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REGULATION OF FATTY ACID SYNTHESIS

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ABSTRACT

All plant cells produce fatty acids from acetyl-CoA by a common pathway localized in plastids. Although the biochemistry of this pathway is now well understood, much less is known about how plants control the very different amounts and types of lipids produced in different tissues. Thus, a central challenge for plant lipid research is to provide a molecular understanding of how plants regulate the major differences in lipid metabolism found, for example, in mesophyll, epidermal, or developing seed cells. Acetyl-CoA carboxylase (ACCase) is one control point that regulates rates of fatty acid synthesis. However, the biochemical modulators that act on ACCase and the factors that in turn control these modulators are poorly understood. In addition, little is known about how the expression of genes involved in fatty acid synthesis is controlled. This review evaluates current knowledge of regulation of plant fatty metabolism and attempts to identify the major unanswered questions.

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INTRODUCTION

All cells in a plant must produce fatty acids, and this synthesis must be tightly controlled to balance supply and demand for acyl chains. For most plant cells, this means matching the level of fatty acid synthesis to membrane biogenesis and repair. Depending on the stage of development, time of the day, or rate of growth, these needs can be highly variable, and therefore rates of fatty acid biosynthesis must be closely regulated to meet these changes. In some cell types, the demands for fatty acid synthesis are substantially greater. Obvious examples are oil seeds, which during development can accumulate as much as 60% of their weight as triacylglycerol. Another example is epidermal cells, which traffic substantial amounts of fatty acids into surface wax and cuticular lipid biosynthesis. In leek, even though the epidermis is less than 4% of the total fresh weight of the leaf, as much as 15% of the leaf lipid is found in a single wax component, a C31 ketone (52). How do cells regulate fatty acid synthesis to meet these diverse and changeable demands for their essential lipid components? We are only beginning to understand the answer to this question.

In this review, we focus on questions about regulation of fatty acid synthesis. Several reviews in recent years provide excellent overviews of fatty acid synthesis, and we do not duplicate those efforts except where necessary for clarity. An excellent and comprehensive review of plant fatty acid metabolism has recently been published (33), and several other recent reviews covering plant lipid metabolism, molecular biology and biotechnological aspects of plant fatty acids have also appeared (10, 44, 57, 64, 97, 105). Because there is much yet to be learned about regulation of this essential and ubiquitous path-

way, we often dwell on what is unknown. This approach is intended to provide the reader with a clearer sense of the major questions of fatty acid metabolism that remain to be answered before a reasonable understanding of this regulation is achieved.

Compartmentalizaton and the Need for Interorganelle Communication

Overall fatty acid synthesis, and consequently its regulation, may be more complicated in plants than in any other organism (Figure 1). Unlike in other organisms, plant fatty acid synthesis is not localized within the cytosol but occurs in an organelle, the plastid. Although a portion of the newly synthesized acyl chains is then used for lipid synthesis within the plastid (the prokaryotic pathway), a major portion is exported into the cytosol for glycerolipid assembly at the endoplasmic reticulum (ER) or other sites (the eukaryotic pathway)

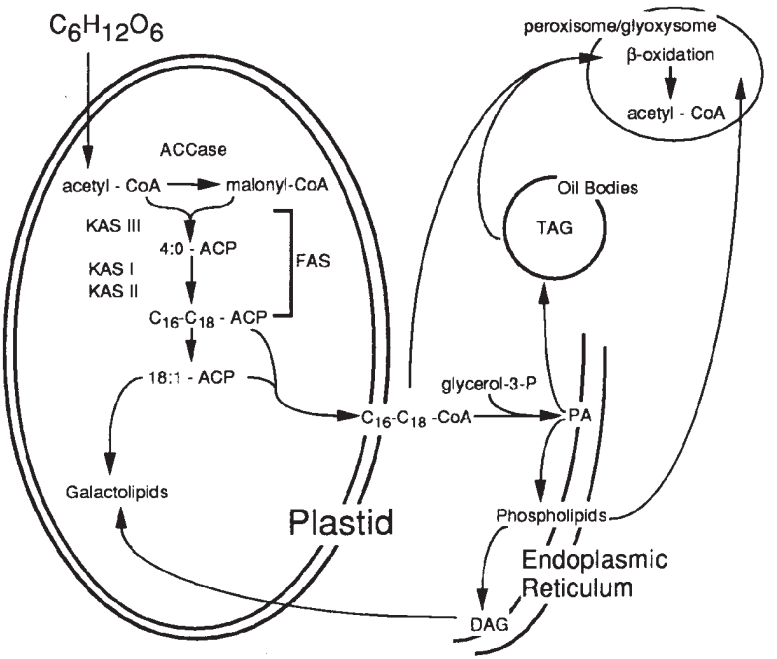

Figure 1 Simplified schematic of overall flow of carbon through fatty acid and lipid metabolism in a generalized plant cell. Because acyl chains are used in every subcellular compartment but are produced almost exclusively in the plastid, interorganellar communication must balance the production and use of these acyl chains.

(82, 100). In addition, some of the extraplastidial glycerolipids return to the plastid, which results in considerable intermixing between the plastid and ER lipid pools. Both the compartmentalization of lipid metabolism and the intermixing of lipid intermediates in these pools present special requirements for the regulation of plant fatty acid synthesis. Foremost is the need for regulatory signals to cross organellar boundaries. Because fatty acids are produced in the plastid, but are principally esterified outside this organelle, a system for communicating between the source and the sinks for fatty acid utilization is essential. The nature of this communication and the signal molecules involved remain an unsolved mystery.

In addition to having regulatory mechanisms that control overall levels of individual lipids, plants must also regulate rates of fatty acid synthesis under circumstances in which there are large shifts in the demand by major pathways of lipid metabolism located both in and out of the plastid. For example, consider the consequence of the Arabidopsis mutant *act1*, which has a mutation in the plastidial acyl transferase that directs newly synthesized fatty acid into thylakoid glycerolipids (49). This mutant has the remarkable ability to compensate for loss of the prokaryotic pathway by diverting nearly all the fatty acids into the phospholipids of the eukaryotic pathway. It then funnels unsaturated diacylglycerol from the ER phospholipids back into the plastidial lipids, with only minor change in the overall composition of either of these membranes.

