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Lipid Biosynthesis

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INTRODUCTION

Lipids are an essential constituent of all plant cells. The vegetative cells of plants contain ~5 to 10% lipid by dry weight, and almost all of this weight is found in the membranes. Although each square centimeter of a plant leaf may contain only 0.2 mg of lipid, this amount can account for ~400 cm^2 of membrane, reflecting the fact that membrane lipids are arranged in layers just two molecules thick (5 to 8 nm). If a leaf mesophyll cell were expanded a million times, the membranes would still be less than 1 cm thick. Despite their slight dimensions, however, the lipid membranes are the major barriers that delineate the cell and its compartments, and they form the sites where many essential processes occur, including the light harvesting and electron transport reactions of photosynthesis.

Some plant cells produce much more lipid than does a leaf mesophyll cell. Lipids are the major form of carbon storage in the seeds of many plant species, constituting up to ~60% of the dry weight of such seeds. Epidermal cells produce cuticular lipids that coat the surface of plants, providing the crucial hydrophobic barrier that prevents water loss and also forming a protection against pathogens and other environmental stresses. In addition to the abundant cellular lipids, minor amounts of fatty acids are important as precursors to the hormone jasmonic acid and in acylation of certain membrane proteins.

Unlike the other major constituents of plants (proteins, carbohydrates, and nucleic acids), lipids are defined on the basis of their physical properties rather than their common chemical structure. Thus, lipids are often loosely defined as those compounds that are insoluble in water and that can be extracted from cells by nonpolar organic solvents (such as chloroform). As such, this class of compound is extremely diverse in structure and actually constitutes the products of several distinct biosynthetic pathways. The most abundant types of lipid in most cells, however, are those that derive from the fatty acid and glycerolipid biosynthetic pathway, and these lipids constitute the major subject of this article. Other recent reviews include Ohlrogge et al. (1993b), Kinney (1994), Miquel and Browse (1994), and Töpfer et al. (1995).

Other classes of lipid include many types of compounds derived from the isoprenoid pathway. Over 25,000 different isoprenoid-derived compounds have been described in the plant kingdom, making this probably the richest store of chemical structures in the biosphere. Most of these compounds are considered "secondary" metabolites because they are not found in all cells and are probably not essential to cell growth. However, the sterols, gibberellins, abscisic acid, and the phytol side chain of chlorophyll are also derived from this pathway. A recent book by Moore (1993) and articles in this issue by Bartley and Scolnick (1995) and McGarvey and Croteau (1995) provide more detailed information on some of these other lipid classes.

The fatty acid biosynthesis pathway is a primary metabolic pathway, because it is found in every cell of the plant and is essential to growth. Inhibitors of fatty acid biosynthesis are lethal to cells, and no mutations that block fatty acid synthesis have been isolated. The major fatty acids of plants (and most other organisms) have a chain length of 16 or 18 carbons and contain from one to three *cis* double bonds. Five fatty acids (18:1, 18:2, 18:3, 16:0, and in some species, 16:3) make up over 90% of the acyl chains of the structural glycerolipids of almost all plant membranes (Figure 1).

Fatty acids in cells are almost never found in the form of "free" fatty acids. Instead, their carboxyl group is esterified or otherwise modified. In membranes, almost all the fatty acids are found esterified to glycerol; this class of lipid is termed glycerolipids. Membrane glycerolipids have fatty acids attached to both the sn-1 and sn-2 positions of the glycerol backbone and a polar headgroup attached to the sn-3 position (Figure 1). The combination of nonpolar fatty acyl chains and polar headgroups leads to the amphipathic physical properties of glycerolipids, which are essential to formation of membrane bilayers. If all three positions on glycerol are esterified with fatty acids, a "triacylglycerol" structure results that is not suitable for membranes but instead constitutes the major form of lipid storage in seeds. The cuticular lipids, which are found on the surface of all terrestrial plants (von Wettstein-Knowles, 1993), contain cutin, which is a polymer of primarily 16- and 18-carbon hydroxy fatty acids cross-linked by esterification of their carboxyl groups to hydroxyl groups on neighboring acyl chains. Wax esters in the cuticular lipids are formed by esterification of fatty acids to fatty alcohols. Finally, many fatty acids are reduced to aldehydes and alcohols that remain embedded in the complex cuticular lipid matrix.

Although fatty acids are major constituents of every membrane in a cell and are also found outside cells in the cuticular

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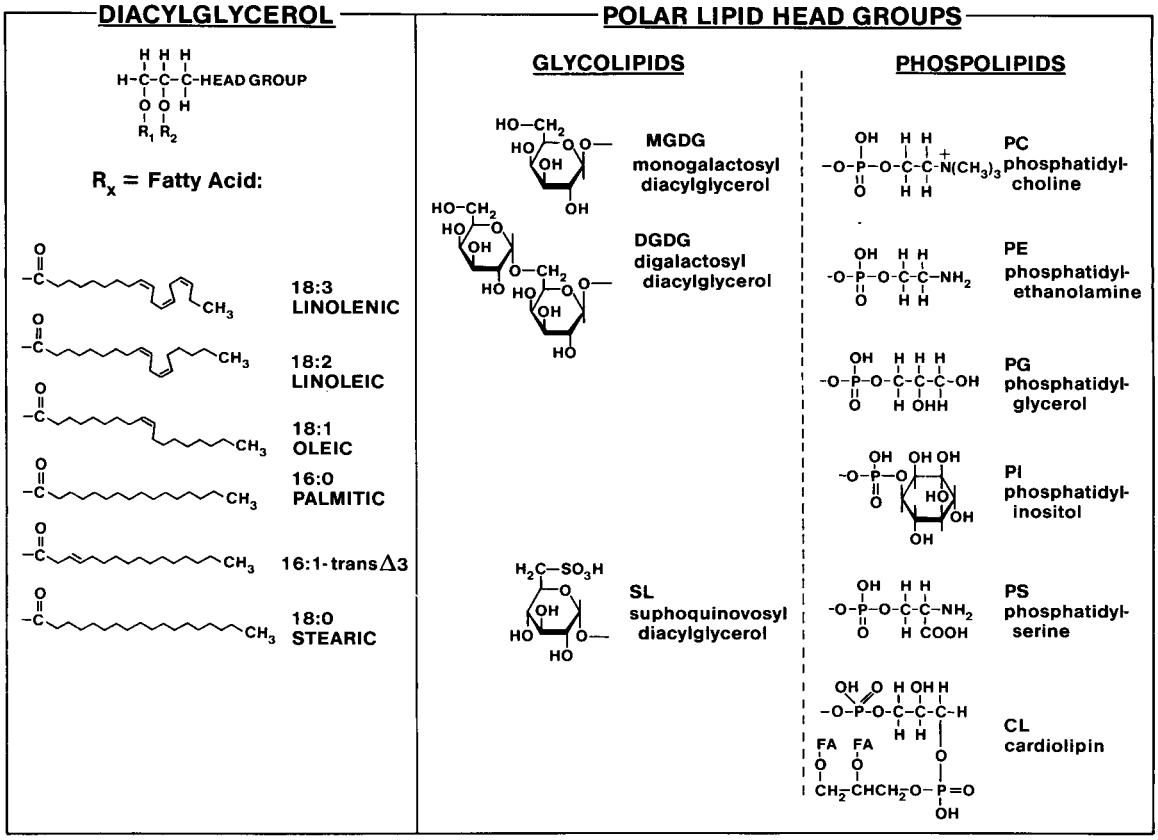

Figure 1. Structures of the Major Fatty Acids and Glycerolipids of Plant Cell Membranes.

