAG TTAGTGATAG GAATGGAGAC
AGTTATTCCA TCTATTTTGA TATTGAAAAT CATATTTTTG AGATTGACAA
CGATCATTCT TTTCTGAGTG AACTAGAAAG TTCTTTTTAT AGTTATCGAA
ACTCGAATTA TCGGAATAAT GGATTTAGGG GCGAAGATCC CTACTATAAT
TCTTACATGT ATGATACTCA ATATAGTTGG AATAATCACA TTAATAGTTG
CATTGATAGT TATCTTCAGT CTCAAATCTG TATAGATACT TCCATTATAA
GTGGTAGTGA GAATTACGGT GACAGTTACA TTTATAGGGC CGTTTGTGGT
GGTGAAAGTC GAAATAGTAG TGAAAACGAG GGTTCCAGTA GACGAACTCG
CACGAAGGGC AGTGATTTAA CTATAAGAGA AAGTTCTAAT GATCTCGAGG
TAACTCAAAA ATACAGGCAT TTGTGGGTTC AATGCGAAAA TTGTTATGGA
TTAAATTATA AGAAATTTTT GAAATCAAA[redacted]TATTT GTGAACAATG
TGGATATCAT TTGAAAATGA GTAGTTCAGA TAGAATTGAA CTTTTGATCG
ATCCGGGTAC TTGGGATCCT ATGGATGAAG ACATGGTCTC TCTAGATCCC
ATTGAATTTC ATTCGGAGGA GGAGCCTTAT AAAGATCGTA TTGATTCTTA
TCAAAGAAAG ACAGGATTAA CCGAGGCTGT TCAAACAGGC ATAGGCCAAC
TAAACGGCAT TCCCGTAGCA ATTGGGGTTA TGGATTTTCA GTTTATGGGG
GGTAGTATGG GATCCGTAGT CGGAGAGAAA ATCACCCGTT TGATTGAATA
CGCTGCCAAT CAAATTTTAC CCCTTATTAT AGTGTGTGCT TTGGGGGGGC
GCGCATGCAG GAAGGAAGTT TGAGCTTGAT GCAAATGGCT AAAATATCGT
CTGCTTTATA TGATTATCAA TTA[redacted]A AGTTATTTTA TGTATCAATC
CTTACATCTC CGACAACTGG TGGAGTGACA GCTAGTTTTG GTATGTTGGG
GGATATCATT ATTGCCGAAC CCAACGCCTA CATTGCATTT GCAGGTAAAA
GAGTAATTGA ACAAACATTG [redacted]ACAG TACCCGAAGG TTCACAAGCA
GCTGAATACT TATTCCAGAA GGGTTTATTC GACCTAATTG TACCACGTAA
TCTTTTAAAA AGCGTTCTGA GTGAGTTATT TAAGCTCCAC GCCTTTTTTC
CTTTGAATCA AAAGTCAAGC AAAATCAAGT AG