OVERVIEW OF FATTY ACID SYNTHESIS: THE ENZYME SYSTEMS

The simplest description of the plastidial pathway of fatty acid biosynthesis consists of two enzyme systems: acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). ACCase catalyzes the formation of malonyl-CoA from acetyl-CoA, and FAS transfers the malonyl moiety to acyl carrier protein (ACP) and catalyzes the extension of the growing acyl chain with malonyl-ACP. In nature, ACCase occurs in two structurally distinct forms: a multifunctional homodimeric protein with subunits >200 kDa, and a multisubunit ACCase consisting of four easily dissociated proteins. Ideas about the structure of plant ACCases have undergone considerable evolution in the past few years. Until 1992, most researchers had concluded that plant ACCase was a large (>200 kDa) multifunctional protein similar to that of animal and yeast. This type of enzyme had been purified from several dicot and monocot species, and partial cDNA clones were available. However, in 1993, Sasaki and co-workers demonstrated that the chloroplast genome of pea encodes a subunit of an

ACCase with structure related to the β subunit of the carboxyltransferase found in the multisubunit ACCase of *Escherichia coli* (88). A flurry of interest in this topic has resulted in rapid extension of these initial studies. It has now been clarified that dicots and most monocots have both forms of ACCase, a >200-kDa homodimeric ACCase (probably localized in the cytosol) and a heteromeric ACCase with at least four subunits in the plastid (2, 48, 80). It is the heteromeric plastid form of the ACCase that provides malonyl-CoA for fatty acid synthesis.

In addition to the β-carboxyltransferase subunit characterized by Sasaki, clones are now available for the biotin carboxylase (94), biotin carboxyl carrier protein (BCCP) (17), and α-subunit of the carboxyltransferase (95). The four subunits are assembled into a complex by gel filtration with a size of 650–700 kDa. However, the subunits easily dissociate such that ACCase activity is lost, which accounts for the failure to identify the multisubunit form of ACCase for many years. Because the β–carboxyltransferase (β-CT) subunit is plastome encoded, whereas the other three subunits are nuclear encoded, assembly of a complete complex requires coordination of cytosolic and plastid production of the subunits. Little is know about this coordination and assembly. However, several-fold overexpression and antisense of the biotin carboxylase subunit does not alter the expression of BCCP, which suggests that a strict stoichiometric production of subunits may not be essential (92).

The structure of ACCase in Gramineae species is different in that these species lack the heteromeric form of ACCase and instead have two types of the homodimeric enzyme (89). An herbicide-sensitive form is localized in plastids, and a resistant form is extraplastidial. Because both Gramineae and dicot plastid FAS are regulated by light and dependent on ACCase activity, it will be of considerable interest to discover whether the two structurally very different forms of ACCase are subject to the same or different modes of regulation.

The structure of FAS has many analogies to ACCase structure. In nature, both multifunctional and multisubunit forms of the FAS are found. In addition, as in the case of ACCase, the plastidial FAS found in plants is very similar to the *E. coli* FAS and is the easily dissociated multisubunit form of the enzyme. It is now well established in both plants and bacteria that the initial FAS reaction is catalyzed by 3-ketoacyl-ACP III (KAS III), which results in the condensation of acetyl-CoA and malonyl-ACP (37, 106). Subsequent condensations are catalyzed by KAS I and KAS II. Before a subsequent cycle of fatty acid synthesis begins, the 3-ketoacyl-ACP intermediate is reduced to the saturated acyl-ACP in the remaining FAS reactions, catalyzed sequentially by the 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and the enoyl-ACP reductase.

In addition to ACCase and FAS, discussion of the regulation of fatty acid synthesis must also consider those reactions that precede and follow these two enzyme systems. It is not fully understood which reactions are responsible for providing acetyl-CoA to ACCase, but extensive experiments with leaf tissue indicate that acetyl-CoA synthetase can rapidly convert acetate to acetyl-CoA, and therefore free acetate may be an important carbon source (83–85). Other possible sources of acetyl-CoA include synthesis from pyruvate by a plastidial or mitochondrial pyruvate dehydrogenase (14, 51) or citrate lyase (61). Because acetyl-CoA is a central metabolite required for synthesis of isoprenoids, amino acids, and many other structures, it is likely that more than one pathway provides acetyl-CoA for fatty acid synthesis, and the source may depend on tissue and developmental stage (40).

The final products of FAS are usually 16:0- and 18:0-ACP, and the final fatty acid composition of a plant cell is in large part determined by activities of several enzymes that use these acyl-ACPs at the termination phase of fatty acid synthesis. The relative activities of these enzymes therefore regulate the products of fatty acid synthesis. Stearoyl-ACP desaturase modifies the final product of FAS by insertion of a *cis* double bond at the 9 position of the C18:0-ACP. Reactions of fatty acid synthesis are terminated by hydrolysis or transfer of the acyl chain from the ACP. Hydrolysis is catalyzed by acyl-ACP thioes-

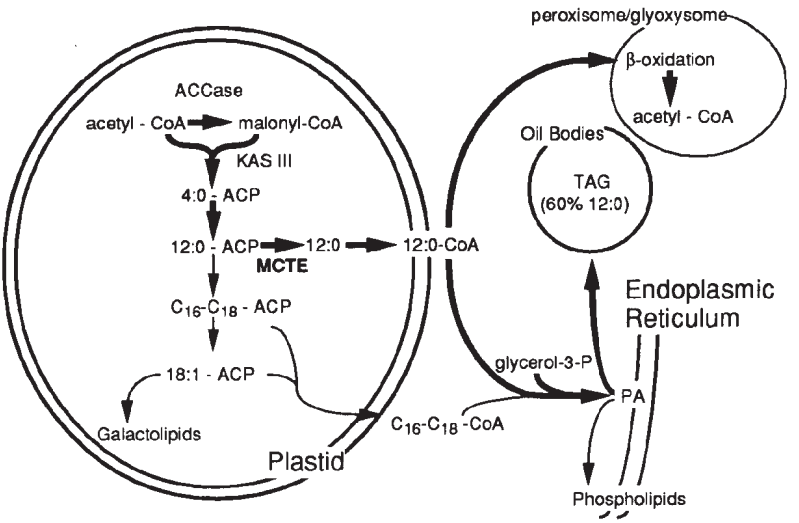

Figure 2 Overexpression of the California Bay medium-chain thioesterase (MCTE) results in activation of β-oxidation as well as increased expression of the enzymes of fatty acid biosynthesis.

terases, of which there are two main types: one thioesterase relatively specific for 18:1-ACP and a second more specific for saturated acyl-ACPs (24, 39). Fatty acids that have been released from ACPs by thioesterases leave the plastid and enter into the eukaryotic lipid pathway (82), where they are primarily esterified to glycerolipids on the ER. In some plants, thioesterases with specificity for shorter chain length acyl-ACPs are capable of prematurely terminating fatty acid synthesis, thereby regulating the chain length of the products incorporated into lipids. For example, thioesterases specific for medium-chain fatty acids are responsible for high levels of C10 and C12 fatty acids in triacylglycerols of California Bay, *Cuphea*, and other species (19, 23, 71). Acyl transferases in the plastid, in contrast to thioesterases, terminate fatty acid synthesis by transesterifying acyl moieties from ACP to glycerol, and they are an essential part of the prokaryotic lipid pathway leading to plastid glycerolipid assembly (82, 100).