The fatty acid and glycerolipid structures are arranged in approximate order of their abundance in plant leaves. Note that the fatty acids are referred to by the number of carbon atoms (before the colon) and the number of double bonds (after the colon).

lipids, their major site of synthesis is within the plastid. In this regard, the process of lipid biosynthesis in plants is fundamentally different from that in animals and fungi, which produce fatty acids primarily in the cytosol. The plastid localization of fatty acid synthesis means that unlike animals and fungi, plants must have mechanisms to export fatty acids from the plastid to other sites in the cell. Furthermore, there must be mechanisms by which the rest of the cell controls the production and export of fatty acids from the plastid. How the demand for fatty acids for assembly of extraplastidial lipids is communicated to the plastid is a major unknown in plant lipid metabolism.

SUBSTRATES FOR FATTY ACID SYNTHESIS

All the carbon atoms found in a fatty acid are derived from the pool of acetyl-coenzyme A (CoA) present in the plastid. The concentration of acetyl-CoA in chloroplasts is only 30 to 50 μM (Post-Beittenmiller et al., 1992), which is sufficient to supply the needs of fatty acid synthesis for only a few seconds. Nevertheless, acetyl-CoA pools remain relatively constant, even when rates of fatty acid synthesis vary greatly, as in light (when synthesis is relatively high) and dark (when synthesis is low). Thus, a system must be available that rapidly produces acetyl-CoA in the plastid for fatty acid production.

A major unresolved question in plant metabolism is how this pool of acetyl-CoA is generated. The most straightforward pathway would be through the action of plastidial pyruvate dehydrogenase (PDH) acting on pyruvate, either derived from the glycolytic pathway or perhaps produced as a side reaction of ribulose bisphosphate carboxylase activity (Andrews and Kane, 1991). However, this route has been questioned on several grounds. First, although PDH activities are generally high in nongreen plastids, PDH activity in isolated chloroplasts of some species is insufficient to account for rates of fatty acid synthesis (Lernmark and Gardeström, 1994, and references therein). In addition, chloroplasts contain an extremely active acetyl-CoA synthetase, and free acetate has been found superior to pyruvate and other substrates as a precursor of fatty acid synthesis by isolated chloroplasts (reviewed by Roughan and Slack, 1982). These considerations have led to suggestions

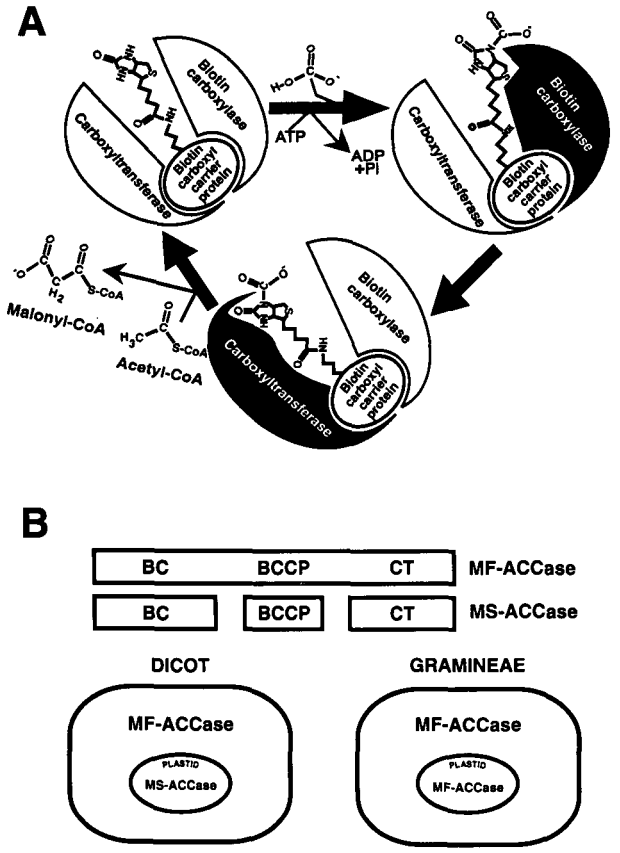

Figure 2. The Acetyl-CoA Carboxylase Reaction.

(A) ACCase has three functional regions: biotin carboxylase, which activates CO_2 by attaching it to biotin in an ATP-dependent reaction; biotin carboxyl carrier protein; and carboxyltransferase, which transfers activated CO_2 from the biotin carboxylase region to acetyl-CoA, producing malonyl-CoA.

(B) Two forms of ACCase occur in plants. A multifunctional structure (MF-ACCase) has the three functional regions shown in **(A)** encoded in a single, large (>200 kD) polypeptide. A multisubunit structure (MS-ACCase) consists of three or more subunits that form a large complex. The MF-ACCase is believed to occur in the cytosol of dicots and in both the plastid and cytosol of graminaceous plants. The MS-ACCase occurs in the plastids of most other plants, including all dicots examined so far.

of a number of alternate pathways, including production of acetyl-CoA by a mitochondrial PDH followed by transport of free acetate or acetylcarnitine to the plastid. Free acetate entering plastids is activated to acetyl-CoA by acetyl-CoA synthetase, an enzyme with 5- to 15-fold higher activity than the in vivo rate of fatty acid synthesis (Roughan and Ohlrogge, 1994). In addition, cytosolic malate and glucose-6-phosphate have been proposed as precursors of the plastid acetyl-CoA pool in oilseeds (Smith et al., 1992; Kang and Rawsthorne, 1994).

Thus, our understanding of how carbon moves from photosynthesis into acetyl-CoA is clouded by an abundance of potential pathways. Furthermore, essentially all of the suggestions on the origin of plastidial acetyl-CoA are based on in vitro analyses of enzyme activities or precursor incorporations. In no case do in vivo data address how photosynthate is metabolized to produce acetyl-CoA for fatty acid synthesis. Because of the central role of acetyl-CoA in many metabolic pathways, it is likely that more than one pathway may contribute to maintaining the acetyl-CoA pool, and which pathway is used may vary with tissue, developmental stage, light/dark conditions, and species.

STRUCTURE AND ROLE OF ACETYL-COA CARBOXYLASE

The enzyme acetyl-CoA carboxylase (ACCase) is generally considered to catalyze the first reaction of the fatty acid biosynthetic pathway—the formation of malonyl-CoA from acetyl-CoA and CO_2. This reaction actually takes place in two steps, which are catalyzed by a single enzyme complex, as shown in Figure 2A. In the first reaction, which is ATP dependent, CO_2 (from HCO_3^-) is transferred by the biotin carboxylase portion of ACCase to a nitrogen of a biotin prosthetic group attached to the ε-amino group of a lysine residue. In the second reaction, catalyzed by the carboxyltransferase, the activated CO_2 is transferred from biotin to acetyl-CoA to form malonyl-CoA.

The structure of the plant ACCase has been a subject of considerable confusion in the past, but recent evidence from several laboratories is starting to provide new insights into the organization of this complex key regulatory enzyme (Figure 2B). The confusion arose in large part because plants contain different forms of the enzyme, one of which easily loses activity during attempts to characterize it.

