TOWARD A BIOCHEMICAL AND MOLECULAR UNDERSTANDING OF THE BIOSYNTHESIS OF FATTY ACID AND TRIACYLGLYCEROL IN PLANTS

By

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ABSTRACT

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In plants, fatty acid synthesis is a primary metabolic pathway and triacylglycerol (TAG) is one of the means for storing energy and carbon in seeds. In this thesis, I addressed four questions dealing with plant lipid metabolisms which span the pathway from the origin of acetyl-CoA to the final accumulation of TAG in oil seeds. First, acetyl-CoA is the ultimate precursor for fatty acid synthesis in all kinds of tissues, but its origin has been a debated topic. Free acetate has been suggested as a possible source of acetyl-CoA in leaf tissues. Pulse-chase and continuous labeling with $^{14}CO_2$ were conducted in leaves of *Arabidopsis*, barley, and pea, and the label rapidly appeared in fatty acids with a lag phase of less than 2 min. These data suggest that either the bulk pool of acetate is not involved in fatty acid synthesis or the concentration of acetate must be less than 0.05 mM in light grown leaves. Second, a genomic sequence of biotin carboxylase (BC), one of four subunits of multisubunit acetyl-CoA carboxylase, was isolated from *Arabidopsis* and its promoter was partially characterized. BC expression was found to be higher in silique and flower than in root and leaf. The region from -140 to +147 contained necessary promoter elements which supported basal gene expression. A positive regulatory region was found to be located between -2100 and -140, whereas a negative element was possibly located in the first intron. In addition, several

conserved regulatory elements were identified in the BC promoter. Third, to examine whether newly synthesized oleic acid is directly elongated to erucic acid in developing seeds of *B. rapa*, embryos were labeled with [^{14}C]acetate, and the ratio of radioactivity of carbon atoms C(5)-C(22) (from plastid) to C(1)-C(4) (from cytosol) of erucic acid was monitored with time. This labeling ratio decreases with time and therefore suggests the existence of an intermediate pool of oleate which contributes at least part of the oleoyl precursor for the production of erucic acid. The malonyl-CoA for elongation of oleate to erucate was produced by homodimeric ACCase. Both light and haloxyfop increased the accumulation of [^{14}C]oleate and the parallel accumulation of [^{14}C]phosphatidylcholine. Taken together, these results show an additional level of complexity in the biosynthesis of erucic acid. Fourth, to examine limiting factors on seed TAG production, developing *C. lanceolata, U. carpinifolia* and *U. parvifolia* embryos were incubated with factors whose availability might limit oil accumulation. The addition of glycerol or sucrose did not significantly influence the rate of TAG synthesis. However, the rate of ^{14}C-TAG synthesis, upon addition of 2.1 mM ^{14}C-decanoic acid (10:0), was approximately four times higher than the *in vivo* rate of TAG accumulation in *Cuphea*, and two times higher than the respective *in vivo* rates in *Ulmus*. ^{14}C-10:0 was incorporated equally well in all three acyl positions of TAG. The results suggest that both *Cuphea* and *Ulmus* embryos have sufficient acyltransferase activities and glycerol-3-phosphate levels to support rates of TAG synthesis in excess of those found *in vivo*. Consequently, the amount of TAG synthesized in these oilseeds may be in part determined by the amount of fatty acid produced in plastids.

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LIST OF ABBREVIATIONS

ACCase ..... aceyl-CoA carboxylase

ACP ..... acyl carrier protein

ADP ..... adenosine diphosphate

ATP ..... adenosine triphosphate

BC ..... biotin carboxylase

BCCP ..... biotin carboxyl carrier protein

C16 and C18 ..... fatty acids with 16 or 18 carbons

chl ..... chlorophyll

CT ..... carboxyltransferase

DAF ..... days after flowering

DAFC ..... days after the first collection

DAG ..... triacylglycerol

DAGAT ..... diacylglycerol acyltransferase

FA ..... fatty acid

FAME ..... fatty acid methyl ester

GC ..... gas chromatography

GC/MS ..... gas chromatography-mass spectrometry

KAS ..... 3-ketoacyl-acyl carrier protein synthase

TLC ..... thin layer chromatography

PC ..... phosphatidylcholine

PDC ..... pyruvate dehydrogenase complex

TAG ..... triacylglycerol

GPAT ..... glycerol 3-phosphate acyltransferse

LPAAT ..... lysophosphatidic acid acyltransferase

FATTY ACID NOMENCLATURE

10:0 ..... decanoic acid

12:0 ..... lauric acid

14:0 ..... myristic acid

16:0 ..... palmitic acid

17:0 ..... heptadecanoic acid

18:0 ..... oleic acid

18:1 $^{\Delta 9}$ ..... decanoic acid

18:1 $^{\Delta 6}$ ..... petroselinic acid

18:1 $^{\Delta 11}$ ..... *cis*-vaccenic acid

18:2 $^{\Delta 9,12}$ ..... linoleic acid

18:1$^{\Delta 9, 12, 15}$ ..... linolenic acid

20:1$^{\Delta 11}$ ..... eicosenoic acid

22:1$^{\Delta 13}$ ..... erucic acid

CHAPTER 1

Introduction

The overall theme of this PhD dissertation has been to obtain a fuller understanding at both the biochemical and molecular biological levels of the biosynthesis of fatty acid and triacylglycerol in plants. The importance of fatty acid and triacylglycerol synthesis is reflected in several aspects of plant biology. Firstly, fatty acids are the essential component of all membrane glycerolipids and form the central hydrophobic barrier at the core of the membrane. Fatty acids are attached to both the *sn*-1 and *sn*-2 positions of glycerol backbone and a polar headgroup to the *sn*-3 position. The resultant amphipathic properties of glycerolipids allow the formation of membrane bilayers which delineate the cell and sub-cellular compartments. These compartments not only are the sites of many essential biochemical processes, like photosynthesis in chloroplasts and respiration in mitochondria, but also separate certain reactions that make overall regulation and coordination of metabolisms possible. Diverse glycerolipids species exists in plant cells through the combination of different headgroups and fatty acids. The membrane of each individual sub-cellular structure has it own unique lipids classes that possess distinct fatty acid composition (Browse *et al.*, 1993), like galactolipids predominantly found in plastids and cardiolipin mainly in mitochondria. The differences in lipid structure of each sub-cellular membrane system are important for them to execute specific functions, even though the exact structure to function relationships are not fully understood.

Secondly, fatty acids are precursors for the synthesis of cuticular wax that are an important defense system against pathogens and prevent water loss for terrestrial plants. A plant hormone, jasmonic acid, is also derived from fatty acid.

Thirdly, fatty acids are rarely found in the form of free fatty acids, instead, and their carboxyl group is esterified, for the most part, to glycerol. In addition, fatty acids cannot be transported between cells, so each cell has to synthesize its own. Therefore, the fatty acid synthesis and utilization systems must be fully coordinated and tightly regulated. The regulation of fatty acid synthesis occurs at several levels. The *de novo* fatty acid synthesis in plastids determines the overall output of total fatty acids to meet the demand at a given growth stage. After *de novo* synthesis, further modifications are required to synthesize certain lipid classes. Proportional distribution of fatty acids among lipid classes and organelles is necessary for keeping the delicate balance in each living cell and the whole plant. Currently, relatively little is known about the mechanism of regulation for fatty acid synthesis in plants.

Finally fatty acid composition of glycerolipids in membranes changes in responding to different environments. Plants grown at low temperature, have higher percent of unsaturated fatty acids relative to that of grown at high temperature. Wax synthesis increases significantly when plants are transferred from humid condition to drier environment. Fatty acid synthesis as a primary metabolic pathway, its regulation and the origin of substrate for fatty acid synthesis have been under extensive study in past twenty years, but many questions remain

to be answered.

Triacylglycerols (TAG), mainly accumulate in seeds, and unlike glycerolipids in membranes, do not perform any structural role but rather serve primarily as storage form of carbon and energy for the growth of next generation. TAGs are a major agricultural commodities with an annual value of more than 50 billion USD (Lühs and Friedt, 1994). More than 80% of vegetable oils are used in the food industry. There are three practical goals which relate to this field of research. First is to increase the oil content and yield of existing oil crops to meet the demand of population growth. Traditional and molecular markers guided breeding methods have been used to achieve higher oil content and yield. However, because of limited germplasm of cultivated crops and extensive inbreeding, it is difficult to further increase yields through traditional plant breeding. Since most pathways and enzymes related to carbon partition are now known, it looks more promising to manipulate carbon distribution among different pathways in order to channel more carbon into fatty acid synthesis, thereby increasing an oil yield. Second is to alter the relative fatty acid compositions in seed oils that are beneficial for human health and replace some animal fat products. With the knowledge of pathways for TAG synthesis and genetic engineering, also the health concerns animal fats in human diets, substantial efforts have been given in altering the composition of vegetable oil to improve nutritional value of oils consumed. Due to the low melting point of vegetable oils, the production of margarine and shortening from vegetable oils requires catalytic hydrogenation to reduce unsaturated fatty acids, and therefore

increase a melting point. This process not only increases the production cost, but also converts certain percentage of natural *cis* configurations to *trans* isomer which are suggested to be bad for health. By reducing the activity of stearoy-ACP desaturase, the percent of saturated fatty acids increased about 20-fold in *Brassica* (Knutzon *et al.*, 1992), whose oil might be directly used for the production of margarine and shortening without the hydrogenation process. On the other hand, unsaturated fatty acids are beneficial in reducing cholesterol levels. Increasing unsaturated fatty acids in vegetable oil was achieved through enhancing the elongation of palmitoyl-ACP to stearoyl-ACP in transgenic *B. napus* (Bleibaum *et al.*, 1993). A third goal is to produce special fatty acids in oil crops for industrial usages and as a renewable raw material to replace fossil fuel. A diverse range of fatty acid structures has been found in seed oils and a number of these are used industrially. For example, erucic acid is used as a slip agent in plastic film and might have a large potential as a precursor to nylon 13,13, a high-temperature thermoplastic. Ricinolenic acid from castor beans is used in coatings and lubricants. Lauric acid (12:0) from palm oil is a major ingredient in soaps and detergents. Because of the limited geographical distribution of palm trees, the incentive to produce lauric acid in temperate oil crops resulted in the high lauric *Brassica* obtained by introducing lauroyl-ACP thioesterase from California bay into *Brassica* (Voelker *et al.*, 1992). Even though considerable progress has been made to understand the synthesis of unusual fatty acids and TAG, we still have a lot of difficulties to achieve the desired fatty acid levels that are high enough for industrial

usages.

In this thesis, four major questions were addressed, which span the lipid biosynthetic pathway, from the origin of acetyl-CoA to the final accumulation of TAG. Each question will be discussed separately in the following paragraphs of this chapter, which are organized to follow the biochemical pathway of carbon from CO_2 to TAG. An overview of these questions is presented schematically in Figure 1-1 to show their relationship to overall lipid metabolisms. Each question also represents a chapter of this thesis and a publication or submitted manuscript.

I. What is the Origin of Plastidial Acetyl-CoA?

As illustrated in Figure 1.1, *de novo* fatty acid biosynthesis in higher plant cells is exclusively located in the plastid (Ohlrogge *et al.*, 1979), except a very small contribution from the mitochondrion (Wada *et al.,* 1997). The immediate carbon precursor for fatty acid biosynthesis is acetyl-CoA, which is used as substrate by KASIII for the acyl-chain primer reaction, and, after carboxylation to malonyl-CoA and transacylation to malonyl-ACP, for the acyl-chain elongation steps. The first question we address in this thesis is: how is acetyl-CoA generated in plastids. Because different tissues may not have the same mechanism to produce their acetyl-CoA, we will limit our scope for this question to green leaf tissue.

The biosynthetic origin of acetyl-CoA for plastid fatty acid synthesis has been addressed by many reports, but remains unsolved for the most part due to lack of definitive evidence to prove or disprove the proposed possibilities (Givan, 1983;

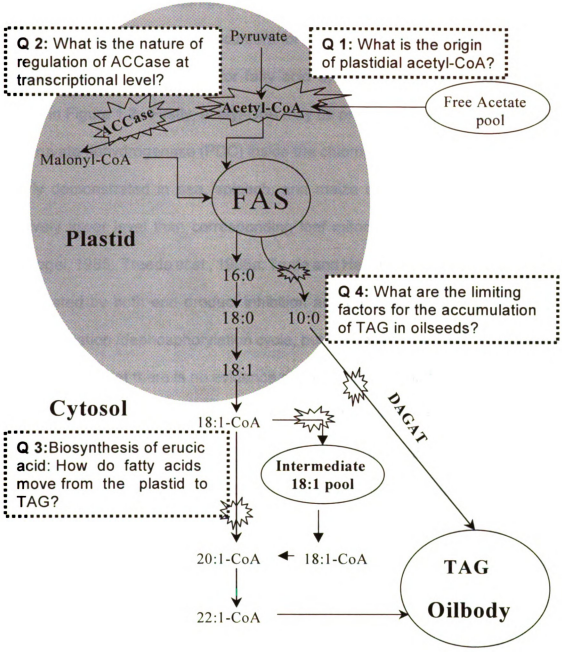

Figure 1-1. The general pathway from acetyl-CoA to the accumulation of TAG. The major questions addressed in this thesis are indicated.

Liedvogel, 1986). Because acetyl-CoA does not readily cross the plastid membrane (Brooks and Stumpf, 1965), it must be synthesized inside the plastid in order to provide the carbon precursors for fatty avid synthesis. Several possible routes generating acetyl-CoA for fatty acid synthesis have been suggested as shown in Figure 1.2. Firstly, acetyl-CoA may be produced from pyruvate and CoA by pyruvate dehydrogenase (PDC) inside the chloroplasts. PDC activity has been clearly demonstrated in pea, spinach, and maize chloroplasts, even though at relatively lower level than corresponding leaf mitochondria (Camp *et al.*, 1985; Liedvogel, 1985; Treede *et al.*, 1986a; Trude and Heise, 1985). Mitochondrial PDC is regulated by both end product inhibition and covalent modification through a phosphorylation /dephosphorylation cycle, but the chloroplast PDC resembles the *E. coli* PDC in that there is no evidence of any covalent modification (Camp *et al.*, 1985). Pyruvate is most likely derived from 3-PGA which can be produced from glycolysis or the initial reactions of the Calvin cycle. Although most of the enzymes needed for glycolysis exists in the chloroplast stroma, phosphoglyceromutase, required to convert 3-PGA to 2-PGA, may not have sufficient activity in chloroplasts to produce enough pyruvate in order to sustain the rate of fatty acid synthesis. Therefore, the participation of cytosolic phosphoglyceromutase may be required (Givan, 1983). This would explain the low incorporation of $^{14}CO_2$ into fatty acids by isolated chloroplasts. An alternative source of pyruvate might be as a direct product of ribulose-biphosphate carboxylase in chloroplasts. However, this source can only count for a quarter of fatty acids synthesized (Andrews *et al.*, 1991).

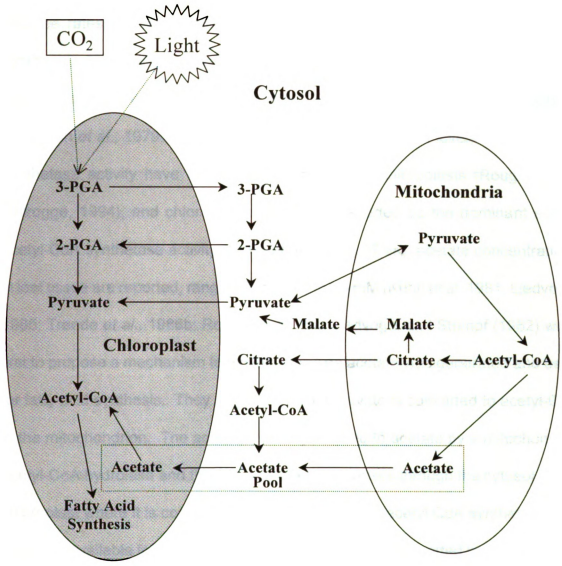

Figure 1-2. Simplified scheme of proposed pathways that might generate acetyl-CoA for fatty acid synthesis in leaf tissue.

Secondly, acetyl-CoA may be synthesized from free acetate which is imported into chloroplasts. Several lines of evidence support this view. First, exogenous acetate is rapidly incorporated into fatty acids of isolated chloroplasts and the rates of incorporation are equivalent to the *in vivo* rate of fatty acid synthesis. In addition, acetate is three fold or more effective than pyruvate (or $^{14}CO_2$) as an *in vitro* substrate for fatty acid synthesis in isolated chloroplasts (Roughan *et al.*, 1979; Springer *et al.,* 1989). Second, high levels of acetyl-CoA synthetase activity have been reported in isolated chloroplasts (Roughan and Ohlrogge, 1994), and chloroplasts have been reported as the dominant site of acetyl-CoA synthetase activity (Kuhn *et al.*, 1981). Third, acetate concentrations in leaf tissue are reported, ranging from 0.05 to1.4 mM (Kuhn et al, 1981; Liedvogel 1985; Treede *et al.*, 1986b; Roughan, 1995). Liedvogel and Stumpf (1982) were first to propose a mechanism to explain how free acetate was generated and used for fatty acid synthesis. They suggested that pyruvate is converted to acetyl-CoA in the mitochondrion. The acetyl-CoA is hydrolyzed to acetate by a mitochondrial acetyl-CoA hydrolase and the free acetate then diffuses through the cytosol to the chloroplast where it is converted to acetyl-CoA by the acetyl-CoA synthetase and becomes available for fatty acid synthesis. This view is supported by the facts that mitochondria have an active pyruvate dehydrogenase and acetyl-CoA hydrolase. But this mechanism of acetyl-CoA production, as of yet, has not been supported by *in vivo* experiments. Roughan (1995) concluded that it seems probable that endogenous acetate would be the direct source of acetyl-CoA for fatty acid

synthesis in leaves. An important part of the argument for acetate as the dominant substrate for fatty acid synthesis *in vivo* is that the bulk tissue concentrations are above the concentration for acetate saturation for fatty acid synthesis. In addition to the mechanisms discussed above, acetylcarnitine was suggested as a possible way to deliver acetyl-CoA into plastids (Woods *et al.*, 1992).

It is clear from the above that a consensus is not yet available concerning the origin of acetyl-CoA for fatty acid synthesis. Although several pathways can be supported based on *in vitro* data, *in vivo* evidence is lacking in almost all cases. In chapter 2, the question of whether free acetate is a substrate for fatty acid synthesis in leaf tissue is examined *in vivo*. We approach this question first by accurately measuring the rate of fatty acid synthesis in leaf tissue. Knowing the synthesis rate and the pool size of free acetate, the turnover time of free acetate pool can be calculated, if it serves as the primary source for acetyl-CoA. As shown schematically in Figure 1.3, when plants are given $^{14}CO_2$, the lag phase of radioactivity accumulation in fatty acids will reflect the pool size of intermediate substrates through which carbon must flow. Thus, by examining the kinetics of ^{14}C incorporation into fatty acids and the calculated turnover time of free acetate pool as shown in Figure 1.3, we can test the possibility that the bulk acetate pool is the main source for the synthesis of acetyl-CoA.

Accurate rate of fatty acid synthesis is crucial to calculate the turnover time of the free acetate pool. In chapter 2 we also demonstrate a new method that can accurately measure the absolute rate of fatty acid synthesis both in the light and in

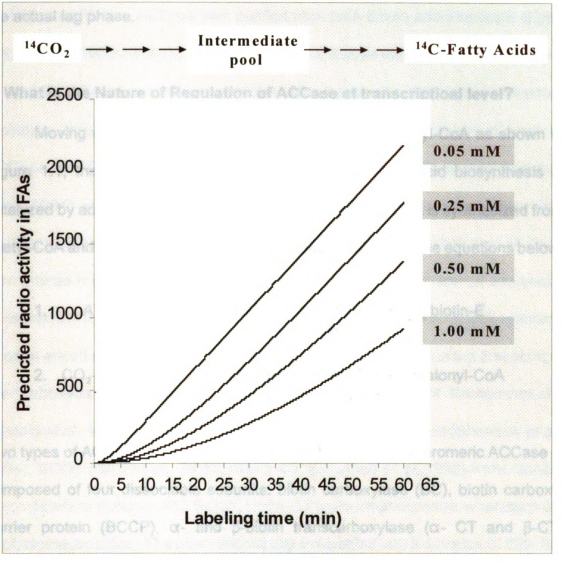

Figure 1.3. Simulated models for kinetics of radioactivity accumulated in fatty acids with different concentrations of intermediate pools. The rate of fatty acid synthesis is 38nmols/min/gfw. The lag phase before linear kinetics is observed will depend on the size of the pools through which carbon must flow before incorporation into fatty acids.

dark. And finally, short and long term carbon dioxide labeling is used to measure the actual lag phase.

II. What Is the Nature of Regulation of ACCase at transcriptioal level?

Moving one step further down the pathway from acetyl-CoA as shown in Figure 1.1, the first committed reaction of *de novo* fatty acid biosynthesis is catalyzed by acetyl-CoA carboxylase (ACCase). Malonyl-CoA is synthesized from acetyl-CoA and bicarbonate via the two step reaction shown in the equations below.

$$1.\quad ATP + HCO_3^- + \text{biotin-BCCP} \overset{Mg^{++}}{\underset{\text{biotin carboxylase}}{\rightleftharpoons}} ADP + Pi + CO_2\text{-biotin-E}$$

$$2.\quad CO_2\text{-biotin-BCCP} + \text{acetyl-CoA} \underset{\text{carboxyltransferase}}{\rightleftharpoons} \text{biotin-BCCP} + \text{malonyl-CoA}$$

Two types of ACCase are found in nature. Multisubunit or heteromeric ACCase is composed of four dissociable subunits: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), α- and β-biotin transcarboxylase (α- CT and β-CT) (Guchhait *et al.*, 1974). Multifunctional or homomeric ACCase is a large single polypeptide containing the same three function domains as the multisubunit ACCase, but in one polypeptide.

The structures of plant ACCases have been under intensive studies in the past 15 years. Early attempts to purify ACCase from plant tissues yielded multiple subunits on PAGE under denaturing conditions but these were thought to have

resulted from endogenous proteinase degradation (Harwood, 1987). However, with proteinase inhibitors ACCase was purified from both dicots and monocots (Egin-Buhler *et al.*, 1980; Finlayson and Dennis, 1983; Slabas and Hellyer, 1985; Hellyer *et al.*, 1986; Charles and Cherry, 1986) and in all cases the ACCases purified had molecular mass in the range 220-240 kDa. Deduced amino acid sequence of ACCase from *Cyclotella cryptica* (Roessler and Ohlrogge, 1993), alfafa (Shorrosh *et al.*, 1994), and *Arabidopsis* (Roesler *et al.*, 1994) also have a molecular mass of 220-250 kDa. So until 1993, most researchers had concluded that plant ACCase was a large (>200 kDa) mutifuntional protein similar to that of animal and yeast. However, in 1993, Sasaki and co-worker demonstrated that the chloroplast genome of peas encodes a subunit of an ACCase with structure related to the β subunit of the carboxyltransferase found in the multisubunit ACCase of *Escherichia coli* (Sasaki *et al.*, 1993). In the following several years, a BC subunit (Shorrosh *et al.*, 1994), BCCP (Choi *et al.*, 1995), and α-CT (Shorrosh *et al.*,1996) were cloned. Among the four subunits, BC, BCCP, and α-CT are nuclear encoded, whereas β-CT is plastome encoded. The four subunits are assembled into a complex of 650-700 kDa which is easily dissociable. It has now been clarified that dicot and most monocots have both forms of ACCase, the multifunctional form localized in cytosol and the mutisubunit form with at least four subunits localized in plastids (Alban *et al.*, 1994; Konishi *et al.*, 1996; and Roesler *et al.*,1996). It is the mutisubunit plastid form of ACCase that provides malonyl-CoA for fatty acid synthesis. The structures of ACCase in *Gramineae* are different in that these species lack the multisubunit

form of ACCase and instead have two type of mutifunctional ACCase. An herbicide-sensitive form is located in plastid and a resistant form in the cytosol.

It has been long known that fatty acid synthesis is tightly regulated. ACCase is the first committed step for de *novo fatty* acid synthesis, therefore, it is logical to speculate that ACCase is an important site of regulation. In animals, ACCase is regulated by covalent modification (phosphoralation/dephosphoralation) and also allosterically regulated by its reaction product and end products of fatty acid synthesis. There are also complex transcriptional and posttranscriptional controls of ACCase expression in animals which involve transcriptional induction and differential splicing of mRNA in response to diet and developmental state (Lopez-Casillas *et al.*, 1989; Luo *et al.*, 1989). The tight overall control of acyl lipid synthesis by ACCase has also been shown in yeast and *E. coli* (Hasslacher *et al.*, 1993; Jackowski *et al.*, 1991).

In plant leaf tissues, the rate of fatty acid synthesis is at least six-fold higher than in the dark. There has been great interest to find out which enzyme(s) play the regulatory role in the pathway of fatty acid synthesis. As mentioned earlier, ACCase is located at the ideal site of regulation. There was early indirect evidence implying that ACCase is an important regulatory enzyme. When isolated chloroplasts were incubated with radio-labeled acetate or pyruvate, acetyl-CoA was formed in the dark, but not malonyl-CoA and fatty acids. Only in the light were malonyl-CoA and fatty acids labeled (Nakamura and Ymada, 1979). ACCase from chloroplast and wheat germ were inhibited by ADP whose concentration was higher in the dark than

in the light (Eastwell and Stumpf, 1983). The direct evidence to prove that ACCase is indeed the regulatory enzyme came from the work of Post-Beittenmiller *et al.* (1990) through measuring the pools of intermediates during manipulation of the rates of fatty acid synthesis by light in spinach leaves. When comparing light vs dark incubations, it was found that there were large increases in the acetyl-ACP and commensurate decrease of malonyl-ACP from light to dark. From these and other observations it was concluded that light/ dark control of leaf fatty acid synthesis resides in ACCase. In a following study (Post-Beittenmiller *et al.*,1992), short chain acyl-CoA pools were measured in isolated pea and spinach chloroplasts. Malonyl-CoA and malonyl-ACP could be detected only during light incubations and increased as fatty acid synthesis increased. In contrast, both acetyl-CoA and acetyl-ACP were detectable in the absence of fatty acid synthesis and acetyl-ACP decreased with the increased rates of fatty acid synthesis. These data provided direct in situ evidence that ACCase plays a regulatory role in fatty acid synthesis. A quantitative measure of the importance of ACCase in the control of flux through the pathway of fatty acid synthesis was obtained by using the metabolic control theory (Page *et al.*, 1994). They took advantage of the fact that plastidial ACCases of barley and maize leaves are sensitive to the herbicide fluazifop and sethoxydim. The sum of all the flux control coeffients in a metabolic pathway is one, and the closer an individual flux control coefficient is to one, the more control that enzyme exerts in regulating the metabolic pathway. Apparent flux control coefficient of about 0.58 and 0.52 were determined for acetyl-CoA

carboxylase in barley and maize leaves, respectively. These results clearly showed that acetyl-CoA carboxylase is a major flux controlling enzyme for light-stimulated lipid synthesis in leaf tissues. ACCase is also the site of feedback regulation by reducing fatty acid synthesis of tobacco suspension cell in response of to exogenous lipids (Shintani and Ohlrogge, 1995). It is clear that plastid ACCase activity is regulated at the biochemical level, but the mechanisms of how ACCase is regulated has not been resolved yet. Savage and Ohlrogge (1999) observed that the phosphorylation of β-CT of pea ACCase resulted in the decrease of ACCase activity. So plant ACCase may be also regulated by covalent modification (phosphoralation/ dephosphoralation).

Control of ACCase gene expression. In tobacco and castor, expression of the BC gene is much higher in developing seeds than in leaf or root (Elborough *et al.*,1996; Roesler *et al.*,1996; Shorrosh *et al.*, 1995). In developing castor and *Brassica napus* seeds, ACCase activity as well as expression levels of BC and BCCP correlate with oil deposition (Roesler *et al.*,1996). Using *in situ* hybridization analysis, BC and BCCP were found to be expressed in a coordinate fashion and were correlated with oil accumulation during seed development in *Arabidopsis* (Choi *et al.*,1996). The plastid ACCase subunits, BC, BCCP, and α-CT are encoded by nuclear genes, whereas β-CT is encoded by a plastid gene. It is not known how overall ACCase expression is controlled or how levels of the four subunits are coordinately regulated. Nor is there research to investigate what *cis* acting elements are responsible for the regulation of ACCase. To begin to address these

questions in chapter 3, we describe the isolation and characterization of a biotin carboxylase genomic clone from *Arabidopsis.* Furthermore, we report initial studies to determine the *cis* acting elements of the promoter of the BC subunit of ACCase.

III. Biosynthesis of Erucic Acid: How do fatty acids move from the plastid to triacylglycerol

The fatty acids synthesized in plastids have two fates; one is to remain inside plastids for glycerolipid synthesis; the other is to be exported outside plastids. The exported fatty acids are usually 16:0, 18:0, and 18:1, and they can be used in several ways, such as the synthesis of membrane lipid, wax, and triacylglycerol. Sometimes the fatty acids go through extensive modification during these processes. In oilseeds, most of the exported fatty acids are used for the synthesis of triacylglycerol as a means of storing carbon and energy. A diverse range of fatty acids can be found in seed oil. Some of the fatty acids are called unusual fatty acids due to their limited distribution among plant species and their chemical structures differing significantly from those of the common C_{16} and C_{18} acyl moieties such as palmitic (16:0) and oleic acids ($18:1^{\Delta 9}$). Structural divergence in these acyl moieties include the variations in carbon chain lengths, position and numbers of double bond, additional function group such as hydroxyl or epoxide ring, and the combination of above. Here, we will limit our discussion to the biosynthesis of erucic acid ($22:1^{\Delta 13}$), one of the unusual very long chain fatty acids.

Erucic acids is an important industrial material used as lubricants or as slip

agents in plastic film manufacture. It may also have a larger potential as a precursor of nylon 13, 13, a high temperature thermoplastic (Ohlrogge, 1994). Erucic acid (*cis*-13-docosenoic acid) and its homolog, *cis*-11-eicosenoic acid, are commonly found in the seed oils of the *Crucifereae*. The seed oil of *Brassica spp., Crambe abyssinica, Sinapsis alba, and Lunaria annua* usually contain 40-60% erucic acid while seed oil of nasturtium (*Tropaeolum majus*) contains as much as 80% erucic acid (Gustone *et al.*, 1994; Luhs and Friedt, 1994). Commercially, erucic acid has usually been derived from rapeseed oil. However, even for high erucic rapeseed oil, the current level of 50% erucate in its triacylglycerol is not sufficient to compete well with alternative source of lubricant oils from petrochemicals because of the high cost of purification (Ohlrogge, 1994). Genetic manipulation to increase the erucic acid content in rapeseed oil has attracted great interests due to its economic potential. To achieve this goal, we have to better understand the biosynthetic pathway of erucic acid.