BIOCHEMICAL CONTROLS: WHICH ENZYMES REGULATE FATTY ACID SYNTHESIS?

> Control in metabolic pathways is not a dictatorship, nor is it an Athenian democracy; rather, it is a halfway house in which (to quote Orwell) "all pigs are equal but some are more equal than others" (101).

A number of approaches have been used by biochemists to identify where regulation occurs in a pathway. One approach depends on examination of the in vitro properties of enzymes of a pathway. Enzymes that have low activity relative to other members of the pathway are frequently considered as potentially rate limiting. Other properties, such as changing activities during developmental regulation of the pathway, or influence (activation or inhibition) on the enzyme by intermediates of the pathway can provide additional evidence toward identification of control points.

Analysis of Rate-Limiting Steps

ACCase is frequently considered the first committed step in the fatty acid biosynthetic pathway. In animals (30) and in yeast (111), there is evidence for ACCase as a major regulatory enzyme in fatty acid production. Therefore, for some time this enzyme was also proposed to be rate determining for plant fatty acid biosynthesis. Several lines of evidence supported this suggestion: Acetate or pyruvate were incorporated into acetyl-CoA in the dark by isolated chloroplasts, but malonyl-CoA and fatty acids were formed only in the light (58). Thus, the light-dependent step of fatty acid synthesis appeared to be at the

ACCase reaction. Eastwell & Stumpf (26) found that chloroplast and wheat germ ACCase were inhibited by ADP and suggested this may account for light-dark regulation of the enzyme. Nikolau & Hawke (63) characterized the pH, Mg, ATP, and ADP dependence of maize ACCase activity and concluded that changes in these parameters between dark and light conditions could account for increased ACCase activity upon illumination of chloroplasts. Finally, ACCase activity and protein levels are coincident with increases and decreases in oil biosynthesis in developing seeds (e.g. 80, 107; but see 40a).

However, in vitro approaches are limited because they show only that the enzyme has in vitro properties consistent with control, not that it actually does control in vivo metabolism. There are numerous examples of enzymes whose biochemical properties imply control but which have subsequently been found to have little role in controlling flux in vivo (96). Thus, there is no obligatory link between enzyme characteristics observed in vitro and regulatory properties in vivo.

The sites of metabolic control of a pathway can be more reliably identified by examination of in vivo properties of enzymes. Although it is often technically difficult, examining the concentrations of the substrates and products of each enzymatic step in a pathway provides information on which reactions are at equilibrium and which are displaced from equilibrium. This information is important because an essential feature of almost all regulatory enzymes is that the reaction that they catalyze is displaced far from its thermodynamic equilibrium. This is a requirement for regulation because if an enzyme has sufficient activity in vivo to bring its reaction to or near equilibrium, then changes in the activity of the enzyme will lead to no net change in flux through the pathway (81). On the basis of this criterion, it is possible to classify enzymes within a pathway as either nonregulatory or potentially regulatory based simply on an examination of the pools of substrates and products for each reaction. Further evidence of actual control can be obtained by examining changes in pool sizes when flux through the pathway is altered. It can be shown both theoretically and experimentally that when flux through the pathway is reduced at the regulatory reaction, the substrate pool for the regulatory step will increase and the product pool will usually decrease. Thus, an important experimental procedure for identifying regulatory steps is to examine changes in the pool sizes of intermediates when flux through the pathway is altered.

How can these principles be applied to evaluating control of plant fatty acid biosynthesis? Most of the substrates and intermediates of plant FAS are attached to acyl carrier protein (ACP). Analysis of acyl-ACPs is aided because the chain length of fatty acids attached to ACP alters the mobility of the protein in native or urea PAGE gels. Because of these alterations in mobility,

most of the acyl-ACP intermediates of fatty acid synthesis can be resolved, and when transferred to nitrocellulose, antibodies to ACP can provide sensitive detection at nanogram levels. Although the acyl-ACP intermediates have a half life in vivo of only a few seconds (37, 99), by rapidly freezing tissues in liquid nitrogen it has been possible to determine the relative concentrations of free, nonacylated ACPs and of the individual acyl-ACPs. Analysis of acyl-ACP pools has been used to study regulation of FAS in spinach leaf and seed (72) in chloroplasts (74) in developing castor seeds (73) and in tobacco suspension cultures (93).

The initial examination of the composition of the acyl-ACP pools provided information about the potential regulatory reactions in plant fatty acid biosynthesis. The various saturated acyl-ACP intermediates between 4:0 and 14:0 occur in approximately equal concentrations. Because the 3-ketoacyl-ACPs, enoyl-ACPs, or 3-hydroxyacyl-ACPs, which are substrates for the two reductases and dehydrase reactions, were not detected, it is likely that these reactions are close to equilibrium and that the in vivo activities of these enzymes are in excess. Thus it is not likely that these enzymes are regulatory. In contrast, the concentration of acetyl-ACP was considerably above that of malonyl-ACP. This result suggests that the acetyl-CoA carboxylase reaction, which has an equilibrium constant slightly favoring malonyl-CoA formation, is significantly displaced from equilibrium and therefore potentially regulatory (37, 72, 74). The condensing enzymes can also be considered displaced from equilibrium because of the concentration of malonyl-ACP and the saturated acyl-ACPs.

Is Acetyl-CoA Carboxylase Rate-Limiting?

To obtain more information on sites of regulation, the changes in pool sizes when flux through the fatty acid biosynthetic pathway changes were examined. The rate of spinach leaf fatty acid biosynthesis in the dark is approximately one sixth the rate observed in the light (9). In the light, the predominant form of ACP was the free, nonacylated form, whereas acetyl-ACP represented about 5–6% of the total ACP (72). In the dark, the level of acetyl-ACP increased substantially with a corresponding decrease in free ACP, such that acetyl-ACP was now the predominant form of ACP. In similar experiments, when chloroplasts are shifted to the dark, malonyl-ACP and malonyl-CoA disappear within a few seconds, and acetyl-ACP levels increase over a period of several minutes. The rapid decrease in malonyl-ACP and malonyl-CoA when fatty acid synthesis slows, together with the increase in acetyl-ACP and lack of change in other intermediate acyl-ACP pools all lead to the conclusion

that ACCase activity is the major determinant of light/dark control over FAS rates in leaves.