Oenothera AccD location on the plastidial genome and sequence

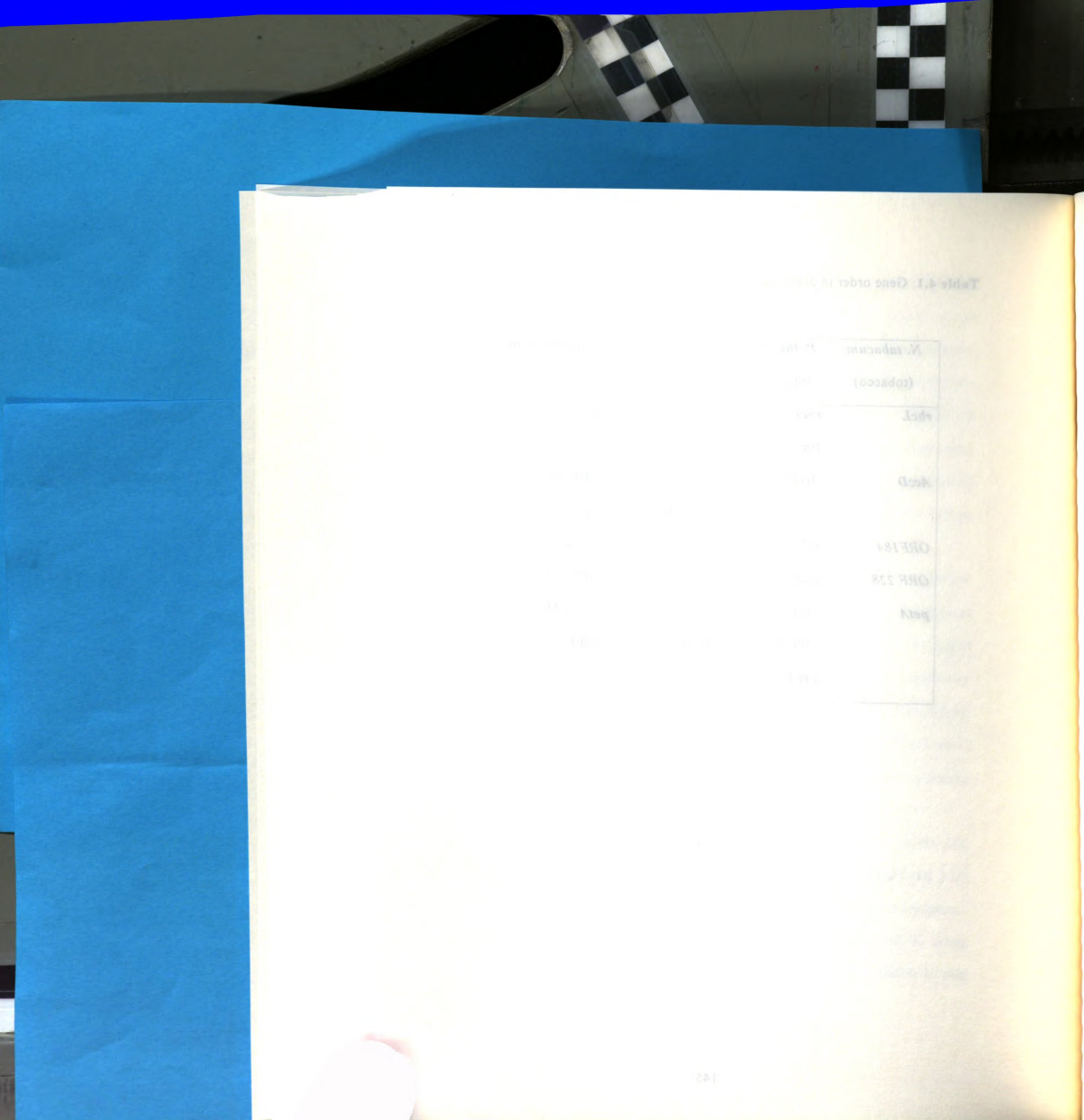
In the plastid genomes which were sequenced at the time this work was done (tobacco, rice, black pine, liverwort), *AccD* is close to *rbcL*, usually separated by one tRNA gene or ORF, and the *AccD* gene precedes the *petA* gene. However, in the *O. hookeri* plastome, *rbcL* and *petA* are at opposite ends of the large single copy region. *AccD* is still next to *petA*, but near *atpA* and far from *rbcL*. The gene order is given in Table 4.1. The region around *AccD* appears to have been an area where the chloroplast genome could recombine and rearrange.

While the *O. hookeri* line used in these studies was a not a line with an active plastome mutator (PM), in the evolution of the four different plastidial genomes found in *Oenothera* repeat amplifications are common (B. Sears, personal communication; Sears et al., 1996). As seen in Figure 4.5, there are many direct repeats in the 5' region of *AccD*. While the significance, if any, of these repeats is not known, the appear to not be tolerated in the 3' region of the gene. Perhaps ancestral rearrangements which occurred in this region were not viable.

The DNA sequence and protein alignments do little to clear up confusion - in fact, they raise more questions. Although the carboxy end of the protein is more conserved than the amino end, the putative *Oenothera* protein does not contain some of the conserved motifs. For example, the first putative phosphorylation site is missing, and the carboxy biotin binding site is poorly conserved. Is the

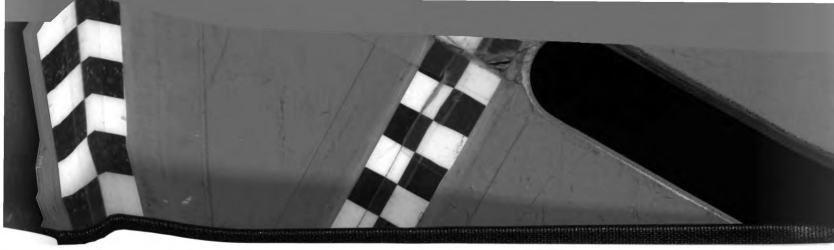
Table 4.1. Gene order in plastidial genomes.