The reactions leading to the synthesis of erucic acid are, for the most part, well understood. In the developing embryos of several erucate accumulating species, it was demonstrated that erucic acid is synthesized by the elongation of oleic acid rather than by *de novo* synthesis (Appleby, 1974; Downey *et al.*, 1964; Ohlrogge *et al.*, 1978; Pollard *et al.*, 1980a and 1980b). This conclusion was deduced from the observation that exogenous [^{14}C]acetate was preferentially incorporated into the carboxyl-terminal carbons of the long-chain fatty acids rather than the methyl terminal 18 carbons. These results inferred that the addition of the

carboxyl-terminal four carbons were catalyzed by different enzymes and at different compartment, comparing to the synthesis of methyl terminal 18 carbons of erucic acid. Many reports have confirmed that the location of oleoyl elongation system was extra-plastidial, being associated with oil bodies or microsomal membranes (Agrawal and Stumpf,1985a and 1985b; Fehling *et al.*, 1991; Imai *et al.*,1995; von Wettstein Knowles, 1993; Créach *et al.*, 1993). In *Arabidopsis*, a mutation (FAE1) result in reduced level of seed very long chain fatty acids and deficiencies in elongation activities (James and Dooner, 1990). The gene has been cloned and the predicted amino acid sequence shares homology with those of other condensing enzymes, suggesting that it encode a fatty acid elongase (James *et al.*, 1995). Thus, as summarized in Figure 1.4, biosynthesis of erucic acid can be described as oleate, synthesized in the plastid, is then exported to the cytosol, where, presumably in the endo-membrane system, it is elongated via malonyl-CoA-requiring elongases to C20 and longer-chain monounsaturated fatty acids. However, the nature of the immediate18:1 donor needs to be clarified.

It is generally believed that the end-product of newly synthesized oleic acid exported from plastids is oleoyl-CoA. This oleoyl moiety can be elongated directly to erucic acid in the cytosol through successive additions of two carbons derived from malonyl-CoA. However, in an oil body fraction from developing rapeseed, Hlousek-Radojcic *et al.*(1995) observed *in vitro* that radioactivity from oleoyl-CoA was incorporated into eicosenoate and erucate at least 2.5-fold more slowly than from malonyl-CoA. Furthermore, radioactivity from oleoyl-CoA was rapidly diluted

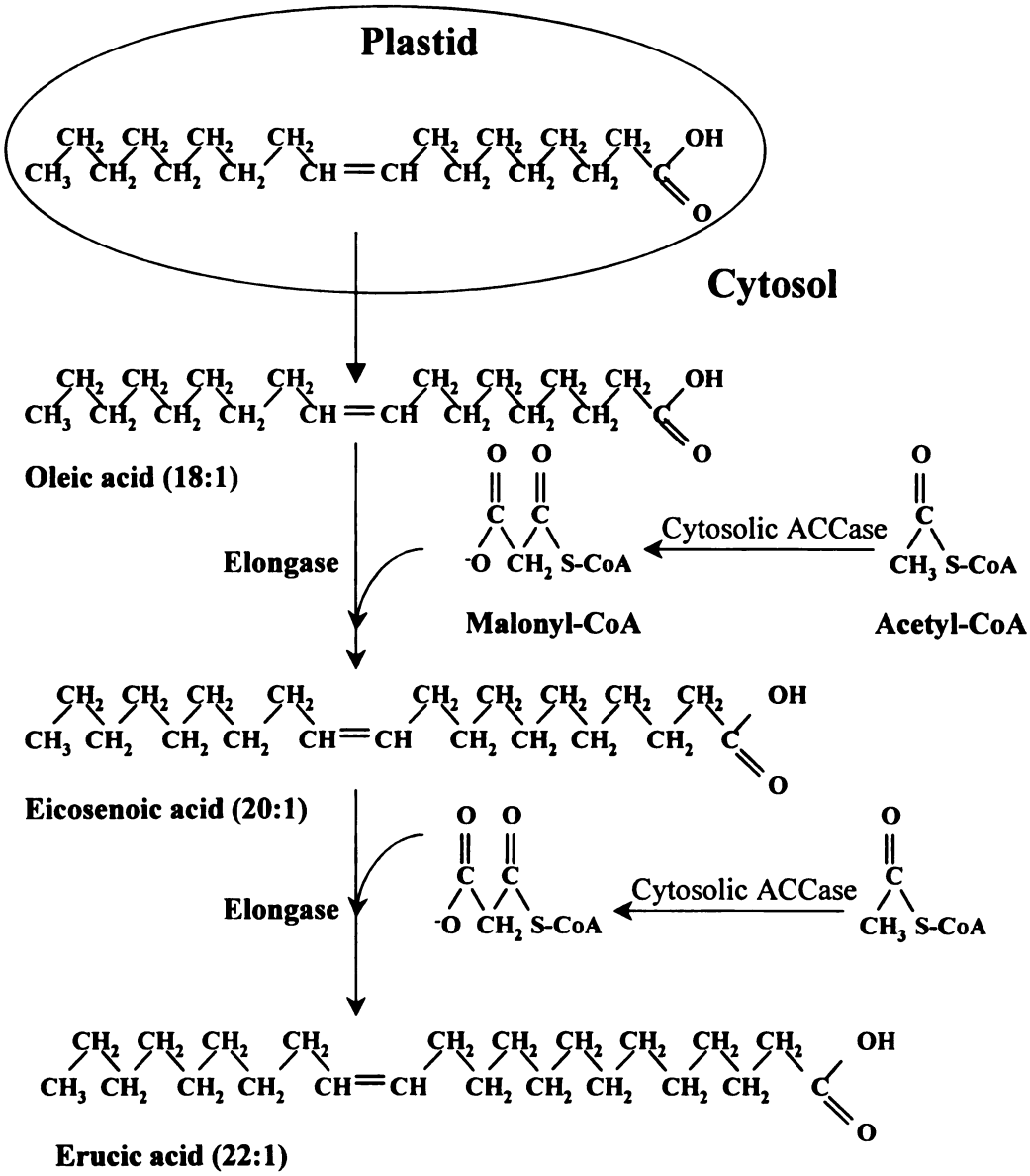

Figure 1.4. The general pathway for the synthesis of erucic acid. Oleic acid is first produced in the plastid and later elongated outside the plastid to produce eicosenoic and erucic acids.

upon the formation of eicosenoyl-CoA and the elongation could proceed without the addition of exogenous oleoyl-CoA. Based on these *in vitro* observations, they concluded that oleoyl-CoA is not the immediate substrate for elongation. Instead they proposed that the intermediate oleoyl donor for the elongase may be either a lipid or unesterified fatty acid. In addition, when intact *Brassica* (high erucic) embryos were incubated with [^{14}C]acetate, phosphatidylcholine (PC) was always heavily labeled at early time points, with oleate constituting over 90% of [^{14}C]labeled fatty acid esterified to PC. We considered that this oleoyl-PC might contribute to the synthesis of erucic acid, either via a mechanism of direct acyl transfer as proposed by Hlousek-Radojcic et al (1995), or via the acyl-exchange between acyl-CoA and PC, as first reported by Stymne and Stobart (1984). The involvement of PC for the synthesis of erucic acid can be envisioned for at least two physiological reasons. First, the newly synthesized oleic acid is not directly accessible to the compartment where the elongase is located, and PC is used as delivering carrier of oleic acid. Second, because free fatty acids are toxic, PC may act as a temporary storage for oleic acid before the elongation to erucic acids.

In this chapter, we have two goals in mind; one is to evaluate the existence of intermediate oleoyl donor pool for the synthesis of erucic acid proposed by Hlousek-Radojcic *et al.* (1995); the other is to examine the possibility that PC serves as oleoyl donor. Developing *Brassica rapa* embryos were incubated with ^{14}C-acetate, labeled tissue would be collected at different time points, fatty acids were separated from each time point, and the ratio of two fragments cleaved at the

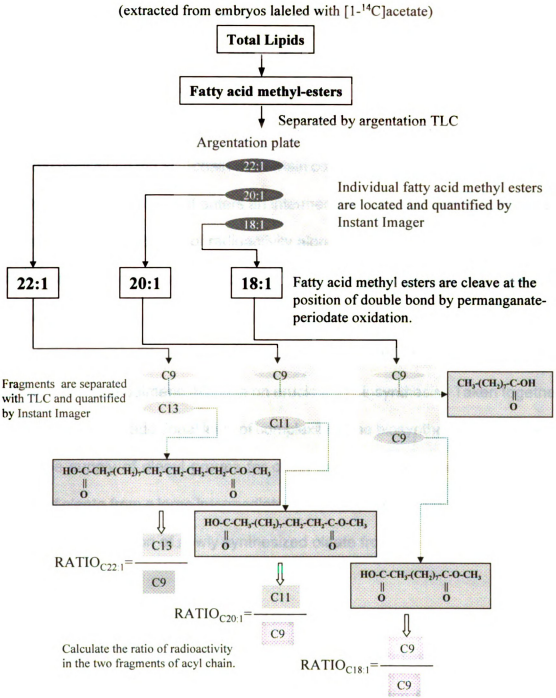

Figure 1.5. Flow chart of how distribution of radioactivity within acyl chain is determined and the ratio of cleaved fragments was obtained.

double bond position would be calculated (Figure 1.5). The analytical method was designed to elucidate these questions as explained in Figure 1.6. Since acetate can diffuse into tissue within 5 seconds, if newly synthesized 18:1 is directly elongated to 22:1, radioactivity should be evenly distributed along the carbon chain. Regardless of whether the carbons are added in plastids or cytosol, the distribution of radioactivity along the acyl chain will remain constant with time course. However, if newly synthesized 18:1 first enters an intermediate pool and then is elongated to 22:1, an uneven distribution of radioactivity along the carbon chain will result from different sites of the carbons added will reflect in the ratio decrease with course for 20: and 22:1.

In addition, in Chapter 4 we have examined the influence of light and of an inhibitor of the homodimeric ACCase on erucic acid biosynthesis. Taken together, our results show an additional level of complexity in the biosynthesis of erucic acid, in that the supply of oleoyl groups for chain elongation is a combination of the release of oleate from a large intermediate lipid pool, probably phosphatidylcholine and the direct provision of newly synthesized oleate from the plastid.

IV. What Are the Limiting Factors for the Accumulation of TAG in Oilseeds?

After considering the origin of acetyl-CoA, the regulatory role of ACCase, and the pathway of erucic synthesis, we move to the end of pathway shown in Figure 1.1, and ask what determines how much oil accumulates in oilseeds. To meet the increasing demands for vegetable oil, considerable research is oriented

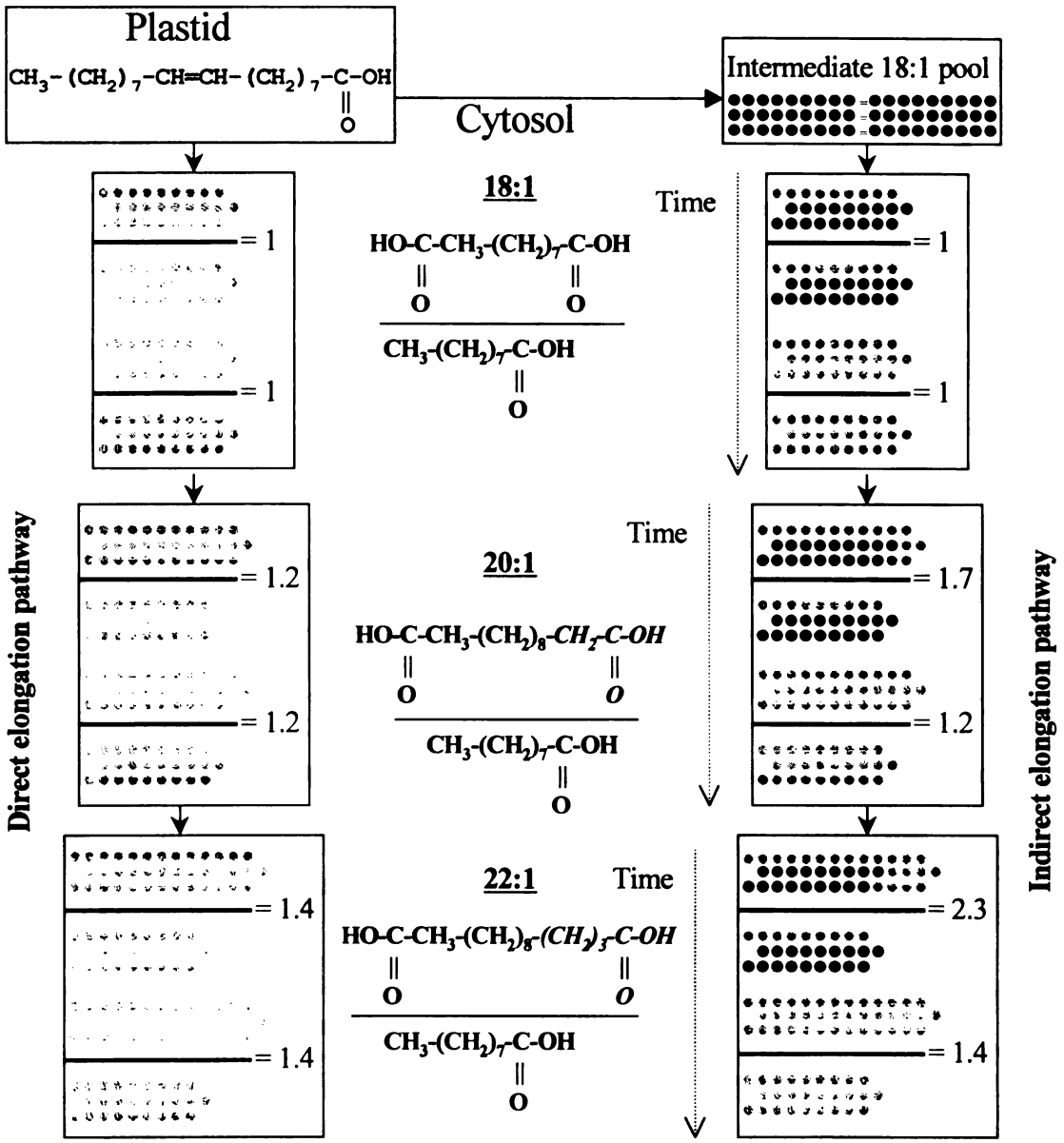

Figure 1.6. A schematic representation of the method and logic used in Chapter 4 to understand the movement of oleic acid from the plastid and its elongation to erucic acid. The ratio of radioactivity in the two fragments of fatty acid change with time for indirect elongation pathway.

in two directions; one is to increase the oil content and yield of existing oil crops; the other is to produce novel fatty acid in oil crop through genetic engineering in anticipation to replace petroleum.

In plants, the biosynthesis of storage triacylglycerol (TAG) occurs at high levels primarily in the seeds, but there is a wide range in the levels of TAG which accumulate in different plant species. For example, seeds of species of *Zea*, *Hordeum* or *Pisum* usually contain less than 5-10 % TAG by dry weight whereas many other species such as castor accumulate over 50% TAG in seeds. On the other hand, leaves, roots, and other vegetative tissues usually contain less 10% lipids by dry weight, most of which is polar lipids. In contrast, neutral lipids constitute over 95% of its lipids in oilseeds. The regulatory or metabolic factors which influence this very wide range of oil accumulation in seeds are currently unknown (Ohlrogge and Jaworski, 1997).

The pathway of TAG synthesis, or the so-called Kennedy pathway (Kennedy, 1961), was found to operate in the synthesis of TAG in plants as early as 1962 (Barron and Stumpf, 1962) as shown in Figure 1.7. The first reaction is catalyzed by glycerolphosophate acyltransferase (E.C.2.3.1.15), the second by 1-acylglycerol-3-phosphate acyltransferase (E.C.2.3.1.51), the third by phosphatidate phosphatase (E.C.3.1.3.4), the fourth by diacylglycerol acyltransferase (E.C.2.3.1.20) (Stymne and Stobart, 1987). Fatty acids are first synthesized in plastids, whereas the acyl-CoAs are formed on the outer membrane of the plastid envelope by the action of acyl-CoA synthetase (Roughan and Slack, 1977; Joyard

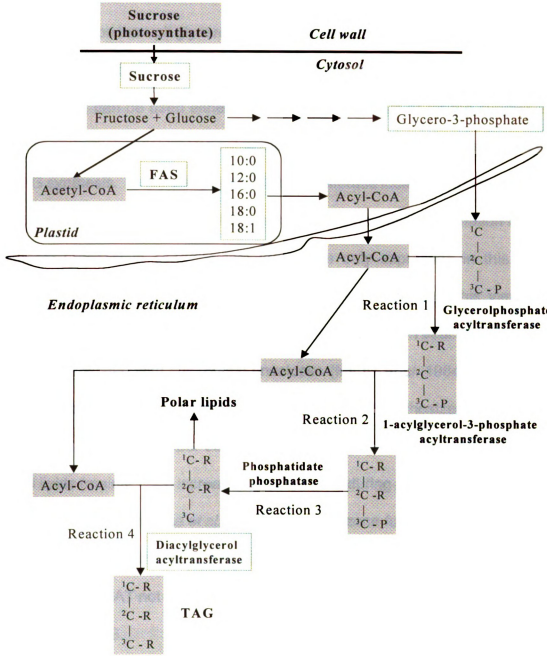

Figure 1.7. Proposed pathway for the synthesis of tricylglycerols in oilseeds. Some possible limiting factors are marked by dotted box.

and Douce, 1977). The glycerol phosphate is mainly derived through dihydroxyacetone oxidoreductase (Finlayson and Dennis, 1980), with perhaps a minor contribution through glycerol kinase (Gurr *et al.*, 1974). The site of sequential addition of three acyl groups to glycerol backbone is located in ER or oilbody. The diacylglycerol, can be used either for polar lipid synthesis or for the synthesis of TAG. Therefore, diacylglycerol acyltransferase (DAGAT) is the only unique enzyme for TAG synthesis. Since diacylglycerol acyltransferase locates at the branch point that channels diacylglycerol to TAG synthesis, one potential explanation for TAG synthesis primarily in seeds is that DAGAT is only expressed in this tissue. However, there are several observations which argue against this view. For example, DAGAT activity was found in spinach leaves (Martin *et al.*,1983) and was primarily associated with chloroplast envelopes (Martin *et al.*,1984). Roughan *et al.*(1987) reported that significant amounts of TAG were synthesized when palmitic acid was applied to the upper surface of expanding spinach leaves. The level of neutral lipids (mainly TAG) increased at least 3 fold during protoplast isolation from *Arabidopsis* leaves (Browse *et al.*, 1988). Finally, ozone-fumigated spinach leaves produced high proportions of TAG (Sakaki *et al.*,1990). These data together suggest that DAGAT not only occurs in leaves, but also that leaves have the ability to synthesize TAG.

Enormous efforts have been put into the isolation of DAGAT, but have not been successful until recently. In 1998, a mouse cDNA sharing similarity with acyl-CoA: cholestrol acyltransferase, when expressed in insect cells, resulted in high

levels of DAGAT activity and it is believed that this cDNA encodes a DAGAT (Cases *et al.*, 1998). Several *Arabidopsis* ESTs were found sharing significant similarity with the mouse DAGAT. Two putative DAGAT genes were isolated from *Mortierella ramanniana* through protein sequencing, which are not related to mouse DAGAT (Lardizabal *et al.*, 1999). Stymne and co-workers (1999) reported that a transacylase can transfer acylgroups from PC or DAG into TAG. So far, all the new findings of plant DAGATs are at prelimary stage, and it is hard to draw any conclusions at present time regarding the quantitative contributions of the enzymes encoded by the newly discovered genes.

Considering that over 30 reactions are required to convert acetyl-CoA to TAG, there could be many steps or genes which control the yield of end product TAG. In order to begin to dissect possible limiting factor(s) in the pathway of TAG biosynthesis, it is useful to conceptually divide the pathway into two parts: 1) the production of acyl chains which occurs in plastids (source), and 2) the utilization of acyl chains for glycerolipid synthesis in the ER and oilbody (sink). The accumulation of TAG by oilseeds may be a response to the high rate of fatty acid synthesis. There is so far no research specifically looking into this possibility, but some observations are in agreement with the concept. In *Chlamydomonas*, addition of exogenous PC liposomes to cultures caused a 10-fold increase in TAG accumulation (Grenier *et al.*, 1991). Another example comes from comparisons of oleaginous (oil-accumulating) versus nonoleaginous yeast (Botham and Ratledge, 1979). They concluded that differences between these two types of species are

controlled by the production rather than utilization of fatty acids. On the other hand, some evidence implies that utilization of fatty acids can increase the rate of fatty acid synthesis. When a plant 12:0-ACP thioesterase was overexpressed in *E. coli* cells, 10-fold more fatty acid accumulated than controls (Ohlrogge *et al.*, 1995). In another experiment, massive overexpression of membrane proteins resulted in no change of membrane's phospholipid to protein ratio, but rather more fatty acid was produced to accommodate the excess proteins (von Meyenburg *et al.*, 1984; Weiner *et al.*, 1984). So far, we don't know whether TAG accumulation is driven more strongly by the supply or the demand for acyl chains or if both mechanisms operate in oilseeds.

In chapter 5, we asked whether the supply of fatty acid can influence the amount of TAG produced in oilseeds. As shown in Figure 1.7, the possible factors that might limit TAG accumulation are marked red. If the supply of fatty acid is a limiting factor for TAG biosynthesis, then providing exogenous fatty acid to the developing embryos should increase the rate of TAG production. Unfortunately, long-chain fatty acids (LCFA) have very low solubility in aqueous solution, and so addition of LCFA at concentrations sufficient to increase rates of lipid synthesis is very difficult. However, embryos of *Cuphea lanceolata, Ulmus carpinifolia*, and *Ulmus parvifolia* contain high levels of decanoic acid in their TAG (80%, 63%, and 71%, respectively). Decanoic acid is easily dissolved in water at mM concentrations, and therefore, these species provided a convenient model system to test the influence of fatty acid supply on TAG accumulation. The supply of

sucrose and glycerol are also evaluated in a similar fashion like fatty acids. Such in vitro model experiments may provide a useful guide toward the selection of targets for future metabolic engineering in transgenic plants.

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CHAPTER 2

Understanding *in Vivo* Carbon Precursor Supply for Fatty Acid Synthesis in Leaf Tissue

Abstract

The principal supply of carbon precursors for fatty acid synthesis in leaf tissue has been a debated topic with some experiments suggesting a direct supply from the C3 products of photosynthetic carbon fixation and others suggesting the utilization of free acetate, for which concentrations in leaves in the range of 0.05 to 1 mM have been reported. To address this issue we first reassessed the *in vivo* rate of fatty acid synthesis using a new method, that of [^{13}C]carbon dioxide labeling of intact *Arabidopsis thaliana* plants with subsequent analysis of fatty acids by GC-MS. This method gave an average value of 2.3 μmoles carbon atoms h^{-1} mg chlorophyll^{-1}. The method was extended by the use of pulse labeling and isotopic dilution analysis to measure the rate of fatty acid synthesis in the dark. There was negligible fatty acid synthesis (<5% of the rate in the light) in the dark. These results extend estimates from other workers. With the *in vivo* rate of fatty acid synthesis in the light defined, if the bulk tissue acetate concentration available for fatty acid synthesis is 1 mM, this acetate pool can sustain fatty acid synthesis for about 60 minutes. When leaves of *Arabidopsis*, barley, and pea were provided a 5 min pulse of [^{14}C]carbon dioxide the label rapidly appeared in fatty acids with a lag phase of 2-3 min. Continuous labeling with [^{14}C]carbon dioxide, for up to one hour, showed a similar result. Furthermore, ^{14}C-label in free acetate was less than

5% of that in fatty acids. In conclusion, these data suggest that either the bulk pool of acetate is not involved in fatty acid synthesis or the concentration of acetate must be less than 0.05 mM under strong illumination.

Introduction

Apart from a very small contribution from the mitochondrion (Wada *et al.*, 1997) *de novo* fatty acid biosynthesis is exclusively located in the chloroplast of leaf mesophyll cells (Ohlrogge *et al.*, 1979). The immediate carbon precursor for fatty acid biosynthesis is acetyl-CoA, which is used as substrate by KAS-III for the acyl-chain primer reaction, and, after carboxylation to malonyl-CoA and transacylation to malonyl-ACP, for the acyl-chain elongation steps catalyzed by KAS-I and KAS-II. The source of acetyl-CoA in chloroplasts and its relationship to the immediate products of photosynthetic carbon dioxide fixation continues to be a matter for debate.

Rates of fatty acid synthesis by intact leaf tissues have been assessed by various methods. Browse *et al.* (1981) note that [^{3}H] water incorporation into fatty acids by spinach leaf discs was equivalent to 2.6 μmol C h^{-1} mg $chlorophyll^{-1}$. However, that was an estimate made without knowledge of either a kinetic isotope effect for tritium and assuming only 68% substitution of hydrogen by tritium. In the same paper the authors measured the net fatty acid accumulation in maize leaves during a 12 hour day at 80 nmol cm^{-2}. Using a conversion factor of 30 cm^2 mg $chlorophyll^{-1}$, (Focke, personnal communication) this translates to 3.6 μmol C h^{-1} mg $chlorophyll^{-1}$. Recently, using [^{13}C] acetate incubations with spinach leaf discs Pollard and Ohlrogge (1999) showed that the total capacity to synthesis fatty acids, that is, from a combination of both endogenous and exogenous carbon sources, ranged from 2 to 8 μmol C h^{-1} mg $chlorophyll^{-1}$ depending on the age of the leaf,

with higher rates for younger leaves. Clearly, rates of synthesis of fatty acids will vary depending on the age of the leaf, the physiological state and growth conditions of the plant, and species, so exact comparisons between studies is difficult, but it is clear that endogenous rates of fatty acid synthesis in leaves of the order of 2-8 μmol C h^{-1} mg chlorophyll^{-1} can be expected.

Exogenous acetate is rapidly incorporated into fatty acids of leaves and isolated chloroplasts (Roughan *et al.*, 1979; Springer *et al.*, 1989; Pollard and Ohlrogge, 1999). In addition high levels of acetyl-CoA synthetase activity have been reported in isolated chloroplasts (Roughan and Ohlrogge, 1994), while chloroplasts have been reported as the dominant site of acetyl-CoA synthetase activity (Kuhn *et al.*, 1981). The reported acetate concentration in leaf tissue varies widely, ranging from 0.05 to1.4 mM (Kuhn *et al.*, 1981; Liedvogel, 1985; Treede *et al.*, 1986; Roughan, 1995). Murphy and Stumpf (1981) and Liedvogel and Stumpf (1982) were first to propose a mechanism to explain how free acetate was generated and used for fatty acid synthesis. They suggested that pyruvate is converted to acetate via acetyl-CoA in the mitochondrion; that acetate then diffuses through the cytosol to chloroplasts where it is converted to acetyl-CoA by the acetyl-CoA synthetase and becomes available for fatty acid synthesis. Roughan (1995) revisited this issue and compared different methods used to measure the acetate concentration in leaf tissues. In his report, the acetate concentration ranged from 0.5 to 0.8 mM in spinach, 0.5 to 0.8 mM in pea, and 0.9 to 1.4 mM in *Amaranthus*, with higher values in younger leaves when compared to older ones.

The acetate concentration of leaves in the dark ranged from 0.35 to 0.70 mM, while that of isolated chloroplasts was 0.4 mM. In conclusion, he suggested that it seems probable that endogenous acetate would be the direct source of acetyl-CoA for fatty acid synthesis in leaves. An important part of the argument for acetate as the dominant substrate for fatty acid synthesis *in vivo* is that the bulk tissue concentrations are above the concentration for acetate saturation of rate of fatty acid synthesis.

Significant labeling of fatty acids from other substrates has been reported for both isolated chloroplasts and for leaf tissue. In leaf tissue Murphy and Leech (1981) noted carbon dioxide was incorporated into lipids after a short (~5 min) lag phase at an average rate of 2.1 μmol C h^{-1} mg chlorophyll^{-1}, while in isolated chloroplasts the rate was 0.6 μmoles C h^{-1} mg chlorophyll^{-1}. In a careful comparison of highly-purified substrates Roughan *et al.* (1979) observed maximum rates of 2.7 μmol C h^{-1} mg chlorophyll^{-1} for acetate, 0.8 μmol C h^{-1} mg chlorophyll^{-1} for pyruvate and 0.35 μmol C h^{-1} mg chlorophyll^{-1} for bicarbonate. Clearly, isolated chloroplasts can convert the carbon they fix into fatty acids, but not at rates equivalent to the endogenous rate of fatty acid accumulation.

In this report, we evaluate the relationship between the rate of fatty acid synthesis in leaves and the total leaf pool of acetate. We demonstrate a new method that can accurately measure the absolute rate of fatty acid synthesis both in the light and in dark. We use this rate of synthesis in the light to estimate the turnover time of the free acetate pool if it is the major source for carbon precursors

of fatty acid synthesis. The size of the free acetate pool will determine any lag phase for added label. And finally, short term carbon dioxide labeling is used to measure the actual lag phase. These results are inconsistent with the model of a high concentration of free acetate (0.5-1.4 mM) supplying most of the carbon for fatty acid synthesis in leaves.

Material and Methods

Plant Material

Arabidopsis thaliana (ecotype Columbia), Pea (*Pisum sativum*, cv Little Marvel), barley (*Hordeum vulgare*,cv Morax or Alexis), and spinach (*Spinacia oleacera*, hybrid spinach No. 7 R Early) were grown under the same environmental conditions as used for the labeling experiments (13.5 h light and 10.5 h dark cycle, at temperatures of 22-25°C). Cotyledons of the pea plants were sometimes removed after 4 or 7 days after sowing to prevent the use of storage starch for fatty acid biosynthesis. Pea plants were used at day 9 after sowing, barley plants at day 5 or 7. *Arabidopsis* plants were 2 to 3 weeks old and at the 4 to 6 leaf stage.