The above experiments on acyl-ACP and acyl-CoA pools have been carried out with dicot plants. Gramineae species such as maize and wheat have a substantially different (homodimeric) structure of ACCase (described above). Is this homomeric enzyme also involved in regulating leaf fatty acid synthesis? This question was addressed by a different approach toward evaluating metabolic control. Page et al (69) took advantage of the susceptibility of maize and barley plastid ACCase to the herbicides fluaxifop and sethoxidim. When chloroplasts or leaves were incubated with herbicides and radiolabeled acetate, a flux control coefficient of 0.5 to 0.6 was calculated for acetate incorporation into lipids. Flux control coefficients of this magnitude indicate strong control by the ACCase reaction over fatty acid synthesis (18, 41). Thus, in a wide variety of species and tissues, both in vivo and in vitro experiments point to ACCase as a major regulatory point for plant fatty acid synthesis.

Other Potential Rate-Limiting Steps

Although the best evidence available so far implicates ACCase activity as a primary determinant of fatty acid synthesis rates, the concept of a single rate-determining reaction is clearly an over-simplification. Control over flux is frequently shared by more than one enzyme in a pathway, and furthermore, the relative contributions of enzymes to control are variable. For example, under low irradience, only ADP-glucose pyrophosphorylase exerted strong control over CO_2 incorporation into starch, whereas under high irradience, phosphoglucoisomerase and phosphoglucomutase also exerted control (101). A similar situation may apply to fatty acid synthesis. For example, chloroplasts incubated in the light have high rates of fatty acid biosynthesis, but these rates can be further stimulated by addition of Triton-X-100. Under these conditions, malonyl-CoA and malonyl-ACP levels increase fivefold or greater, but the increase in fatty acid synthesis is only 30–80% (74). One possible implication of this observation is that under high flux conditions, biochemical regulation of ACCase may be most effective in decreasing fatty acid synthesis rates, and a large increase in ACCase activity may yield a relatively small increase in the overall flux through the pathway. These results further suggest that under high flux conditions through ACCase, the condensing enzymes or other factors may begin to limit FAS rates. All the saturated acyl-ACP intermediates of fatty acid synthesis from 4:0 to 14:0 are detected in approximately equal quantities in extracts from leaf or seed. This suggests that activities of all the KAS isoforms are approximately equal. Furthermore, it suggests that if the rate of any one of

the KAS isoforms was to increase, the effect would be small, because the other KAS isoforms would be limiting. Evidence to support this has come from the increased expression of spinach KAS III in tobacco under the control of the CaMV 35S promoter (104). KAS III activity was increased 20- to 40-fold, with no effect on either the quantity or composition of the fatty acids. Analysis of the ACP and acyl-ACP intermediates revealed that the major form of ACP in the transgenic leaves was 4:0-ACP, which suggested that KAS I was now the limiting condensing enzyme in this tissue, and prevented any significant change in the overall flux through the pathway.

FEEDBACK REGULATION

Most biochemical pathways are controlled in part by a feedback mechanism which fine-tunes the flux of metabolites through the pathway. Whenever the product of a pathway builds up in the cell to levels in excess of needs, the end product inhibits the activity of the pathway. In most cases this inhibition occurs at a regulatory enzyme which is often the first committed step of the pathway. When the activity of the regulatory enzyme is reduced, all subsequent reactions are also slowed as their substrates become depleted by mass-action. Because enzyme activity can be rapidly changed by allosteric modulators, feedback inhibition of regulatory enzymes provides almost instantaneous control of the flux through the pathway.

Evidence from leaves, isolated chloroplasts, and suspension culture cells strongly implicates ACCase as a major point of flux control. If ACCase is the valve that determines flow of carbon toward fatty acids, the key question now becomes “How is this enzyme’s activity regulated?” In animals and yeast, it has long been considered that fatty acid synthesis is partly controlled by feedback on ACCase by long-chain acyl-CoAs. Since acyl-CoAs are one end product of the FAS pathway, they were tested in vitro and found to strongly inhibit ACCase at submicromolar concentrations (29). Although this inhibition seems logical, it has been called into question by the discovery that acyl-CoA–binding proteins exist at high concentrations in the cytosol of animals (76), yeast (46), and plants (28, 34). Because these proteins have extremely high affinity for acyl-CoAs, the concentration of free acyl-CoA in the cytoplasm may be only nanomolar, a level unlikely to inhibit ACCase. These studies further emphasize the difficulty in extrapolating in vitro enzyme studies to in vivo conditions.

The in vitro analyses of plant ACCase have indicated that pH, Mg, and ATP/ADP can explain much of the light-dark modifications of ACCase activity observed in leaves (63). However, other modifications in the enzyme’s

activity are apparently involved. For example, light activation of fatty acid synthesis in chloroplasts does not require ATP synthesis by light (58) and has been reported to be stimulated by photosystem (PS) I but not PS II activity (70). In addition, Sauer & Heise (90) reported that ACCase activity was higher after rapid lysis and assay of light-incubated chloroplasts than after similar treatment of dark-incubated chloroplasts. The initial ACCase activity from chloroplasts incubated in the light is three- to fourfold higher than from either chloroplasts incubated 5 min in dark or from chloroplasts incubated in the light followed by 2 min in dark (K Nakahira & JB Ohlrogge, unpublished information). After lysis, ACCase activity from the dark-incubated chloroplasts increased until it reached light levels within 3–4 min. Thus, ACCase activity appears to be transiently inactivated or inhibited in dark-incubated chloroplasts. Because the lysis of chloroplasts into assay buffer would result in over a 100-fold dilution of stromal contents, any differences in the ACCase activity can not be attributed to contributions by the stroma contents (such as ATP) during the assay. At present there is not an explanation for this transient inactivation. Animal ACCase is known to be inactivated by phosphorylation (43). However, addition of protein kinase and phosphatase inhibitors to chloroplast lysates did not substantially alter the patterns.