N. tabacum (tobacco)	*P. thunbergii* (black pine)	*O. sativa* (rice)	*M. polymorpha* (liverwort)
rbcL	***rbcL***	***rbcL***	***rbcL***
	trnR	*ORF133*	*trnR*
AccD	***AccD***	***ORF106***	***ORF316***
		(AccD)	***(AccD)***
ORF184	*ORF119*	*ORF36*	*ORF36*
ORF 228	*psaI*	*ORF185*	*ORF184*
petA	*ORF184*	*ORF230*	*ORF434*
	ORF261	***petA***	***petA***
	petA		



Oenothera protein longer than most? Could the protein be truncated? Although the earliest start site is at base 319, the sequence presented here differs from unpublished sequence generated elsewhere from the same clone (R. G. Herrmann, Ludwig Maximilians University). This difference is due to a difference in the repeated region.One of the imperfect repeats presented in Figure 4.5 is a perfect repeat in the Herrmann et al. sequence. This difference causes a frameshift. Thus, Herrmann et al. assign the start codon to base 556 (B. Sears, personal communication). This difference may be due to sequencing errors.

Analysis of the regions 5' to the open reading frame in search of a promoter region does not shed more light on the start codon or length of the *Oenothera* transcript. There are two different plastidial RNA polymerases, one nuclear encoded (NEP), the other encoded by the plastidial genome (PEP) (Allison et al., 1996; reviewed in Weihe and Börner, 1999). The PEP uses a eubacterial-type promoter and primarily transcribes photosynthetic genes. The NEP usually transcribes *AccD*, and the promoter from tobacco has been described (Leire and Maliga, 1999). The tobacco *AccD* promoter is a class Ia NEP promoter. Due to the high GC content of the repeated motifs, there are few truly AT-rich regions in this area. The tobacco *AccD* promoter has an AT content of 85%. In the *Oenothera* sequence there are many YRT motifs (Y = C or T, R = A or G) in the region 5' to the potential start codons. The AT content of the surrounding bases ranges from 65% to 30%, so no region strongly suggests itself as a promoter.



Based on the discussion above, some of the traits of *AccD* which may interfere with relocation of the *AccD* gene do not appear to be confined to any one species. All of the genes are AT-rich and there is little conservation in the 5' region. All the species discussed in this chapter have retained a functional *AccD* gene in their plastidial genomes. However, neither the tobacco nor the pea gene was successfully relocated, and the *Oenothera* gene has a unique 5' end and a possibly unique location on the plastidial genome. The true significance of these differences and gene sequences are unknown; however, they may have a significant bearing to the ability of *AccD* to be easily engineered.

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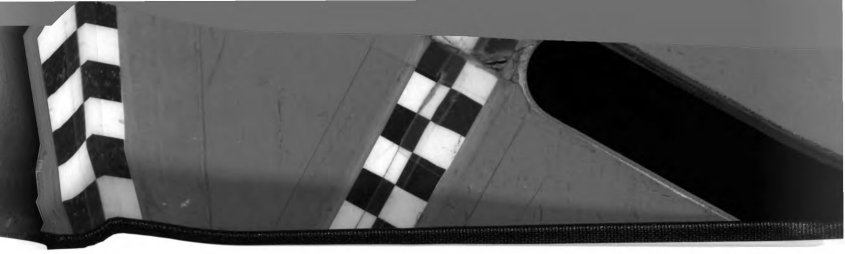

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Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

The work described herein used a variety of methods and approaches in an attempt to understand the regulation of FAS and to develop strategies to manipulate FAS. Even as the amount and types of fatty acids made and stored in a plant are being genetically engineered and grown as crops (e.g., Facciotti et al., 1999), the work presented in Chapter 2 demonstrates that physiologically important *in vitro* biochemical properties of individual enzymes can still be discovered. For example, in Chapter 2, new information about the metabolic regulation of ACCase was presented: in a lysed chloroplast system ACCase is activated by its substrate, acetyl-CoA. This activation occurs between 10-100 μM acetyl-CoA. Since the concentration of acetyl-CoA in the chloroplast is approximately 10-20 μM, this activation may be physiologically relevant. Less is known about the *in vivo* behavior of individual enzymes, let alone enzyme systems or complexes.