It is now understood that there are at least two different types of ACCase structures. In one type of organization (frequently referred to as prokaryotic), ACCase consists of several separate subunits assembled into a 700-kD complex (Sasaki et al., 1993; Alban et al., 1994). At present, we know some details about three of the subunits. The biotin carboxylase is an ~50-kD polypeptide that is nuclear encoded (Shorrosh et al., 1995). The biotin carboxyl carrier protein (BCCP) is a 34- to 38-kD protein that is almost certainly also nuclear encoded. A gene for a third subunit (~65 to 80 kD) has been identified in the plastid genome by its homology to one of the carboxyltransferase subunits of *Escherichia coli* ACCase. This is the only component of plant lipid metabolism known to be encoded in the plastid genome. Furthermore, it may be unusual among plastome-encoded proteins in that its expression does not seem highly regulated by light. Antibodies to this carboxyltransferase subunit inhibit ACCase activity and coprecipitate the BCCP subunit (Sasaki et al., 1993). This result indicates that, unlike in *E. coli*, the separate subunits associate in a complex whose components can be coprecipitated. At present, it is not clear if the three known subunits are sufficient to produce an active

ACCase complex. Most likely, other components of the ACCase complex exist that have yet to be characterized, because even dimers of the described subunits do not add up to a 700-kD complex. The 700-kD complex remains associated during gel filtration experiments, but attempts by several groups to purify it to homogeneity have resulted in the loss of activity, presumably due to dissociation of the subunits. An intensive research effort is currently under way to characterize further the structure of this complex.

In the second type of ACCase organization, the three components of the reaction are present on a single large multifunctional polypeptide. This structure is termed eukaryotic because it is similar to that found in the cytosol of yeast and animals. Several genes and cDNA clones have been isolated for this type of ACCase from plants, animals, and fungi, all of which encode proteins with the biotin carboxylase domain at the N terminus, the BCCP domain in the middle, and the carboxyltransferase at the C terminus.

The two ACCase isozymes have several important differences in their biochemical properties. The multifunctional enzyme has a much lower K_m for acetyl-CoA than the multisubunit complex and has the ability to carboxylate propionyl-CoA at substantial rates, which the multisubunit complex does not. In addition, the multifunctional enzyme is sensitive to several important herbicides of the aryloxyphenoxy–propionic acid and cyclohexane–1,3-dione classes that have no effect on the multisubunit ACCase.

Which type of ACCase structure is present depends on its subcellular localization and the type of plant. Dicots have both types of enzyme. The prokaryotic multisubunit form is found in plastids, whereas the eukaryotic multifunctional polypeptide structure occurs outside the plastids, most likely in the cytosol. The plastidial isozyme of ACCase is involved primarily, if not exclusively, in supplying malonyl-CoA for de novo fatty acid synthesis. A second isozyme of ACCase is presumably needed in the cytosol to supply malonyl-CoA for a variety of pathways, including fatty acid elongation for cuticular lipid production and flavonoid biosynthesis. Both pathways are found primarily in leaf epidermal cells, and the epidermis is indeed the main location of the multifunctional ACCase in leaves (Alban et al., 1994). In addition, the elongation of oleic acid (C18) to erucic acid (C22) is a major malonyl-CoA–dependent pathway in some oilseeds, such as *Brassica napus*. This elongation occurs outside the plastid and presumably depends on the cytosolic ACCase isozyme. Finally, substantial concentrations of malonic acid that may derive from a cytosolic ACCase isozyme occur in the leaf and root of soybean (Stumpf and Burris, 1981). Although a cytosolic location seems most reasonable for the multifunctional ACCases that have been cloned from dicots (Shorrosh et al., 1994), such a location has yet to be demonstrated directly.

Although the evidence is still fragmentary, many monocots share with dicots the occurrence and localization of the two types of ACCase. However, the Gramineae family of plants is different in that both the plastid and cytosolic ACCase isozymes are large multifunctional polypeptides (Egli et al., 1993; Konishi and Sasaki, 1994). Coincident with this evolutionary difference, the chloroplast genomes of rice and maize have lost the gene that encodes the putative carboxyltransferase subunit of the prokaryotic-type ACCase. The difference in ACCase organization in the Gramineae has now provided an explanation for the action of the grass-specific herbicides, which inhibit only the eukaryotic form of the enzyme (Konishi and Sasaki, 1994). Although both the cytosolic and plastid eukaryotic ACCases are inhibited by these herbicides, the plastid form in the Gramineae is much more sensitive than is the cytosolic form, and the plastid fatty acid synthesis pathway is more essential to growth than are the secondary pathways dependent upon the cytosolic ACCase.

REGULATION OF FATTY ACID SYNTHESIS

In animals, yeast, *E. coli*, and plants, ACCase is a regulatory enzyme that controls, at least in part, the rate of fatty acid synthesis. Light/dark regulation of ACCase activity is responsible for the light/dark modulation of fatty acid synthesis rates of spinach leaves (Post-Beittenmiller et al., 1991, 1992). In addition, fatty acid synthesis in tobacco suspension cells is subject to feedback inhibition by lipids provided exogenously in the media, and this feedback appears to act at the level of ACCase activity (Shintani and Ohlrogge, 1995). Although the regulatory role of ACCase is well established in some tissues, several important questions remain about how flux through the fatty acid synthesis pathway is controlled.

(1) What regulates ACCase activity? Although ACCase activity may determine the rate of fatty acid synthesis, understanding the regulation of lipid metabolism requires an understanding of what factors control ACCase. In animals and fungi, ACCase is regulated by several biochemical mechanisms, including phosphorylation, activation by citrate, and feedback inhibition by acyl-CoA. None of these mechanisms has yet been shown to occur in plants; nevertheless, clearly biochemical regulation occurs. The rate of fatty acid synthesis in leaves is six-fold higher in the light than in the dark. Although part of the light/dark control in vivo is likely to arise from alterations in cofactor supply, ACCase rapidly extracted from light-incubated chloroplasts is two- to fourfold more active than that from dark-incubated chloroplasts, even when in vitro conditions and cofactors are identical (Ohlrogge et al., 1993a). At present, we have no explanation for this difference in activity.

(2) What other enzymes control the flux of fatty acid synthesis? ACCase may be only one of a number of enzymes that can be considered rate limiting. The condensing enzymes (see later discussion), in particular, 3-ketoacyl-ACP synthase III (KAS III), are also logical control points. In some metabolic pathways, control is spread over several regulatory enzymes, and the flux control coefficient of each varies with the conditions. Now that clones are available for ACCase and most of the other enzymes of fatty acid synthesis, transgenic plant experiments

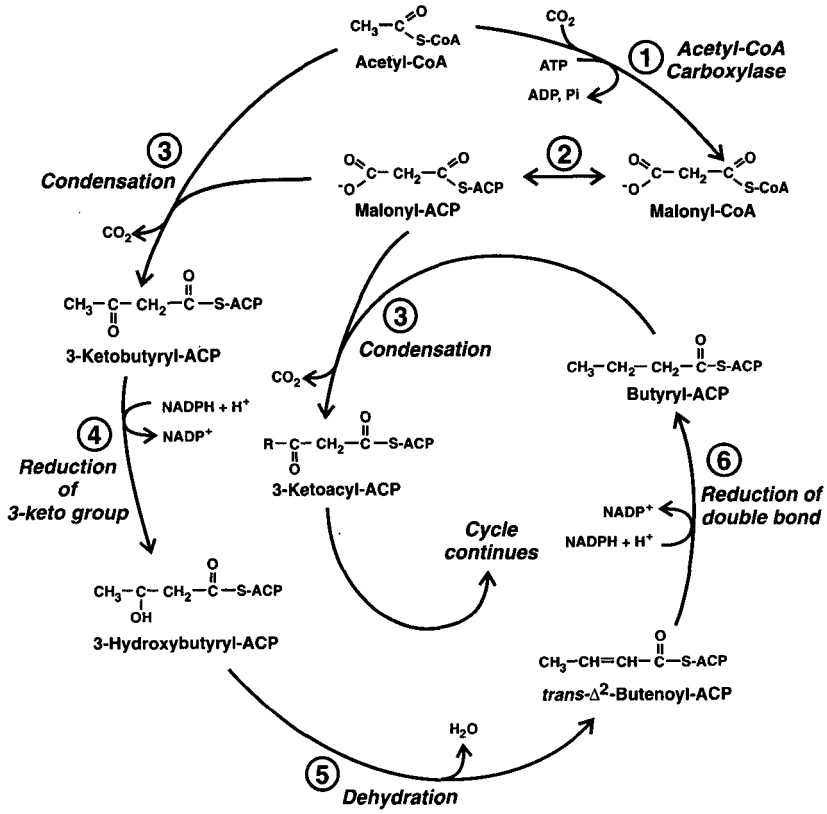

Figure 3. The Reactions of Saturated Fatty Acid Biosynthesis.