Labeling with Carbon Dioxide: General Conditions

All labeling experiments were performed in a transparent glove bag (40 liter gas space, $I^2R^{®}$,Instruments for Research and Industry, Cheltenham, PA, USA) with one gas inlet and one gas outlet and a larger sleeve closed by a clamp to put the plant material inside the bag. Gloves were used to manipulate plants and to liberate $^{14}CO_2$ inside the bag. A CO_2 atmosphere inside the bag was achieved by

injecting the appropriate mix of ^{14}C-$NaHCO_3$ (ICN) and unlabeled Na_2CO_3 solutions into a non-volatile acid (lactic or sulfuric acid). Air circulation was maintained by using a small battery-driven fan inside the bag. The gas inlet and outlet of the bag were connected via plastic tubes and stopcocks to an air pressure system and a gas trap at the outlet in the case of the radioactive labeling experiments or with an $^{13}CO_2$/air gas tank (compressed air, scrubbed to remove carbon dioxide and replaced with $^{13}CO_2$ at 450-480 ppm, BOC gases, Riverton, NJ, USA) at the inlet-side in the case of the ^{13}C-labeling experiments. During incorporation of labeled CO_2 the plants were illuminated (150 µmols second $^{-1}$ $m^{2\,-1}$) with flourescent tubes (6 high-output light tubes, 8 feet long, Hubbell Lighting Inc.) at room temperature.

Labeling with [^{13}C]Carbon Dioxide

Continuous labeling to determine the rate of fatty acid synthesis in the light. Two hundred and thirty 18-day-old *Arabidopsis thaliana* seedlings were transferred to the glove bag under illumination. The flow rate of $^{13}CO_2$/air mix was 2L min^{-1}. 20 seedlings each were taken at 30 or 60 min time intervals after the start of labeling, beginning at 30 min and ending after 8 h. The tissue was frozen in liquid N_2, ground to a powder and stored at -70°C until aliquots were removed for lipid analysis.

Pulse labeling to determine the rate of fatty acid synthesis in the dark. *Arabidopsis thaliana* plants (3-week-old) were labeled with 400µCi of $^{14}CO_2$ for 20 min in the light; then labeled with $^{13}CO_2$ for 12 hours under the light. The labeled plants were removed from the bag and grown under a regular atmosphere for two

and half days with a cycle of 13.5 hr light and 10.5 hr dark, after which a sample of 20 plants was taken at several time points both during light and dark period. The length of this sampling period was 5 days. The tissue was frozen in liquid N_2, ground to a powder and stored at -70°C until aliquots were removed for lipid analysis.

Labeling with [^{14}C]Carbon Dioxide

Pulse-chase. After a labeling phase of 5 min in which plants were exposed to 700 ppm CO_2 generated with the inclusion of 2 mCi of $^{14}CO_2$ (pulse period), the radioactive atmosphere was rapidly vented and the plants were placed in a normal (non-radioactive) atmosphere (chase period) both in the light and dark. Directly after the 5 minute pulse period a sample of the plant material (20 plants for *Arabidopsis*) was taken and frozen in liquid nitrogen. Further samples were taken for up to 5 h and frozen.

Continuous labeling. One hundred *Arabidopsis* plants were incubated in the bag with 5.4 mmol CO_2 and 2 mCi $^{14}CO_2$ to give a nominal carbon dioxide concentration of 3050 ppm. At 5 or 10 minute intervals up to 60 minutes, 12 plants were transferred into tubes with sulfuric acid, which were previously placed inside the bag, to quench the reaction, thereby allowing a continuous labeling without changing abruptly the labeling conditions.

Lipid Analysis

Before the extraction of fatty acids, 0.5 mg of pentadecanoic acid (15:0) was added to each sample as an internal standard. Lipids were extracted with

hexane:isopropanol according to Radin (1981). The organic phase was evaporated under a stream of nitrogen and the lipids were dissolved in methanol. Chlorophylls and carotenoids were determined according to Lichtenthaler (1987). In experiments with [^{14}C]carbon dioxide, radioactivity in lipid, fatty acid and/or isoprenoid fractions was determined by liquid scintillation counting. Lipids were saponified by heating at 90°C for one hour in 2.2 M of KOH in 50% aqueous methanol. On cooling isoprenoids were extracted three times with hexane. After acidification with 10N HCl the fatty acids were extracted three times with hexane. The pooled fatty acid fractions were dried with nitrogen. Fatty acid methyl esters were prepared by heating fatty acids at 90°C for 45 min in 0.3 ml of toluene and 1 ml of 10% (v/v) boron trichloride/methanol.

To simplify GC-MS analysis, unsaturated FAME were converted to saturated FAME by catalytic hydrogenation. After hydrogenation, the sum of 16:0 and 18:0 constitute over 99% percent of total fatty acids in *Arabidopsis* leaf tissue, allowing rates of fatty acid synthesis to be expressed simply as the sum of C16 and C18 fatty acids. FAME were hydrogenated using hydrogen at slightly greater than atmospheric pressure with a platinum (IV) oxide catalyst in methanol, to convert all 16 and 18 carbon unsaturated fatty acids to 16:0 and 18:0, respectively. Total C16 and C18 fatty acids in tissues was determined by GC analysis of the hydrogenated FAME, against the 15:0 internal standard.

Stable Isotope Analysis of [^{13}C]-Fatty Acid Methyl Esters.

The percentage of newly synthesized fatty acids in total fatty acids was

determined by GC-MS analysis. A Hewlett Packard 5890 gas chromatography configured with an autosampler and HP MSD 5972 mass analyzer (quadruple, operating in electron impact mode) was used in this study. Separations of FAME were carried out on a 30m×0.25mm i.d., 0.25μm thickness DB-23 capillary column (J&W), with helium carrier gas and using a temperature ramp from 100°C to 240°C at 5°C sec^{-1}. Injector and GC-MS interface temperatures were 250°C, with GC injection in the "splitless" mode. Mass spectra were obtained in "scan" mode (scanning across 50 to 500 atomic mass units, 3 scans per data point, to give 1.03 spectra per second). Molecular ion clusters of 16:0 and18:0 were monitored for each time point. The percent of newly synthesized fatty acids of 16 carbon was calculated by the sum of intensities for $(M+3)^+$ (m/z=273) to $(M+16)^+$ (m/z=286) divided by the sum of intensities over the entire molecular ion cluster, namely M^+ (m/z=270) to $(M+16)^+$ (m/z=286). For 18 carbon fatty acids, a similar calculation was used, that is, the sum of the intensities for $(M+3)^+$ (m/z=301) to $(M+18)^+$ (m/z=316) divided by the sum of intensities over the entire molecular ion cluster, namely M^+ (m/z=298) to $(M+18)^+$ (m/z=316). The value of $(M+3)^+$ (m/z=273) and (m/z=301) was corrected by subtracting the natural abundance in the calculation of percentage of newly synthesized fatty acids. The amount of newly synthesized fatty acid was obtained by multiplying the percentage newly synthesized fatty acid by the total amount fatty acid as measured by GC.

[^{14}C] Acetate Analysis

To determine whether acetate was labeled during the $^{14}CO_2$ pulse-chase

experiments *Arabidopsis* plants from the time points of 10, 20, 40, and 120 min (chase period, after 5 min pulse with $^{14}CO_2$) were extracted and analyzed according to the following protocol. 100 mg of frozen plant tissue was homogenized in 1.6 ml isopropanol and then in 2.4 ml of hexane. The organic extracts were combined and shaken with 150 mg of anhydrous sodium sulphate. After centrifugation the supernatant was partitioned against 0.4 ml of 0.5 M glycine/HCl (pH2.0) saturated with sodium sulphate and then against 0.2 ml of 0.1M potassium hydrogencarbonate. The top phase containing the lipids were used to determine the pigment content and the labeled fatty acids. The aqueous phase was used to measure the radioactivity in acetate. As a control radioactive acetate was added to frozen, un-labeled *Arabidopsis* seedling tissues which then underwent the same extraction and derivatization procedure as the $^{14}CO_2$ labeled materials. Acetate in the aqueous extracts was analyzed by one of two methods. In the first method, aqueous extracts were derivatized by heating 0.1 ml of the extract with 0.9 ml of acetonitrile containing 10 mM 2-bromoacetophenone, 1 mM 18-crownether-6 and 1% triethylamine, for one hour at 90°C in a closed tube. The phenacyl ester reaction products were extracted with hexane/isopropanol (2:3, v/v) and separated by TLC (Whatman K6F silica plates developed with hexane:diethylether (7:3, v/v)). The radioactive spots on the TLC plates were localized with an Instant Imager (Canberra Instruments) using the lanes with the exogenously added acetate samples as markers. The corresponding areas of acetate derivative were scrapped and radioactivity assayed by liquid scintillation counting. In the second method,

the aqueous extracts were directly applied to silica TLC plates (Whatman K6) which were then developed with 95% ethanol :25% ammonia: water 156:25:19, v/v/v). A radioactive band with a similar but not identical Rf value to the authentic acetate band was observed. In order to determine if this radioactive band was indeed acetate, the band was scrapped out, derivatized and analyzed as its phenacyl ester.

Results

Measurement of the rate of fatty acid synthesis in *Arabidopsis* in the light using [^{13}C]carbon dioxide

In order to determine whether the free acetate pool is involved in fatty acid synthesis or not it is crucial to have accurate measurements of the rate of fatty acid synthesis and the pool size of free acetate. The rate of fatty acid synthesis should be the absolute rate, and not the net rate of fatty acid accumulation (rate of synthesis less rate of degradation). Each of the methods described in the Introduction for the measurement of rates of fatty acid synthesis in leaf tissues has some drawback. Measuring rates with radioisotopes has the problem that although the rate of synthesis from the exogenous carbon source can be readily quantified, the contribution from endogenous substrates is much harder to measure accurately. To gauge the endogenous contribution small mass increments occurring during the labeling period (which are net accumulations) must be measured. Here, [^{13}C]carbon dioxide labeling was employed in attempt to measure the rate of fatty

acid synthesis in *Arabidopsis*.

By monitoring the molecular ion clusters of 16:0 and18:0 FAME, which were obtained after hydrogenation of total FAME, the newly synthesized fatty acids are readily distinguishable from pre-existing fatty acids. As an example, the molecular ion cluster of 18:0, at one hour labeling time point, is shown in Figure 2.1. An ion corresponding to methyl [$^{13}C_{18}$] stearate (m/z = 316) is clearly visible, showing that all the carbon atoms in the chain are derived from the added [^{13}C]carbon dioxide. More important, the distribution of species is such that methyl [$^{13}C_{14}$] stearate is the dominant species in the distribution, indicating that dilution with endogenous carbon is small (less than 30%). The source of this endogenous contribution may be from leaks of $^{12}CO_2$ into the system, and from [^{12}C]carbon dioxide released by the system, especially through respiration of the plants themselves and soil microorganisms. The point is that it can be easily seen and measured. Ignoring it would underestimate the rate of fatty acid synthesis by about 30%. In unlabeled methyl stearate, natural abundance isotopic peaks can be readily observed for (M + 1), (M + 2) and (M + 3) ions, at relative intensities of 21.3%, 2.6% and 0.25% respectively (experimental values, not calculated), when compared to the molecular ion peak (M, m/z = 298, at 100% intensity). Thus the ions from 303 to 316 (m/z) are clearly derived from only newly synthesized fatty acid containing 4 to 18 ^{13}C-carbons. Ions from m/z = 298 to 302 have only a very small component from the labeling period, which is less than 2% of the total newly fatty acid synthesis, and so can be ignored. Thus the percent of newly synthesized fatty acids was obtained

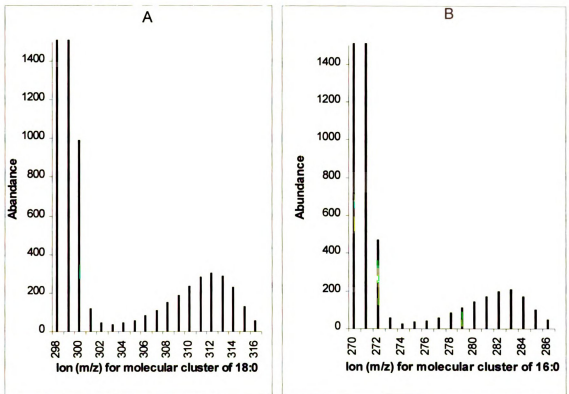

Figure 2.1. The molecular ion distribution of 18:0 and 16:0 from *Arabidopsis* labeled by $^{13}CO_2$ at one hour time point. The molecular ion clusters of 18:0 (A) and16:0 (B) FAME, which were obtained by the hydrogenation of total FAME from *Arabidopsis* leaves, at one hour labeling time point with $^{13}CO_2$. The 18:0 represents all 18 carbon fatty acids, and so as for 16:0, due to the hydrogenation.

through the sum of ion abundances derived from newly synthesized fatty acids (m/z = 303 to 316) divided by the total ion abundance for the molecular ion cluster. The percent of newly synthesized 16 and 18 carbon fatty acids were plotted with time and are shown in Figure 2.2 (A) and (C). After 8 hour labeling with [^{13}C]carbon dioxide, 16% of 16 carbon fatty acids and 14% of 18 carbon fatty acids are newly synthesized. The data are also expressed as mg newly synthesized fatty acids mg chlorophyll^{-1} (Figure 2.2 (B) and (C)). In both experiments shown in Figure 2.2 the accumulation of both 16 and 18 carbon fatty acids showed linear increases throughout the 8 hour labeling period. The synthesis rates of 16 and 18 carbon fatty acids were 12 and 24 μg fatty acids h^{-1} mg chlorophyll^{-1}, respectively. Combining 16 and 18 carbon fatty acids, the rate of fatty acid synthesis is converted to 2.3 μmol C h^{-1} mg chlorophyll^{-1} for the whole aerial part of the plant (stem plus leaves).

Measurement of the rate of fatty acid synthesis in *Arabidopsis* in the dark with [^{13}C]carbon dioxide

It is well known that the rate of fatty acid synthesis of leaf tissues is lower in the dark than in the light. Estimates made using labeled precursors fed to leaf discs suggest 12-20% of the rate in the light (Browse *et al.*, 1981). The same authors show that fatty acid synthesis from acetate by isolated chloroplasts is essentially zero. Since we can accurately measure the rate in the light as demonstrated in the previous section, we should be able to determine the rate in the dark, if the dark rate relative to light can be evaluated. This can be done with an isotope dilution

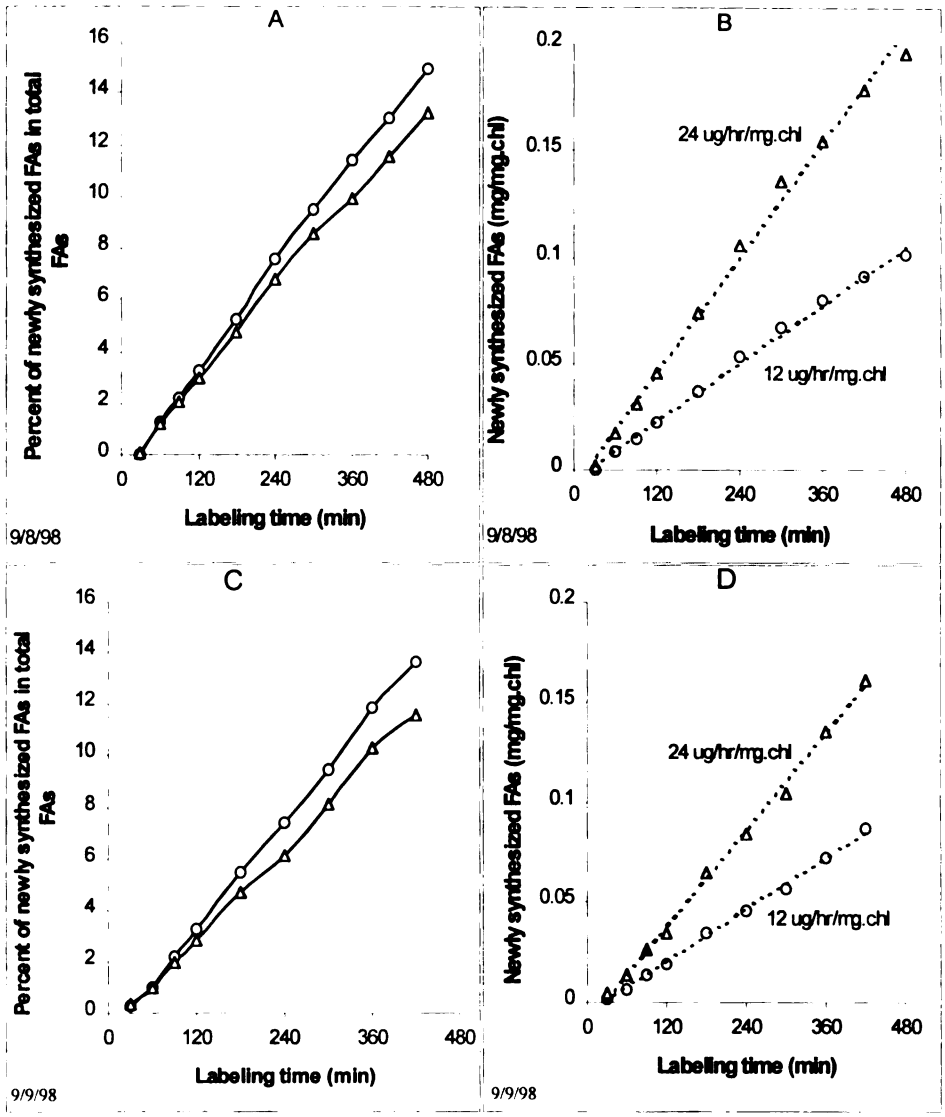

Figure 2.2. Determining the rate of fatty acid synthesis in *Arabidopsis* by continuos labeling with $^{13}CO_2$. (A) (B) and (C) (D) represent two independent experiments. (A) and (C) show the percentage of newly synthesized fatty acids in total fatty acids with labeling time. The calculation of newly synthesized fatty acids was described in Methods. (B) and (D) gave the amount of newly synthesized fatty acids with time course. At given time point, the value was obtained by multiplying percent of newly fatty acids with the absolute amount of total fatty acids measured by GC. (○)and (△) represent 16 and 18 carbon fatty acids, respectively.

strategy, again employing [^{13}C]carbon dioxide. *Arabidopsis* plants were labeled with [^{14}C]carbon dioxide for 20 min and then with [^{13}C]carbon dioxide for 12 hours under the light. The labeled plants were removed from the labeling chamber and grown under a normal atmosphere for two and half days prior to sampling both during light and dark periods. The first sample was taken just before the light was turned on, and this time point was designated as zero time. The sampling period lasted for 5 days. The purpose for growing the labeled plants under a normal atmosphere for 60 hours was to deplete the labeled carbon intermediates, particularly sucrose and starch, in order to minimize further incorporation of labeled carbons into fatty acids during the sampling period. Thus it would be expected that any turnover of label from any source into fatty acids would be highly diluted, contributing on average only one or two ^{13}C or ^{14}C atoms per newly synthesized fatty acid. As shown in Figure 2.3, ^{14}C label in fatty acids measured on a per plant basis decreased slightly during the sampling period, by about 22% (140 to110 units on Figure 2.3). The turnover of fatty acids could be greater, however (since we are measuring a net figure for total labeled fatty acids), if there is significant recycling of label. Considering C16 and C18 fatty acids, there is about a 100% increase in mass per plant over the 120 h sampling period. Also in Figure 2.3, fatty acids, fresh weight and chlorophyll all showed linear increase throughout the period, indicating the labeled plants grew normally.

The ion distribution of species in the ion molecular cluster of 18:0 and 16:0 was analyzed for each sampling point. For the purpose of this analysis m/z = 309

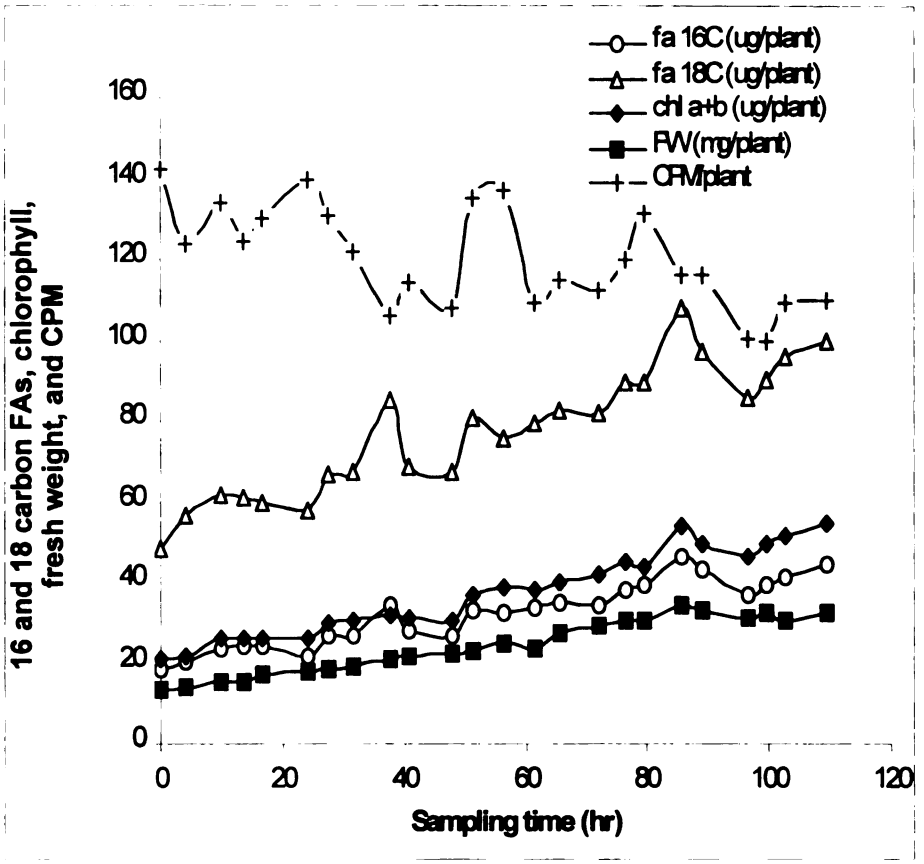

Figure 2.3. Some important parameters concerning plant growth during the sampling period in the pulse-chase experiment to determine the rate of fatty acid synthesis in the dark.

was chosen to represent the ^{13}C labeled C18 fatty acids, m/z = 280 to represent the ^{13}C labeled C16 fatty acids. The base peak for each molecular ion cluster, namely m/z = 298 for C18 fatty acids and m/z = 270 for C16 fatty acids, represents unlabeled fatty acids. If there was any fatty acid synthesis during the sampling period, it would be reflected as a percentage increase of m/z=298 for 18:0 and m/z=270 for 16:0, or in a percentage decrease of m/z=309 for 18:0 and m/z=280 for 16:0 at any given time point. Conversely, if there was any degradation there would be no change in the percentages. These data are plotted over the sampling period in Figure 2.4. As demonstrated in Figure 2-4 (A) and (B), m/z=298 and m/z=270 (representing unlabeled C18 and C16) increased during the light periods, but not during the dark periods. On the other hand, Figure 2.4 (C) and (D) showed that m/z=309 and m/z=280 (representing labeled C18 and C16) decreased during the light periods, but not during the dark periods. This result clearly demonstrated that fatty acid synthesis was halted during the dark period. Averaging the data for four dark periods gave a rate of zero.

Short term labeling with [^{14}C]carbon dioxide

As measured in the experiments described above, the rate of fatty acid synthesis was 2.3 μmol C h^{-1} mg chlorophyll^{-1} for the whole aerial part of the plant. If the free acetate concentration in aerial plant tissues is 1 mM (about 1.2 μmol mg chlorophyll^{-1}) and is completely available, this acetate pool would be able to sustain 63 min of fatty acid synthesis. This would cause a lag phase prior to incorporation of exogenously labeled substrate. Since pool filing is a first order kinetic process,

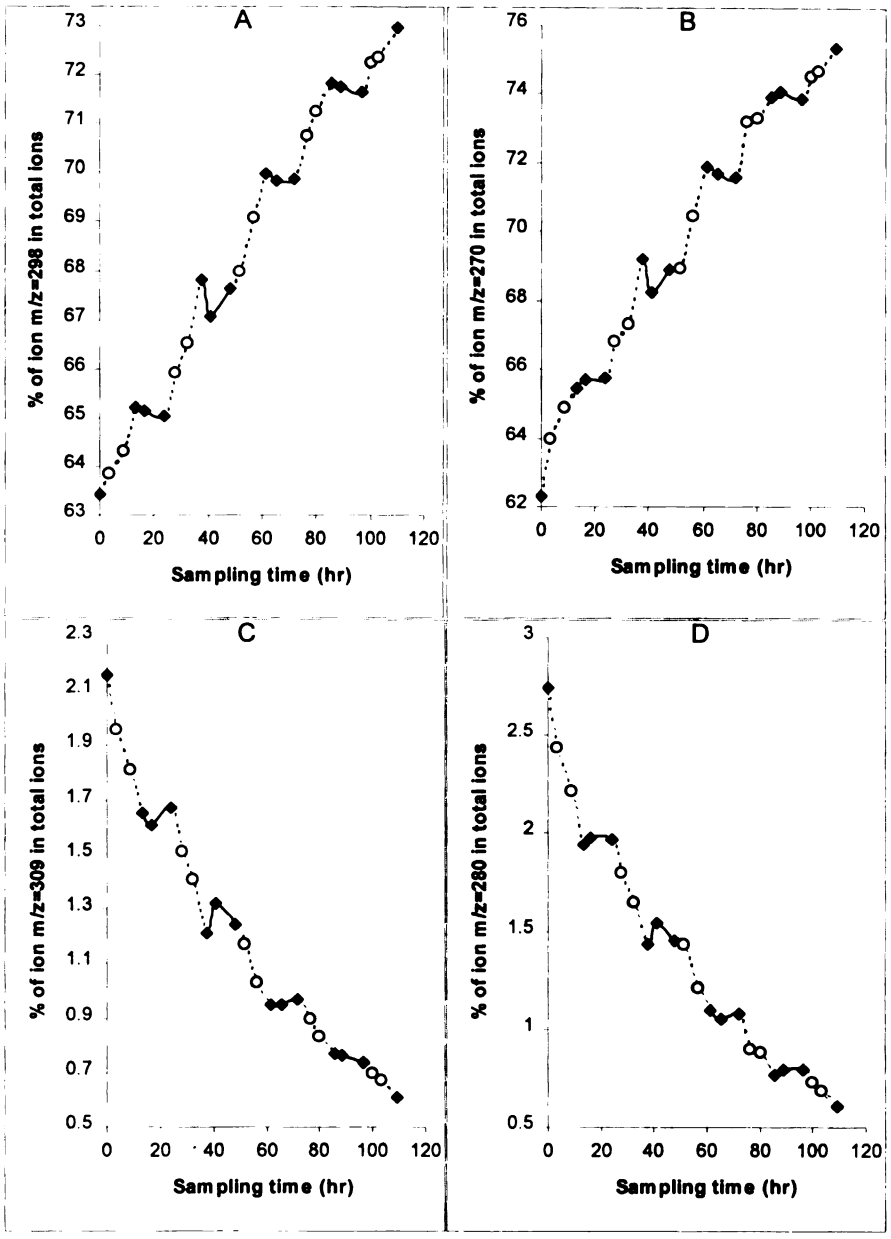

Figure 2.4. The pulse-chase labeling with $^{13}CO_2$ to determine the rate of fatty acid synthesis in the dark. The same set of samples were used both in this figure and figure 3. FAMEs were hydrogenated, so 18:0 and 16:0 represented 18 and 16 carbon fatty acids, respectively. The sampling time was the time after the first sample taken which designated as the zero time point. (A) (C) and (B) (D) are for 18:0 and 16:0, respectively. (-○-) and (-♦-) represent light and dark period, respectively.

with a rate constant k = 0.95 hr^{-1}, calculated from the above values, the half life for pool filing is 44 minutes ($t_{1/2}$ = 0.693/k) (Segel, 1968). Thus the extrapolation of the linear rate of fatty acid synthesis to the x-axis (time axis) would result in a lag measured on the x-axis of 44 min. These calculations suggest that very significant lag phases would occur with [^{14}C]carbon dioxide labeling if a large free acetate or other intermediate pool did indeed supply fatty acid synthesis *in vivo*.

Arabidopsis seedlings were pulse labeled for 5 min with [^{14}C]carbon dioxide, then chased with unlabeled carbon dioxide for up to 270 min. The time course for label accumulating in fatty acids is shown in Figure 2.5 (A). Extrapolation of the labeling rate at the end of the 5 minute pulse period to the beginning of the pulse period gives a lag period of about 2-3 minutes. This can be explained, at least in part, by label moving through C(1) to C(2) to C(3) in triose-phosphate intermediates in the Calvin cycle. Such intermediates will be the source of pyruvate and then acetyl-CoA. After 40 minutes of chase period movement of label from precursor pools into fatty acids is almost complete. In addition to *Arabidopsis* a similar kinetic behavior of fatty acid labeling was observed in the dicotyledonous plants spinach and pea, and the monocotyledonous plant barley (data not shown). We therefore believe that this labeling pattern is a general property of fatty acid biosynthesis in higher plants.

In another experiment, *Arabidopsis* seedlings were labeled with [^{14}C]carbon dioxide for 5 min, then chased with unlabeled carbon dioxide for 270 min in either the light or the dark. These pulse-chase time courses are shown in Figure 2.5 (B).