In an effort to examine under in vivo conditions whether feedback inhibition of fatty acid synthesis occurs in plants, Shintani & Ohlrogge (93) added exogenous lipids to tobacco suspension cultures. The incorporation of ^{14}C-acetate into fatty acids (but not sterols) was rapidly decreased by the addition of lipids to the cultures. Thus, the cells apparently have a mechanism that senses the supply of fatty acids and responds by decreasing the de novo production of more fatty acid. Furthermore, examination of the pools of acyl-ACP intermediates indicated increases in acetyl-ACP, decreases in long-chain acyl-ACPs, and no change in medium-chain acyl-ACPs. These responses are identical to those observed when fatty acid synthesis is decreased by shifting leaves or chloroplasts to the dark and are expected if ACCase activity is decreased. Therefore, ACCase regulation can also account for the decrease of fatty acid production in response to exogenous lipids. Immunoblot analysis indicated that ACCase protein levels did not change during the feedback inhibition, indicating that biochemical controls are primarily responsible for the reduced ACCase and fatty acid synthesis activity.

What Is the Feedback System?

The first end products of plastid fatty acid synthesis are the long-chain acyl-ACPs, and therefore these seem logical candidates for feedback regulators of

fatty acid synthesis. However, when fatty acid synthesis slows in the dark or in response to exogenous lipids, the long-chain acyl-ACP pools drop significantly. Therefore, these molecules have the opposite concentration response expected from a feedback inhibitor. Furthermore, in vitro assays have failed to reveal substantial inhibition of ACCase by acyl-ACPs (80).

Acyl-ACPs may play a role in the regulation of KAS III, however. When KAS III in seed homogenates of *Cuphea* was challenged with acyl-ACPs, as little as 0.5 μM 10:0-ACP was able to cause 50% inhibition (12). Similar results were obtained using 1 μM 10:0-ACP with homogenates of *Brassica* seed or spinach leaf. In each case, maximum inhibition was observed with the 10:0-ACP, compared with longer-chain acyl-ACPs, and the source of the ACP was *E. coli.* We have incubated a purified preparation of spinach KAS III with 10:0-ACP (ACP source: spinach) at concentrations up to 0.6 μM and failed to observe any inhibition (B Hinneberg-Wolf & J Jaworski, unpublished information). This suggests that inhibition of KAS III activity observed in the plant homogenates was indirect. A corresponding study carried out using purified preparations of *E. coli* KAS III and 100-μM 16:0- or 18:1-ACP resulted in a 50% inhibition (77). Because these levels far exceed the intracellular levels observed in *E. coli*, the physiological significance of this inhibition remains to be demonstrated.

Several other potential feedback inhibitors such as acyl-CoA, free fatty acids, and glycerolipids also fail to strongly inhibit the plant ACCase at physiological concentrations (80). Because FAS occurs inside the plastid but the major utilization of the products of fatty acid synthesis is at the ER membranes, it is likely that feedback regulation must allow communication across the plastid envelope. At this time we do not have any clear indications of what molecules are involved in feedback regulation of plastid fatty acid synthesis, and their discovery remains a major challenge for plant biochemists.

Control of Substrate and Cofactor Supply

Another way to control flux through a pathway is to regulate delivery of substrates and cofactors to the pathway. In animals (30) and some oleaginous yeast (7), there is evidence that the accumulation of acetyl-CoA via the ATP:citrate lyase reaction is a major determinant of fatty acid synthesis rates. Although conclusive evidence is not yet available for plants, indications are that acetyl-CoA supply does not usually limit plastid fatty acid production. If acetyl-CoA levels were limiting rates of fatty acid production, a decrease in acetyl-CoA level would be expected during high rates of fatty acid synthesis. However, almost all CoA found in chloroplasts is in the form of acetyl-CoA,

and despite very large differences in rates of fatty acid synthesis in light or dark, acetyl-CoA levels in chloroplasts remain almost unchanged (74). In developing seeds, levels of acetyl-ACP are higher than in light-grown leaves, and the level does not change substantially throughout seed development, despite major changes in rates of FAS (73). Again, these results suggest that acetyl-CoA concentrations in seed plastids are high and do not change during development.

Although we tentatively conclude that carbon supply does not limit fatty acid production in leaves and most seeds in normal plants, there are examples of transgenic plants that may give some indication of what is required for carbon limitation to occur. The targeting of the enzymes of the polyhydroxybutyrate (PHB) pathway into the chloroplasts of *Arabidopsis thaliana* resulted in an accumulation of PHB of up to 14% dry weight of the plant (59). Synthesis of this large carbon sink also requires the plastidial acetyl-CoA pool, and yet there was no detectable deleterious effect on fatty acid biosynthesis. Thus, it was concluded that a mechanism must exist that allows the plastid to synthesize the required acetyl-CoA in response to additional metabolic demand for PHB production. In another example, over-expression of the *E. coli* ADP:glucose pyrophosphorylase in developing *Brassica napus* seeds leads to a large increase in starch content of seeds and a 50% decrease in oil content (6). One interpretation of this result is that in this case, diverting carbon to starch storage was sufficient to "starve" the fatty acid pathway for available carbon precursors.

As in the case of substrates, consideration should be given to cofactors or energy as potentially limiting under certain conditions. Fatty acid synthesis is an energy-demanding pathway that requires at least 7 ATP and 14 NAD(P)H to assemble an 18-carbon fatty acid. However, energy is usually not a limitation in overall plant growth. In chloroplasts, ATP and NADPH can be derived from photophosphorylation and electron transport. In the dark or in nongreen tissues, glycolysis and the oxidative pentose phosphate pathway can provide the ATP and reductant (1, 21).

WHAT DETERMINES HOW MUCH OIL IS PRODUCED BY A SEED?

The oil content of seeds of different plant species varies from under 4% of dry weight (e.g. *Triticum sativum*) to over 60% (e.g. *Ricinus communis*). Furthermore, whereas leaves, roots, and other vegetative tissues usually contain less than 10% lipid by dry weight, most of which is polar membrane lipids, seed lipids usually contain over 95% neutral storage lipids in the form of triacyl-

glycerol (TAG). An understanding of how plants regulate fatty acid metabolism to achieve these major differences in lipid content and composition is not yet available.

The only enzyme unique to TAG biosynthesis is diacylglycerol acyltransferase (DAGAT). Several lines of evidence suggest that tissue-specific expression of DAGAT is not sufficient to explain either the high proportion of TAG in seeds or the high level of oil in some seeds. 1. DAGAT activity is found not only in seeds but also in leaves (15, 55). 2. Certain types of stress such as ozone cause leaves to produce high proportions of TAG (87). Even detaching leaves and floating them in buffer plus 0.5 M sorbitol is sufficient to increase the accumulation of neutral lipids several fold (11). 3. Addition of fatty acids to the surface of spinach leaves results in a substantial proportion being incorporated into TAG (86). Clearly, leaves as well as seeds have the capacity for TAG synthesis, and therefore specific expression of DAGAT seems insufficient to explain abundant TAG synthesis in seeds. To understand the high proportion of TAG in seeds, it may be useful to consider whether oil synthesis is controlled by the supply (source) of fatty acids or fatty acid precursors or by the demand (sink) for fatty acids.