Acetyl-CoA is the basic building block of the fatty acid chain and enters the pathway both as a substrate for acetyl-CoA carboxylase (reaction 1) and as a primer for the initial condensation reaction (reaction 3). Reaction 2, catalyzed by malonyl-CoA:ACP transacylase, transfers malonyl from CoA to form malonyl-ACP, which is the carbon donor for all subsequent elongation reactions. After each condensation, the 3-ketoacyl-ACP product is reduced (reaction 4), dehydrated (reaction 5), and reduced again (reaction 6), by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, respectively.

will provide crucial in vivo tests of the role of each enzyme in controlling flux through the pathway.

THE FATTY ACID SYNTHESIS PATHWAY

Plants are fundamentally different from other eukaryotes in the molecular organization of the enzymes of fatty acid synthesis. Overall, to produce a 16- or 18-carbon fatty acid from acetyl-CoA and malonyl-CoA, at least 30 enzymatic reactions are required. In animals, fungi, and some bacteria, all of these reactions are catalyzed by a multifunctional polypeptide complex located in the cytosol. In plants, the individual enzymes of the pathway are dissociable soluble components located in the stroma of plastids. Although the component enzymes of plant fatty acid synthesis can be separated easily in vitro, an intriguing question is whether they may be organized in vivo into a supramolecular complex.

The central carbon donor for fatty acid synthesis is the malonyl-CoA produced by ACCase. However, before entering the fatty acid synthesis pathway, the malonyl group is transferred from CoA to a protein cofactor, acyl carrier protein (ACP). From this point on, all the reactions of the pathway involve ACP until the 16- or 18-carbon product is ready for transfer to glycerolipids or export from the plastid (Figure 3). ACP is a small (9 kD) acidic protein that contains a phosphopantethein prosthetic group to which the growing acyl chain is attached as a thioester. After transfer to ACP, the malonyl-thioester enters into a series of condensation reactions with acyl-ACP (or acetyl-CoA) acceptors. These reactions result in the formation of a carbon–carbon bond and in the release of the CO_2 that was added by the ACCase reaction. Removal of this CO_2 helps to drive this reaction forward, making it essentially irreversible.

At least three separate condensing enzymes (also known as 3-ketoacyl-ACP synthases) are required to produce an 18-carbon fatty acid. The first condensation of acetyl-CoA and malonyl-ACP to form a four-carbon product is catalyzed by

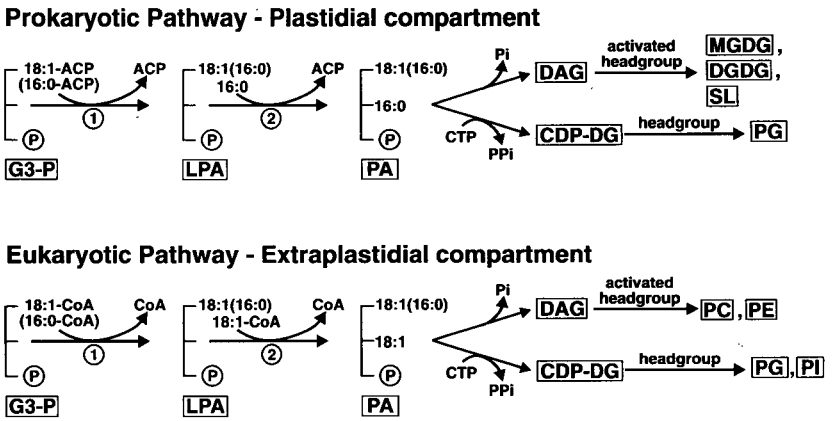

Figure 4. The Prokaryotic and Eukaryotic Pathways of Glycerolipid Synthesis.

The prokaryotic pathway occurs in plastids, uses acyl-ACPs as substrates, and esterifies predominantly palmitate (16:0) at position 2 of glycerol. The eukaryotic pathway occurs outside the plastid (primarily at the ER), uses acyl-CoAs as substrates, and positions 18-carbon fatty acids at position 2 of glycerol-3-phosphate. The amount of lipid synthesized by the prokaryotic pathways varies in angiosperms from 5 to 40% depending on the plant species and the tissue. Reactions 1 and 2 are catalyzed by glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase, respectively. CDP-DG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; G3-P, glycerol-3-phosphate; LPA, monoacylglycerol-3-phosphate; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SL, sulfoguinovosyldiacylglycerol.

KAS III (Jaworski et al., 1989). A second condensing enzyme, KAS I, is believed responsible for producing chain lengths from six to 16 carbons. Finally, elongation of the 16-carbon palmitoyl-ACP to stearoyl-ACP requires a separate condensing enzyme, KAS II. The initial product of each condensation reaction is a 3-ketoacyl-ACP. Three additional reactions occur after each condensation to form a saturated fatty acid (Figure 3). The 3-ketoacyl-ACP is reduced at the carbonyl group by the enzyme 3-ketoacyl-ACP reductase, which uses NADPH as the electron donor. The next reaction is dehydration by hydroxyacyl-ACP dehydratase. Each round of fatty acid synthesis is then completed by the enzyme enoyl-ACP reductase, which uses NADH or NADPH to reduce the *trans*-2 double bond to form a saturated fatty acid. The combined action of these four reactions leads to the lengthening of the precursor fatty acid by two carbons while it is still attached to ACP as a thioester.

The fatty acid biosynthesis pathway produces saturated fatty acids, but in most plant tissues, over 75% of the fatty acids are unsaturated. The first double bond is introduced by the soluble enzyme stearoyl-ACP desaturase. This fatty acid desaturase is unique to the plant kingdom in that all other known desaturases are integral membrane proteins. The cloning of this soluble enzyme has recently led to its crystallization and the determination of its three-dimensional structure by x-ray crystallography (J. Shanklin, personal communication). This and other structural studies have led to the first detailed insights into the mechanism of fatty acid desaturation and the nature of the active site. The enzyme is a homodimer in which each monomer has an independent active site consisting of a diiron-oxo cluster. The two iron atoms are coordinated within a central four helix bundle in which the motif (D/E)-E-X-R-H is represented in two of the four helices. During the reaction, the reduced iron center binds oxygen and a high valent iron-oxygen complex likely abstracts hydrogen from the C–H bond (Fox et al., 1993).

The elongation of fatty acids in the plastids is terminated when the acyl group is removed from ACP. This can happen in two ways. In most cases, an acyl-ACP thioesterase hydrolyzes the acyl-ACP and releases free fatty acid. Alternatively, one of two acyltransferases in the plastid transfers the fatty acid from ACP to glycerol-3-phosphate or to monoacylglycerol-3-phosphate. The first of these acyltransferases is a soluble enzyme that prefers oleoyl-ACP as a substrate. The second acyltransferase resides on the inner chloroplast envelope membrane and preferentially selects palmitoyl-ACP. Whether the fatty acid is released from ACP by a thioesterase or an acyltransferase determines whether it leaves the plastid. If a thioesterase acts on acyl-ACP, then the free fatty acid is able to leave the plastid. It is not known how free fatty acids are transported out of the plastid, but it may occur by simple diffusion across the envelope membrane. On the outer membrane of the chloroplast envelope, an acyl-CoA synthetase is thought to assemble an acyl-CoA thioester that is then available for acyltransferase reactions to form glycerolipids in the endoplasmic reticulum (ER). How the acyl-CoA moves from the outer chloroplast envelope to the ER is also unknown, but it may involve acyl-CoA binding proteins, small abundant proteins recently found to be present in plants (Hills et al., 1994).