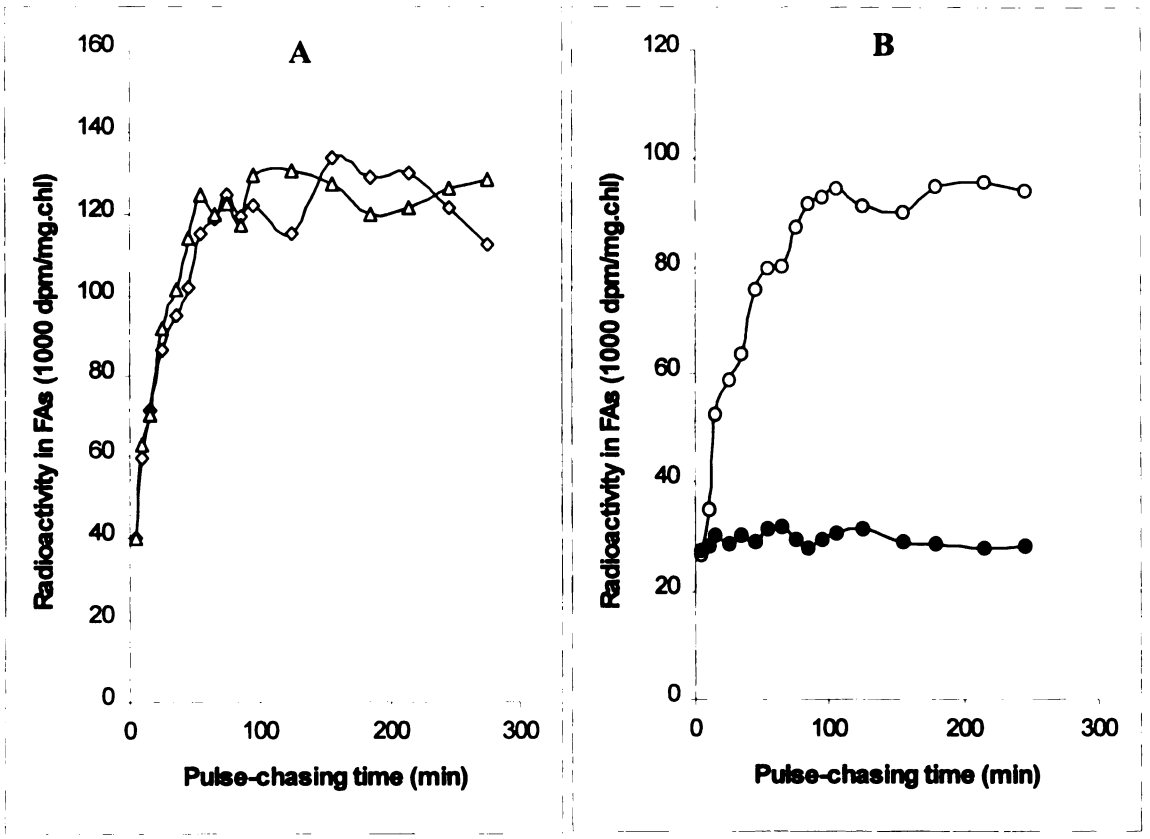

Figure 2.5. Pulse-chase labeling of Arabidopsis with $^{14}CO_2$ to determine the lag phase. (A) *Arabidopsis* seedlings were pulse labeling with for 5 min, then chase for up to 280 min in the light. Radioactivity in fatty acids from two independent experiments was plot with pulse-chase time. (B) *Arabidopsis* seedlings were pulse labeling with for 5 min in the light, then chase for up to 260 min either in the light (-○-) or in the dark (-●-).

In the light, radioactivity accumulated in fatty acids continued increasing up to 40 min; but in dark, incorporation of radiolabel stopped immediately after plants are transferred to the dark, demonstrating a complete cessation of fatty acid synthesis.

A third type of experiment used continuous [^{14}C]carbon dioxide labeling for one hour. This experiment was designed to look for a slow response pool in addition to the rapid labeling kinetics seen in the previous two experiments by short time pulses. The results are shown in Figure 2.6. The fatty acids shows a linear labeling without upward hyperbolic kinetics at later time points, indicating that there is not a second more slowly responding pool of precursors for fatty acid biosynthesis in mesophyll cells. Consistent with [^{14}C]carbon dioxide pulse-chase experiment, extrapolation of the labeling rate to the x-axis gives a lag period of about 3 minutes. Radioactivity accumulated into total isoprenoids (largely sterols and plastidic isoprenoids) also showed a similar linear labeling pattern, with a similar lag phase (Figure 2.6).

[^{14}C] Acetate Analysis

In order to assay [^{14}C]acetate recovered from the aqueous extracts of incubations with [^{14}C]carbon dioxide the aqueous extracts were derivatizaed to convert volatile carboxylic acids into non-volatile phenacyl esters which could be analyzed by TLC. Samples from the above pulse-chase experiment (shown in Figure 2.5) at 10, 20, 40, and 120 minutes were analyzed using the Instant Imager and by liquid scintillation counting. No radioactivity could be detected in the region corresponding to the phenacyl acetate standard. Taking into account the efficiency

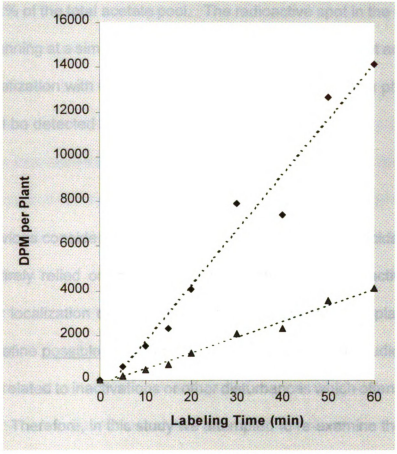

Figure 2.6. Continuos labeling of *Arabidopsis* with $^{14}CO_2$ to determine whether there is a slow filling pool. One hundred *Arabidopsis* plants were incubated in the bag with 5.4 mmol CO_2 and 2 mCi $^{14}CO_2$. After 5, 10, 15, 20, 30, 40, 50, and 60 minutes 12 plants were transferred into tubes with sulfuric acid, then radioactivity in fatty acids (◆) and isopprenoids (▲) in each sample was plot with labeling time.

of extraction and derivatization, if the acetate concentration in leaves is 1 mM, the detection limits for the protocol suggest that the amount of labeled acetate must be less than 2% of the total acetate pool. The radioactive spot in the non-derivatized samples running at a similar Rf value to acetate standard was not acetate, because after derivatization with bromoacetophenone no corresponding phenacyl acetate band could be detected by TLC.

Discussion

Previous considerations of the path of carbon into fatty acids in leaves have almost entirely relied on *in vitro* measurements of enzyme activities and their subcellular localization or on experiments with isolated chloroplasts. Although useful to define possible reactions which occur *in vivo*, such studies have several limitations related to inactivations or other disturbances which often occur upon cell disruption. Therefore, in this study we attempted to re-examine the flux of carbon into fatty acids by methods which could more closely reflect *in vivo* metabolism.

Measurements of *In Vivo* Rates of Fatty Acid Synthesis

The first step in assessing the flux of intermediates through a pool for any pathway is to know the *in vivo* rate of synthesis of the product of interest, not just the net rate of accumulation of the product. For fatty acids, the use of exogenous tracers presents uncertainties because it is impossible to assess endogenous contributions without measuring specific activities. Such measurements over short periods give a high degree of statistical variability, since the increase in mass is

small, while over extended periods they introduce the uncertainty of net accumulation. A way around the dilemma in the investigation of carbon fluxes is to perform *in vivo* [^{3}H]water labeling to estimate the rate of fatty acid synthesis (Browse *et al.*,1981). The water should exchange rapidly with the leaf tissue water and is expected to label both endogenous and exogenous carbon substrates equally. Injury to the tissue during preparation of the leaf discs may introduce some uncertainty into applying the resultant rate to intact plants. Also, the use of [^{3}H]water requires corrections for isotope effects and a knowledge of the maximum value for substitution of hydrogen by tritium. These corrections were not made by Browse *et al.,* (1981). Nevertheless, these workers obtained as estimated *in vivo* rate of fatty acid synthesis of 2.6 μmol C h^{-1} mg chlorophyll^{-1}.

Using [^{13}C]carbon dioxide labeling to measure the rate of fatty acid syntheses by GC-MS has some obvious advantages. First, plants are labeled intact under natural growth conditions, rather than as detached parts floating in incubation medium. Second, the newly synthesized fatty acids are easily distinguishable from pre-existing fatty acids (Figure 2.1). Thirdly, the method removes the problem of measuring the small mass increments for fatty acid accumulation over short assay periods. In the light, the rate of fatty acid synthesis of *Arabidopsis* obtained with [^{13}C]carbon dioxide labeling was 2.3 μmol C h^{-1} mg chlorophyll^{-1}. Exact comparisons with the 2.6 μmol C h^{-1} mg chlorophyll^{-1} obtained by [^{3}H]water labeling (Browse *et al.*,1981) are not possible because of the species difference and the fact that our work represents a whole plant (aerial parts) average rather than selected

leaves. It is of interest that of the total rate of fatty acid synthesis about 30% was not accountable to the added [^{13}C]carbon dioxide label. What is important is that we can see the mixing of the exogenous and endogenous label since the distributions are essentially smooth curves. The conclusion that exogenously and endogenously derived carbon substrates mix randomly for fatty acid synthesis was also reached in a GC-MS analysis of fatty acids from the labeling of spinach leaf discs with [1-^{13}C] acetate (Pollard and Ohlrogge, 1999). The work presented here is based on the validity of this assumption.

It is clear that the majority (at least 70%) of carbon in newly synthesized fatty acids is derived from recently fixed [^{13}C]carbon dioxide. At this time we cannot define whether the 30% ^{12}C contribution represented dilution of the [^{13}C]carbon dioxide with unlabeled carbon dioxide (generated by respiration either internally within the tissues or from the system, particularly the soil) or represented non-photosynthetic parts of the tissues, or represented an additional carbon source for fatty acid synthesis from previous fixed carbon pools such as starch or sucrose. Delwiche and Sharkey (1993) noted that isoprene labeling from carbon dioxide in isolated oak leaves saturated at about 80% contribution from the carbon dioxide, which was very similar to the saturation level of the phosphoglycerate pool in beet leaves described by Canvin (1979). Both results suggested an approximately 20% contribution of carbon from a source other than the immediately fixed carbon in the chloroplast.

The rate of fatty acid synthesis in the dark in leaf tissue is known to be much

reduced, but the question remains whether, *in vivo*, it is still significant (10-20% of light rate) or much closer to zero (<5%). Estimates made using labeled precursors fed to leaf discs suggest 12-20% of the rate in the dark (Browse *et al.*, 1981). The same authors show that fatty acid synthesis from acetate by isolated chloroplasts is essentially zero. Since we can accurately measure the rate in the light, we should be able to determined the rate in the dark, if the dark rate relative to light can be evaluated. This can be done with an isotope dilution strategy, again employing [^{13}C]carbon dioxide (Figures 2.3 and 2.4). In this experiment we demonstrate that there is a slow fatty acid degradation, of the order of 4-5% per day. The label released is not significantly recycled back into fatty acids to complicate the analysis. As shown in Figure 2.4, during the dark period, ions representative of the ^{13}C labeled C18 fatty acids (m/z = 309) and of the ^{13}C labeled C16 fatty acids (m/z=280), and ions representative of the unlabeled C18 fatty acids (m/z = 298) and of unlabeled C16 fatty acids (m/z=280) remain unchanged in relative proportion. Fatty acid synthesis will result in the latter set increasing at the expense of the former, whereas there was a very slight statistical average in the other direction. These result clearly demonstrated that, for *Arabidopsis* seedlings, there was negligible fatty acid synthesis in the dark. In support of this observation, when *Arabidopsis* seedlings were transferred to dark after 5 min labeling with [^{14}C]carbon dioxide in the light, radioactivity in fatty acids remained constant throughout the dark period (Figure 2.5(B)).

Rapid Movement of Label from Carbon Dioxide to Fatty Acids and Isoprenoids

The labeling from [^{13}C]carbon dioxide suggests that at least 70% of the carbon for fatty acid synthesis arises from newly fixed carbon, while the [^{14}C]carbon dioxide pulse chase and time courses (Figures 2.5 and 2.6) show that the lag phase for the movement of photosynthetically fixed carbon into fatty acids occurs very rapidly, within 2-3 minutes. This lag time is about half that reported by Murphy and Leech (1981). Thus, if acetate is the dominant source of acetyl-CoA for fatty acid synthesis, newly fixed carbon (with all three triose-P carbon atoms labeled) will have to move through to acetate very quickly, by a mechanism that is still debatable. A particularly important consideration for this putative flux is the rate at which label from [^{14}C]carbon dioxide moves into isoprenoids that are synthesized in the chloroplast, most notably phytol (Figure 2.6). This discovery that isopentenyl pyrophosphate for isoprenoid biosynthesis in the chloroplast is not derived from acetyl-CoA via mevalonate, as it is in the cytosol, but via the glyceraldehyde-3-phosphate:pyruvate pathway with 1-deoxy-D-xylulose-5-phosphate as an intermediate, is important (Lichtenthaler *et al.,* 1997). Because the synthesis of phytol, via the chloroplastic isopentenyl pyrophosphate pathway used C3 precursor that are "upstream" of acetyl-CoA any lag phase for phytol biosynthesis will include part of the same lag phase for acetyl-CoA production, that is mixing time for [^{14}C]carbon dioxide in the bag, diffusion to chloroplasts and flushing through of the label in the Calvin cycle. In fact, as shown in Figure 2.6, the lag phases for the movement of label from [^{14}C]carbon dioxide into fatty acids and into isoprenoids are experimentally indistinguishable. Thus the three minute lag phase for label moving

into fatty acids is an upper limit in considering an acetate pool, and may be significantly less than that value. Delwiche and Sharkey (1993) make estimates of the lifetime of carbon in the photosynthetic carbon reduction cycle of the order of two minutes. Pyruvate in plastids is not only used for the generation of acetyl-CoA for fatty acid biosynthesis but also for isoprenoid biosynthesis and for the synthesis of branched-chain amino acids (Hoppe *et al.*, 1993; Schulze-Siebert *et al.*, 1984). Pyruvate concentrations of 40 to 120 μM in the leaves of dicot plants (Treede *et al.*, 1986) are low enough to allow the observed kinetic pattern of fatty acid labeling.

Issues of Acetate Supply of Fatty Acid Synthesis *in Vivo*

We know that the endogenous rate of fatty acid synthesis in *Arabidopsis* plants is 2.3 μmol C h^{-1} mg chlorophyll^{-1} for the whole aerial part of the plant (stem plus leaves), that most of the carbon for fatty acid synthesis comes from carbon dioxide that has been recently fixed, and that the movement of label from carbon dioxide to fatty acids reaches a steady state level within about 2 minutes. If all this carbon passed through an acetate pool, then the lifetime of acetate in this pool would be about 3 minutes, and amount to 0.114 μmol C mg chlorophyll^{-1}. In the "Results" section we used a conversion factor of 1 mM acetate bulk tissue concentration equivalent to 1.2 μmol acetate mg chlorophyll^{-1}. Thus the putative acetate pool supplying fatty acid synthesis will be 0.0475 mM, or more likely <0.0475 mM. Consistent with this conclusion, the results of [^{14}C]-acetate analysis demonstrated that if there was a 1 mM bulk acetate pool less than 2% of it was labeled, which is equivalent to <0.02 mM. This result should be compared with

the range of bulk acetate concentrations measured in leaf tissues, from 0.05 to 1.4 mM (Kuhn *et al.*, 1981; Liedvogel 1985; Treede *et al.*, 1986; Roughan, 1995). Roughan found higher values in younger leaves when compared to older ones, and that acetate concentration of leaves in the dark ranged from 0.35 to 0.70 mM, while that of isolated chloroplasts was 0.4 mM.

Several points must be kept in mind in interpreting the results. First, in isolated chloroplasts the rate of fatty acid synthesis from exogenous acetate saturates at between 0.1 and 0.2 mM (Roughan *et al.*, 1979). Second, a bulk concentration of 1 mM does not imply that each compartment in the cell will be at 1 mM. For example, the mesophyll cell has about 80% of its volume as vacuole (Winter *et al.*, 1994), with a pH of about 5.5. Thus at equilibrium most of the cellular free acetate is likely to be partitioned into the cytoplasm, and even more so into the chloroplast stroma. Thus, even at a bulk tissue concentration of 0.05 mM acetate, it is probable that the concentrations of acetate in proximity to the chloroplast are enough to sustain high rates of fatty acid synthesis. And third, high concentrations of acetate can drive high rates of fatty acid synthesis in spinach leaf discs ($K_{0.5}$ for exogenous acetate at pH 5.6 is about 2 mM, with V_{max} of about 2 μmol C h^{-1} mg chlorophyll^{-1}; Pollard and Ohlrogge, 1999). This confirms the concept that acetate will readily compete with endogenous substrate to supply fatty acid synthesis *in vivo*, and is suggestive of the idea that acetate in leaf tissues is freely diffuseable, not tightly bound, and therefore available.

Thus we reach a conclusion which seems almost counter-intuitive. If the *in*

vivo bulk concentration of acetate in leaf tissues is at the upper end of the range reported in the literature (0.5-1.4 mM), we must conclude from our kinetic analyses that this acetate is not a major substrate for fatty acid synthesis. The only possible mechanism for this is that all this acetate is so tightly bound or sequestered that it is unavailable. Because of the rapid movement of exogenously added acetate into tissue fatty acids, regardless of how it is applied, these alternatives seems unlikely. Possibly the measurements of the *in vivo* concentration of acetate in leaf tissues are incorrect (Kuhn *et al.*, 1981; Liedvogel, 1985; Treede *et al.*, 1986; Roughan, 1995). On the one hand, acetate is volatile and might be lost during the preparation and clean-up of the extracts. On the other hand, during extract preparation artificially high acetate concentrations might be generated due to chemical or enzymatic hydrolysis of endogenous acetyl or to breakdown of molecules yielding acetate (e.g. decarboxylation of malonate). All the methods quoted above (Kuhn *et al.*, 1981; Liedvogel, 1985; Treede *et al.*, 1986; Roughan 1995) use heating steps at some stage in the extraction. By contrast, if the *in vivo* bulk concentration of acetate in tissues is even lower than the range reported in the literature, (e.g. <0.05 mM), it is still possible that acetate is a major supplier of substrate for fatty acid biosynthesis. However, if the level of free acetate in tissues is in actuality low, then the issue of its contribution to fatty acid synthesis becomes a quantitative one. At a certain point the lower steady-state free acetate concentration in leaf tissues will no longer be able to support the total carbon flux into fatty acids. The question then arises as to whether the rapid movement of

newly fixed carbon into fatty acids is solely a flux of intermediates within the plastid, a flux involving the plastid and the cytosol, or includes movement of carbon through other organelles, especially the mitochondrion.

Origin of Acetyl-CoA *in Vivo* for Fatty Acid Synthesis.

The question regarding the origin of acetyl-CoA used for fatty acid biosynthesis has been discussed for several decades. Starting with the observation by Smirnov (1960) that acetate is a very good precursor for incorporation into fatty acids in isolated chloroplasts, acetate has been used for many labeling studies both *in vivo* and with isolated plastids. Researchers then looked for mechanisms that would explain how free acetate might be generated in the plant cell. In one model acetyl-CoA is generated via oxidative decarboxylation of pyruvate in the mitochondria. Acetyl-CoA, which is known not to be transported across membranes, is hydrolyzed by an acetyl-CoA hydrolase in the mitochondrial matrix to yield free acetate, which can diffuse freely through membranes and so enter the chloroplast (Murphy and Stumpf 1981; Liedvogel and Stumpf 1982). An alternative pathway for acetate generation has also been proposed, namely through the action of ATP:citrate lyase (Nelson and Rinne, 1977; Fritsch and Beevers, 1979). In chloroplasts, the existence of an acetyl-CoA synthetase using free acetate, CoA, and ATP to synthesize acetyl-CoA, AMP, and pyrophosphate has been demonstrated (Kuhn *et al.*, 1981) and its maximum activity is over 5 fold higher than the flux of fatty acid synthesis (Roughan and Ohlrogge, 1994). It should be noted that cellular anabolism produces free acetate by at least three mechanisms of which

we are aware. These are in the biosynthesis of cysteine *via* O-acetylserine, the biosynthesis of ornithine and hence the polyamines via *N*-acetylglutamate, and the acetylation and deacetylation of histones, each yielding acetate in a subsequent deacetylation step. Thus the efficient use of free acetate for acetyl-CoA biosynthesis (and hence fatty acid biosynthesis) in the chloroplast may simply reflect an efficient scavenging mechanism for free acetate used by the plant cell.

An alternative pathway for the formation of acetyl-CoA in plastids has been proposed via the plastidial pyruvate dehydrogenase complex (pPDC) (Reid *et al.*, 1977; Elias and Givan, 1979; Williams and Randall, 1979). There are two origins for the pyruvate needed for the pPDC reaction. First the existence of a complete glycolytic sequence inside the chloroplasts has been proposed (Schulze-Siebert and Schulz, 1989; Hoppe *et al.*, 1993). This is supported by the observation that isolated chloroplasts can synthesize fatty acid from [^{14}C]bicarbonate (Murphy and Leech, 1977; Murphy and Leech, 1978), albeit with a lower rate than that required for the *in vivo* rate of fatty acid synthesis (Roughan, 1993; Murphy and Leech,1981). This pathway requires the conversion of 3-phosphoglyceric acid to acetyl-CoA via 2-phosphoglyceric acid, phosphoenolpyruvate and then pyruvate. It is sometimes called the C_3-C_2 pathway. A modification of this C_3-C_2 pathway occurs if not all the glycolytic steps occur in the plastid but instead some reactions are located in the cytosol and that PEP (Fischer *et al.*, 1997) or pyruvate are reimported into the chloroplast.

A third, but less generally accepted hypothesis is that acetate units may be

moved by an acetylcarnitine-shuttle into different compartments. In such a scheme pyruvate in the mitochondrial is converted to acetyl-CoA, which is exported from the mitochondria as acetyl-carnitine, and imported by the chloroplast (Masterson *et al.*, 1990a and 1990b).

Although the majority of acetyl-CoA in plastids is used for fatty acid biosynthesis, the supply of plastidial acetyl-CoA must be flexible enough to support plastidial polyhydroxybutyric acid biosynthesis in *Arabidopsis thaliana* transgenics from acetyl-CoA, since it accumulates at up to 14% of dry weight of the leaf, whereas no significant changes of membrane lipids was observed (Nawrath *et al.*, 1994). In conclusion, the results presented in this paper provide in vivo data which distinguish in part between the alternative pathways suggested for acetyl-CoA production in leaves. Our data clearly indicate that fatty acid synthesis does not depend on a high tissue concentration (0.5-1.4 mM) of acetate for a carbon source. Furthermore, the involvement of other large pools of metabolic intermediates between carbon dioxide and acetyl-CoA is ruled out. The kinetic analysis of the rapid movement of label from carbon dioxide into both fatty acids and plastidial isoprenoids is certainly suggestive of direct utilization of newly fixed carbon via the C_3-C_2 pathway into fatty acids. However, although unlikely, the type of experiments we have performed do not provide enough data to eliminate with certainty the possibility that a small pool of acetate, metabolically distinct from bulk acetate, is an intermediate in leaf fatty acid synthesis. We do not know the time constant for transport processes and for filling intermediate pools through other organelles,

especially the mitochondria, which is the only other site of PDC in the plant cell. This will require rapid kinetic studies for which the use of leaf tissue may or may not be adequate to give the required time resolution, and generally can only give bulk cellular pool information.

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CHAPTER 3[1]

Isolation and Characterization of an Arabidopsis Biotin Carboxylase Gene and its Promoter

Abstract

In the plastids of most plants, acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) is a multisubunit complex consisting of biotin carboxylase (BC), biotin-carboxyl carrier protein (BCCP), and carboxyltransferase (α-CT, β-CT) subunits. To better understand the regulation of this enzyme, we have isolated and sequenced a BC genomic clone from Arabidopsis and partially characterized its promoter. Fifteen introns were identified. The deduced amino acid sequence of the mature BC protein is highly conserved between Arabidopsis and tobacco (92.6% identity). BC expression was evaluated using Northern blots and BC/GUS fusion constructs in transgenic Arabidopsis. GUS activity in the BC/GUS transgenics as well as transcript level of the native gene were both found to be higher in silique and flower than in root and leaf. Analysis of tobacco suspension cells transformed with truncated BC promoter/GUS gene fusions indicated the region from -140 to +147 contained necessary promoter elements which supported basal gene expression. A positive regulatory region was found to be located between -2100 and -140, whereas a negative element was possibly located in the first intron. In addition, several conserved regulatory elements were identified in the BC promoter.

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[1]Material in this chapter was published previously [Bao X, Shorrosh BS, and Ohlrogge J (1997) Plant Molecular Biology 35: 539-550].

Surprisingly, although BC is a low abundance protein, the expression of BC/GUS fusion constructs was similar to 35S/GUS constructs.

Introduction

Acetyl-CoA carboxylase catalyzes the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA. This reaction is the first committed step of *de novo* fatty acid biosynthesis and is a major point of flux control for this pathway (Harwood,1988; Ohlrogge and Jaworski, 1997; Wakil *et al.*,1983). Most plants contain two structurally distinct forms of ACCase (Sasaki *et al.*, 1995). One form, termed "homodimeric" (HO), "eukaryotic" or "multifunctional" is a >200kD polypeptide in which biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT) occur as functional domains (Gornicki *et al.*,1994; Roesler *et al.*,1994; Roessler and Ohlrogge, 1993; Shorrosh *et al.*, 1994). The other form of ACCase, termed "heteromeric" (HE), "multisubunit" or "prokaryotic" is an enzyme composed of easily dissociable subunits. Most evidence suggests the HO-ACCase occurs in the cytosol of all plant families whereas HE-ACCase is localized in the plastids. However, within the *Gramineae* family the HO-ACCase exists in the plastid as well as the cytosol (Baldet *et al.*,1993; Gornicki and Haselkorn,1993; Konishi and Sasaki,1994; Roesler *et al.*,1994; Shorrosh *et al.*,1995).

Plastid ACCase activity is regulated at the biochemical level by light and by feedback regulation (Post-Beittenmiller *et al.*,1991; Post-Beittenmiller *et al.*,1992; Shintani and Ohlrogge, 1995). There is also evidence which suggests plastid ACCase is regulated at the level of gene expression. For example, in tobacco and castor, expression of the BC gene is much higher in developing seeds than in leaf

or root (Elborough *et al.*, 1996; Roesler *et al.*,1996; Shorrosh *et al.*,1995). In developing castor and *Brassica napus* seeds, ACCase activity as well as expression levels of BC and BCCP correlate with oil deposition (Roesler *et al.*,1996). Using *in situ* hybridization analysis, BC and BCCP were found to be expressed in a coordinate fashion and were correlated with oil accumulation during seed development in Arabidopsis (Choi *et al.*,1996). The plastid ACCase subunits, BC, BCCP, and α-CT are encoded by nuclear genes, whereas β-CT is encoded by a plastid gene. It is not known how overall ACCase expression is controlled or how levels of the four subunits are coordinately regulated. However, recent results indicate that antisense or over-expression of the BC subunit in tobacco did not alter expression of the BCCP subunit (Shintani *et al.*, 1995). Nor is there research to investigate what *cis* acting elements are responsible for the regulation of ACCase. To begin to address these questions we describe the isolation and characterization of a biotin carboxylase genomic clone from *Arabidopsis*. Furthermore, we report initial studies to determine the *cis* acting elements of the promoter of the BC subunit of ACCase.

Materials and Methods

Isolation of genomic clones and DNA sequencing

A biotin carboxylase cDNA clone from tobacco (Shorrosh *et al.*, 1995) was used as probe to screen an *Arabidopsis thaliana* 'Columbia' genomic library in λ GEM 11(Promega) vector. Approximately 10^6 plaques were screened using

Colony/Plaque Screen (New England Nuclear) membranes according to the manufacturer's instructions. Hybridization with the ^{32}P probe was carried out under conditions as described (Shorrosh *et al.*, 1994). Positive clones were purified and then subcloned into pBluescript SK(+/-) phagemid (Stratagene) using *Sac*I, *Hin*dIII, and *Eco*RI sites. Both strands of plasmid clones were sequenced using universal and synthetic primers and AmpliTaq DNA polymerase (Boehringer Mannheim).

Plant RNA isolation and Northern blotting

The RNAs from leaf, root, flower, and silique, were prepared as described (Sambrook *et al.*, 1989). Total RNA (20 µg) was then fractionated on a formaldehyde gel and blotted to Zeta-Probe nylon membranes (Biorad, Richmond CA) as described (Sambrook *et al.*, 1989). The membranes were prehybridized overnight at 42^{0}C in 5X SSC, 10X Denhardt's solution, 0.1% SDS, 0.1 M potassium phosphate pH 6.8, 100 µg/ml salmon sperm DNA. The membranes were then hybridized overnight with 1x10^{6}CPM/ml random primer ^{32}P-dCTP labeled probei n 5X SSC, 10X Denhardt's solution, 0.1 M potassium phosphate pH 6.8, 100 µl/ml salmon sperm DNA, 10% dextran sulfate, 30% formamide. Blots were washed 3 times at 65^{0}C in 0.1X SSC, 0.1% SDS.

Primer extension and RT-PCR

Primers were labeled by ^{32}P in 10 µl labeling solution (1x Kinase buffer, 20 pmols of primer, 30 µCi γ-^{32}P-dATP, 10 units T4 polynucleotide kinase). The reaction was incubated 30 min at 37^{0}C and terminated by heating to 70^{0}C for 10 min. Labeled primer (3 pmols) was added into annealing solution (10 mM Tris·Cl

pH 8.3, 50 mM KCl, 10 µg of total Arabidopsis RNA from developing seeds) in a final volume of 15 µl. The rest of the procedure was conducted as described (Ausubel,1990). The cDNA was collected by centrifuging at top speed for 15 min in a microcentrifuge and the pellet was washed twice with 70% ethanol and dried in speed vacuum. The dried pellet was dissolved in 6 µl sequencing loading buffer and denatured at 90^{0}C for 5 min, then cooled quickly on ice. A 3 µl sample was analyzed on 8% polyacrylamide sequencing gel containing 7 M urea.

For RT-PCR, first-strand cDNA synthesis and cDNA amplification were performed using total RNA from developing seeds and CapFinder™PCR cDNA library construction kit (Clontech, Catalog# k1051-1). The primers used for amplifying the fragment of interest were one near the transcription start (5') and a set of 3'-primers located in the second and third exon (Figure 3.1). The PCR products were cloned into pBluescript SK(+/-) for sequencing.

Promoter-GUS plasmid construction

As shown schematically in Figure 3.3, four promoter/GUS fusion constructs were prepared to examine the expression pattern of the BC promoter and its *cis* acting elements. Because there were no convenient restriction sites between -140 and +345, the *Hin*dIII/*Eco*RI fragment (-140 to 748) was subcloned into the polylinker of pBluescriptSK (+/-). Two PCR primers (JO380 and JO379) with addition of *Xho*I and *Xba*I sites at the end (Fig. 1) in combination with T7 primer on the vector, were used to amplify two promoter fragments (-140 to +345 and -140 to +147) by PCR. The two PCR fragments, which were digested by *Hind*III and *Xba*I,

were designated 380 and 379, and then directionally cloned into *Hin*dIII/*Xba*I site of pBI101.1 preceding GUS. Subsequently, the 380 and 379 fragments were ligated back to the -2100 to -140 fragment at its *Hin*dIII site and the two fragments, -2100 to 345 and -2100 to 147, were then inserted into the *Xba*I site of pBI101.1 which generated the constructs designated as BF380 and BF379. The binary vectors were transferred (An, 1987) from *E. coli* into *Agrobacterium tumefaciens* strain LBA4404 (for tobacco suspension cells) or strain C58C1 (for Arabidopsis).