Control by Fatty Acid Supply (Source)

Control of oil production in seeds may reflect a response to the high rate of fatty acid synthesis in this tissue. If DAGAT is present at similar levels in most tissues, then high levels of TAG synthesis may occur in seeds because high levels of fatty acid are produced. Data that support this concept are available for *Cuphea* and *Chlamydomonas.* Addition of excess exogenous fatty acid and glycerol to developing *Cuphea* cotyledons rapidly resulted in rates of TAG accumulation several-fold higher than observed on the plant (5). In *Chlamydomonas,* addition of exogenous lipids (PC liposomes) to cultures resulted in up to 10-fold increases in TAG accumulation (32). Thus, it appears to be the fatty acid substrates, not the utilization enzymes that limit TAG synthesis in *Cuphea* and *Chlamydomonas,* and the capacity for TAG synthesis is greater than actually used. Further examples supporting this theory come from comparisons of oleaginous (oil-accumulating) versus nonoleaginous yeast. Extensive studies by Ratledge and coworkers have led to the conclusion that differences between these two types of species are controlled by the *production* rather than utilization of fatty acids (7). In particular, the production of acetyl-CoA through the action of ATP:citrate lyase is considered to control the flux of carbon into storage lipids.

Control by Demand (Sink)

Evidence that utilization of fatty acids can increase rates of fatty acid synthesis is available from experiments with *E. coli* (38, 65, 108). When a plant l2:0-ACP thioesterase is overexpressed in *E. coli* cells, fatty acid synthesis is increased substantially. Such cultures accumulate at least 10-fold more total fatty acid (most in the form of free lauric acid) than control cultures. In these experiments, the removal of the products of fatty acid synthesis to a metabolically inert end product (free fatty acid) appeared to release feedback inhibition on acetyl-CoA carboxylase, resulting in higher malonyl-CoA and fatty acid production (65). In a completely different type of experiment, massive overexpression of cloned membrane proteins resulted in no change of the membrane's phospholipid to protein ratio, but rather more fatty acid was produced to accommodate the excess proteins (110, 112). Therefore, the rate of fatty acid synthesis can apparently be regulated by the demand for fatty acids needed for membrane synthesis. Extrapolating this concept to plant seeds: Perhaps fatty acid synthesis increases in response to the high demand for acyl chains brought on by the depletion (or increase) of a key metabolic intermediate. A hypothetical example to illustrate one possibility might be that high expression of DAGAT (or some other acyl transferase) leads to a depletion of acyl-CoAs. This could in turn lead to release of feedback inhibition (probably indirectly) of ACCase by acyl-CoA and thus provide a mechanism whereby removal of acyl-CoA by the acyltransferases could stimulate fatty acid production.

Additional recent evidence suggests that both of the mechanisms above may be involved in determining seed oil content. When a transit peptide is added to the cytosolic ACCase of Arabidopsis and this chimeric gene is expressed in *B. napus* under control of the napin promoter, the oil content of the seeds is increased approximately 5% (79). Thus, increasing the supply of fatty acids at the first step in the pathway leads to increased oil content. Even larger increases (>20%) in Arabidopsis and *B. napus* seed oil content were achieved when a yeast lysophosphatidic acid acyltransferase (LPAAT) was expressed in developing seeds (114). Because overexpression of coconut (60) or *Limnanthes* (50) LPAAT did not alter oil content, the ability of the yeast enzyme to increase oil may involve its lack of regulation in the plant host.

Analysis of developing *B. napus* seeds expressing high levels of the California Bay 12:0-ACP thioesterase (MCTE) provided intriguing and surprising results that emphasize how much we need to learn before we have a good understanding of what determines the amount of oil in a seed (see Figure 2). Voelker et al (109) found that increased expression of the MCTE in seeds

resulted in linear increases of lauric acid (12:0) in TAG up to ~35%. However, for quantities beyond 35%, the correlation with MCTE began to reach a plateau. To achieve 60 mol% 12:0, a further 10-fold increase in MCTE expression was required. At high levels of MCTE, expression of fatty acid β-oxidation as well as enzymes of the glyoxylate cycle were induced (68). Thus, these seeds appear to produce more 12:0 than can be metabolized to TAG by the *Brassica* acyltransferases or other enzymes whose normal substrates are C16 and C18 fatty acids, and the excess 12:0 signals the induction of the catabolic pathway. Thus, a portion of the fatty acid synthesis in these seeds is involved in a futile cycle of synthesis and breakdown of 12:0. Surprisingly, despite induction of fatty acid β-oxidation, the total amount of TAG in these MCTE-expressing seeds is not substantially reduced. How is oil content maintained if a significant amount of the lauric acid produced by FAS is being broken down in a futile cycle? Analysis of the fatty acid biosynthetic enzymes revealed that the levels of ACP and several of the fatty acid biosynthetic enzymes (acetyl-CoA carboxylase, 18:0-ACP desaturase, KAS III, etc) had increased two- to threefold at midstage development of high MCTE-expressing seeds. These results have several important implications. First, a coordinate induction of the enzymes of the fatty acid pathway had occurred, presumably to compensate for the lauric acid lost through β-oxidation or the shortage of long-chain fatty acids in these seeds. This suggests that the enzymes for the entire FAS pathway may be subject to a system of global regulation perhaps similar to lipid biosynthesis genes of yeast (16, 91). Second, these results indicate that although *B. napus* seeds are relatively high in oil content (ca 40%) the expression of the FAS enzymes is not at a maximum and can be induced a further two- to threefold over the levels found at midstage of seed development. Finally, the results suggest that these seeds might be preprogrammed to produce a particular amount of oil, and the levels of expression the fatty acid pathway may adjust to meet the prescribed demand for TAG synthesis.

What determines the level of TAG in these cells and what are the signals that result in increased expression of the FAS pathway? One major difficulty with interpreting the above data occurs if we attempt to fit it to the minimal model comprised of the currently understood roles of the pathway enzymes. However, recent results clearly indicate that there are still major gaps in our understanding of lipid and TAG biosynthesis. Analysis of the pathway for petroselinic acid ($18:1\Delta^6$) from 16:0 in coriander indicated that this plant has evolved a specialized 3-ketoacyl-ACP synthase (KAS), not found in other plants, that has high activity with $16:1\Delta^4$-ACP (13). Similarly, a KAS from *Cuphea wrightii* that has homology to plant KAS II was coexpressed with the *C. wrightii* MCTE in Arabidopsis, and this resulted in dramatic increase in the

levels of 10:0 and 12:0 in the seeds compared with seeds expressing the MCTE alone. Both studies suggest that there are additional specialized enzymes for seed lipid metabolism that were not predicted on the basis of previous biochemical understanding. At this time there is no clear idea how these enzymes interact or what additional specialized functions they may have in oil synthesis. Presumably other enzymes or regulatory interactions are yet to be discovered that have a role in determining the quantity as well as quality of the oil.