GLYCEROLIPID SYNTHESIS

The major fate of 16:0 and 18:1 acyl chains produced in the plastid is to form the hydrophobic portion of glycerolipid

molecules, which are components of all cellular membranes. The first steps of glycerolipid synthesis are two acylation reactions that transfer fatty acids to glycerol-3-phosphate to form phosphatidic acid (PA; Figure 4). Diacylglycerol (DAG) is produced from PA by a specific phosphatase; alternatively, a nucleotide-activated form of DAG (CDP-DAG) is produced from the reaction of PA with cytidine 5′-triphosphate (CTP). The energy to drive attachment of the polar headgroup during de novo glycerolipid synthesis is provided by nucleotide activation. When DAG is the lipid substrate, it is the headgroup that is activated. Thus, cytidine 5′-diphosphate (CDP)-choline, CDP-ethanolamine, and CDP-methylethanolamine can be substrates for phospholipid synthesis, and UDP-galactose and UDP-sulfoquinovose are substrates for monogalactosyldiacylglycerol (MGDG) and sulfoquinovosyldiacylglycerol (SL) synthesis, respectively. Conversely, when CDP-DAG is the lipid substrate, reactions with myo-inositol, serine, and glycerol-3-phosphate result in formation of the phospholipids phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidyl glycerol phosphate (the precursor of PG), respectively. Digalactosyldiacylglycerol (DGDG) is synthesized from MGDG (Joyard et al., 1993).

Although the synthetic pathways are presented here as a linear series of simple enzymatic steps, the actual biochemistry involved is complicated by the possibilities of headgroup modification (for example, the synthesis of phosphatidylcholine [PC] from phosphatidylmethylethanolamine [PE] by two rounds of methylation) and headgroup exchange. The details of the reactions involved and the synthetic routes that probably operate in higher plants have been reviewed previously (Browse and Somerville, 1991; Joyard et al., 1993; Kinney, 1993).

Two Pathways for Membrane Lipid Synthesis

As outlined in Figure 4, higher plants possess two distinct pathways for the synthesis of glycerolipids: the prokaryotic pathway of the chloroplast inner envelope, and the eukaryotic pathway, which begins with phosphatidic acid (PA) synthesis in the ER. Because of the specificities of the plastid acyltransferases for certain acyl-ACP substrates (Frentzen, 1993), the PA made by the prokaryotic pathway has 16:0 at the sn-2 position and, in most cases, 18:1 at the sn-1 position. This PA is used for the synthesis of PG or is converted to DAG by a PA-phosphatase located in the inner plastid envelope. This DAG pool can act as a precursor for the synthesis of the other major plastid membrane lipids, MGDG, DGDG, and SL (Joyard et al., 1993). In contrast with the plastid isozymes, the ER acyltransferases use acyl-CoA substrates to produce PA that is highly enriched in 18-carbon fatty acids at the sn-2 position; 16:0, when present, is confined to the sn-1 position. This PA gives rise to phospholipids such as PC, PE, and PI, which are characteristic of the various extrachloroplast membranes. In addition, however, the DAG moiety of PC can be returned to the chloroplast envelope, where it enters the DAG pool and contributes to the synthesis of plastid lipids (Figure 5). Evidence from several Arabidopsis mutants indicates that lipid exchange between the ER and the chloroplast is reversible to some extent (Miquel and Browse, 1992; Browse et al., 1993) because extra chloroplastic membranes in mutants deficient in ER desaturases (see later discussion) contain polyunsaturated fatty acids derived from the chloroplasts.

In many species of higher plants, PG is the only product of the prokaryotic pathway, and the remaining chloroplast lipids are synthesized entirely by the eukaryotic pathway. In other species, including Arabidopsis and spinach, both pathways contribute about equally to the synthesis of MGDG, DGDG, and SL (Browse and Somerville, 1991), and the leaf lipids of such plants characteristically contain substantial amounts of hexadecatrienoic acid (16:3), which is found only in MGDG and DGDG molecules produced by the prokaryotic pathway. These plants have been termed 16:3 plants to distinguish them from the other angiosperms (18:3 plants), whose galactolipids contain predominantly linolenate. The contribution of the eukaryotic pathway to MGDG, DGDG, and SL synthesis is reduced in lower plants, and in many green algae the chloroplast is almost entirely autonomous with respect to membrane lipid synthesis. One problem presented by the two-pathway model is the need to move hydrophobic lipid molecules from the ER to other sites, particularly the chloroplast. Until recently, a class of soluble proteins characterized (from in vitro experiments) as lipid transfer proteins had been considered to be the intracellular transporters. However, biochemical and immunohistochemical evidence has made it clear that these proteins are extracellular and therefore cannot fulfill this proposed role (Sterk et al., 1991; Thoma et al., 1993).

Membrane Desaturases

In all plant tissues, the major glycerolipids are first synthesized using only 16:0 and 18:1 acyl groups. Subsequent desaturation of the lipids to the highly unsaturated forms typical of the membranes of plant cells (Figure 1) is carried out by membrane-bound desaturases of the chloroplast and the ER (Browse and Somerville, 1991; Heinz, 1993). Investigation of these desaturases by traditional biochemical approaches has been limited because solubilizing and purifying them have proven very difficult (Schmidt et al., 1994). Our understanding of the mechanisms and regulation of the chloroplast and ER desaturases has benefited considerably from the characterization of seven classes of Arabidopsis mutants, each deficient in a specific desaturation step (Browse and Somerville, 1991; Somerville and Browse, 1991). The loci defined by four of these classes were originally called *fadA*, *fadB*, *fadC*, and *fadD* (for fatty acid desaturation), but these have now been renamed *fad4*, *fad5*, *fad6*, and *fad7*, respectively. Mutations in two loci, *fad2* and *fad3*, primarily affect desaturation of the extrachloroplast lipids, whereas mutations in the remaining five loci, *fad4*, *fad5*, *fad6*, *fad7*, and *fad8*, affect chloroplast lipid desaturation (Figure 5).