Tobacco suspension cell and Arabidopsis transformation

Tobacco suspension cells (*Nicotiana tabacum* L. cv. bright yellow 2) were maintained in liquid medium containing Murashige and Skoog basal salts (Gibco, Grand Island, NY), 3% sucrose, 2.5 mM MES/KOH pH 5.7, 1 mg/ml thiamine, 1 mg/ml myo-inositol, and 1 μM 2,4-D. Cultures were subcultured weekly with a 5% (v/v) inoculum from a 7-day-old culture and shaken at 28^{0}C in 50 ml flasks. *Agrobacterium* transformation was carried out as described (Rempel and Nelson 1991). The cells were pelleted by centrifuging at 1000g for 5 min at room temperature in a swinging bucket rotor and were washed two additional times before spreading on selection plates (liquid medium with addition of 0.7% phytagar, 100mg/L kanamycin, and 500mg/L carbenicillin). After three weeks, independent transformants were transferred to liquid medium containing 100mg/L kanamycin and maintained as described above.

Arabidopsis thaliana 'Columbia' plants were grown with 16 hours light/8 hours dark at a temperature of 22^{0}C. After 4-6 weeks, when the primary

inflorescence were 10-15 cm tall and the secondary inflorescence were appearing at the rosette, the vacuum infiltration method was used for transformation as described (van Hoof and Green,1996). The transformed plants were grown until harvest under the same conditions as before transformation. Collected seeds were screened on kanamycin (50mg/L) plates and resistant seedlings were transferred to soil for further GUS assays.

Fluorometric β-glucuronidase (GUS) assays and histochemical assays

Protein extraction and GUS assays were carried out as described (Jefferson, 1987) with Arabidopsis tissues (root, leaf, flower, and silique) of independent transformants and transformed tobacco suspension cells. Assays were conducted at four time points (0, 15, 30, 45 min) with each time point containing 20 µl protein extract and 20 µl assay buffer. Reactions were terminated by addition of 1 ml of stop buffer and after all reactions were terminated, another 0.5 ml of stop buffer was added to each tube. Methyl umbelliferyl (MU) concentration was determined with a spectrofluorimeter (excitation at 365 nm, emission at 455 nm) and GUS activity was expressed in terms of pmol MU/min/mg protein.

GUS activity was localized in different organs of F_2 transgenic Arabidopsis transformed with the BF380 construct by histochemical staining with X-gluc as described (Jefferson, 1987) with the following modifications. Fresh tissue was incubated in reaction solution (1 mM X-gluc, 50 mM sodium phosphate buffer pH 7.0, 1 mM K^+ ferricyanide/ferrocyanide mixture, 0.01% Triton X-100, 10 mM β-Mercaptoethanol, 20% methanol, 1 mM EDTA) at 37°C for times which varied from

1 to 12 hour. After staining the tissue and rinsing with 70% ethanol, observation and photographs were taken under a dissecting microscope.

Results

Analysis of the biotin carboxylase genomic clone

The complete Arabidopsis BC genomic sequence was obtained from multiple overlapping genomic subclones (Figure 3.1). To determine the position of introns and the translation start site, we compared the Arabidopsis genomic sequence to the tobacco BC cDNA deduced amino acid sequence (Shorrosh *et al.*, 1995). The predicted amino acid sequence of Arabidopsis BC deduced from the genomic DNA sequence shared a very high (92%) sequence identity to the tobacco BC deduced amino acid sequence. Further evidence substantiating the placement of introns is that the predicted protein from the genomic sequence is 100% identical to a deduced 120 amino acid sequence of an Arabidopsis EST (150M20T7). Based on these analyses, we concluded that the isolated genomic clone is the Arabidopsis BC gene and contains 15 introns. Placements of introns-2 through intron-14 were made based on comparison to the tobacco BC protein, in addition to the presence of the conserved intron boundary sequence GT/AG (Figure 3.1). Because of a lack of sequence similarity between Arabidopsis BC and tobacco BC in the transit peptide region, intron-1 was identified by comparison of the genomic sequence to that of RT-PCR products prepared from developing seed RNA (see Materials and Methods). The Arabidopsis genomic BC predicted protein and the tobacco BC

sequence also shared no significant similarity over the last 15 amino acids. Therefore, placement of the 3'-most intron was based on comparison with additional sequence obtained from EST clone 150M20T7.

The translation start site (indicated in Figure 3.1) begins with the first Met codon after the predicted transcriptional start site. The first 70 amino acids following this MET have a high SER/THR content, only one acidic amino acid, and a high proportion of hydrophobic amino acids. These characteristics correspond to a typical chloroplast transit peptide. We have tentatively identified the transit peptide cleavage site between amino acids 71 and 72 based on the consensus cleavage site described by Gavel *et al.* (1990). The transcriptional start site was identified by primer (JO381, Figure 3.1) extension of RNA extracted from seedlings. A putative TATA motif was found 49 bp upstream from the transcriptional start site and a putative polyadenylation signal was identified at +4353. Several interesting sequence motifs were also found in the BC promoter and the 5' untranslated region. First, there are three short CT stretches (TCTCTGTTTCTCTGTTTCTCTGTTT, TCTCTCTCTCTCTCTC, and TCTCTCTCTCTCTC) located at position +310, +115, and -745, respectively (Figure 3.1). Second, two clusters similar to the SEF4 (soybean embryo factor) binding motif (Lessard, 1991) were identified in the BC promoter. Each cluster contains three copies of binding sites and the two clusters were separated by about 950 bp (Figure 3.1). Lastly, two large (approximately 480 and 280 bp long) segments of repetition were found in the BC promoter; one repeat was between -1289 to -888 and -478 to -84, and the two regions share 75%

*Eco*RI

-2051 GAATTCAAATTATTTTTCTATGA

-2028 TTTTGTCCCTTAAATTATAACTGATCTACTTCATATAGACTATTATAACTAAAATCATTT

-1968 CTAGTCAATAATAGAAGATGCTACGAACAAAAGCACTTAATTTTGAAACCTTAAATAAAA

-1908 TACATAGTATAACGATTTTTATATACTTATCTATTAGATCACAAAAACTAAATTCAATGT

-1848 TAAAAATAAAGTACATAAATTTATTTGAGATATGGAAGAGGTAAGAAAATATTATTTGAT

-1788 AAATATATCACAACAATTATTTTGTTAAATATTACATAAATAAAATGACACACGAATTAT

-1728 TATCGAAAATAGTCAAATATCCAAAATAGATAATAGAGATAGATCTTTTCAACTTCATTT

-1668 CCTTGCATGATGGTCAAAATTGATATTTTGTTACATGTATCAAGACTTCCAGTTTACAAA

-1608 GGTGAATTTGAACTAAAATAAATATGAAAATCAAAGAAGAAATAAGTATAATCTAGTAAC

-1548 ACAATATAAATAAAGGGTTTTGTGCAAAAAAAACTGAAATTGTATATTGTTGCAGCTTTA

-1488 CCCTTCACGTTAAAAAAATGCAAATAATCCTATAAATTTTAAAAATAATGCGAAATATAG

SEF-4

-1428 CCATTTTTTTGCAAATTTACATATATTGTAAATATTTTCATGATCACATACATTTGTTTT

SEF-4

-1388 TGAATTAGACTCTTCCAATATAGCAATCTATTGAAGAAATAAGTATTATTTATCAAAAAA

-1328 TTGAATTTTTGACAATAGCAAAAAAGTTAATTTGCGTATTATTTTGAAATTCATAAGTAT

-1268 TTTGCATTTTTTTATGTGAAAGATAAAATTGCAAAAATAGACGAATTTGAAAGTTGTTTT

SEF-4

-1208 TGCACAAAACCCAAAATAAAATACAACAATATAGGATATATAGATCATAGCCTCTAAAAA

-1148 TAAATTTAAAATTAGATTCTTCCAATGATGTTTCAACCCATATAACAACAGAAAAGCAAA

-1088 ACTAAAATATTTAAAATTTGAGATAGGTTCGAAGATGATTATCTTTGGAACTTGGTTTTC

-1028 TTGATGTATATGATTAAATTTGGTGCATATGTAATTAAGTGTGCACTGGACCAATAAAAA

-968 ACATTGAAACCTTATATAATATATCTTCTTATGACACATTATTATTTAAATAAACAAACA

-908 AACAAAACTTTAAAAGCGTTTGCGTTCAGCGTTCAGCGTTCAGCGTTTGCGTTCGACGTT

-848 TAGAGTTGGCACAAAATCCACCAAATTAAAACCATTCNGGCGGAGGATTCAATATATCTC

-788 ACAGTTTCACTCTCAACATTTCAATATCTCTCTCTCTCTCTGGGAAGAACAAATACAGCG

-728 TTTCTTCTGCGATTAGATCTATTTCGATACTCACGAGGATTGCCTCCTGAGGAGACTACA

-668 TATACTAAACAAAATACAAGCTGAGGAGACTAAATATATTTAGTTAATACCAATAATATA

-608 TGATATACAACTACATATTTACAAACAAAGAAAATATAGTAGATTTGTAATATATACATA

-548 ATTGAGATTTGATGATTGATTAAACCAAGAATACCGTGCGTAGCACGGGTACTGACATAG

-488 TATGTGTATTATTCTGAACTTCATAAGATTATTTTTCATTTTTGATGTGAAAGATAAAGT

SEF-4

-428 TGCAAAAATGGTCGAATTTGAAGGTTTTTTGCACAAACCCAGAAATAAAATAAAACAATA

SEF-4

-368 TAAGATATGATCATAGCCTCTGAAAATAAATTTAGAACTAGACTCTTCCATTGATGTTTC

-308 AACCCATATAACAACATAAAAGCAAAGCAATAAGTTGAAAGATGATAATCAAAATTAAAA

-248 AAATGAGTATCTTTGCAACTTGGTTTTCTTGATGTATATAATTAAATTTGTTACATATGT

SEF-4 *Hin*dIII

-188 AATTAAGTGTAAACTGGACCCATAAGACCTTTTTCATCCTTGTAAGCTTAATGGGCTTTA

-128 TCAATATAATGTTGGCCGACAAAAAAAAAAAAAAAAAGACCCATAAGAAACATTGAAACC

-68 AGTCTGGCCCATGACCCA**TATAAT**TTATCTTTAAAAGAGTCTGCGTTCCACGTTTGCGTT

▼ JO381 3'-GGTC

-8 CCGAGTTTGCGTTCCGTGTTTGCGTTTAGAGTTGGCACAAAATCCACCAAATCAAACCAG

AGCCCGCCTCCTAAGTTATAG-5'

52 TCGGGCGGAGGATTCAATATCTCAGAGTTTCACTCTCAACATTTCATTCTCTCTCTCTCT

JO379

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          3'-ATCTAGACAAAGCTATTAGTGCCTTagatctgagctcggc-5'
112   CTCAACAATTAGATCTGTTTCGATAATCACGAGGTTTCCCTCACTTCCTTTCCATTGCTA
172   AACCAGGATTTGCCTCCTGCATTTGGAAATGGACGCTTCTATGATTACCAATTCCAAATC
                                    M  D  A  S  M  I  T  N  S  K  S    11
232   CATTACTTCTCCACCCgtaagcaccaaaacgctattaatttgaaattcgtcaatctctgt
       I  T  S  P  P                                                  16
                            JO380
                        3'-AGAGACTTAGACAATTTAGACAATATACTTC
                                                    agatctgagctcggc-5'
292   ttctctgtttctctgtttcatgtctctgaatctgttaaatctgttatatgaagTCTTTAG
                                                          S  L  A    19
352   CCTTAGGGAAGTCAGGAGGAGGAGGAGTTATCAGAAGTTCACTATGTAACTTAATGATGC
        L  G  K  S  G  G  G  G  V  I  R  S  S  L  C  N  L  M  M  P  39
412   CAAGCAAAGTTAACTTCCCTAGACAAAGAACTCAAACTCTAAAGGTTAGCCAGAAGAAAC
        S  K  V  N  F  P  R  Q  R  T  Q  T  L  K  V  S  Q  K  K  L  59
472   TCAAGAGAGCTACTAGTGGTGGTCTTGGTGTTACATGCAGTGGTGGTGATAAGATTCTCG
        K  R  A  T  S  G  G  L  G  V  T  C//S  G  G  D  K  I  L  V  79
                                                  -  -  -  -  -      76
532   TAGCGAATCGAGGTGAAATTGCAGTTCGTGTTATCAGAACTGCTCATGAAATGGGGATTC
        A  N  R  G  E  I  A  V  R  V  I  R  T  A  H  E  M  G  I  P  Ara
99
        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  96
592   CTTGTGTTGCTGTGTATTCTACTATAGATAAAGATGCTCTTCATGTTAAATTAGCTGATG
        C  V  A  V  Y  S  T  I  D  K  D  A  L  H  V  K  L  A  D  E  119
        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  116
652   AAGCTGTTTGTATTGGTGAAGCTCCTAGTAACCAGTCgtaagtgtggaacaaaatgattg
        A  V  C  I  G  E  A  P  S  N  Q  S                          131
        S  -  -  -  -  -  -  -  -  -  -  -  -                       128
                                  EcoRI
712   atcttgntgtgtagagaatgaactagtgttgaattcttgtccttttgtgtgtagGTATTT
                                                          Y  L       133
                                                          -  -       130
772   GGTGATTCCGAATGTTCTGTCTGCGGCTATTAGCCGTGGATGTACAATGCTTCATCCTGG
       V  I  P  N  V  L  S  A  A  I  S  R  G  C  T  M  L  H  P  G    153
       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  150
832   ATATGGTTTTCTTTCGGAGAACGCTCTCTTTGTTGAAATGTGTAGAGACCATGGGATCAA
        Y  G  F  L  S  E  N  A  L  F  V  E  M  C  R  D  H  G  I  N  173
        -  -  -  -  A  -  -  -  V  -  -  -  -  -  -  E  -  -  -  -  170
892   TTTTATTGGACCTAATgtaagtgtcttatatctcttcaaagactttatagactttgcaga
        F  I  G  P  N                                                178
        -  -  -  -  -                                                175
952   acttcattagtctcagttcagccctagctttcttttgttggagagaaggaagatttcagt
1012  atctgatgtatcgtattcgtctttcctatctgtagCCTGATACCATCCGTGTTATGGGAG
                                          P  D  S  I  R  V  M  G  D  187
                                          -  -  -  -  -  -  -  -  -  184
1072  ACAAAGCGACTGCAAGAGAGACAATGAAAAATGCAGGGGTTCCCACTGTACCAGGGAGTG
        K  A  T  A  R  E  T  M  K  N  A  G  V  P  T  V  P  G  S  D  207
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        - S - - - D - - - - - - - - - - - - - - -   204
1132    ATGGGCTATTGCAGgtatttttttagattatcagtttagnatttcaagttttgcatgtc
          G  L  L  Q                                  211
          -  -  -  -                                  208
1192    aagagangtcttgggatttgaatgttttattttgtcttattgcagAGTACAGAAGAAGCT
                                                S  T  E  E  A   216
                                                -  -  -  -  G   213
1252    GTCAGGGTCGCCAATGAGATTGGTTTCCCTGTAATGATCACGgtaatattaacttgattg
        V  R  V  A  N  E  I  G  F  P  V  M  I  T              230
        -  -  L  -  E  -  -  -  Y  -  -  -  -  K              227
1312    gatttggagttaaattcgtatttcatactttcttttatgtaaccgataaactggattctc
1372    aaaattggtctgcttgaaaagacacaaatttaagtatgtttattttatgctcagcttgtc
1432    tccctgtacttattgatatgcttatagttacaaaaactgactgttctcacttttctggtt
1492    tcaagGCAACAGCTGGTGGTGGTGGACGTGGAATGCGTCTTGCTAAGGAACCGGGAGAGT
            A  T  A  G  G  G  G  R  G  M  R  L  A  K  E  P  G  E  F  249
            -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  D  -  -  246
1552    TTGTAAAACTGTTGCAGgtatgacaatcatgtaaatctgaaccaacaattcctctgatag
          V  K  L  L  Q                                  254
          -  -  -  -  -                                  251
1612    aatttttaatgagtccagggatcaagttctccataagcataattgcagnattgncacaga
1672    ttctcnatnnctnnctaactcagacaatgtaaaggtgtnatctcatgatgnttncccaca
1732    nnncgnaaannaagccncctgnatagttgtgaaanctggaagtgtagtancntaaatcta
1792    gtaatnctgaccattgatatcaantacnccaaccagattgtatgctttccgcttgccttt
1852    atagtcacagttttcttaaatgaaaattccacaaaagattcgacttccctttattgaact
1912    tggcttaatttttttatcgacagCAAGCTAAGAGCGAGGCTGCTGCTGCTTTTGGGAATG
                              Q  A  K  S  E  A  A  A  A  F  G  N  D  267
                              -  -  -  -  -  -  -  -  -  -  -  -  -  264
1972    ATGGATGTTATCTGGAGAAGTTCGTTCAAAACCCTAGACATATTGAGTTCCAGgtatgca
          G  C  Y  L  E  K  F  V  Q  N  P  R  H  I  E  F  Q        284
          -  V  -  -  -  -  Y  -  -  -  -  -  -  -  -  -  -        281
2032    ttttatcaggagaagttgncaaaacagttgaggttttancaaaagatgattcttgtgagt
2092    tgatggtctagtgatacacattattgatcccaaagccatattttctctcngtacctctng
2152    tatttcagGTGCTGGCAGATAAATTTGGAAATGTTGTTCACTTTGGTGAGCGTGACTGCA
                V  L  A  D  K  F  G  N  V  V  H  F  G  E  R  D  C  S  302
                -  -  -  -  -  Y  -  -  -  -  -  -  -  -  -  -  -  -  299
2212    GCATCCAGgtatcttcctcttaggtttttataatttcattaccatcaattgttgcatag
          I  Q                                           304
          -  -                                           301
2272    tgctccaagaataaatgtaactgaatatatcaaaacaggaataaatttacttcagcctaa
2332    ttactgcacttgcatgtcatatcccagctgcatgatctgctaaactgatttttcctgta
2392    ctaattgctaagataactcgaacttcgatactagttcacagaatttgagtatacctcttg
2452    ctgctttctttcaattcttgtatgacatctaaaaccatctggatcatttaattatatctt
                                  HindIII
2512    cccctgacaacagAGGCGTAACCAAAAGCTTCTGGAAGAACCTTCTCCAGCATTGACCGC
                      R  R  N  Q  K  L  L  E  E  P  S  P  A  L  T  A  320
                      -  -  -  -  -  -  -  -  -  -  A-  -  -  -  -  -  P  318
2572    TGAATTGCGAAAAGCCATGGGTGATGCAGCAGTCGCAGCAGCAGCATCCATTGGGTACAT
```

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      E  L  R  K  A  M  G  D  A  A  V  A  A  A  A  S  I  G  Y  I    340
      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -    338
2632  TGGTGTTGGTACTGTGGAGTTTCTTTTAGATGANAGAGGTTCCTTCTACTTCATGGAAAT
      G  V  G  T  V  E  F  L  L  D  E  R  G  S  F  Y  F  M  E  M    360
      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -    358
2692  GAACACTAGGATCCAGgttgaattttattatgactgctaagtatatttataagcatctga
      N  T  R  I  Q                                                 365
      -  -  -  -  -                                                 363
2752  agtatactgaatgcgaaccatttcttgaatctaaactcagGTGGAGCATCCTGTGACAGA
                                          V  E  H  P  V  T  E       372
                                          -  -  -  -  -  -  -       370
2812  GATGATTTACTCTGTTGATTTGATAGAGGAGCAGATTCGTGTTGCAATGGGAGAGAAACT
      M  I  Y  S  V  D  L  I  E  E  Q  I  R  V  A  M  G  E  K  L    392
      -  -  S  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -    390

2872  TCGTTACAAACAGgtttgaatagtgttgtatatgatgttttgagtggataccaaaaaagt
      R  Y  K  Q                                                    396
      -  -  -  -                                                    394
2932  tatttcaaaatttaattttgtttgtttacagGAAGATATTGTGCTCAGAGGGCACTCAAT
                                    E  D  I  V  L  R  G  H  S  I    406
                                    -  -  -  -  -  -  -  -  -  -    404
2992  TGAATGTCGTATCAATGCAGAAGATCCATTTAAAGGATTCAGACCTGGACCTGgtatatt
      E  C  R  I  N  A  E  D  P  F  K  G  F  R  P  G  P  G         423
      -  -  -  -  -  -  -  -  -  A  -  -  N  -  -  -  -  -  -      422
3052  tcagtagacttttatatgttaacgtcttcgaattttagcagattggctttctttaaaata
3112  acgtcactcttacaacaacagGCAGAATAACATCATACCTGCCATCTGGAGGTCCTTTCG
                          R  I  T  S  Y  L  P  S  G  G  P  F  V    437
                          -  -  -  A  -  -  -  A  -  -  -  -  -    435
3172  TTAGAATGGACAGCCATGTCTATTCCGACTATGTTGTGCCTCCAAGCTATGATTCTCTTC
        R  M  D  S  H  V  Y  S  D  Y  V  V  P  P  S  Y  D  S  L  L  457
        -  -  -  N  -  -  -  P  -  -  -  -  -  -  -  D  -  -  -  -  455
3232  TTGGGAAGgtaatatctgtcttttactttctctggatagccatttagtatctgtttggtt
        G  K                                                        458
        -  -                                                        457
3292  atttaggaacaaattttgtggcttcatggtaaaataatcactcgggtgttttggaatctt
3352  aaacaaatattggaaaagtcttcctggtgttgttcatttgcttctatagcagcagcttac

3412  tggaacttatagctgatgtgttgacacactaaatctcttgtagCTTATTGTGTGGGCTCC
                                          L  I  V  W  A  P          465
                                          -  -  -  -  -  -          463
3472  AACGAGGGAAAAAGCAATTGAACGGATGAAGCGGGCGCTTAATGACACTATCATTACAGg
      T  R  E  K  A  I  E  R  M  K  R  A  L  N  D  T  I  I  T  G    485
      -  -  -  G  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -    483
3532  tatgtcgagtactacttttgttaattccatattgtttatgcttcttcattgtctgtttt
3592  gtttttcttttattctgtgtaagatgtgacatttcgttacattggacatctggtggaatg
3652  ttaaatcaaatttcgttttcttggtttcatcagGGGTTCCAACGACTATCAATTACCACA
                                    V  P  T  T  I  N  Y  H  K    494
```

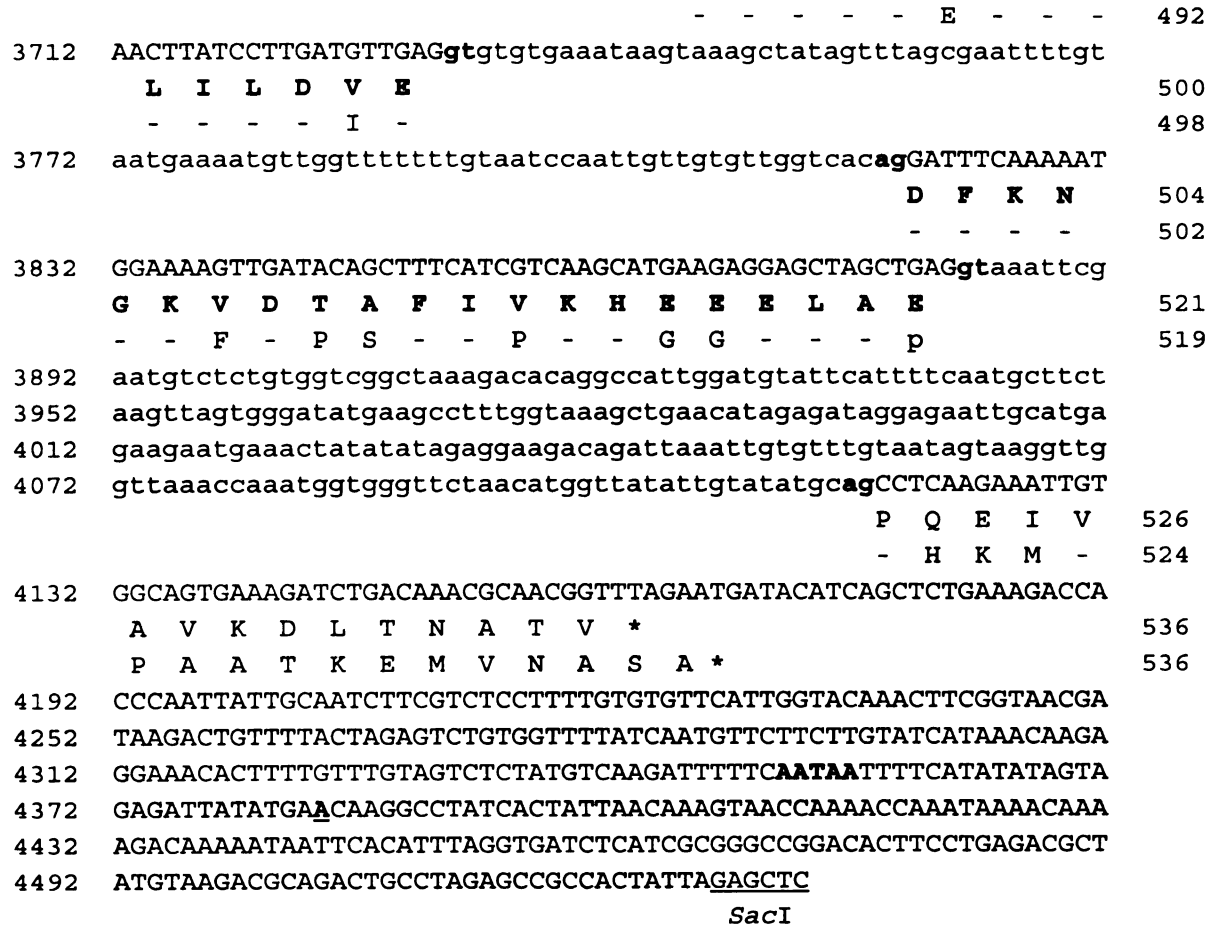

Figure 3.1. Structure and sequence of the biotin carboxylase gene from *Arabidopsis*. The transcription start site is marked with a ▼, and a putative TATA box is shown in bold and underlined. A putative polyadenylation signal is shown in bold. Underlined regions are discussed in the text. Introns are shown in lower case and GT/AG nucleotides corresponding to the intron boundaries are shown in bold. The derived amino acid sequence is presented in single-letter code. In the alignment with tobacco BC, identical amino acids are presented by` -` otherwise the single-letter code is used. Primers JO380 and JO379 were used to make promoter/GUS constructs. Primer JO381 was used for primer extension.

identity. A different repeat occurs between -972 to -670 and -86 to 181, and these regions share 67% identity.

Gene expression pattern and BC promoter expression analysis

An Arabidopsis Northern blot was probed with the 2.7 kb *Hin*dIII genomic fragment of BC and a single band was detected in all tissues tested (Figure 3.2). Expression levels of BC were quantified, and variability attributable to unequal RNA loading was estimated by probing with EIF4a (eukaryotic initiation factor). The relative expression level of biotin carboxylase based on the ratio of BC/EIF4a signal was found to be highest in siliques (0.54), five fold lower in leaves (0.10), and at intermediate levels in flowers (0.39) and roots (0.27).

The expression pattern of the BC promoter during early seedling development and in mature plants was examined in *Arabidopsis* plants transformed with the BF380 GUS construct (Figure 3.3). To locate BC promoter activity by GUS expression, seedlings were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) at various development stages. GUS staining was found in roots of young seedlings (48 hours after germinating), but not in cotyledons (Figure 3.4). After 3 days, GUS staining appeared in roots and radicles, but still not in cotyledons. At 4 days, GUS activity could be detected throughout the seedlings, but was still highest in root tips. GUS staining was observed in all tissues of mature plants but the most intense staining occurred in anthers. Within leaves, staining was strongest in the vascular tissues. Because staining rates varied in tissues of mature plants, and may in part reflect rates of substrate access to the enzyme, it is

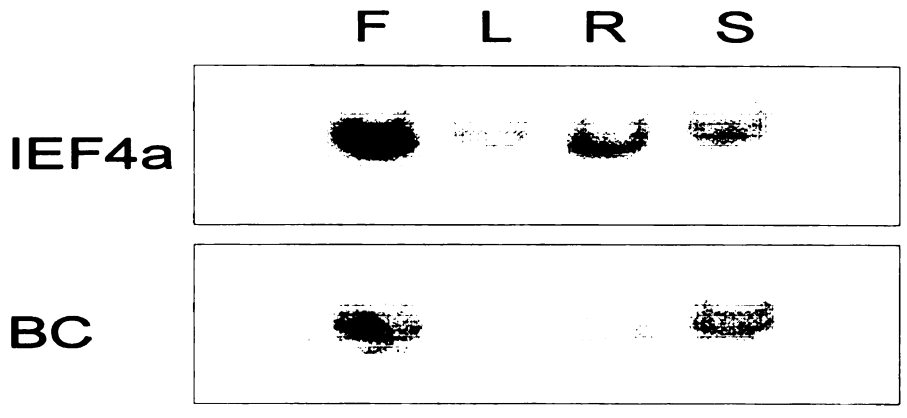

Figure 3.2. Northern analysis of BC mRNA levels in F (flower), L (leaf), R (root), and S (silique) from *Arabidopsis*. Total RNA (20 µg/sample) was separated on a formaldehyde gel and blotted to a nylon membrane. This blot was hybridized with a ^{32}P-labeled probe of the *Hin*dIII fragment from BC genomic clone. The same blot was stripped and hybridized again with ^{32}P-labeled EIF4a probe as a loading control.

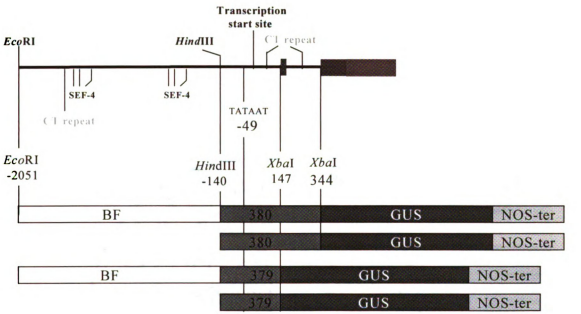

Figure 3.3. Structure of BC promoter-GUS deletion constructs used for Arabidopsis and tobacco transformation. The numbers are relative to the transcription start site; a putative TATA box at position -49 is also indicated. ATG represents the translation start codon of the GUS gene.