Chapter 2 also provides more fodder for the sometimes-forgotten truism that laboratory experiments done in test tubes with artificial systems do not automatically transfer to the living organisms under study. While previously reported work (Sauer and Heise, 1984; Sasaki et al., 1997) clearly present results that DTT and thioredoxin stimulate ACCase, these results could not be

duplicated in a lysed chloroplast system when ACCase was activated (Chapter 2). It is likely that both thioredoxin and acetyl-CoA activate ACCase, but the effect is not additive. Given the data of Sasaki et al. (1997), it appears that both thioredoxin and acetyl-CoA can activate ACCase to a similar extent. It is not surprising that there are multiple methods of activating ACCase. It is, after all, a major determinant of the rate of FAS (Post-Beittenmiller et al., 1992).

In view of the results presented in Chapter 2 (Table 2.1); namely that *in vitro* results do not accurately reflect *in vivo* conditions, the computer simulation approach presented in Chapter 3 may initially appear overly simplistic. This is true: a modeling approach is simplistic if built without *in vivo* data or followed up by *in vivo* experiments. In Chapter 3, a model of the major plastidial reactions of FAS and its predictions were presented. This model was validated using the non-intuitive results of KAS III overexpression. *In vivo* experiments in spinach show that KAS III overexpression alters the 16 carbon to 18 carbon fatty acid ratio (Jaworski and Hinneburg-Wolf, 1998). Although the quantitative values of *in vivo* results and model predictions differ, the model qualitatively predicts the *in vivo* result. In the artificial and simpler system of the model, the behavior is easy to dissect - the results are due to increased competition for malonyl-ACP by KAS III, KAS I and KAS II. Further validation of the model was provided by comparison of model predictions with *in vivo* pool size changes during a light/dark transition and other antisense/overexpression experiments.

Data presented in Chapter 3 also questions some assumptions that workers have made about the regulation of FAS. Many workers have reasonably assumed that the regulation of 16:0 and 18:1 production is essentially similar and they have based the interpretation of their results on this assumption, either consciously or unconsciously. The model does not make this assumption, and in fact, predicts that the production of the two fatty acids has different co-limiting steps. Thus, the model has brought out an assumption we have been using. Which is correct: the assumption or the model? We don't know yet. The model presented in Chapter 3 also predicts that the most efficient way to increase FAS would be to overexpress three enzymes in conjunction with each other - ACCase, KAS III, and KAS I. KAS II, the enzyme which does the final elongation from 16:0 to 18:0, is apparently not required. Although this is a logical result, to our knowledge it has not (yet) been attempted.

Finally, Chapter 4 presents two unsuccessful attempts to accomplish a seemingly simple genetic engineering project - relocation of the plastidial *AccD* gene to the nucleus. This is not novel; the plastidial gene which codes for the large subunit of RuBisCO has been moved to the nuclear genome (Kanaveski and Maliga, 1994). But evidence presented in Chapter 4 indicates that the *AccD* transcript is not functional in a nuclear background. Thus, while the data presented in Chapter 3 encourages efforts to overexpress ACCase, this may be technologically difficult for the heteromeric ACCase. The plastidial ACCase is comprised of four subunits, and the homomeric ACCase by itself increases oil yield 5% (Roesler et

al., 1997). Chapter 4 also presents the sequence of *Oenothera hookeri AccD*. This region is a hotspot for recombination in *O. hookeri* (B. Sears, personal communication) and *AccD* has been deleted from the plastidial genome in many lineages (Martin et al., 1998). Furthermore, mRNA instability motifs and polyadenylation signals may exist in the coding region. These issues make relocating the *AccD* gene as outlined in chapter 4 risky proposition.

Future Directions

The questions that started these research projects, "what regulates FAS in a plant?" and "how can we alter FA composition, quality, and yield in a plant?" are still unanswered, although progress has been made. Answering these questions is imperative if we are to realize the vision of biological plants as large scale special purpose chemical factories (Ohlrogge, 1994; Brown, 1996; Ohlrogge 1999; Kinney, 1998). Therefore, future research should focus in this direction. One of the questions involves very basic research and the other question very applied research. The knowledge gained from probing either question suggests solutions for (or more often, invalidates approaches being tried by) the other approach.