WHAT HAVE WE LEARNED FROM TRANSGENIC PLANTS AND MUTANTS?

Fatty Acid Composition of Seeds Can Be More Radically Altered Than Other Tissues

The composition of fatty acids produced in plants is primarily determined by thioesterases, condensing enzymes, and desaturases. Manipulation of the thioesterases and desaturases in transgenic plants has been highly successful in producing major modifications of the chain length and level of unsaturation of plant seed oils (for reviews, see 44, 57, 66, 105). An additional new insight into regulation of fatty acid metabolism was recently obtained from expression of enzymes producing unusual fatty acids in other nonseed tissues. For example, transgenic expression of the 12:0-ACP thioesterase in *B. napus* under control of the constitutive 35S promoter resulted in lauric acid production in the seeds but not in leaves or other tissues (27). However, chloroplasts isolated from the *B. napus* leaves produced up to 35% lauric acid. These results indicate that some tissues expressing MCTE produce lauric acid but that it is subsequently degraded. In support of this hypothesis, the activity of isocitrate lyase of the glyoxylate cycle was induced in leaves expressing the California Bay thioesterase. A similar mechanism may apply to the production of hydroxy fatty acids which accumulate in seeds but not leaves of Arabidopsis transformed with the castor oleate hydroxylase under control of the 35S promoter (8). Together, these results imply that nonseed tissues may have general mechanisms to degrade unusual or excess fatty acids and thereby prevent their incorporation into membranes.

Manipulation of Oil Quantity

Plant breeding and mutation studies have demonstrated that the amount of oil in a seed can be varied over a wide range. A classic example is the selection for high and low oil maize that over a period of almost 100 years resulted in

lines ranging from 0.5% to 20% lipid (25). Arabidopsis mutants with both increased seed oil (36) and reduced triacylglycerol (42) have been reported. In the latter example, not only was oil content reduced but 18:3 levels were doubled, 18:1 and 20:1 levels were reduced, and several enzymes of lipid metabolism had altered activity. These pleiotropic effects of a single gene mutation illustrate the complexities and inter-relationships of lipid metabolism which are difficult to explain based on our current models of seed oil biosynthesis.

Many Enzymes of Fatty Acid Synthesis Are Present in Excess

Although there has been much success in manipulating chain length and unsaturation of plant seed oils in transgenic plants, directed alterations in oil quantity are just beginning to be achieved. A number of the core enzymes of fatty acid synthesis have been overexpressed or underexpressed in transgenic soybean or *B. napus* seeds (45). Overexpression of ACP, KAS III, KAS I, KAS II, oleoyl-ACP thiosterase (FatA), or saturate-preferring acyl-ACP thioesterase (FatB) individually has not resulted in increased seed oil content. It is not known whether increased expression of combinations of these components might be more effective. As discussed above, increased ACCase and a yeast acyltransferase have been reported to increase oil in *B. napus* seeds.

Complete suppression of any of the core enzymes of FAS would be expected to reduce fatty acid synthesis and seed oil content. In support of this, cosuppression of FatA or KAS I in soybean resulted in reduced embryo oil content (45), and antisense of enoyl-reductase in *B. napus* gave shrunken seeds (113). Antisense of the tobacco biotin carboxylase under control of the 35S promoter was found to result in stunted plants with a 26% reduction in leaf fatty acid content (92). However, these effects were only observed when the reduction in BC level was 80% or greater. At 50% reductions in BC, no phenotype could be detected. Similar results were obtained with antisense of stearoyl-ACP desaturases (47). Many other experiments on antisense of enzymes of plant carbohydrate metabolism have also found that phenotypes (if observed at all) only occur when enzyme level is reduced 80% or more (102). Furthermore, the impact of an enzyme's reduction is usually dependent on growth conditions. These results suggest that under most conditions, many of the enzymes of plant metabolism are present in functional excess. Additional evidence that enzymes of lipid metabolism are expressed in excess comes from crosses of mutants. For several mutants of Arabidopsis fatty acid desaturation (*fad2*, *fad5*, and *fad6*), crosses with wild-type give a near wild-type phenotype rather than a fatty acid composition intermediate between parents. Thus, for

several desaturases and other enzymes, gene dosage is not the primary determinant of the enzymes activity, and more complex controls must operate in vivo. In the case of highly regulated enzymes such as ACCase, a variety of mechanisms, such as increased enzyme activation, may compensate for reductions in expression brought on by antisense or mutation.

CONTROL OF GENE EXPRESSION

Multigene Families

A puzzling aspect of the molecular biology of plant fatty acid synthesis is the role of multiple genes. Even within Arabidopsis with its small genome, there is considerable range in the number of genes encoding the different proteins of plant fatty acid synthesis. Acyl carrier protein and the 18:0-ACP desaturases are each encoded by at least five genes, whereas many other enzymes are encoded by a single gene. The expression of the ACP genes has been studied in some detail, and both constitutive and tissue-specific patterns of expression have been observed (3, 35). In several other plant species, with genomes larger than Arabidopsis, tissue-specific desaturases and other enzymes are known to control seed fatty acid composition (67). Although it might be considered advantageous to have multiple genes to allow fine tuning of expression in different tissues, it is clear that this is not essential for many of the genes of fatty acid metabolism as several of the proteins in the pathway are encoded by a single gene. In the case of the glycerolipid desaturases, one gene encodes a plastid isozyme, whereas a second gene encodes a presumably ER localized isozyme. However, two genes encode the 18:2 desaturase of plastids, one of which is temperature regulated (10).