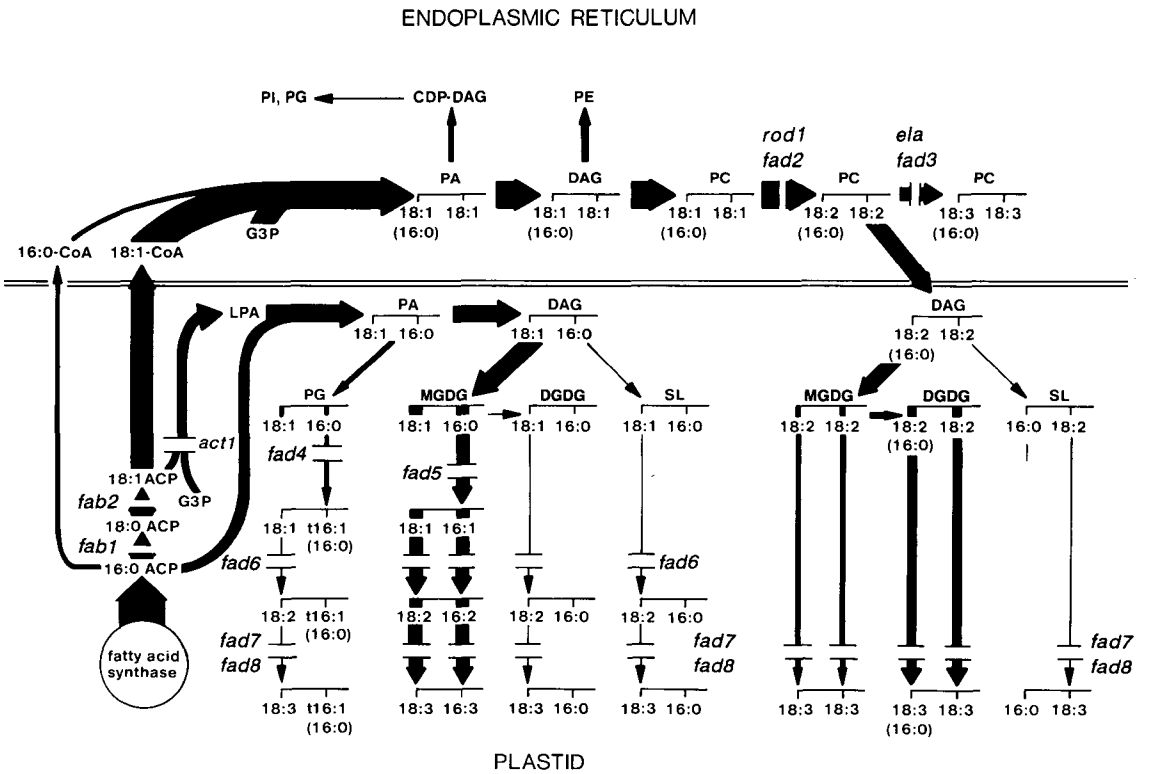

Figure 5. An Abbreviated Diagram of Fatty Acid Synthesis and Glycerolipid Assembly in Arabidopsis Leaves.

Widths of the lines show the relative fluxes through different reactions. The breaks indicate the putative enzyme deficiencies in various mutants. Adapted from Browse and Somerville (1991) and used with permission of Annual Reviews. Abbreviations for lipid structures are defined in Figures 1 and 4. *ela*, enhanced linolenate accumulation; *rod1*, reduced oleate desaturation.

Two of the chloroplast desaturases are highly substrate specific. The *fad4* gene product controls a Δ3 desaturase that inserts a *trans* double bond into the 16:0 esterified to position sn-2 of PG, whereas the *fad5* gene product is responsible for the synthesis of Δ7 16:1 on MGDG and possibly DGDG (Browse et al., 1985; Kunst et al., 1989a). In contrast, the other two chloroplast desaturases act on acyl chains with no apparent specificity for the length of the fatty acid chain (16 or 18 carbon), its point of attachment to the glycerol backbone (sn-1 or sn-2), or the nature of the lipid headgroup. The 16:1/18:1 desaturase is encoded by the *fad6* gene (Browse et al., 1989), whereas two 16:2/18:2 isozymes are encoded by *fad7* and *fad8* (Browse et al., 1986; Gibson et al., 1994; McConn et al., 1994). The ER 18:1 (*fad2*) and 18:2 (*fad3*) desaturases (Miquel and Browse, 1992; Browse et al., 1993) also act on fatty acids at both the sn-1 and sn-2 positions of the molecule. These enzymes have been characterized as PC desaturases, but it is possible that they act on other phospholipids as well.

The overall level of glycerolipid unsaturation is also affected by mutations that control other steps of fatty acid biosynthesis. For instance, the *fab1* and *fab2* (for fatty acid biosynthesis) mutants of Arabidopsis are characterized by increased levels of 16:0 or 18:0, respectively, in seed and leaf tissues (James and Dooner, 1990; Lightner et al., 1994a; Wu et al., 1994). In the *fab1* mutant, the biochemical defect appears to be a reduction in the activity of the condensing enzyme (KAS II) responsible for the elongation of 16:0 to 18:0. In *fab2*, it is assumed that 18:0-ACP desaturase activity is reduced. In both mutants, saturated fatty acids are incorporated into all the major membrane glycerolipids (although MGDG contains relatively low proportions of them). Both mutants appear to be leaky, so the changes in overall membrane fatty acid composition are moderately small. Nevertheless, the changes have profound effects on the growth and development of these plants (see later discussion).

Characterization and cloning of the membrane-bound fatty acid desaturases were for many years unattainable and frustrating goals. However, isolation of most of the genes encoding membrane-bound desaturases has been possible due to recent advances in molecular biology and genetics, especially with the creation of T-DNA tagged mutants of Arabidopsis (Feldmann et al., 1989). This T-DNA tagging method allowed *FAD2* to be cloned (Okuley et al., 1994). *FAD3* was isolated by both map-based chromosome walking (Arondel et al., 1992)

and T-DNA tagging. The *FAD6* (Hitz et al., 1994), *FAD7* (Iba et al., 1993; Yadav et al., 1993), and *FAD8* (Gibson et al., 1994) genes were all cloned based on their homology to *FAD2* and *FAD3*. The homology of these genes with gene products from other organisms that have been characterized at the biochemical level indicates that the *FAD* loci represent the structural genes for desaturases rather than proteins that control the desaturation reactions in any other way. The power of the approaches available to Arabidopsis researchers is evidenced by the fact that clones are available now for more than six different desaturases, whereas research in animal lipid metabolism has yielded only a single, membrane-bound desaturase clone.

Regulation of Membrane Lipid Synthesis

Analyses of several of the Arabidopsis lipid mutants (Browse and Somerville, 1991), particularly *act1*, demonstrate that powerful mechanisms mediate the regulation of glycerolipid biosynthesis to meet the demands for particular lipid molecular structures for optimal membrane function. For example, *act1* mutants are deficient in activity of the chloroplast acyl-ACP *sn*-glycerol-3-phosphate acyltransferase—the first enzyme of the prokaryotic pathway (Figure 5). This deficiency is compensated for by increased synthesis of chloroplast lipids via the eukaryotic pathway (Kunst et al., 1988). There remain the fundamental questions of precisely what signals communicate this demand to the lipid biosynthetic machinery and what mechanism transmits the signal from the many different membranes of the cell to the major sites of lipid and fatty acid synthesis in the ER and the chloroplast envelope and stroma. Currently, no aspect of these regulatory mechanisms is understood in higher plants, so this area represents a challenging and potentially rewarding subject for future research.

FURTHER METABOLISM OF MEMBRANE GLYCEROLIPIDS

Little is known about the cell biology of lipid turnover and degradation. The enzymes involved, including phospholipases, galactolipases, and lipoxygenases, have been thoroughly investigated for a number of years (Galliard, 1980; Vick, 1993; Wang, 1993). However, it is not at all clear how these and other enzymes are regulated during the senescence of an organ. For example, up to half of the lipid content of a leaf is metabolized during senescence, presumably through β-oxidation of the fatty acyl chains. Lipids are probably not transported out of an organ; therefore, the whole process of lipid turnover must be controlled to bring about the orderly dismantling of cells and organelles and to facilitate the export of those lipid breakdown products that can be used as precursors elsewhere in the plant.

The further metabolism of membrane lipids can result not only in nonspecific breakdown products but also in the production of specific cellular signals. One of these is phosphatidylinositol 4,5-bisphosphate, whose presence in higher plants has been established (Cote and Crain, 1993). By analogy to the well-studied phosphoinositide system in animal cells (Majerus, 1992), this product may act as a precursor for signal molecules (that is, DAG and inositol 1,4,5-trisphosphate, or IP3). The action of IP3 in releasing Ca^{2+} into the cytoplasm and thereby regulating cellular processes has been demonstrated in several plant systems (Alexandre and Lassalles, 1992; Cote and Crain, 1993). On the other hand, it is not at all certain that the DAG arm of the phosphoinositide signaling pathway is present in plants (Cote and Crain, 1993).