How does a plant control how much and what types of fatty acid are synthesized? What regulates plant FAS? Some pertinent questions are: how many different FAS pathways exist, and are they regulated in the same manner? Specific ACP's

(Suh et al., 1999), ferredoxins (Schultz and Ohlrogge 1998), desaturases (Cahoon et al., 1992) and thioesterases (Pollard et al., 1991) exist for the synthesis of unusual fatty acids. This is ample evidence that unusual fatty acids are synthesized by a different set of enzymes, but it is unknown how much overlap exists between the set which produces normal fatty acids and the set specific to each different unusual fatty acid. Unusual fatty acids are excluded from membranes by some unknown mechanism and found almost completely in seed oils. While it is possible that plants which make unusual fatty acids have specific pathways only for the unusual fatty acid and another pathway for 18:1 and 16:0 production, another scenario is that fatty acids destined for membranes are synthesized by a separate pathway or system than fatty acids destined for storage triacylglycerols in all plants. Another alternative is that the separation occurs in the cytosol, outside the chloroplast, and fatty acids are funneled into either membranes or oil bodies. In any case, there must be some manner of sensing and signaling demand for, or supply of, fatty acids. When MCTE is overexpressed, laurate is synthesized and degraded, but due to presumed lack of 18C fatty acids, overall fatty acid production is induced (Eccleston and Ohlrogge, 1998).

How will this knowledge help to manipulate the products of FAS? If enzymes are specific to membrane, oil biosynthesis, or housekeeping functions, they are essentially in different pathways. This is important information when attempting overexpression experiments because overexpressing an enzyme outside of the pathway targeted for manipulation is unlikely to yield productive results. For

example, if the role of homomeric ACCase is to provide malonyl-CoA for further elongation of fatty acids, phytoalexins, and flavonoids (Sasaki et al., 1995), this enzyme may not respond to signaled demand for fatty acids destined for membranes or TAG. On the other hand, if enzymes are not specific to storage or membrane pathways there are fewer to find and target. This question will not be easy to answer at this point in time. If a set of enzymes which make unusual fatty acids can be localized in the chloroplast *and* that localization is different than the set of enzymes which make common fatty acids, this is a good indication that the pathways are separated. This would require specific antibodies or other probes to many enzymes, some of which may be undiscovered. These probes would have to be labeled differently for common and unusual FAS. However, insight to the question may be received by further investigation into one of the lipid field's hotly debated topics: channeling.

Is FAS channeled?

Currently, of the three intermediates in the reduce/dehydrate/reduce cycle, only hydroxyacyl-ACP's have been detected (Roughan, 1998, personal communication). This has led Roughan and others to argue that FAS is channeled. Evidence for this hypothesis consists of 1) chloroplasts permeabilized with low concentrations of Triton X-100 lose RuBisCO activity, but retain FAS competence (Roughan and Ohlrogge, 1996); 2) *in vivo* concentrations of CoA and its esters are too low to support *in vivo* rates of FAS (Roughan, 1997); 3) the 3-ketoacyl-ACP and 2-enoyl-ACP intermediates have yet to be detected

(Roughan, 1998, in preparation); 4) both ACP (Slabas and Smith, 1998) and ACCase are membrane associated (Sasaki et al, 1993; Shorrosh et al., 1995); 5) estimates of the concentration of the stearoyl-ACP desaturase enzyme exceed the known concentrations of stearoyl-ACP (Mehkedov et al., in preparation); finally, 6) data in Chapter 2 clearly demonstrate that an *in vitro* ACCase assay with *in vivo*-like conditions will not support the known *in vivo* rate of FAS. While none of the lines of evidence are definitive, they all suggest that FAS may, in fact act as a complex *in vivo*.

Channeling can occur in both stable and dynamic forms (e.g. Cornish-Bowden, 1997). A stable complex is, of course, easier to find than a dynamic one. In dynamic channeling, an enzyme-substrate (E_1-S) complex diffuses to meet a second enzyme (E_2) faster than the free intermediate product diffuses to meet E_2. This can occur if the concentration of E_1-S is much higher than the free intermediate product (Cornish-Bowden, 1997). If enzymes remain membrane-associated, this is not far-fetched. Two membrane-associated proteins would diffuse in two-dimensions, over the area of a membrane, while a soluble substrate, or protein-substrate complex, such as an acyl-ACP, would diffuse in three dimensions, in the volume of the stroma. Furthermore, some FAS enzymes, KAS III and perhaps α-CT, require detergent for maximal recovery during extraction (Gulliver and Slabas, 1994; Shorrosh et al., 1996). If channeling exists, it could be the mechanism by which fatty acids destined for storage products and for membranes are segregated.