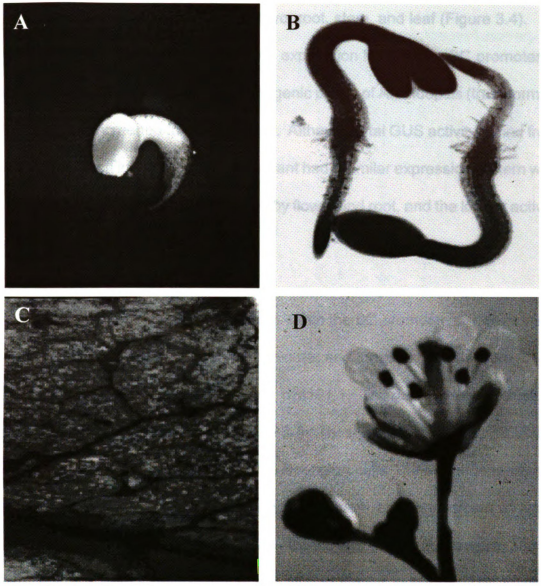

Figure 3.4. Localization of GUS expression in transgenic Arabidopsis transformed by construct BF380. (A) Two-day-old seedling stained for 3 hr. (B) Three- (left) and four-day-old seedlings stained for 2 hr. (C) Mature leaf stained for 12 hr. (D) Flower stained for 1 hr.

difficult to make an accurate comparison of staining levels in different tissues. However, as judged by the time required for staining, the order from the fastest to the slowest was anther, silique and embryo, root, stem, and leaf (Figure 3.4).

To further characterize the relative expression level of the BC promoter in different tissues, ten independent F_2 transgenic plants of Arabidopsis (transformed by BF380) were screened for GUS activity. Although total GUS activity varied from plant to plant, each independent transformant had a similar expression pattern with the highest activity in the silique, followed by flower and root, and the lowest activity was in leaf (Figure 3.5).

BC promoter deletion studies

To identify functionally significant domains within the BC promoter, deletions were made from the 3' and/or 5' ends of the promoter and these constructs were fused to GUS in the plant transformation vector, pBI101.1. The deletions and structure of each derivative are diagramed in figure 3-3. These constructs and a 35S/GUS control were used to transform tobacco suspension cells. Fifteen independently transformed lines were recovered for each construct, and then grown in liquid medium with subculturing every 7-8 days. Constructs BF380 (full length), 380 (5' deletion), 379(5' and 3' deletion) and 35S all had similar levels of GUS activity. However, construct BF379 which is deleted in the promoter 3' end but not 5' showed approximately 5 fold higher GUS activity (Figure 3.6).

The expression of ACCase has previously been found to be tightly associated with growth rate in *E. coli* (Li and Cronan, 1993). Therefore, we

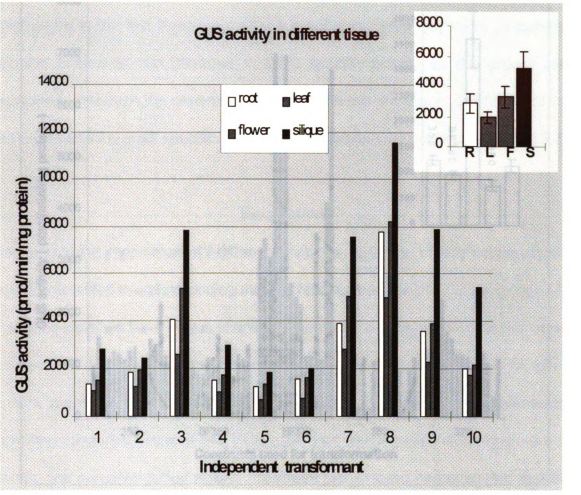

Figure 3.5. GUS activity in roots, leaves, flowers and siliques of independently transformed *Arabidopsis* plants. The construct used for transformation was BF380. The insert (upright) presents the average GUS activity (±S.E.) in different tissues from the ten independent plants.

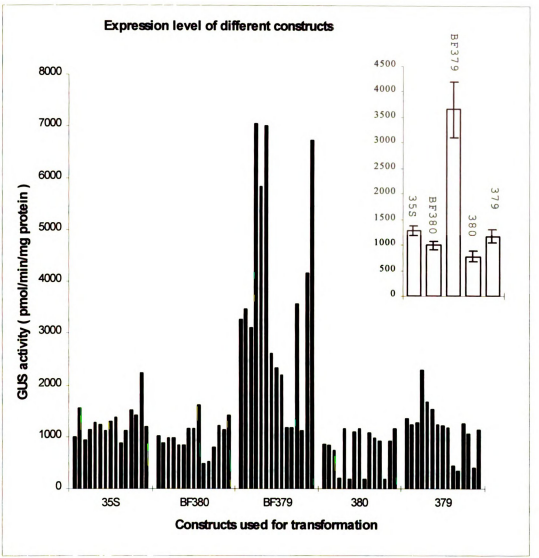

Figure 3.6. Comparison of GUS activity in transgenic tobacco suspension cells transformed with different BC promoter- and 35S GUS constructs. GUS activity of 15 independent lines from each construct was fluorometrically assayed and the specific activities calculated based on extract protein concentration. The insert (upright) presents the average (±S.E.) GUS activity of 15 cell lines transformed by different constructs.

examined this relationship in the tobacco suspension cells. The growth rate of suspension cells increased over the first four days after subculturing and then decreased (Figure 3.7). GUS specific activity of all constructs remained almost unchanged in the first three days after subculturing. After day three all cultures showed a several fold increase in GUS specific activity as the growth rate increased. Although the general patterns of increase are similar, the timing of the increases and the peak specific activities varied among the constructs (Figure 3.7).

Discussion

Because of the importance of ACCase activity for regulation of fatty acid synthesis we are interested in understanding the control of expression of ACCase genes. As a starting point we have begun characterization of the BC promoter. In this paper we describe the isolation, sequence and expression of the Arabidopsis BC gene and its promoter. We were able to identify a number of *cis*-genetic elements, including transcriptional start site, translation start codon, distribution of introns and exons, and polyadenylation signal. Northern blot analysis demonstrated that BC is expressed at higher levels in silique and flower and lower levels in root and leaf. The histochemical staining of Arabidopsis plants transformed with the BF380 construct demonstrated that anthers (pollen) stained most rapidly followed by silique, root and leaf. Quantitative GUS assays of tissue extracts provided comparable results (Figure 3-5). The higher level of GUS activity in silique and embryo is understandable as a high rate of fatty acid synthesis is required to meet

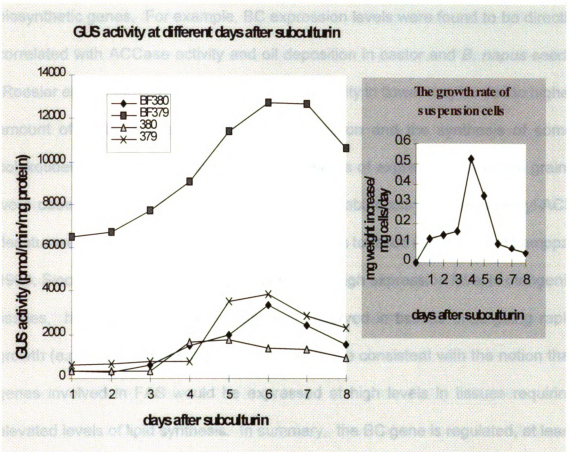

Figure 3.7. Changes of GUS activity during growth of tobacco suspension cells transformed by different constructs (A). The growth rate of suspension cells is shown in the right box in terms of mg weight increase/mg cells/day (B).

triacylglycerol synthesis in oil storing Arabidopsis seeds.

Histochemical analysis and quantitative GUS assays presented here are consistent with previous analysis of expression of ACCase and other lipid biosynthetic genes. For example, BC expression levels were found to be directly correlated with ACCase activity and oil deposition in castor and *B. napus* seeds (Roesler *et al.*,1996). The high level of GUS activity in flower may be due to higher amount of lipids required during pollen formation and the synthesis of some polyketides (Evans *et al.*, 1992). Similar patterns of expression in pollen grains were observed when the *A1* ACP gene from Arabidopsis and the stearoyl-ACP desaturase gene from *B. napus* were expressed in tobacco (Baerson and Lamppa, 1993; Slocombe *et al.*, 1994). In addition to the high expression level in oleogenic tissues, high levels of activity were also observed in tissues undergoing rapid growth (e.g. root tip, meristem). These results are consistent with the notion that genes involved in FAS would be expressed at high levels in tissues requiring elevated levels of lipid synthesis. In summary, the BC gene is regulated, at least in part, at the level of gene transcription in a pattern similar to other FAS genes and which is apparently correlated to the rate of tissue fatty acid synthesis.

To date only a few studies have focused on fatty acid biosynthesis gene promoters (Baerson *et al.*, 1994; de Boer *et al.*,1996; de Silva *et al.*,1992; Slocombe *et al.*, 1994). One consistent trend, found with Arabidopsis BC and previous studies of promoters of FAS genes, is that such promoters show the highest expression levels in rapidly growing tissues and developing seeds. Two

conserved elements (GCCCAT and ATGGGC), found in the *B. napus* ACP05, ACP09, and an Arabidopsis ACP gene were also identified in the Arabidopsis BC promoter (located at -62 to -57 and -138 to -133, respectively) (Figure 3.1). These elements have previously been observed in seed-expressed genes (Doyle *et al.*,1986). The (CT)n stretch, which we found in the Arabidopsis BC promoter, also occurs in the 5'-untranslated leader sequences of BC from tobacco (Shorrosh *et al.*,1995), three ACP genes from Arabidopsis (Lamppa and Jacks,1991; Post-Beittenmiller *et al.*, 1989), two ACP genes from *B. napus* (de Silva *et al.*, 1990), four ACP genes from other species (Ohlrogge *et al.*,1991), stearoyl-acyl carrier protein desaturase gene (Slocombe *et al.*, 1994), 1-acyl-sn-glycerol-3-phosphate acyltransferase gene (Knutzon *et al.*, 1995), and *Arabidopsis* HMG2 gene (Enjuto *et al.*, 1995). The $d(CT)_n$ tracts in eukaryotic genomes may be involved in recombination (Weinreb *et al.*, 1990), replication (Caddle *et al.*, 1990), transcription (Wells *et al.*,1988), and chromatin structure (Lu *et al.*, 1993). Proteins which specifically bind (CT)n(GA)n promoter elements have also been reported (Gilmour *et al.*, 1989). So far, almost all the characterized promoters or 5' UTR of genes involved in the pathway of fatty acid synthesis possess this (CT)n tract. The significance of these CT tracts is unknown, but its conservation in genes involved in fatty acid synthesis suggests that it may have a universal regulatory function. The importance of the SEF4 binding motif found in the BC promoter is also not clear. SEF (soybean embryo factor) is a nuclear DNA binding protein whose expression begins to appear in mid-maturation soybean embryo and increases

moderately during embryo development (Lessard *et al.*, 1991). Based on the change in SEF4 activity during seed development, in parallel with accumulation of the β-subunit mRNA of β-conglycinin, it was proposed that the binding of SEF4 to the promoter region could activate the expression of the β subunit of β-conglycinin. These motifs may also play some role for the higher expression level of BC in the seed.

To our knowledge there are no previous reports showing large segment repeats in promoter regions such as those found in the BC promoter (Figure 3.1). We do not yet know any regulatory function of these promoter repeats; however, in comparing the GUS activity of construct 379 and BF379, the repetition region located in region from -2048 to -140 may enhance the expression of the BC gene.

As a first approach to identify *cis* regulatory elements involved in the control of Arabidopsis BC gene expression, we analyzed the effect of BC promoter deletions/GUS fusion constructs in transgenic tobacco suspension cells. We found that deletions removing sequences from either the 5' and/or the 3' end of the full length BC-GUS construct BF380 resulted in altered expression. The first exon and intron were included in this construct because there are two repeat elements in the first intron. The first element (CTCTGTTT) has three repeats and the second (ATCTGTTAA) has two repeats. We suspect that the first intron may play a functional role in the regulation of BC expression and in this regard, the deletion of the first exon and first intron (construct BF379) resulted in a several-fold increase of GUS activity. There are at least two possible reasons for the increase of GUS

activity. The first is that the intron can not be recognized and properly excised in tobacco cells. This seems unlikely because the first intron possesses conserved intron boundary sequences (Figure 3.1) and in numerous previous examples introns from one dicot species can be properly processed in other species. Furthermore, intron junctions throughout the plant kingdom are highly conserved (Laliberte *et al.*,1992; Paszkowski *et al.*,1992; Quigley *et al.*,1988; Vancanneyt *et al.*,1990). The second possibility is that the intron does play a role in the regulation of BC expression and functions to depress the expression of BC. The GUS activity of construct 379 (5' deletion) compared with construct BF379 is decreased several-fold. This implies that other regulatory element(s) which could enhance the GUS expression may exist between the region corresponding to -2048 and-140. In addition, this also suggests that the region between +140 and -147 contains the basal promoter elements which can support the expression of BC gene. Construct 380 (-140 to +344) shows essentially the same GUS expression level as BF380 and 379. Taken together, it appears as though positive element(s) are located between -2048 and -147, and a negative element(s) is located between +147 and +344. Considering the relatively low GUS expression of construct BF380 (full length promoter), we conclude that the negative element is dominant over the positive element. The presence of a negative element in the first intron is similar to a recent report that deletion of the first intron from the enoyl-ACP reductase promoter of Arabidopsis resulted in increased expression in roots (de Boer *et al.*,1996). All our deletion experiments were performed in tobacco suspension cells, Thus we do not

yet know if these elements regulate BC expression in *Arabidopsis* tissues in the same manner.

We have observed that GUS activity of BF380 is comparable to the GUS activity observed in constructs under the control of the 35S promoter. This is surprising because BC is a low abundance protein in plant cells with levels estimated to be <0.05% of protein in leaf or root (Roesler *et al.*,1996). The fact that GUS activity under the control of the BC promoter is comparable to that of 35S promoter suggests that translation of the BC mRNA may be inefficient or that the protein may have a high rate of turnover *in vivo*.

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The sequence of the BC gene described in this report is registered in the EMBL database under accession number Y09061.

CHAPTER 4[2]

The Biosynthesis of Erucic Acid in Developing Embryo of *Brassica rapa L.*

Abstract

The prevailing hypothesis on the biosynthesis of erucic acid in developing seeds is that oleic acid, produced in the plastid, is activated to oleoyl-CoA for malonyl-CoA-dependent elongation to erucic acid in the cytosol. Several *in vivo* labeling experiments designed to probe and extend this hypothesis are reported here. To examine whether newly synthesized oleic acid is directly elongated to erucic acid in developing seeds of *Brassica rapa L*, embryos were labeled with [^{14}C]acetate, and the ratio of radioactivity of carbon atoms C(5)-C(22) (*de novo* fatty acid synthesis portion) to C(1)-C(4) (elongated portion) of erucic acid was monitored with time. If newly synthesized oleate immediately becomes a substrate for elongation to erucic acid, this ratio would be expected to remain constant with time. However, if erucic acid is produced from a pool of pre-existing oleic acid the ratio of ^{14}C in the four elongation carbons to ^{14}C in the methyl terminal 18 carbons would be expected to decrease with incubation time. This labeling ratio indeed decreases with time and therefore suggests the existence of an intermediate pool of oleate which contributes at least part of the oleoyl precursor for the production of erucic acid. Addition of haloxyfop, which inhibits the homodimeric acetyl-CoA

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[2] Material in this chapter was published previously [Bao X, Pollard M, and Ohlrogge J (1998) Plant Physiol. 118: 183-190].

carboxylase, severely inhibited the synthesis of [^{14}C]erucic indicating that essentially all malonyl-CoA for elongation of oleate to erucate was produced by homodimeric ACCase. Both light and haloxyfop increased the accumulation of [^{14}C]oleate and the parallel accumulation of [^{14}C]phosphatidylcholine. Taken together, these results show an additional level of complexity in the biosynthesis of erucic acid.

Introduction

Erucic acid (*cis*-13-docosenoic acid) and its homolog, *cis*-11-eicosenoic acid, are commonly found in the seed oils of the *Crucifereae*. The oils and the corresponding fatty acids are produced by high erucic acid cultivars of *Brassica and Crambe* species and are oleochemical commodities. The reactions leading to the synthesis of erucic acid are, for the most part, well understood. In the developing embryos of *Brassica napus* (Downey *et al.*, 1964), *Crambé abyssinica* (Appleby, 1974), *Simmondsia chinensis* (Ohlrogge *et al.*, 1978), *Tropaeolum majus* (Pollard *et al.*, 1980a) and *Limnanthes alba* (Pollard *et al.*, 1980b), it was demonstrated that erucic acid is synthesized by the elongation of oleic acid rather than by *de novo* synthesis. This conclusion was deduced from the distribution of label in the long-chain fatty acids after incubating seed tissue with exogenous [^{14}C]acetate. The label was preferentially incorporated into the carboxyl-terminal carbons of the long-chain fatty acids rather than the methyl terminal 18 carbons. Subsequently, the work of Ohlrogge *et al.* (1979) demonstrated that *de novo* fatty acid synthesis was almost exclusively located in the chloroplast in spinach leaves. Thus the hypothesis on the biosynthesis of erucic acid was extended to a description in which oleate, synthesized in the plastid, was exported to the cytosol, where, presumably in the endo-membrane system, it was elongated via malonyl-CoA-requiring elongases to C20 and longer-chain monounsaturated fatty acids. Several reports (e.g. Imai *et al.* ,1995; von Wettstein Knowles, 1993) have confirmed that the location of oleoyl elongation system was extra-plastidial, being associated with

oil bodies or microsomal membranes. Créach *et al.* (1993) and Fehling *et al.* (1991) showed that oleoyl-CoA is readily elongated *in vitro* and the intermediates of the elongation reaction are acyl-CoA thioesters.

It is generally accepted that the end-product of newly synthesized oleic acid exported from plastids is oleoyl-CoA. This oleoyl moiety can be elongated directly to erucic acid in the endoplasmic reticulum or oil body-associated membranes through successive additions of two carbons derived from malonyl-CoA. However, in an oil body fraction from developing rapeseed, Hlousek-Radojcic *et al.* (1995) observed *in vitro* that radioactivity from oleoyl-CoA was incorporated into eicosenoate and erucate at least 2.5-fold more slowly than from malonyl-CoA. Furthermore, radioactivity from oleoyl-CoA was rapidly diluted upon the formation of eicosenoyl-CoA and the elongation could proceed without the addition of exogenous oleoyl-CoA. Based on these *in vitro* observations, they concluded that oleoyl-CoA is not the immediate substrate for elongation. Instead they proposed that the intermediate oleoyl donor for the elongase may be either a lipid or unesterified acid. Furthermore, in unpublished experiments in our lab, when intact *Brassica* (high erucic) embryos were incubated with [^{14}C]acetate, phosphatidylcholine was always heavily labeled at early time points, with oleate constituting over 90% of [^{14}C]labeled fatty acid esterified to phosphatidylcholine. We considered that this oleoyl-phosphatidylcholine might contribute to the synthesis of erucic acid, either via a mechanism of direct acyl transfer as proposed by Hlousek-Radojcic *et al.* (1995), or via the acyl-exchange between acyl-CoA and

phosphatidylcholine, as first reported by Stymne and Stobart (1984). In this study, we examined whether oleoyl-CoA produced by plastids is directly elongated to erucic acid, or if the oleoyl-CoA enters another intermediate pool before it is elongated. In addition we have examined the influence of light and of an inhibitor of the homodimeric ACCase on erucic acid biosynthesis. Taken together, our results show an additional level of complexity in the biosynthesis of erucic acid, in that the supply of oleoyl groups for chain elongation is a combination of the release of oleate from a large intermediate lipid pool, probably phosphatidylcholine and the direct provision of newly synthesized oleate from the plastid.

Materials And Methods

Plant material and biochemicals

Developing embryos of *Brassica rapa L.* (high erucic) were obtained from plants grown in growth chamber with 16 hours illumination at 25°C. Four-week-old siliques were taken from plants and after removal of seed coats, the resulting embryos were used immediately for labeling experiments.

[1-^{14}C]acetate (1.74GBq/mmol) and [U-^{14}C]oleic acid (33.3GBq/mmol) were purchased from New England Nuclear-DuPont (Wilmington, DE). The herbicide, haloxyfop [2-((3-chloro-5-(trifluoromethyl)-2-pyridinyl)oxy)phenoxy) propanoic acid], was a gift from DowElanco (Indianapolis, IN 46268).

[1-^{14}C]Acetate incubations of rapeseed embryos

In the [1-^{14}C]acetate labeling experiments, three four-week-old embryos were

incubated at 25°C with gentle shaking either in light (300 µmols/s/m^2) or in the dark in 200µl of 0.1 mM MES-NaOH (pH 5.0) containing 5 mM sodium [1-^{14}C]acetate (1.74Gbq). Assays were terminated by removing the incubation buffer, washing the embryos twice with water, and initiating the lipid extraction. For experiments with the herbicide haloxyfop the embryos were pretreated in 200µl of 0.1 mM MES-NaOH (pH 5.0) with addition of different concentration of herbicide for 30 minutes before the addition of 2 mM [1-^{14}C]acetate substrate.

Lipid Analysis

Lipids were extracted from the embryos according to the method of Bligh and Dyer (1959). Radioactivity in lipids at each time point was quantified by liquid scintillation counting. Lipid classes were separated by TLC (20 x 20 cm K6 silica, 60Å plates, Whatman) to heights of 4 and 12 cm in chloroform:methanol:acetic acid (75:25:8, v/v), allowing the plates to air dry between developments. The TLC plates were subsequently developed to 20 cm in hexane:diethyl ether:acetic acid (60:40:1, v/v). Radioactivity of the separated lipid classes on the plates was assayed with an Instant Imager (Packard Instrument Company). Labeled triacylglycerol and phosphatidylcholine bands were eluted from the silica gel by elution with chloroform:methanol (1:2, v/v). For transmethylation of total lipids or lipid classes, the lipids were heated at 90°C for 45 minutes in 0.3 ml of toluene and 1 ml of 10% boron trichloride/methanol (Sigma). The recovered [^{14}C] fatty acid methyl esters were separated by argentation TLC (Morris *et al.*,1967). Argentation plates (15% silver nitrate) were developed sequentially at -20°C to heights of 10, 15, and 20 cm

in toluene. Separated [^{14}C] fatty acid methyl esters were located and quantified by Instant Imager. The oleate, eicosenoate and erucate bands were scraped into test tubes, and recovered by elution with 6 ml of hexane:ethyl ether (2:1, v/v). In order to characterize the distribution of label in 18:1, 20:1, and 22:1, these fatty acid methyl esters were cleaved at the position of the double bond by permanganate-periodate oxidation (Christie, 1982). The resulting nonanoic acid (C9A) and 1,ω-nonane-, undecane- or tridecane-dioic monomethyl ester fragments (C9AE, C11AE and C13AE respectively) were separated by silica TLC in hexane:ethyl ether:acetic acid (90:10:1) and quantified using the Instant Imager. The relative amount of [1-^{14}C]acetate incorporated into the *de novo* portion (C5-C22) of 22:1 is calculated simply as C9A+1.25C9A or 2.25C9A.

In order to measure the *in vivo* fatty acid accumulation rate, twenty embryos were taken from plants at different stages from 20 DAF (days after flowering) to 43 DAF. At the initiation of lipid extraction, 500 μg 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (Sigma) was added to each sample as internal standard. Lipid extraction was as described above. Fatty acid methyl esters from total lipids at different DAF were separated and quantified by GC analysis.

Results

Accumulation of fatty acids during *B. rapa L.* seed development.

As shown in Figure 4.1, developing *B. rapa* seeds accumulate fatty acids which are primarily derived from oleic acid. The modifications of oleic fall into two mutually

exclusive types: further desaturation and further chain-elongation. Elongation is the more prevalent modification, as erucic acid is the most abundant fatty acid, reaching a level of 56 mol % in seeds at 43 DAF, whereas 18:2 plus 18:3 total <20% at the same stage. Palmitate and stearate with a content of <3 % in mature seeds are the only significant fatty acids which do not derive from oleic acid. The studies described below were conducted on seeds at 28 DAF when total fatty acid and erucic acid were accumulating at maximum rates. At this stage, the rate of total 18:1 production, including its elongated and desaturated derivatives, was 16 nmol $hr^{-1}embryo^{-1}$.

Light alters relative proportions of oleic and erucic acid synthesized

To monitor how light influences the accumulation of oleate and erucate, and triacylglycerol versus phosphatidylcholine, incubations with [^{14}C]acetate were carried out either in light or dark. As shown in Figure 42, incubation of embryos in the light increased radioactivity in oleate approximately two-fold, whereas the radioactivity in erucate was only fractionally higher in light incubations. The comparatively small impact of light on radioactivity of erucate, which is highly labeled at the carboxyl end, suggests that the homodimeric acetyl-CoA carboxylase and fatty acid elongation are not strongly influenced by light, whereas the 50% reduction of radioactivity in oleate in dark versus light implies that a major site of light regulation is located in the plastids which are responsible for the *de novo* fatty acid synthesis. An alternative interpretation of these experiments is that reduction in label of oleate in the dark reflects changes in the endogenous pools of acetate.

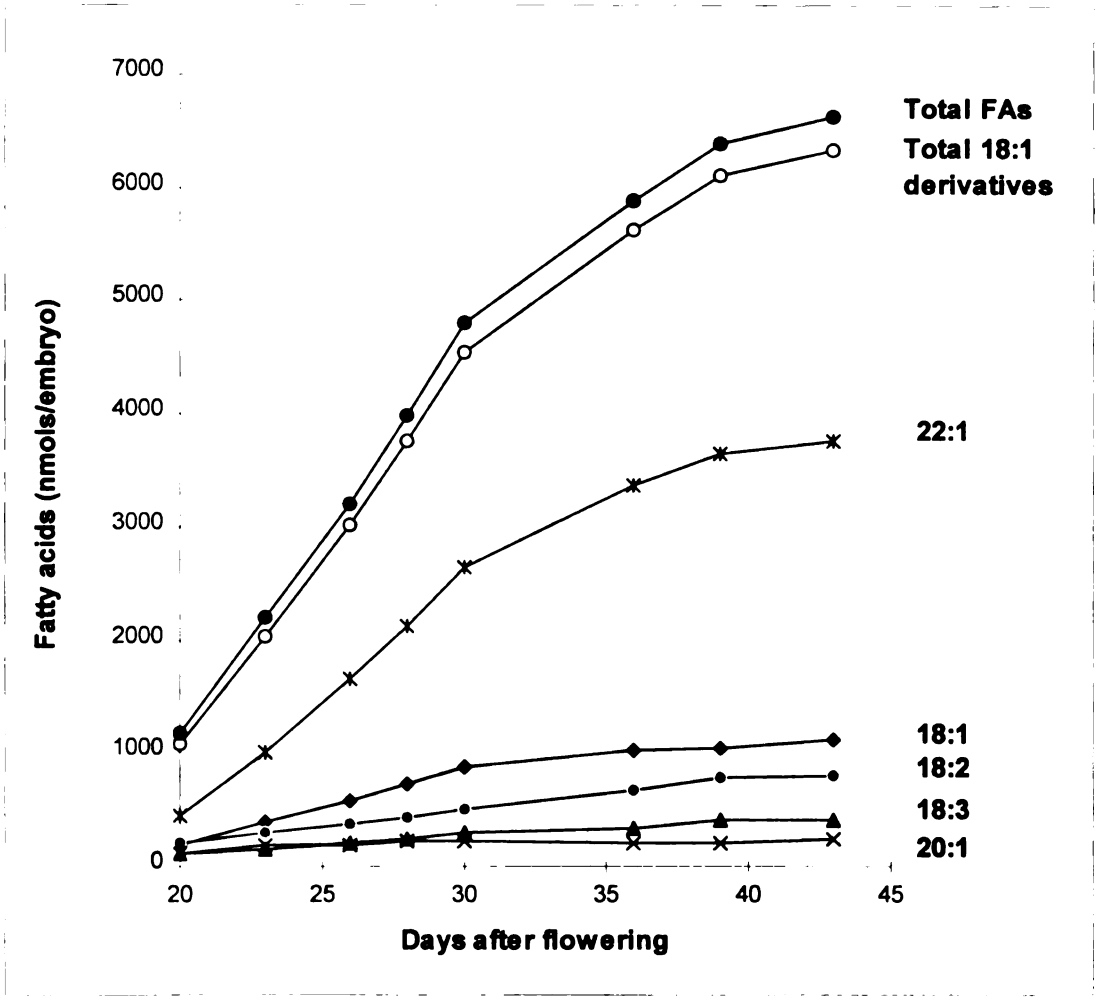

Figure 4.1. Accumulation of fatty acids during embryo development of *B. rapa*. Lipids were extracted from 20 pooled embryos at times indicated and fatty acid methyl-esters were separated and quantified by GC. The total 18:1 derivatives were obtained by adding 18:1, 18:2, 18:3, 20:1, and 22:1 together. The accumulation rate rate of 18:1 derivatives (16 nmol/hr/embryo) was calculated for the embryos at 28 DAF when labeling experiments were conducted.

This explanation was ruled out because very similar light dependence was also observed when [^{14}C]sucrose or [^{3}H]-water were used as the precursors for fatty acid synthesis (not shown). These observations suggest that an endogenous pool of oleate might contribute to the synthesis of *cis*-11-eicosenoate and erucate. If the newly synthesized oleate was the predominant source of oleoyl moeities for chain elongation and the synthesis of oleate was reduced by half in dark, we would expect that the labeling of erucate would also be reduced similarly. In fact, very little reduction of erucate labeling occurs in the dark.

Analysis of the distribution of label from [1-^{14}C]acetate in long-chain fatty acids with time.

Erucic acid is synthesized from oleate by addition of two two-carbon units from two molecules of malonyl-CoA. When exogenous [^{14}C]acetate is used as substrate for the biosynthesis of erucate the two-carbon units from the chain elongation of oleate have a higher specific activity than the two-carbon units of the methyl terminal 18 carbons, which are derived from *de novo* fatty acid synthesis of oleate. The same applies to *cis*-11-eicosenate, except that only one elongation cycle occurs from oleate. This differential labeling of C20 and longer fatty acids from exogenous acetate was previously documented for four different oilseed species: *Brassica napus* (Downey *et al.*, 1964), *Simmondsia chinensis* (Ohlrogge *et al.*, 1978) *Tropaeolum majus* (Pollard *et al.*, 1980) and *Limnanthes alba* (Pollard *et al.*, 1980) and has been interpreted as reflecting different pools of acetate supplying the *de novo* fatty acid synthesis and the chain elongation reaction. What

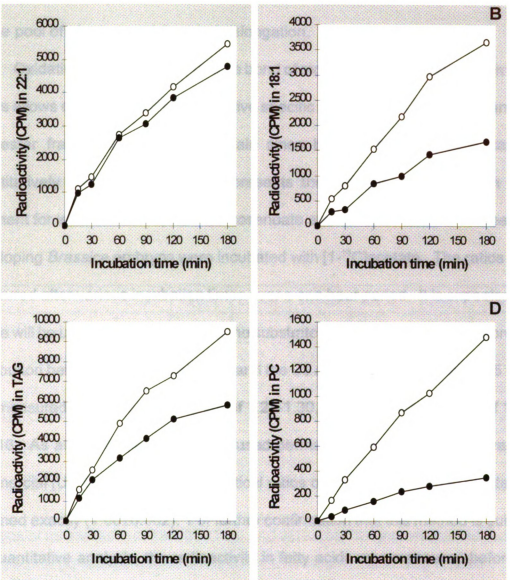

Figure 4.2. Effects of light on the synthesis of 22:1 (A) 18:1 (B), TAG (C), and PC (D). ○, light; ●, dark. Radioactivity is expressed per embryo.

has not been examined, however, is the time dependency of this differential labeling. As we will demonstrate below, this time dependency provides information on the pool of oleate supplying chain elongation.