Another specific signal is jasmonate, a plant growth regulator derived from 18:3. Recent investigations of jasmonate action have revealed that at very low concentrations it is able to strongly induce specific genes, including proteinase inhibitors and other plant defense genes (Farmer and Ryan, 1990; Gundlach et al., 1992) and vegetative storage proteins (Mason and Mullet, 1990). The structure and biosynthesis of jasmonate have intrigued plant biologists because of their parallels to some eicosanoids central to inflammatory responses and other physiological processes in mammals (Creelman et al., 1992). In plants, jasmonate is synthesized from linolenate (which presumably is released from membrane lipids by a phospholipase A2) by a pathway that starts with the action of lipoxygenase on 18:3 (Figure 5). The proposed pathway has not been fully characterized at the level of purified enzymes. Several lipoxygenase isozymes have been purified and thoroughly studied (Vick, 1993), but it is not yet clear which of these acts in jasmonate synthesis. Allene oxide synthase has also been purified and the corresponding genes cloned. Both the biochemistry and cell biology of jasmonate synthesis are now being given more attention so that we can look forward to having a more complete picture of this extremely potent lipid-derived signal molecule.

PHYSIOLOGY AND CELL BIOLOGY OF MEMBRANE LIPID COMPOSITION

The central issue with respect to the role of glycerolipids in membranes is framed by the observations that each membrane of the cell has a characteristic and distinct complement of glycerolipid types and that, within a single membrane, each class of lipids has a distinct fatty acid composition (Browse et al., 1993). Furthermore, the composition of each membrane and lipid class is largely conserved throughout the plant kingdom. This diversity and its conservation throughout evolution imply that differences in lipid structure are important for membrane function. However, despite considerable effort, the details of this structure/function relationship have remained elusive, in large part because there have been no instructive biological examples available in which changes in lipid composition have been clearly shown to have specific effects on membrane processes.

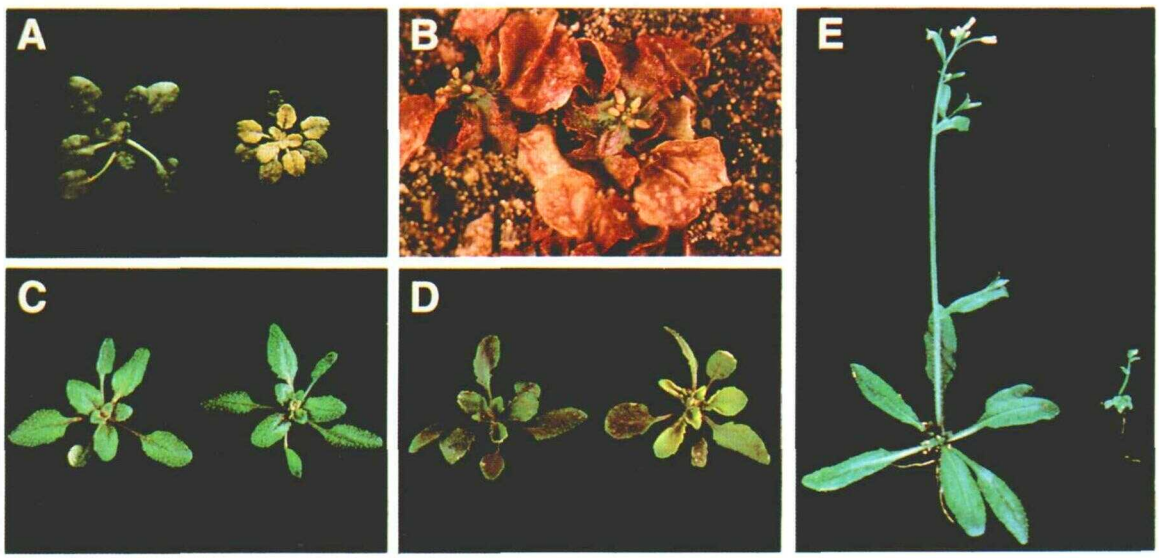

Figure 6. Some Phenotypic Consequences of Changes in Membrane Lipid Composition.

(A) Compared with wild-type plants (left), the Arabidopsis *fad6* mutant (right) becomes chlorotic after 3 weeks at 5°C.
(B) *fad2* Arabidopsis plants die after 7 weeks at 6°C.
(C) Compared with wild-type plants (left), the *fab1* mutant (right) is unaffected by up to 1 week of exposure to chilling at 2°C.
(D) After 4 weeks at 2°C, however, *fab1* plants (right) show clear symptoms of chlorosis and reduced growth.
(E) The *fab2* mutant (right) is a severe dwarf at 22°C compared with the wild type (left), as a result of an increase in stearic acid (18:0) in its membrane lipids.

In the last few years, investigations of several Arabidopsis lipid mutants have begun to provide such examples (Hugly et al., 1989; Kunst et al., 1989b; Hugly and Somerville, 1992). The *fad2* mutants of Arabidopsis are deficient in the activity of the ER 18:1 desaturase responsible for production of polyunsaturated lipids on the eukaryotic pathway of lipid synthesis (Miquel and Browse, 1992). When grown at 22°C, *fad2* plants are similar in growth and appearance to the wild type. However, these mutants show a dramatic chilling-sensitive phenotype when plants are transferred to 6°C (Miquel et al., 1993). At this temperature, wild-type Arabidopsis continues to grow and develop normally, but the leaves of three allelic *fad2* mutants gradually deteriorate, displaying patches of necrosis and extensive accumulation of anthocyanins. Death of the leaves and of the whole plants eventually follows (Figure 6). Therefore, even though the lesions at the *fad2* locus are largely irrelevant to the growth and survival of plants at normal temperatures, polyunsaturated membranes are essential for maintaining cellular function and plant viability at low temperatures. Although the deficiency in polyunsaturated lipids has severe consequences, several lines of evidence indicate that the change(s) in membrane function in *fad2* plants at 6°C begins to compromise cell viability only after several days (Miquel et al., 1993). This gradual development of symptoms at 6°C argues against a general collapse of membrane integrity as the cause of the lethal phenotype. Rather, it indicates that the decrease in polyunsaturated membrane lipids may initially have relatively limited effects in disrupting cellular function.

Additional Arabidopsis mutants have helped to clarify the role of lipid composition in chilling sensitivity, a phenomenon of considerable agricultural significance. The increased 16:0 content in leaves of *fab1* plants includes the synthesis of molecular species of chloroplast PG that contain only 16:0 plus 16:1–*trans* plus 18:0, and these molecular species account for 42% of the PG in leaves of the mutant. Such "high melting point" molecular species of PG have been correlated with chilling sensitivity of plants (Murata, 1983). Experiments with transgenic plants engineered to express increased levels of high–melting-point PG have also been interpreted as supporting a major role for high–melting-point PG species in determining plant chilling sensitivity (Murata et al., 1992; Wolter et al., 1992). However, *fab1* mutants are able to grow and complete their life cycles normally at 10°C. They are also unaffected (as compared with wild-type controls) by more severe chilling treatments that lead quickly to the death of cucumber and other chilling-sensitive plants. These treatments include exposure to 2°C for up to 7 days and freezing to −2°C for 24 hr (Wu and Browse, 1995). Following these treatments, mutant plants returned to 22°C remain indistinguishable from wild-type controls (Figure 6). If grown continuously at 2°C, *fab1* plants eventually show damage (Figure 6; Wu and Browse, 1995), but the *fab1* mutant does not show classic chilling sensitivity, despite its increased 16:0 content. This suggests that high–melting-point species of PG cannot alone account for chilling sensitivity of plants.