Light/dark regulation of FAS

The mechanism of light/dark regulation and fatty acid synthesis in the light and dark needs to be further examined. ACCase activity is lower in the dark than the light (Chapter 2). Are other enzymes of FAS also influenced by light? If only ACCase is affected, it would be wise to look into the specific mechanism and target the specific mechanism. If other enzymes also have higher activity in the light than in the dark, then the light-regulation is likely to be a global mechanism or signal. Recent evidence where $^{13}CO_2$ was used to label fatty acids (Bao et al., in preparation) indicates that the rate of fatty acid synthesis in the dark is essentially zero. However, while the demand for fatty acids may decrease in the dark, it does not disappear - plant cells still expand. Are new lipids for membrane biogenesis needed for expansion and if so are they stored?

Another approach for manipulating metabolism is to find and alter transcription factors. This approach has worked in maize cell cultures where three transcription factors were overexpressed (Grotewold et al., 1998). Over-expression of two of the transcription factors led to an increase of one set of flavonoid products, overexpressing the other led to an increase of a different flavonoid product. In another recent example, Thomashow and coworkers overexpressed a transcription factor for cold acclimation which led to an increase in the expression of genes involved in cold-acclimation (Jaglo-Ottensen et al., 1998). There are two tasks to accomplish before this method can be applied to

fatty acid synthesis. First, the appropriate transcription factor is necessary, and none have yet been identified. More importantly, neither of the other pathways were primary metabolic pathways. Secondary pathways likely have fewer interconnections to other pathways and may be more amenable to metabolic engineering. There are numerous connections between carbon and nitrogen metabolic pathways. Genes for nitrogen metabolism are directly and indirectly controlled by sugars (Coruzzi, 1999) and carbon and nitrogen metabolism are extensively linked (reviewed in Huppe and Turpin, 1994). Although largely unexplored, interconnections between FAS and other metabolic pathways may exist and may need to be controlled. In spite of our metabolic goals when engineering an organism, the organism will generally attempt to optimize its survival and reproduction, regardless of our goals (Varner and Ramkrishna, 1998). Thus, in order to successfully manipulate FA metabolism, discovering the nature of interconnections between FAS and other pathways and deciphering the resultant interconnections and signals may be necessary.

A more complete computer simulation model could help to address a number of the above issues and increase progress in the manipulation of FAS products and quantity. The model presented in Chapter 3 is immature. It lacks one control point we know exists: feedback on ACCase (Shintani and Ohlrogge, 1995). It does not incorporate cofactor limitations of NADPH and NADH. The concentration of NADH is not significantly different in illuminated and non-illuminated chloroplasts, but the concentration of NADPH in a non-illuminated

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A more complete computer simulation model is also likely to address a number of the above issues and increase predictive power. The manipulation of FAS products and quantity. The model presented in Chapter 3 is unstable. It lacks one control point we know exists: feedback on ACCase (Shintani and Ohlrogge, 1995). It does not incorporate cofactor limitations of NADPH and NADH. The concentration of NADH is not significantly different in illuminated and non-illuminated chloroplasts, but the concentration of NADPH in a non-illuminated

chloroplast is half the concentration of NADPH in an illuminated chloroplast (Winter et al., 1994). These factors, if incorporated into the model, could affect the model's predictions. Furthermore, if connections to other metabolic pathways are made, competition for cofactors could substantially alter model predictions.

Any model, at best, can only be as good as the data of which it is comprised. The model is currently imprecise and only qualitatively matches reality. Precision can be increased by improving the biochemical inputs to the model: specifically, re-checking K_m and v_{max} values, and confirming that *in vitro* data has realistic physiological meaning. To increase precision, work on detecting changes in pool sizes should also continue. Current methods to determine concentration are based on lengthy TLC and HPLC methods as well as evaluation of product partitioning via immunoblotting (e. g., Roughan 1998, in preparation; Post-Beittenmiller et al., 1991, 1992). A method has recently been developed to rapidly and concurrently measure metabolites in crude rabbit extracts using capillary electrophoresis (Dillon and Sears, 1998). If the precision of measuring metabolite pools can be increased by either increased throughput or a new method, the quality of the model inputs and, therefore, outputs can be improved.

Models should be insensitive to unavoidable unknowns. The model in Chapter 3 has not been tested for sensitivity to the accuracy of kinetic values. This can probably be easily remedied. GEPASI is another software package for modeling (Mendes, 1993). It was designed for use in biochemical networks - precisely the

application which was described in Chapter 3. GEPASI was not used to develop the Chapter 3 model because it requires the biological system to be in a steady state before simulation. Furthermore, it is difficult to build a model in incremental stages with GEPASI. Due to those limitations, Stella II was used to build the model described in Chapter 3. But now that the model is built it could be moved to GEPASI. GEPASI has the ability to alter parameters, such as K_m and v_{max} in a sequential series of simulations. This feature can be used to test whether or not the model's predictions vary with changes in these unknown parameters.