Promoter Analysis

Currently, the promoter of acyl carrier protein genes has been examined in the greatest detail. de Silva et al (22) fused 1.4 kB of a *B. napus* ACP gene (ACP05) to β-glucuronidase (GUS) and determined expression levels in transgenic tobacco. GUS activity increased during seed development, concurrent with lipid synthesis and at its maximum was 100-fold higher than in leaves. Surprisingly, although ACP is not an abundant protein, the activity of the ACP/GUS construct was comparable to that obtained from the strong 35S promoter. Several constructs of a promoter from another Arabidopsis ACP gene, *Acl1.2*, have been fused to GUS and examined after transformation into tobacco (4). Fluorometric analysis indicated strongest expression in developing seeds. However the promoter was active at lower levels in all organs

(approx. 50 fold lower in leaves). Histochemical analysis indicated highest *Acl1.2* expression in the apical/meristematic regions of vegetative tissues. During initial flower development, *Acl1.2* promoter activity was detected in all cell types, but as the flower matured, GUS staining was lost in the sepals, epidermis of the style, and most cells of the anther. Intense staining remained in the ovary, stigma, stylar transmitting tissue, and tapetal and pollen of the anther. Thus, the expression pattern of this particular gene appears complex. Similar analysis of other promoters is needed to determine whether common signals are responsible for such patterns.

Six deletions of the *Acl1.2* promoter revealed distinct regions of the promoter involved in vegetative and reproductive development. Expression of *Acl1.2* in young leaves dropped to a basal level when an 85-bp domain from −320 to −236 was removed, but expression in seeds was not altered by this deletion. A protein factor was detected in leaves and roots, but not seeds, which binds to the −320 to −236 domain. Seed expression was reduced ~100-fold when the −235 to −55 region was removed. This same region was also essential for high expression in the flower tissues described above.

2.2 kB of a *B. napus* stearoyl-ACP desaturase promoter was fused to GUS (98). Expression was approximately 2.5-fold higher in developing seeds than in young leaves and thus did not show the dramatic differences reported for the ACP promoters above. However, similar to the ACP promoters, strong activity was also observed in tissues undergoing rapid development, including immature flowers, tapetum, and pollen grains.

Recently the enoyl-ACP reductase promoter of Arabidopsis has undergone similar GUS fusion and deletion analysis (20). Unlike ACP and stearoyl-ACP desaturase, there appears to be only a single gene in Arabidopsis encoding the enoyl-ACP reductase. High expression of enoyl-ACP reductase promoter-GUS fusions was again observed in youngest leaf tissues, with vascular tissue and shoot or root apical meristems highest. As each new leaf matured, GUS activity faded. Three domains of the promoter were identified. Seed expression was unchanged by deletion to −47 bp of the transcription start site, indicating that all elements needed for high level seed expression are present in this relatively small region. Removal of an intron in the 5′ untranslated region resulted in increased expression in roots, suggesting the presence of negative regulatory elements in this region.

Although the studies described above suggest that quantitative differences occur between relative expression levels of ACP, stearoyl-ACP desaturase, and enoyl-ACP reductase promoters in different tissues, all of the analyses of FAS promoters so far have reached similar conclusions about highest expression in apical meristems, developing seeds, and flowers. Such a pattern is not

surprising, because these tissues are the most rapidly growing or are producing lipids in high amounts for storage. Furthermore, expression of the mRNA for most components of FAS probably is under coordinant control. For example, in situ hybridization of the mRNA for biotin carboxylase, BCCP, and carboxyltransferase subunits of Arabidopsis ACCase indicates close coordination of these three subunits, which is coincident with oil deposition (62). It should also be emphasized that promoters of lipid biosynthetic proteins are likely not unusual in these expression patterns. Highest expression in rapidly growing cells and coordinant regulation would be expected for promoters involved in most primary biosynthetic pathways.

What Controls Promoter Activity of FAS Genes?

A major challenge for the future is to discover how the level of expression of genes of lipid synthesis is controlled. The initial studies of promoters reviewed above have identified domains involved in control of gene expression levels. Efforts are under way to identify transcription factors that may bind to these elements. In addition, genetic approaches to search for mutants with altered regulation of fatty acid metabolism may allow identification of additional controls. By analogy to the study of other organisms, the control of plant FAS genes is likely to involve a complex array of *cis* and *trans* acting factors. For example, promoters of animal fatty acid biosynthetic genes are controlled by hormones such as insulin (56), by dietary fatty acids (78), by glucose levels (75), and by differentiation [particularly to adipocytes (54)]. Repression of transcription by negative regulatory elements has been suggested for both yeast (16) and animal lipid biosynthetic genes (103), and in *Saccharomyces*, common DNA sequences have been identified in the promoters of many genes involved in lipid metabolism (16, 91). As with genes of the glyoxylate and many other pathways, transcription of plant FAS genes is likely dependent on both developmental and metabolic signals (31). Because all indications are that the enzymes of fatty acid synthesis are coordinately regulated, it seems probable that global transcriptional signals may control expression of many or all genes of the pathway [perhaps similar to the R and C locus products that control transcription of the anthocyanin biosynthetic pathway (53)]. Identification of such global controls may provide the most effective means toward manipulating the amount of fatty acid produced in transgenic plants. If metabolic control over oil synthesis is shared among several enzymes, there will be limits to how much the flux through this pathway can be manipulated in transgenic plants using overexpression of one or a few genes. Thus, more

complete control may come from identifying transcription factors that can increase expression of the entire pathway.

SUMMARY AND PERSPECTIVES

Fatty acid synthesis is a primary metabolic pathway essential for the function of every plant cell. Its products serve as the central core of membranes in every plant cell, and in specialized cells, fatty acids or fatty acid derivatives act as signal or hormone molecules, as carbon and energy storage, and as a surface layer protecting the plant from environmental and biological stress. Despite these very diverse functions, essentially all fatty acids in a cell are produced from a single set of enzymes localized in the plastid. Understanding how cells regulate the production of these fatty acids and direct them toward their different functions is thus central to understanding a large range of fundamental questions in plant biology. In addition, much interest has recently developed in genetic engineering of the fatty acid biosynthetic pathway to produce new or improved vegetable oils and industrial chemicals. Therefore, knowledge of how cells control the amount of fatty acid they produce may be essential for optimal commercial production of fatty acids.

Our understanding of regulation of fatty acid metabolism is much less developed than that of carbohydrate or amino acid biosynthetic pathways. We now have convincing evidence that ACCase is one enzyme that is involved in regulating fatty acid synthesis rates, and there are indications for other control points. However, this is only the beginning. ACCase might be considered one "slave" enzyme that controls flux into fatty acids but whose activity is dependent upon higher level master control systems in the cell. But what molecules regulate ACCase by feedback or other mechanisms and what metabolic signals or mechanisms control those molecules? How is the global regulation of dozens of genes for lipid synthesis accomplished? As discussed in this review, we have only fragmentary information about the nature of these controls. Thus, understanding regulation of fatty acid synthesis is a rich and relatively unexplored field with much work left to be done.

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