Oxidative cleavage at the double bond of monounsaturated fatty acid methyl esters allows determination of the relative specific activity of ^{14}C in the acid and the acid-ester fragments of the acyl chain since both of the fragments can be quantitatively recovered. Table 4.1 presents the ratio of radioactivity in each fragment for isolated oleate, *cis*-11-eicosenoate and erucate, when four-week-old developing *Brassica* embryos were incubated with [1-^{14}C]acetate. The ratios were measured after various incubation times over a one hour period. It is expected that oleate will be uniformly labeled, and if the substrate is [1-^{14}C]acetate, the theoretical distribution between the C9 acid-ester and the C9 acid fragments will be 1.25 (5/4). The measured ratios fell in the range of 1.24-1.30, with an average value of 1.259 ±0.016. As an additional control, we used permanganate-periodate cleavage of commercial [U-^{14}C]oleate. The theoretical ratios of 1.0 (C9AE/C9A) in oleate was obtained exactly (1.00±0.002). For further confirmation that this method is suitable for quantitative analysis, the radioactivity in fatty acids was measured before the oxidation, and was measured again after extracting cleavage products from the reaction solution. Loss of radioactivity was negligible throughout the procedure. All these controls indicate the high degree of accuracy and reproducibility of the technique which is essential, since the variations in the ratios with time are the key experimental data.

Table 4-1. The ratio of radiolabel in oxidative cleavage fragments of [^{14}C]labeled fatty acids from incubation of *B. rapa* embryos with [1-^{14}C]acetate.

	5 min	10 min	20 min	30 min	40 min	50 min	60 min
Light:C13/C9 of 22:1	9.81[a]	9.68	8.65	8.29	7.92	7.42	6.42
C11/C9 of 20:1	5.58	4.77	4.52	4.39	4.38	4.14	3.63
C9/C9 of 18:1	1.28	1.25	1.27	1.26	1.24	1.25	1.25
Dark: C13/C9 of 22:1	14.42	13.23	10.87	9.73	9.51	9.38	8.93
C11/C9 of 20:1	8.01	7.21	5.48	5.44	5.34	5.28	5.02
C9/C9 of 18:1	1.30	1.27	1.26	1.25	1.25	1.26	1.24

[a]. Ratios of radioactivity in the 13C, 11C, and 9C carbon fragments was determined after permanganate-periodate oxidation at the double bond and isolation of the cleavage products.

Table 4.1 presents the direct measurements of the ratio of [^{14}C]label in acid and ester fragments. The results indicate that the [^{14}C]ratio in the oxidative cleavage fragments of oleate remained constant with time and was close to the expected value. In contrast, the ratio of ^{14}C in the C13AE/C9A fragments of erucate and the C11AE/C9A fragments of eicosenoate both decreased with time in both light and dark incubated embryos. The experiment was repeated ten times, and although the absolute values of the acid to acid-ester ratios at each time point showed some degree of variation between experiments, the trend was always the same. Also noted in Table I and consistent with other experiments described below was the observation that the acid-ester to acid ratios for erucate and eicosenoate were always higher in dark incubated embryos than in light at any given time point. Thus, in the dark the relative specific activity of C2 units used for elongation when compared to C2 units derived from *de novo* fatty acid synthesis was increased by a factor of 1.5-1.6.

It is also instructive to consider these labeling data in terms of the proportion of total plastid-produced [^{14}C]oleate units which appear in erucic acid. As calculated from the fatty acid compositions shown in figure 4.1, over 55 % of 18:1 fatty acids synthesized by 28 DAF developing seeds are elongated to erucic acid. Determination of the ^{14}C in the oxidative cleavage products of 22:1 allows calculation of the ^{14}C content of the *de novo* synthesized 18 carbons. Comparison of this value to the total 18:1 radioactivity accumulated in the incubation (18:1 plus 18 carbon portion of 20:1 and 22:1) gave the values plotted in Figure 4.3A. After

5 min incubation in the light, only 21% of the total 18:1 produced in the incubation appears in 22:1 and this value increases to 35% by 60 min. Thus, there is a substantial lag in the appearance of ^{14}C in the 18:1 portion of 22:1. In dark incubations, a higher proportion of [^{14}C]18:1 initially appears in erucic, but the increase with time is similar. The labeling of the 20:1 *de novo* and elongation carbons showed parallel patterns in light and dark (data not shown).

Two general explanations can be proposed for the change in labeling within the very long chain fatty acids over time. The first considers that there are different sized acetate-accessible pools supplying malonyl-CoA for *de novo* fatty acid synthesis and for chain elongation. The pool supplying chain elongation would be relatively small, rapidly reaching a steady state contribution from exogenous acetate. The pool supplying *de novo* fatty acid biosynthesis would be large and equilibrate more slowly. A variant of this hypothesis is that the pools for elongation and *de novo* fatty acid synthesis that utilize exogenous acetate directly are both small, but that acetate can also be used to sustain *de novo* fatty acid synthesis via an indirect metabolic pathway that slowly reaches steady state labeling. In all cases, oleate synthesized at early time points would be of lower specific radioactivity compared with later time points, and thus the acid-ester to acid ratio would decrease with time. A corollary from this hypothesis is that the synthesis of total [^{14}C]oleate would show a lag at early time points with respect to elongation, and with a time scale similar to the change in differential labeling within the very long-chain fatty acids. Figure 4.3B shows the time-dependent accumulation of label

in total oleate derivatives (18:1 plus 18:1 portion of 20:1 and 22:1. 18:2 and 18:3 contributed less than 2% of the total label in these short term incubations and were not included). The accumulation of [^{14}C]oleate is essentially linear, with no lag phase. Furthermore, the ratio of label in *total* oleate to the label in the elongation portion of erucate plus *cis*-11-eicosenoate remains approximately constant (data not shown), indicating that the first general mechanism, acetate-accessible pool sizes, is not responsible for the changes in [^{14}C]ratios of *de novo* to elongation carbons (Table 4.1)

The second general explanation for the changing ratios of label in the *de novo* and elongation carbons considers that another oleate source besides the newly synthesized [^{14}C]oleate was also used as substrate to synthesize the very long-chain fatty acids. As shown in Figure 4.3A, the radioactivity accumulated in the *de novo* (18:1) portion of erucate lagged significantly to the radioactivity accumulation of total oleate. This result supports the concept that there is an endogenous source of oleate for the synthesis of erucate besides the newly synthesized [^{14}C]oleate. If only newly synthesized oleate was directly used for elongation, the [^{14}C]ratios of C13AE/C9A and C11AE/C9A (Table 4.1), and the percentage of total labeled oleate in erucate would remain constant with time. The contribution of newly synthesized oleate is guaged by the extrapolation of the curves of Figure 4.3A to zero time. At zero time the amount of the total labeled oleate produced which contributes to erucic acid biosynthesis is only 20% in the light (32% in the dark), whereas the theoretical maximum is 55%. Thus 36% (light)

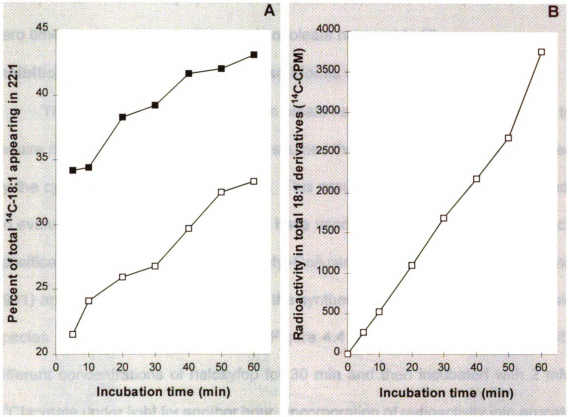

Figure 4.3. Labeling of fatty acids by [^{14}C]acetate in developing *B. rapa* embryos. [A]. The percentage of [^{14}C]acetate incorporated into total 18:1 (18:1 plus derived 18:1 portion of 20:1 and 22:1) which appears in erucate is plotted vs. time. Light (◊) or dark (♦) indicates that the incubation was conducted either under the light or in dark. [B] Time course of ^{14}C accumulation into total 18:1 derivatives (18:1 plus 18:1 derived portion of 20:1 and 22:1) under the light. All the fatty acids were derived from total lipid extracts, and the numbers in the figure represent radioactivity calculated per embryo.

and 58% (dark) of the oleyl flux through the chain elongation system to erucate is directly utilized from newly synthesized (^{14}C-labeled) oleate in the plastid, since at zero time any large intermediate pools of oleate have yet to fill.

Inhibition of the homodimeric ACCase blocks erucic acid production

The synthesis of erucic acid from oleate has been demonstrated *in vitro* to require malonyl-CoA, and it has been assumed that this malonyl-CoA is produced by the cytosolic homodimeric ACCase. This assumption has never been tested. To evaluate this hypothesis *in vivo* we have used the herbicide haloxyfop, which specifically inhibits the homodimeric acetyl-CoA carboxylase (Burton *et al.*, 1987 and 1991) and examined how it influences the synthesis of erucic acid and other lipid species. In the experiment shown in Figure 4.4 embryos were pre-treated with different concentrations of haloxyfop for 30 min and then incubated with 2 mM [^{14}C]acetate under light for another hour. Incorporation of radioactivity into erucate decreased with increased concentrations of haloxyfop. In contrast, radioactivity in oleate increased with haloxyfop concentrations lower than 100 μM. At a concentration of 50 μM haloxyfop, synthesis of erucate was inhibited by 70%, but ^{14}C accumulation in oleate increased almost two-fold. These results demonstrated that the elongation of oleate to erucate is dependent on homodimeric ACCase to supply malonyl-CoA, and that haloxyfop can inhibit the elongation without inhibition of *de novo* synthesis of oleate. Also plotted in Figure 4.4 are the accumulations of ^{14}C in PC and TAG. In response to the addition of haloxyfop, radioactivities in erucate and triacylglycerols were inhibited in parallel whereas the accumulation of

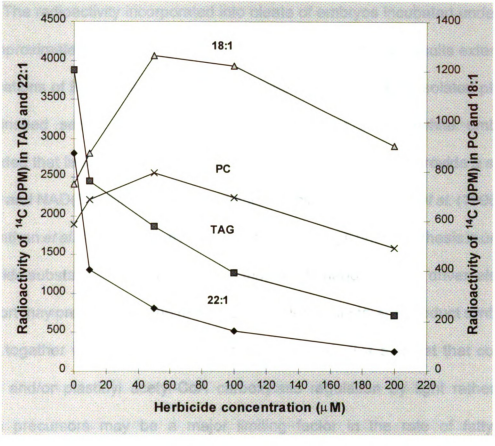

Figure 4.4. Effect of increasing concentrations of haloxyfop on fatty acid and lipid synthesis. *B. rapa* embryos were pre-treated with indicated concentration of herbicide for 30 min, then incubated for another hour under light with addition of [^{14}C]acetate. Radioactivity of lipid and fatty acid are quantified on a basis of DPM per embryo.

radioactivities in PC and oleate showed parallel patterns of increase.

Discussion

The radioactivity incorporated into oleate of embryos incubated under light was approximately two fold higher than that in the dark. These results extend the observations of Browse and Slack (1985), who, in a study using isolated plastids from linseed seeds (green embryos) and safflower seeds (white embryos) concluded that linseed plastids are photosynthetically active and provide a source of ATP and NAD(P)H for fatty acid synthesis. Similarly, Eastmond *et al.* (1996) and Asokanthan *et al.* (1997) recently concluded that although photosynthesis is unlikely to provide substantial net carbon for *B. napus* seed anabolism, light driven electron transport may provide ATP and reducing equivalents for storage product synthesis. Taken together with the data in Figure 4.2, these studies suggest that cofactor supply and/or plastidyl acetyl-CoA carboxylase regulation by light rather than carbon precursors may be a major limiting factor in the rate of fatty acid accumulation in *Brassica* embryos. This hypothesis is consistent with the observation that acetyl-ACP and malonyl-ACP pools are relatively high at all stages of active fatty acid synthesis in developing spinach seeds or castor endosperm (Post-Beittenmiller *et al.*, 1991 and 1992a).

The strong inhibition of acetate incorporation into erucate by haloxyfop, a known non-competitive inhibitor of the cytosolic homodimeric acetyl-CoA carboxylase, confirms the hypothesis that acetate that is utilized for chain

elongation is in the cytosolic compartment and distinct from acetate utilized for *de novo* fatty acid synthesis. In all dicots so far examined the cytosolic acetyl-CoA carboxylase is the homodimeric form, whereas the plastid acetyl-CoA carboxylase is the heteromeric form (Konishi *et al.*, 1994). However, a homodimeric form of ACCase has also recently been reported in *B. napus* plastids (Schulte *et al.*, 1997; Roesler *et al.*, 1997). Although the function of this ACCase is at this time unclear, it appears to be less abundant than the heteromeric form. Furthermore, if this plastid homomeric ACCase is as sensitive to haloxyfop as the cytosolic form, our observation that 50 μM herbicide caused no inhibition of *de novo* FAS while strongly inhibiting elongation suggests that the homomeric ACCase in the plastid has a quantitatively minor role in *de novo* FAS.

As presented in Table 4.1 and Figure 4.3 we observed differential labeling with time of the elongation and *de novo* carbons within the very long chain fatty acids. We considered two explanations which could cause these changes. The explanation whereby different sized acetate-accessible pools supplied malonyl-CoA for *de novo* fatty acid synthesis and for chain elongation was ruled out from kinetic and pool size considerations. This is not surprising if acetate is being utilized directly for acetyl-CoA synthesis in both plastids and the cytosol. Data are not available for oilseeds, but in leaf tissue the chloroplast pool of acetyl-CoA, the direct substrate for the synthesis of malonyl-CoA, ranges from 10 to 20 μM, and it can be equilibrated with exogenously supplied acetate within several seconds (Post-Beittenmiller *et al.*, 1992b; Roughan, 1997). The chloroplast pool of acetyl-CoA

dominates the total leaf acetyl-CoA pool, and, indeed total leaf CoA, so the cytosolic pool of acetyl-CoA is expected to also be small. Extrapolating to seed tissue, even if the cytosolic pool of acetyl-CoA is depleted at a rate of about one tenth of that of the plastid pool, as calculated from the mass composition of the oil, it is expected that the direct utilization of exogenous acetate by either pool will reach steady state very quickly. As endogenous free acetate pools have not been measured in developing seeds it is not possible to estimate the endogenous contribution of free acetate to fatty acid synthesis in seeds. However, utilization of exogenous acetate clearly reaches a steady state rate very quickly (Figure 4.3B), within one minute. Acyl-CoA and acyl-ACP pools might also contribute to a lag in labeling. In *Brassica* embryos, and in developing seeds in general, there is limited information on acyl-ACP levels and a dearth of information on acyl-CoA levels. We can make some extrapolations from the situation in leaves and from the limited seed data. In chloroplasts isolated from leaf tissues (Soll and Roughan, 1982; Roughan and Nishida, 1990), it was estimated that acyl-ACP half lives were of the order of 10 seconds. In seeds, ACP levels of the order of 1 µg/gfw were noted (Hannapel and Ohlrogge, 1988), while in spinach seeds and leaves up to 60% of the ACP is in the free form. Similarly, acyl-CoA pools in *Cuphea* (Singh *et al.*, 1986) and developing *B. napus* (unpublished observations) indicate levels of <20 µM. The *B. rapa* seeds in the present study accumulated about 16 nmoles fatty acid per hour per seed. Using these numbers, the acyl-CoA and acyl-ACP pool turnover time is calculated to be less than one minute. Clearly, accumulation of oleate in the acyl-thioester

pools cannot explain any lag in the labeling kinetics of erucate. This indicates that the second general explanation, that another oleate source besides the newly synthesized [^{14}C]oleate is also used as substrate to synthesize the very long-chain fatty acids, is the correct one.

Three distinct scenarios can be envisaged for the supply of oleate for chain elongation to *cis*-11-eicosenoate and erucate in developing oilseeds. In the first, oleoyl moieties are exported from the plastid, activated to oleoyl-CoA, and can immediately become substrates for elongation. In this scenario the newly-synthesized oleoyl groups can be considered channeled directly to the cytosolic elongation system. In the second model, the newly exported oleoyl-CoA is rapidly equilibrated with the bulk cytosolic pool of oleoyl-CoA. The acyl exchange mechanism first reported by Stymne and Stobart (1984) can be envisaged to dilute the [^{14}C]oleoyl-CoA pool with oleoyl groups from the *sn*-2 position of phosphatidylcholine, while [^{14}C]oleate will enter the *sn*-2 position of phosphatidylcholine and be diluted. The bulk pool of oleoyl-CoA is then available for chain elongation. The third hypothesis is based on the observations of Hlousek-Radojcic *et al.* (1995) and requires that newly synthesized [^{14}C]oleate acylate an acceptor lipid, as yet unidentified, and then be transferred directly or indirectly from this lipid to the elongase. An analysis of the kinetics of differential labeling within the acyl chain of very long-chain fatty acids suggests that an endogenous oleate pool contributes to the biosynthesis of erucate, though the labeling itself cannot be used to distinguish between models two and three. Our results do not rule out

model one or other combinations of these models as a contributing pathway until the details of the endogenous pool can be demonstrated and quantified. However, extrapolating figure 4-3A to zero time indicates that only 20 % of total 18:1 synthesis in light (or 32% in dark) is directly elongated to 22:1. Comparing this to the *in vivo* conversion of >55% of 18:1 to 22:1 (Figure 4-1) suggests that at least half of the oleate enters an intermediate pool before elongation to erucate.

The question follows as to what is the endogenous pool which can accept newly synthesized oleate from the plastid and also provide oleate as substrate for elongation. A logical approach to obtain information on such intermediate pools is to perform pulse chase experiments. However, with intact *B.rapa* embryos we found it was not possible to remove the [^{14}C]acetate sequestered inside the embryos, which continued to sustain fatty acid synthesis and hence confound interpretation. In the absence of pulse-chase data, we decided to employ an indirect approach in which we inhibited the elongation from 18:1 to 22:1 with haloxyfop without reducing the *de novo* synthesis of oleate, and then monitored where the extra 18:1 accumulated. As shown in Figure 4.4, there is an increasing amount of oleate accumulated in phosphatidylcholine when erucate synthesis is inhibited, indirectly supporting the concept that the oleate esterified to phosphatidylcholine could be a source of oleoyl moeities for the synthesis of erucate. Consistent with this observation, Hlousek-Radojcic *et al.* (1995) found that when [^{14}C]oleoyl-CoA was incubated with *B. napus* oil bodies, over 50% of radioactivity was found in phosphatidylcholine.

In a study of petroselinic acid biosynthesis in developing coriander and carrot endosperm, Cahoon and Ohlrogge (1994) concluded that phosphatidylcholine was an intermediate in the movement of petroselinic acid from its site of biosynthesis in the plastid into triacylglycerol. Similar observations have recently been made for the accumulation of $16:1^{\Delta 6}$ in developing seeds of *Thunbergia* (Shultz, Cahoon and Ohlrogge, unpublished). Because neither of these unusual fatty acids are synthesized or further modified on PC, a rationale for their movement through a PC pool before incorporation into TAG is not immediately obvious. The present study on erucic acid biosynthesis adds another example where PC may be an "intermediate" in the flux of fatty acids into TAG, even though no metabolism of the fatty acid may be directly associated with the PC. The observation of a large flux of fatty acids through PC without modification in three diverse oilseed species may simply indicate that acyl exchange between the acyl-CoA pool and PC is very rapid. The low accumulation of unusual fatty acids in PC may further reflect specific mechanisms for their removal (e.g. phospholipases). However, it is interesting to speculate that PC may play some more general role in TAG assembly, perhaps as a carrier of acyl chains toward a subcellular site of TAG assembly. This role might be analogous to the major flux of acyl chains through PC in leaves followed by their movement from the ER to the chloroplast.

Because of its commercial value, several attempts have been made to increase erucic acid content in transgenic plants. However, the factors which limit erucic acid content in *Cruciferae* species are still largely unknown. Expression of

an *sn*-2 acyltransferase from *Limnanthes* in transgenic *B. napus* led to accumulation of erucic in the *sn*-2 position, but no increase in total erucate content of the oil (Lassner *et al.*, 1995). Similarly, over-expression of elongases has resulted in increased chain-length of VLCFAs but not an increase in mole % VLCFA (Lassner *et al.*, 1996). The recent report that a mutated yeast SLC1 gene expressed in *Arabidopsis* or *B. napus* gave increased erucic levels (Zou *et al.*, 1997) is at this time difficult to interpret in light of the results with the *Limnanthes* enzyme. Reduction of 18:1 desaturation by mutation of the oleoyl-desaturase might be expected to increase 18:1 availability for elongation. However, in a mutant of *Arabidopsis* which is deficient in desaturation of 18:1 (Lemieux *et al.*, 1990) 18:2 and 18:3 content of seeds decreased from 53% to 8.7%, 18:1 increased from 15.4% to 53.5%, but 20:1 and 22:1 just slightly increased from 20.2% to 26.7%. Likewise, elimination of the elongation of 18:1 might be expected to increase 18:1 availability for desaturation. However, neither low erucic lines of *Brassica* (Daun, 1983) nor the *fae1* mutant of *Arabidopsis*; (Kunst *et al.*, 1992) exhibit corresponding increases in 18:1 desaturation. Although several interpretations are possible, one hypothesis consistent with these results is that the pathways for 18:1 elongation and desaturation may draw on different pools of 18:1. In summary, our results together with those of Hlousek-Radojcic *et al.* (1995) and those cited above suggest that the pathway for erucic acid biosynthesis may be more complex than originally envisaged. Furthermore, the flux of oleate through distinct intermediate lipid pools prior to elongation or desaturation may be one factor which limits the availability of

oleate for elongation.

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CHAPTER 5[3]

Supply of Fatty Acid Is One Limiting Factor in the Accumulation of Triacylgycerol in Developing Embryos

Abstract

The metabolic factors which determine oil yield in seeds are still not well understood. To begin to examine limits on triacylglycerol (TAG) production, developing *Cuphea lanceolata, Ulmus carpinifolia* and *Ulmus parvifolia* embryos were incubated with factors whose availability might limit oil accumulation. The addition of glycerol or sucrose did not significantly influence the rate of TAG synthesis. However, the rate of ^{14}C-TAG synthesis, upon addition of 2.1 mM ^{14}C-decanoic acid (10:0), was approximately 4 times higher than the *in vivo* rate of TAG accumulation in *Cuphea*, and two times higher than the respective *in vivo* rate in *Ulmus*. In *Cuphea* embryos, the highest rate of ^{14}C-TAG synthesis (14.3 nmols/hr/embryo) was achieved with addition of 3.6 mM of 10:0. ^{14}C-10:0 was incorporated equally well in all three acyl positions of TAG. The results suggest that both *Cuphea* and *Ulmus* embryos have sufficient acyltransferase activities and glycerol-3-phosphate levels to support rates of TAG synthesis in excess of those found *in vivo*. Consequently, the amount of TAG synthesized in these oilseeds may be in part determined by the amount of fatty acid produced in plastids.

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[3]Material in this chapter was published previously [Bao X and Ohlrogge J (1999) Plant Physiol. 120: 1057-1062].

Introduction

In plants, the biosynthesis of storage triacylglycerol (TAG) occurs at high levels primarily in the seeds, but there is a wide range in the levels of TAG which accumulate in different plant species. For example, seeds of species of *Zea, Hordeum* or *Pisum* usually contain less than 5-10 % TAG by dry weight whereas many other species such as castor accumulate over 50% TAG in seeds. The regulatory or metabolic factors which influence this very wide range of oil accumulation in seeds are currently unknown (Ohlrogge and Jaworski, 1997). Considering that over 30 reactions are required to convert acetyl-CoA to TAG, there could be many steps or genes which control the yield of end product TAG. In order to begin to dissect possible limiting factor(s) in the pathway of TAG biosynthesis, it is useful to conceptually divide the pathway into two parts: 1) the production of acyl chains which occurs in plastids, and 2) the utilization of acyl chains for glycerolipid synthesis in the ER and oilbody. In the second part, the only unique enzyme for TAG synthesis is diacylglycerol acyltransferase (DAGAT), which is responsible for the acylation of 1,2-diacylglycerol at the *sn*-3 position (Roughan *et al.*, 1982; and Stymne *et al.*, 1987). Since diacylglycerol acyltransferase locates at the branch point that channels diacylglycerol to TAG synthesis, some reports have suggested that DAGAT may be one rate limiting enzyme for the accumulation of TAG (Griffiths *et al.*, 1988 and 1991; Ichihara *et al.*, 1988; Perry *et al.*, 1993a and 1993b). In this study we asked whether the supply of fatty acid can influence the amount of TAG produced in oilseeds. If the supply of fatty acid is a limiting factor

for TAG biosynthesis, then providing exogenous fatty acid to the developing embryos should increase the rate of TAG production. Unfortunately, long-chain fatty acids (LCFA) have very low solubility in aqueous solution, and so addition of LCFA at concentrations sufficient to increase rates of lipid synthesis is very difficult. However, embryos of *Cuphea lanceolata, Ulmus carpinifolia*, and *Ulmus parvifolia* contain high levels of decanoic acid in their TAG (80%, 63%, and 71%, respectively). Decanoic acid is easily dissolved in water at mM concentrations, and therefore, these species provided a convenient model system to test the influence of fatty acid supply on TAG accumulation. Such in vitro model experiments may provide a useful guide toward the selection of targets for future metabolic engineering in transgenic plants.

Materials And Methods

Plant materials and Chemicals

Cuphea lanceolata plants were grown in Beal garden on the Michigan State University campus. *Cuphea* plants typically begin to flower in mid-July and our experiments were performed in the month of August. Flowers were hand-pollinated and seeds were harvested at various stages of development. After removal of the seed coat, the resulting embryos were used immediately for labeling experiments or stored at -20°C for later lipid analysis. Embryos were also collected from two species of elm trees (*Ulmus carpinifolia* and *Ulmus parvifolia*) growing on the Michigan State University campus. Elm trees begin flowering in early May and we

collected embryos when they were big enough to dissect, until embryo maturity. The embryos were removed from seed coat and used for labeling experiments immediately or stored at -20°C for later lipid analysis.

[1-^{14}C]Octanoic acid (55μCi/mmol), [1-^{14}C]decanoic acid (55μCi/mmol), and [1-^{14}C]oleic acid (55μCi/mmol) were purchased from American Radiolabeled Chemicals, Inc.(11624 Bowling Green Drive, St. Louis, MO 63146). Tritridecanoin (C13:0), L-dipentadecanoyl (C15:0) α-phosphatidylcholine, and lipase (from *Rhizopus arrhizus*) were obtained from Sigma.

Lipid analysis

Lipids were extracted from 20 embryos at each developmental stage according to the method of Bligh and Dyer (1959). Prior to extraction, tritridecanoin (C13:0) and L-dipentadecanoyl (C15:0) α-phosphatidylcholine were added to each sample as internal standards for GC analysis. Triacylglycerol (TAG) was separated from polar lipids by TLC (20×20 cm K6 silica, 60Å plates, Whatman) in hexane:diethyl ether:acetic acid (70:30:1, v/v). TAG bands were eluted from the silica gel with chloroform:methanol (1:2, v/v). Fatty acid methyl esters from TAG were prepared by heating lipids at 90°C for 45 min in 0.3 ml of toluene and 1 ml of 10% (v/v) boron trichloride/methanol (Sigma). The resulting fatty acid methyl esters were separated and quantified by GC analysis.

Feeding developing embryos with exogenous fatty acid

Ten pairs of *Cuphea* cotyledons (10 days after flowing) were cut in half and incubated at 28°C with gentle shaking in 200 μl of 0.1 M phosphate (pH7.2)

containing 2.1 mM [1-^{14}C]decanoic acid with the presence or the absence of 0.125 mM of glycerol. The incubation buffer was changed once after one hour incubation. Assays were terminated by removing the incubation buffer, washing the embryos twice with water, and initiating lipid extraction. Triacylglycerol was separated by TLC using a solvent system composed of hexane:diethyl ether:acetic acid (70:30:1, v/v). Labeled TAG was quantified with both Instant Imager (Packard Instrument Company) and liquid scintillation counting. In some experiments, other factors which may influence TAG synthesis, such as, exogenous fatty acid concentration (2.1 to 80.1 mM), glycerol (with/without 0.125mM), sucrose (0 to 200 mM), and pH (6 to 8), were tested. Only one factor was changed in each different treatment. The position of exogenous fatty acid incorporated in TAG was determined using TAG lipase from *Rhizopus arrhizus* which cleaves fatty acid from *sn-1* and *sn-3* positions of TAG, and then the radioactivity remaining in the *sn-2*-monoacylglycerol was compared to free fatty acids released from the *sn-1* and *sn-3* of TAG by the action of TAG lipase. Purified TAG was dissolved in 0.5 ml diethyl ether in 13 ml screw cap glass tube. 1 ml of 0.1 M Tris-HCl (pH7.8) buffer containing 5 mM $CaCl_2$ was added to the tube, then 43,000 units lipase was added to the bottom of the tube, bubbled in N_2, and shaken for ten min at room temperature. Monoacylglycerol and free fatty acid products were extracted and resolved by TLC in hexane:diethyl ether:acetic acid (35:70:1.5, v/v). The radioactivity in the monoacylglycerol and free fatty acid bands on the TLC plate was quantified using an Instant Imager.

In similar experiments, ten pairs of cotyledons from *U. carpinifolia* and *U.*

parvifolia embryos at the mid-stage of development were also used for feeding experiments as described above, except incubation buffer was changed every half hour.

Results

Fatty acid deposition, composition, and in vivo rate of TAG accumulation during embryo development

Mature *C. lanceolata* seeds accumulate TAG in which decanoic acid is the predominant fatty acid, reaching a level of 80 mol% (Bafor *et al.*, 1990). Embryos were large enough to isolate at 6 days after flowering (DAF), and the seeds reached maturity about 20 DAF. As shown in figure 5-1, TAG deposition in the *Cuphea* embryos was linear from 8 to 12 DAF and during this period TAG accumulated at the rate of 2.9 nmols/hr/embryo. This result was close to the 2.3 nmols/hr/embryo measured by Bafor *et al.* (1990). During this same period, the relative amount of decanoic acid in TAG increased from 40% to 75% mol percent. The fatty acid composition of TAG in *Cuphea* embryos at the mid-stage of TAG accumulation (11 DAF) are similar to the results obtained by Bafor *et al.* (1990).