To be an efficient prediction tool, the model needs to incorporate as much experimental data as possible. The changes above should be incorporated. The model can also be rebuilt in several ways: alternative control pathways can be incorporated, and model predictions which are different between alternatives should be tested. The genetic expression experiments proposed by the model should also be investigated.

Will biotechnology be able and allowed to actually help?

A final area where further work needs to be done is in the acceptance and public perception of and knowledge about biotechnology. The direction and discussion of the work presented here and its usefulness presume that transgenic plant products will be accepted by the public and their governments. This is not a given. There have been unintended consequences from some applications of

biotechnology. Transgenic canola comprises 70% of the crops grown in Canada (Ohlrogge, 1999), but the Roundup Ready® gene has escaped to wild mustard, another member of the Brasssicaceae family (R. Allison, personal communication). Pollen from corn expressing the *B. thuringiensis* toxin is lethal to Monarch butterflies (Losey et al., 1999; Milius, 1999). Although tomato paste from transgenic tomatoes was marketable in the United Kingdom, public acceptance of transgenic products in that country is low (Gavaghan, 1999). There is great concern and debate about the escape of genes used to make crops resistant to herbicides and pests (e.g. Roundup Ready® soybeans and/or *B. thuringiensis* toxin producing plants), However, technology such as the "terminator" gene, which not only makes the plant sterile but also makes gene escape highly improbable, is also publicly unacceptable. (Service, 1999) In addition, there is debate about whether or not biotechnology will increase or decrease sustainability (e.g. Crouch, 1995; Zechendorf 1999). These issues of societal (mis)understanding of biology, what it can and can not accomplish, and the converse issue of scientists not understanding or accepting societal concerns are important future considerations.

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APPENDIX A

Appendix A

NUMERICAL METHODS

This section describes and discusses the numerical methods to solve the equations generated by Stella II. The point of all these methods is to solve a set of differential equations, balancing computational efficiency against error. A mathematical function is "well-behaved" if it is both differentiable and continuous. Since the system is a biological one, it is quite probably described by a system of well-behaved functions. The model generates a system of rate equations that are a set of simultaneous differential equations, and numerous methods exist to solve these equations numerically. The system of equations is too complex to be solved analytically (which would give an exact solution).

All the methods described below are iterative, single-step methods. That is, they evaluate the function at some specific value, in this case, time. The resultant value is used to evaluate the function at some further point in time. The method needs a function, an initial condition, and the first derivative of the function. In general, the closer the time intervals are, the more closely the value obtained from the numerical integration matches the value that would be obtained if the equations were solved analytically.

These methods are based on series expansions of functions. A series expansion allows evaluation of a function, $f(x)$ at some value, i.e. x, given an initial value

(x_0) and the function. Euler's and Runge-Kutta numerical methods are based on the Taylor series expansion. This expansion is shown below (Swokowski, 1978).

$$f(x) = f(x_0) + f'(x_0)(x - x_0) + \frac{f''(x_0)}{2!}(x - x_0)^2 + \frac{f'''(x_0)}{3!}(x - x_0)^3 + \ldots$$

Essentially, the value of the function at the point x is equal to the value of the function at an initial value, x_0, plus an infinite number of terms. These terms are adjustments based on the difference between the two values (x-x_0) and multiplied by the factor (n^{th} derivative/n!), which is an indication of the difference in value between $f(x)$ and $f(x_0)$. These additional correction factors decrease in size due to the increasing denominators (n! = n * (n-1) * (n-2) * (n-3) ... * 1). So, to approximate $f(x)$, one uses as many terms in the Taylor series as desired and as small a step size (x-x_0) as necessary to get the desired accuracy.