The *fab2* mutant, which contains elevated levels of 18:0 in

membrane lipids, shows an extreme dwarf growth habit, with mutant plants growing to less than one-tenth the size of the wild type (Figure 6). Many cell types in the mutant, including leaf mesophyll and epidermal cells, fail to expand, whereas others, including stomatal guard cells and leaf trichomes, develop to the normal size. The relationship between the dwarf and high 18:0 phenotypes was established by identifying a mutation, a suppressor of high stearate, that causes the *fab2* mutant to grow to normal size and to have normal levels of 18:0. Moreover, when *fab2* plants are grown at high temperatures, which ameliorates the effects of increased saturation of membrane lipids, tissue 18:0 levels remain high but plant growth is more normal (Lightner et al., 1994b). This result suggests that increased saturation of membrane lipids is the direct cause of the dwarf phenotype of *fab2* mutants.

BIOSYNTHESIS OF STORAGE TRIACYLGLYCEROLS

A plant stores reserve material (oil, protein, and carbohydrate) in its seed to allow growth of the next generation. The amount of oil in different species may vary widely, from as little as 1% to as much as 60% of the total dry weight of the seed. In almost all plant species, the chemical form in which oil is stored is triacylglycerol (TAG). Unlike the glycerolipids found in membranes, TAGs do not perform a structural role but instead serve primarily as a storage form of carbon.

In the mature seed, TAG is stored in densely packed lipid bodies that are roughly spherical in shape, with an average diameter of 1 μm (Huang, 1992; Murphy, 1993; Herman, 1994). This size does not change during seed development, and accumulation of oil is accompanied by an increase in the number of lipid bodies. These organelles appear to be surrounded by a type of membrane that is only a monolayer rather than a normal bilayer membrane. The membranes of isolated lipid bodies contain both phospholipids and major characteristic proteins, termed oleosins. However, these constituents are less than 5% of the weight of the oil body, with TAG constituting by far the major component (90 to 95%).

The Pathway of Triacylglycerol Synthesis

TAG biosynthesis, at least in some species, has been proposed to take place by the comparatively straightforward Kennedy pathway. As in the eukaryotic pathway for synthesis of the major membrane glycerolipids (Figure 4), fatty acids are sequentially transferred from CoA to positions 1 and 2 of glycerol-3-phosphate, resulting in the formation of the central metabolite, PA. Dephosphorylation of PA releases DAG. In the final step of TAG synthesis, a third fatty acid is transferred to the vacant position 3 of DAG. This step is catalyzed by diacylglycerol acyltransferase (DAGAT), the only enzymatic reaction unique to TAG biosynthesis.

The assembly of three fatty acids onto a glycerol backbone is not always as straightforward as outlined for the Kennedy pathway, however. In many oilseeds, most fatty acids produced in the plastid are not immediately available for TAG biosynthesis. Instead, the acyl chains enter into phosphatidyl choline (and PE to a lesser extent), where they become desaturated or otherwise modified. Fatty acids from PC may then become available for TAG synthesis by one of two mechanisms. In the first mechanism, a fatty acid attached to CoA and a fatty acid on PC may essentially trade places. Such an acyl exchange probably occurs by the combined reverse and forward reactions of an acyl-CoA:PC acyltransferase (Stymne and Stobart, 1987). The resulting acyl-CoA may then be used as an acyl donor in TAG synthesis. The exchange reaction allows 18:1 newly produced and exported from the plastid to enter PC, while desaturated or otherwise modified fatty acids depart for TAG or other lipids. The second mechanism by which PC can participate in TAG synthesis is by donation of its entire DAG portion (Stymne and Stobart, 1987). In many plants, the synthesis of PC from DAG and CDP-choline appears to be rapidly reversible as catalyzed by the CDP-choline phosphotransferase (Slack et al., 1983). The reversibility of this reaction allows the DAG moiety of PC, including any modified fatty acids, to become available for TAG synthesis.

Because DAG is a common precursor to both membrane PC and storage TAG, identification of these enzymatic reactions responsible for TAG biosynthesis does not satisfactorily address two potentially related questions of plant lipid metabolism: How do plants control which fatty acids are stored in triacylglycerol and which are restricted to membranes? Is the synthesis of TAG spatially distinct from the synthesis of membrane glycerolipids?

The first question is particularly relevant to those oilseeds that store "unusual" fatty acids. The fatty acid composition of storage oils varies much more than that of membrane glycerolipids. More than 300 different fatty acids are known to occur in seed TAG (Harwood, 1980; van de Loo et al., 1993). Their structures may vary in chain length from as few as eight carbons to >22 carbons. In addition, the position and number of double bonds may be unusual, and various functional groups such as hydroxy or epoxy may be added to the acyl chain. The reason for this great diversity in plant storage oils is unknown, but the special physical or chemical properties of some of these fatty acids may explain why (if not how) these unusual fatty acids are excluded from membranes. If they were to become major components of the membranes, then the fluidity or other physical properties of the membrane might be disrupted.

SUMMARY AND PERSPECTIVES FOR FUTURE RESEARCH

The basic metabolic pathways that lead to the synthesis of the major plant glycerolipids are now mostly understood. Much

of the research effort in plant lipid biosynthesis over the past five years has been directed toward obtaining clones for enzymes in the pathway. A number of industrial laboratories have participated in this effort because of the potential economic value that might derive from genetic engineering of vegetable oils. Plant TAGs are a major agricultural commodity, with a value of ~$25 billion annually. Recently, several successes have been achieved in genetically engineering oilseed fatty acid composition to produce new or improved vegetable oils (reviewed by Kinney, 1994; Ohlrogge, 1994; Töpfer et al., 1995). Almost all of the many soluble enzymes involved in producing oleic acid from acetyl-CoA have now been cloned (Töpfer and Martini, 1994). Several of the membrane-bound enzymes that were more difficult to obtain using biochemical approaches have now yielded to alternative strategies, such as map-based cloning (Arondel et al., 1992), T-DNA or transposon tagging (Okuley et al., 1994; James et al., 1995), and complementation of mutants (Brown et al., 1994; Dewey et al., 1994). Most of the already cloned genes have significant homology to their microbial or animal counterparts. Within the near future, most of the expressed genes of Arabidopsis and rice will be sequenced (Newman et al., 1994; Sasaki et al., 1994), making it likely that clones for many more of the enzymes in lipid metabolism can be identified by appropriate analysis of the sequence databases.

Although we now understand the basic pathways of glycerolipid synthesis, a number of major questions remain to be answered, among them: (1) What is the source of plastidial acetyl-CoA? Does the pathway for acetyl-CoA production vary with different tissues and during tissue development? (2) What determines how much lipid is produced in a cell? Is control exerted primarily through the level of gene expression or through metabolic controls over enzyme activity? (3) How is the production of fatty acids in the plastid coordinated with their utilization outside the plastid? Are there signals that communicate between the ER and the plastid fatty acid synthesis pathway, and, if so, what are these signals? (4) How and in what form do lipids produced in the ER return for incorporation in plastidial membranes? (5) How do cells determine the subcellular distribution of different lipid classes and the different fatty acid compositions associated with different polar headgroups? Are there specific carrier proteins that allow lipids to move between organelles? (6) How are lipid biosynthesis genes regulated? Are there "global" *trans*-acting factors that simultaneously control the expression of many genes? (7) Are membrane lipid desaturases controlled by signals that respond to membrane fluidity? (8) What roles do phospholipases and lipoxygenases play in lipid metabolism? How is the synthesis of jasmonic acid controlled? (9) Are the precursors for membrane lipids and storage TAGs synthesized by the same enzymes and in the same subcellular location?

Many of these questions have been very difficult to approach in the past. The recent availability of a large number of clones for enzymes of lipid metabolism, together with the ability to manipulate their expression in transgenic plants, now offers plant lipid scientists the potential to design robust new experiments to address these topics.

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