In *U. carpinifolia* and *U. parvifolia*, it is difficult to tag flowers on a daily basis, because the elm trees are very tall. Therefore, embryos were collected only when they reached a stage when lipid analysis was feasible. The first collection was designated as the zero time point, and later time points were recorded as days after the first collection (dafc). The rate of TAG accumulation of *U. carpinifolia* and *U.*

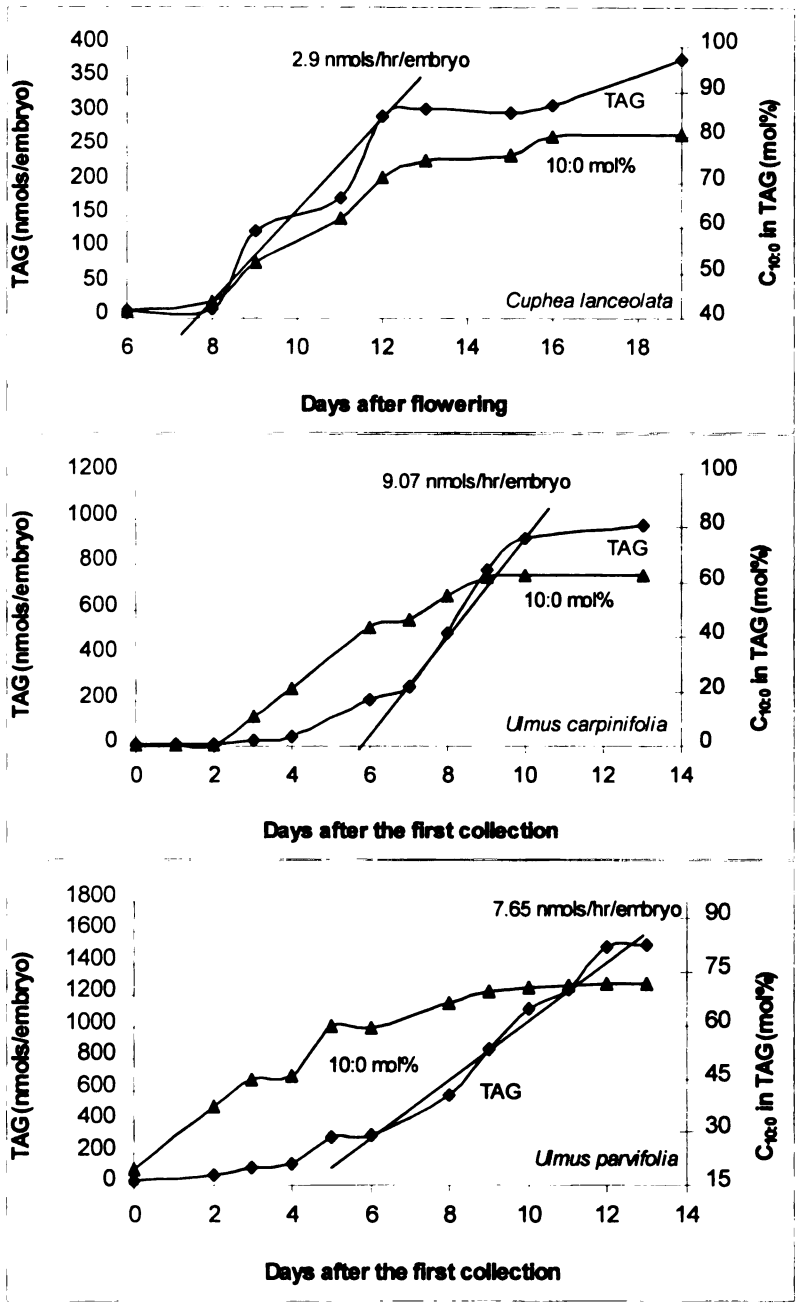

Figure 5.1. TAG accumulation and percentage of decanoic acid in TAG in developing embryos of *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*. 20 embryos at each stage were analyzed for TAG content. The left scale represents the TAG content (◆), and the right scale the percent of 10:0 in TAG (▲)

parvifolia embryos was linear from 7 to 10 dafc, and the relative amount of decanoic acid in TAG increased from 40% to 65% (Figure 5.1). The fatty acid composition of TAG in both elm species at 10 dafc is given in Table 5.1. At this stage, medium-chain fatty acids constitute 77% and 85% of the TAG fatty acids in *U. carpinifolia* and *U. parvifolia*, respectively.

Rates of TAG synthesis by developing embryos with addition of exogenous fatty acid

Bafor *et al.* (1990) observed that, when *Cuphea* developing embryos were incubated with exogenous decanoic acid and glycerol, the rate of exogenous decanoic acid incorporated into TAG was 33.9 nmol/hr/embryo. Assuming that decanoic acid could be esterified to all the three position of glycerol, the rate of ^{14}C-TAG synthesis was at least 11.3 nmols/hr/embryo which is four time higher than the *in vivo* rate of TAG accumulation (2.9 nmols/hr/embryo). To further examine and extend these results, we tested whether addition of exogenous fatty acid alone could increase the rate of ^{14}C-TAG synthesis in *Cuphea* developing embryos, and whether these results could be extended to other species. Ten pairs of cotyledons of *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, harvested when TAG accumulation was in the linear range, were incubated in buffer containing 2.1 mM [1-^{14}C]decanoic acid with or without glycerol. As shown in Table 5.2, in the absence of glycerol, the average rates of ^{14}C-TAG synthesis were 12.5, 20.4, and 12.9 nmols/hr/embryo for *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, respectively. Compared to their respective *in vivo* rates of TAG accumulation, which were 2.9 (*C.*

lanceolata), 9.1 (*U. carpinifolia*), and 7.7 (*U. parvifolia*) nmols/hr/embryo, the rates of ^{14}C-TAG synthesis were approximately 4 times higher for *C. lanceolata* and 2 times for elm tree embryos upon the addition of exogenous decanoic. The addition of 0.125 mM glycerol to the incubation solution did not strongly influence the rates of ^{14}C-TAG synthesis (Table 5.2). This suggests that there is an adequate endogenous supply of glycerol for higher TAG synthesis in developing *Cuphea* and *Ulmus* embryos. The level of ^{14}C-decanoic acid incorporated into TAG was used to calculate the rate of ^{14}C-TAG synthesis in each species (Table 5.2). This calculation did not include the contribution derived from the endogenous *de novo* TAG synthesis from non-radioactive precursors, and so the values given in Table II represent an underestimation of the total TAG synthesis from both exogenous and endogenous fatty acids.

The TAG derived from *Cuphea* embryos incubated with [1-^{14}C] decanoic acid for one hour was treated with TAG lipase (from *Rhizopus arrhizus*). The resulting *sn-2*-monoacylglycerol and the non-esterified fatty acid (derived from *sn-1* and *sn-3* positions of TAG) were separated by TLC. Radioactivity was detected in both *sn-2*-monoacylglycerol and free fatty acid fractions, indicating that labeled decanoic acid was esterified to the *sn-2* as well as to the *sn-1* and *sn-3* positions of the glycerol backbone. The ratio of radioactivity from *sn-2*-monoacylglycerol to that of free fatty acid was 1:2. This suggests that exogenous decanoic acid was equally distributed among the three positions of TAG.

We recently observed that lauric acid produced in transgenic *B. napus* can

Table 5.1. Fatty acid composition of TAG from *U. carpinifolia* and *U. parvifolia* embryos at 10 days after first collection.

		Fatty acid distribution in TAG (mol%)[1]								
TAG[1] (nmol/embryo)		8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3
U. carpinifolia	2750	6.2	62.	4.0	3.8	6.7	0.8	7.4	6.6	1.8
U. parvifolia	3170	7.5	71	4.2	2.6	4.2	0.9	4.9	4.4	0.6

[1]20 embryos were used for lipid extraction and isolation of TAG.

Table 5.2. Rate of ^{14}C-TAG synthesis upon addition 2.1 mM ^{14}C-decanoic acid.

	C. lanceolata		*U. carpinifolia*		*U. parvifolia*	
^{14}C-10:0 (mM)	2.1	2.1	2.1	2.1	2.1	2.1
Glycerol (mM)	0	0.125	0	0.125	0	0.125
Rate of TAG synthesis (nmol/hr/embryo)	12.5±1.3	11.1±1.9	20.4±2.2	21.5±1.0	12.9±2.0	13.8±1.3

Notes:

1. Ten pairs of cotyledons at mid-stage of development were incubated with 2.1 mM decanoic acid in 0.2 ml of 0.1 M phosphate buffer (pH7.2) at 28 °C for 2 hours in the absence or presence of 0.125 mM glycerol. The incubation buffer was changed once at one hour for Cuphea, and every half hour for Elm.
2. Rates of ^{14}C-TAG synthesis are calculated as nmols of ^{14}C-decanoic acid found in TAG (per hour) divided by three.
3. Data represent the average of three independent experiments.

be subject to β-oxidation (Eccleston and Ohlrogge, 1998). If this had occurred in the embryos supplemented with decanoic acid, a loss of lipid soluble ^{14}C would occur and ^{14}C would be detected not only in decanoic, but also in other fatty acids isolated after the incubations. Neither event was observed and furthermore recoveries of added decanoic acid were at least 75-85%. Therefore β-oxidation was not a major fate of the added decanoic acid.

Factors which influence the incorporation of exogenous fatty acid into TAG.

As shown in Table 5.2, addition of glycerol to the incubation buffer had no significant effect on the rate of ^{14}C-TAG synthesis. We also examined if other factors such as exogenous fatty acid concentrations, sucrose (0 to 200 mM), and pH may influence the rate of ^{14}C-TAG synthesis by *Cuphea* developing embryos. Decanoic acid concentrations were varied over the range from 2.1 to 80 mM. As shown in Figure 5.2A, the highest rate of ^{14}C-TAG synthesis (14.3 nmol/hr/embryo) was obtained with addition of 3.6 mM of decanoic acid. Concentrations higher than 3.6 mM of decanoic acid apparently had a deleterious effect on embryos and resulted in lower rates of TAG deposition.

Developing *Cuphea* embryos are non-photosynthetic tissue, so the ultimate carbon source for TAG synthesis is derived from sucrose. To determine if sucrose concentrations may limit TAG formation in these experiments, different concentrations of sucrose (0 to 200 mM) were added to the basic incubation solution. Despite minor variations among samples, the rates of ^{14}C-TAG synthesis in all samples were close to 12 nmol/hr/embryo. This result suggests that carbon

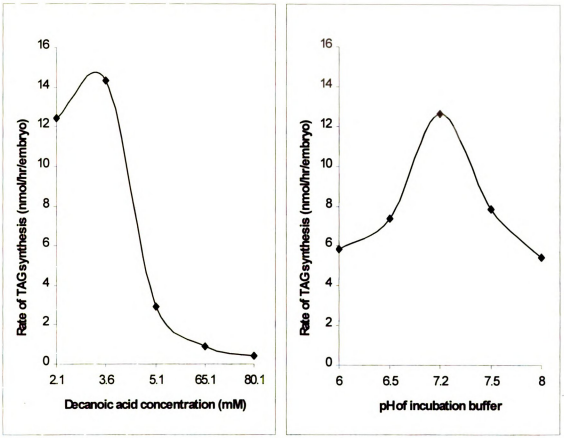

Figure 5.2. Rate of ^{14}C-TAG synthesis under different conditions. (A) ten pairs of cotyledon incubated in 0.2 ml of phosphate buffer (pH7.2) with concentration of ^{14}C-decanoic acid as indicated. (B) ten pairs of cotyledon incubated in 2.1 mM ^{14}C-decanoic acid plus 0.2 ml of phosphate buffer, whose pH ranged from 6 to 8.

supply in the form of sucrose is not limiting TAG accumulation in these short term experiments.

Developing *Cuphea* embryos were also incubated under a range of pH from 6.0 to 8.0. As shown in Figure 5.2B, the optimal pH for TAG deposition was 7.2 whereas at pH6.5 or 7.5, the rate of ^{14}C-TAG synthesis decreased to half of that at pH7.2.

Utilization of other exogenous fatty acids by Cuphea cotyledons for TAG synthesis

Each ten pairs of cotyledons of developing *Cuphea* embryos were separately supplied with 2.1 mM of [1-^{14}C]octanoic acid, [1-^{14}C]decanoic acid, or [1-^{14}C] oleic acid in 200 µl 0.1 M phosphate (pH7.2). 2.0 mM Triton X-100 was used to increase solubility of the oleic acid. After one hour incubation, 5.8 nmols of octanoic acid, 37.5 nmols of decanoic acid, and a trace amount of oleic acid were incorporated into TAG per embryo, respectively. One simple interpretation of this result is that one or more of the acyltransferases of *Cuphea* display a strong selectivity in favor of decanoic acid (Bafor *et al.*, 1992; Vogel *et al.*, 1996). However, *Brassica napus* embryos were also incubated with oleic acid under the same conditions and the incorporation of oleic acid into TAG was significantly lower than the *in vivo* rate (data not shown). So, for oleic acid, low aqueous solubility or transport into the tissue may also prevent its rapid incorporation into TAG by embryos.

Discussion

TAG normally accumulates to a high level only in seeds but a metabolic understanding of the tissue specificity of oil accumulation is not yet available. One potential explanation is that DAGAT, which catalyzes the acylation of position 3 of 1,2-diacyl-*sn*-glycerol, is specifically expressed in seed. However, there are several observations which argue against this view. For example, DAGAT activity was found in spinach leaves (Martin *et al.*,1983) and was primarily associated with chloroplast envelopes (Martin *et al.*,1984). Roughan *et al.* (1987) reported that significant amounts of TAG were synthesized when palmitic acid was applied to the upper surface of expanding spinach leaves. The level of neutral lipids (mainly TAG) increased at least 3 fold during protoplast isolation from *Arabidopsis* leaves (Browse *et al.*, 1988). Finally, ozone-fumigated spinach leaves produced high proportions of TAG (Sakaki *et al.*,1990). These data together suggest that DAGAT not only occurs in leaves, but also that leaves have the ability to synthesize TAG.

Although expressed in several tissues, higher expression of DAGAT during seed development might represent one explanation for TAG accumulation in oilseeds. Ichihara *et al.* (1988) measured the specific activity of DAGAT from safflower *in vitro* and found DAGAT activity was lower than expected. They concluded that the DAGAT reaction may be rate-limiting. When developing safflower and sunflower cotyledons were incubated with exogenous radiolabeled fatty acid tracers, substantial amounts of labeled fatty acids were esterified to DAG (Griffiths *et al.*, 1988). Since DAG is the direct substrate of DAGAT, it was suggested that DAGAT could be a rate-limiting step. Perry *et al.* (1993a, 1993b)

found that, when developing seeds of *Brassica napus* were incubated with [1-^{14}C]acetate and [2-^{3}H]glycerol, very low accumulation of the Kennedy pathway intermediates occurred apart from DAG. These results were also interpreted as indicating that DAGAT is likely to exert significant flux control over TAG accumulation. A similar conclusion was drawn by Griffiths *et al.* (1991) from studies of TAG synthesis in cocoa. However, the accumulation of DAG might also be explained as a shortage of acyl chain supply rather than flux control at DAGAT. Because both DAG and acyl-CoA are direct substrates of DAGAT, lack of one substrate (acyl-CoA) can lead to the accumulation of the other (DAG) if the DAGAT K_m for acyl-CoA is higher than the other acyltransferases. Thus, the accumulation of DAG does not necessarily imply that DAGAT exerts flux control for TAG synthesis.

In order to begin to examine the limiting step(s) in TAG production for this study we considered the pathway of TAG biosynthesis in two parts. The first half can be characterized as fatty acid production inside plastids; the second half can be considered as the assembly of TAG in the ER or oilbodies (Cao *et al.*, 1986; Settlage *et al.*, 1995). If the supply of fatty acid is a limiting factor for TAG synthesis, addition of excess exogenous fatty acid should increase the rate of TAG synthesis. As shown in Figure 5.1, TAG accumulated at the rate of 2.9, 9.07, and 7.65 nmols/hr/embryo *in vivo* for developing embryos of *Cuphea lanceolata*, *U. carpinifolia*, and *U. parvifolia*, respectively. With addition of exogenous decanoic acid, their rate of ^{14}C-TAG synthesis was two to four fold higher than the *in vivo*

accumulation rate. This result clearly indicates that the supply of fatty acid can be one limiting factor for TAG accumulation. In agreement with these observations in seeds, in *Chlamydomonas*, addition of exogenous PC liposomes to cultures caused 10-fold increases in TAG accumulation (Grenier *et al.*, 1991). Thus, it appears that the capacity of these systems for TAG accumulation is greater than actually used and that fatty acid supply, rather than the utilization enzymes may limit TAG accumulation in *Cuphea*, elm trees, and *Chlamydomonas*. In addition, we found that sucrose and glycerol had no significant influence on the rate of ^{14}C-TAG synthesis in *Cuphea*. This implies that both the endogenous carbon source and glycerol backbone are in excess and not limiting ^{14}C-TAG synthesis during these incubations. However, it is important to emphasize that such short-term incubations may not reflect factors which control overall long-term accumulation of storage oils. For example, over the time scale of seed development, many other factors such as the ability of oilbodies to accommodate increased TAG might become limiting.

In this study, the exogenous decanoic acid was almost equally distributed among the three positions of TAG from *Cuphea*. This result implied that not only diacylglycerol acyltransferse, but also glycerol 3-phosphate acyltransferse and lysophosphatidic acid acyltransferase activities could incorporate exogenous decanoic acid at rates several fold above their *in vivo* activity with endogenous substrates. Although our studies support the concept that increased fatty acid supply can increase TAG accumulation, they do not rule out that other factors or enzyme expression levels may have a similar effect. Flux through a metabolic

pathway can often be driven by either stronger source inputs and/or by stronger sinks pulling on the pathway. The observations of Zou *et al.* (1997) that in *B. napus* expression of a yeast acyltransferase can increase oil yields may represent an example of sink driven increases in oil accumulation. Furthermore, we recently found that transgenic *B. napus* seeds which express very high levels of medium chain acyl-ACP thioesterase and produce high levels of lauric acid induce the beta-oxidation pathway to degrade some of the lauric acid (Eccleston and Ohlrogge, 1998). Because oil yields are not reduced in these seeds, fatty acid synthesis apparently increased to provide a constant oil yield.

In oilseeds, fatty acids esterified to TAG can be generally divided into two groups. One (such as 18:3, 22:1, 18:1-HO) needs post-plastidial modification, while the other (10:0, 12:0, 18:1) does not. This work clearly shows that the supply of fatty acids is one limiting factor for the rate of ^{14}C-TAG synthesis in *Cuphea* and *Ulmus*, which might be generalized representatives of the second category. However, for the synthesis of TAG containing high level of post-plastidially modified fatty acids, the involvement of phospholipids, desaturases, elongases, hydrolases, etc. may be additional factors which limit TAG accumulation. In addition, the observation that increased expression of acetyl-CoA carboxylase resulted in increased oil content of high-erucic rapeseed (Roesler *et al.*, 1997) suggests that also in rapeseed, where fatty acids are modified by elongation, increased fatty acid supply can increase TAG accumulation.

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CHAPTER 6

Conclusions and Future Research Perspective

Conclusions

Studies presented in this thesis have clarified several questions along the pathway from the origin of plastidial acetyl-CoA to the final accumulation of triacylglycerol. A method was developed to accurately measure the net rate of fatty acid synthesis, in photosynthetic leaf tissues in short period time. Using the combination of $^{13}CO_2$ labeling and GC/MS analysis, the rate of fatty acid synthesis for 3-week-old *Arabidopsis* seedlings (whole aerial part of plant) was 2.3μmol C h^{-1} mg chlorophyll^{-1} with 150 μmol $s^{-1}m^{2-1}$ lumination. Employing isotope dilution strategy, the rate of fatty acid synthesis in the dark was less than 5% of light rate. Both in pulse-chase and continuous $^{14}CO_2$ labeling experiments, the maximum of lag phase was less than two minutes, not the predicted 60 min lag if free acetate is involved in fatty acid synthesis and its concentration is 1 mM. Also in consistent with this observation, radioactivity recovered in free acetate after one hour continuous labeling was below detection limits or below a concentration of 0.02mM. Based on our kinetic analysis and radioactivity in free acetate, we conclude that the bulk pool of acetate is not a major substrate for fatty acid synthesis or the concentration of acetate is below 0.05 mM.

A biotin carboxylase genomic clone from *Arabidopsis* was isolated and its promoter was partially characterized. The gene contains 16 exons and 15 introns;

the transit peptide is 71 amino acid long and there is one intron inside the transit peptide. Northern blot and BC/GUS fusion constructs indicated that BC was regulated at expression levels which correlated with the rate of fatty acid synthesis. For example, the highest expression level was found in seeds. A positive and a negative element were also identified in the promoter region.

Work described in chapter 4 let to the revision of the biosynthetic pathway for erucic acid in the developing embryos of *Brassica rapa*. This revised pathway indicates that newly synthesized 18:1 enters into an intermediate pool before it is elongated to 20:1, then to 22:1. The results of manipulating the rate 18:1 synthesis and blocking the elongation from 18:1 to 22:1 imply that PC might serve as the intermediate pool of 18:1 for elongation reactions.

In chapter 5, the pathway of TAG synthesis was dissected into the supply and the utilization of fatty acids. The addition of exogenous fatty acid resulted in a 2 to 4 fold increase of the rate of TAG synthesis in *Cuphea* and elm developing embryos. This result suggests that the amount of TAG synthesized in oilseeds may be a response of a high rate of fatty acid synthesis. The acyltrasferases and glycerol-3-phosphate levels seem to not limit the accumulation of TAG in the embryos studied.

Future Research Perspective

1. The source of acetyl-CoA for fatty acid synthesis in plastids

Even though our results indicate that the bulk acetate pool is not involved in

fatty acid synthesis, the question of how the acetyl-CoA in plastids is generated remains unclear. We still can not rule out the possibility that a very small pool of acetate (less than 0.05mM) serves as intermediate for fatty acid synthesis. Due to the short lag of carbon flowing from CO_2 to fatty acids, pool size analysis with isotope labeling can not distinguish acetyl-CoA generated from different pathways. In addition, because common precursors are shared by pathways that produce acetyl-CoA, carbon position analysis can not provide informative data either. With these limitations, manipulating the key enzymes in each individual pathway is a logical next approach to further clarify the relative contribution of plastidial acetyl-CoA from several pathways. As shown in figure 1-2, pyruvate and acetate are the two main candidates as immediate precursors to generate acetyl-CoA inside chloroplast for fatty acid synthesis. The plastidial pyruvate dehydrogenase and acetyl-CoA synthetase are responsible to convert pyruvate and acetate to acetyl-CoA, respectively. It is ideal to inhibit each enzyme, and then measure the rate of fatty acid synthesis to evaluate its relative contribution. Unfortunately, no inhibitors that specifically inhibit acetyl-CoA synthetase or plastidial PDC have been reported. Therefore, more emphasis should be placed on isolating and analyzing mutants of acetyl-CoA synthetase and plastidial PDC. The drawback of this approach is that mutations could be lethal if plastidial acetyl-CoA is generated solely by one pathway, but it is still possible to obtain leaky mutants. If there are more than one pathway contributing to acetyl-CoA in plastids, it should be easier to obtain mutants for each enzyme, considering that different pathways might compensate for each

other. Alternatively, since the DNA sequences of acetyl-CoA synthetase and most subunits of plastidial PDC are available, antisense of either or both enzymes should provide more information about the origin of plastidial acetyl-CoA. Back *et al.* (1999) reported that they obtained transgenic *Arabidopsis* containing a sense or antisense construct of acetyl-CoA synthetase, pPDHα or pPDHβ created from Arabidopsis cDNA. Their research is still at the preliminary stage, information about fatty acid synthesis and lipid contents of those transgenic plants is not available yet.

2. Analyzing promoters related to lipid synthesis

The BC promoter was only broadly analyzed in this study, and fine deletions are needed to further define regulatory factors. Promoter analysis primarily serves two purposes; one is to identify *cis* elements responsible for certain expression patterns and further to isolate the *trans* regulation factors; the other is to design a promoter under whose control the target gene can be expressed as desired. With the onset of *Arabidopsis* genomic project and microarray technique, conceptual changes are necessary to adapt new technologies, so it may not be a wise approach to analyze single promoter. To establish the relationship between gene expression level and enzymatic activity, several layers of regulation have to be put into consideration. At a given condition, the transcript level, the stability of the transcript, the efficiency of translation, the stability of the translated polypeptide, and proper targeting are all contributing factors for the final enzyme activity. Therefore, high transcript level does not necessarily mean high enzymatic activity. It is difficult to obtain information about the stability of the transcript, the efficiency

of translation, the stability of the polypeptide for large number of genes, but it is now relatively easy to get transcript levels and the flux through corresponding enzymes. The microarray chips containing all the known ESTs from *Arabidopsis* will soon be available. As a result, the transcript levels of all the genes related to fatty acid and TAG synthesis in roots, leaves, and seeds can be obtained very quickly; followed with the comparison of gene expression in different tissues. We are interested in seed specific promoters and *cis* and *trans* elements that are responsible for the up regulation of certain genes in oilseeds. Future promoter analysis should be conducted , at least in my opinion, in step wise fashion as outlined below. 1) The transcript levels from seeds will be compared with that from roots or leaves. 2) The genes will be classified into several categories according to changes of expression patterns as following: specifically expressed, upregulated, unchanged, and down regulated, and completely shut down in seeds. 3) Promoter regions of seed specific genes will be retrieved from *Arabidopsis* genomic data bank, multiple comparison of these promoters will be done to identify seed specific *cis* elements, and the secondary structures and locations of these genes will be examined to determine whether position effects also play a role for seed specific gene expression. 4) The strong seed specific promoter will be made through combinations of different *cis* elements from different seed specific expressed gene promoters. 5) For the promoters that are completely shut down in seeds, the similar analysis will be performed. In this group, we are interested in finding out whether these promoters lack certain seed specific elements or have certain repression elements. 6) For the

up, unchanged, and down regulated genes in the seeds, we need calibrate transcript level on per cell basis, and compare their corresponding enzyme activity in terms of flux. Only those gene promoters where transcript level changes are correlated with their enzyme activity changes are deemed for further *cis* elements analysis. Genes that their transcript levels are not correlated with their enzyme activities, are candidates subject to other levels of regulation rather than on the transcription level. In summary, with the complete genomic sequence of *Arabidopsis* and microarray technique, above goals can be achieved in relatively short period of time, for which we dare not dream two years ago.

3. Development of transgenic *Brassica* with high erucic acid content

As mentioned in chapter 4, the level of 50% erucic acid in high erucic rapeseed oil is not sufficient to compete well with alternative source of lubricant oils from petrochemicals because of the high cost of purification (Ohlrogge, 1994). In order to replace petrochemicals, erucic acid content should be over 75% in the rapeseed oil. In the TAG of high erucic *Brassica*, because most of *sn*-2 position is occupied by 18 carbon fatty acids such as 18:1, 18:2, or 18:3, most research is oriented toward replacing 18 carbon fatty acids with erucic acid at *sn*-2 position hoping to achieve higher overall erucic acid content. Unusual fatty acids can be esterified to *sn*-2 position of TAG. Decanoic acid composes 80% of the total fatty of Cuphea and elm seeds, 85% petroselinic acid in coriander seeds, 80% Δ6 hexadecanoic acid in *Thunbergia alata*. This group of fatty acids are synthesized in plastids and do not need any further modification in the cytosol before esterified

to glycerol and they occupy about 30-50% of *sn*-2 position in TAGs. Castor oil contains 85% of ricinoleic acid, 65% of vernolic acid in *Euphorbia* seed, 65% linolenic in linseed oil. These fatty acids need further modification before incorporated into TAG, and the modification reactions take place on the *sn*-2 position of PC. Then the unusual fatty acids would be presumably cleaved from the *sn*-2 position and made available for TAG synthesis. This group of fatty acids also constitute about 20-50% at *sn*-2 positions in their respective TAGs. The enzyme responsible to remove fatty acid from *sn*-2 position of PC is still a debated issue. Lysophosphatidylcholine: acyl-CoA acyltransferase (LPCAT) catalyzes the transfer of a acyl group from acyl-CoA to the *sn*-2 lysophosphatidylcholine as a alternative to form PC (Stymne and Stobart, 1984; Ichihara *et al.*, 1995). This reaction is fully reversible and does not require ATP (Stymne ans Stobart, 1984). In safflower, the activity of LPCAT was correlated with TAG synthesis during seed development (Ichihara *et al.*, 1995). So it is suggested that LPCAT also catalyze the removal of modified fatty acid from *sn*-2 position of PC. The pathway of erucic acid synthesis is different from above two group of unusual fatty acids. As indicated in chapter 4, the newly synthesized 18:1 first enters the PC pool, then it is later removed from PC, and finally elongated to erucic acid. We speculate that insufficient LPCAT activity might be the cause of high 18 carbon fatty acid content at *sn*-2 position of TAGs in high erucic rapeseed. If there is not sufficient LPCAT activity, the 18:1 esterified to the *sn*-2 position of PC most likely have two fates; one is being further desaturated to 18:2 or 18:3; the other is along with PC being converted to DAG

directly and then to TAG. Both scenarios would result in higher 18 carbon fatty acid content than otherwise. Our hypothesis may explain why the failure to increase erucic content by expression of an *sn*-2 acyltransferase from *Limnanthes* (Lassner *et al.,* 1995), over-expression of elongases (Lassner *et al.,* 1996), and reduction of 18:1 desaturation (Lemieux *et al.*, 1990). On the contrary, nasturtium (*Tropaeolum majus*) seed oil contains as much as 80% erucic acid (Gustone *et al.*, 1994; Luhs and Friedt, 1994) . In order to increase erucic acid content in *Brassica*, we need to first carefully compare the elongation system of nasturtium with *Brassica*, especially determining whether 18:1 also enters PC pool before being elongated. Second, LPCAT activities from developing seeds of both species should be assayed. Third, if LPCAT activity of nasturtium is significantly higher than that of *Brassica*, we should try to isolate the LPCAT gene from nasturtium (*Tropaeolum majus*) and transfer this gene into high erucic acid *Brassica* lines. The bigest difficulty for isolating lyso-PC acyltransferase from nasturtium is that there is not single gene sequence from plant available yet. But considering the economic potential and understanding the differences of the two elongation system, it is worth for further investigation.

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