All the methods will be described using the graph shown in figure A.1. This is a graph of initial enzyme velocity vs. substrate concentration for an enzyme which follows Michaelis-Menten Kinetics. That is:

$$v = \frac{v_{max} \times [S]}{[S] + k_m} \qquad \text{or} \qquad f(x) = \frac{v_{max} \times x}{x + k_m}$$

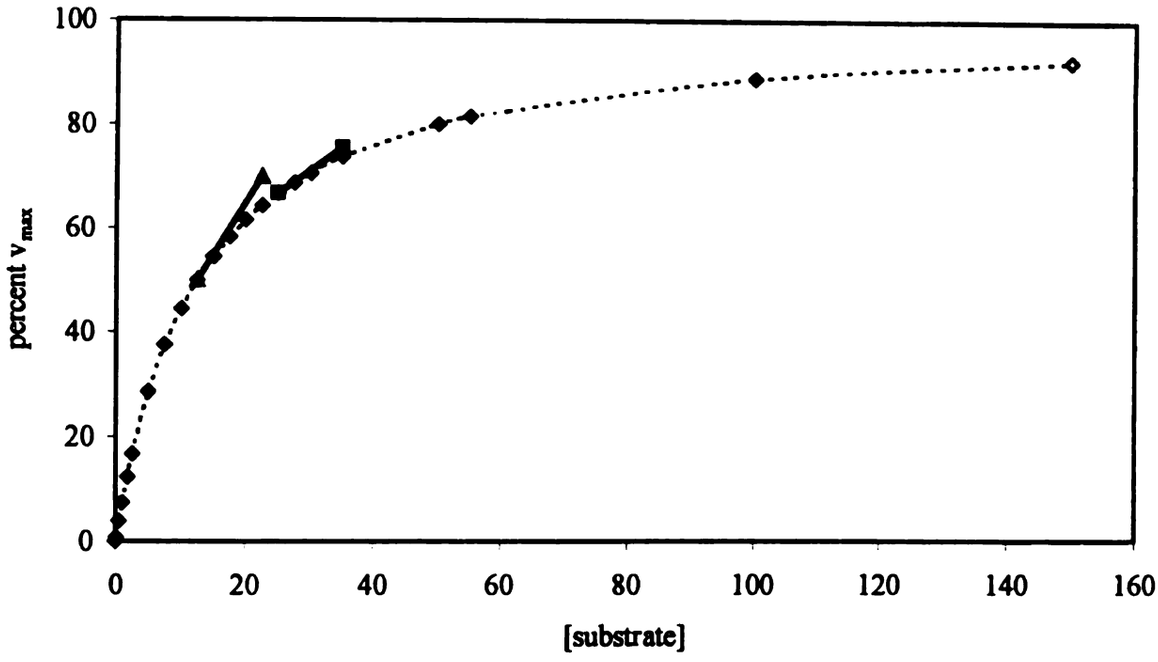

Figure A.1. Graphical representation of Euler's method.

Michaelis-Menten equation with tangent lines showing the values (▲) of approximations using Euler's method. Using the same step size, the approximation at 22.5 is far worse than the approximation at 35 due to the large change in the graph around 22.5.

The methods below need the first derivative of the function, $f'(x)$. Applying the quotient rule:

$$f'(x) = \frac{v_{max} * k_m}{(k_m + x)^2}$$

Euler's method uses only the first two terms of the Taylor expansion series, $f(x_0)$ and $f'(x_0)(x - x_0)$ (Gerard, 1978). The application of this method is also shown in figure A.1. The actual value of the f(x) is shown by the dotted line. The first derivative of a function at a point x is the slope of the line tangent to the function at that point. Thus, Euler's method uses this tangent line and the difference between two values to approximate a new $f(x)$. Approximations using Euler's method are shown for the value $f(22.5)$ using 12.5 as x_0 and a step size of 10 on the left and for the value of $f(35)$ using 25 as x_0 with the same step size. It is apparent from the graph that the latter approximation is closer to the real value than the first approximation. This is because the difference between the first derivatives of the function at x=25 and x=35 is small compared to the difference of the first derivative at x=12.5 and x=2.5. It is also apparent from the graph that smaller step sizes will yield more accurate estimations.

Euler's method, while useful, tends to compound errors (Gerald, 1978) and in some iterations of the Stella models, the step size required for accuracy was smaller than computational error. That is, there are usually truncation errors in numerical calculations done on a computer. These errors were larger than the step size required to get a good approximation via Euler's method.

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This problem is solved by the Runge-Kutta methods. These methods are based on Euler's method, but use more than one approximation for the slope of the line. Runge-Kutta 2 averages the first derivatives at both x_0 and x. Runge-Kutta 4 uses more internal points. Both these methods increase accuracy and are self-correcting (Gerald, 1978). No numerical truncation problems were encountered using either Runge-Kutta method in Stella II.

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